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Editors

Vogel and Motulsky's
Human Genetics

Problems and Approaches

4th Edition

Springer

Speicher • Antonarakis • Motulsky

VOGEL AND MOTULSKY'S HUMAN GENETICS

Problems and Approaches

Fourth Edition

Speicher Antonarakis Motulsky

VOGEL AND MOTULSKY'S HUMAN GENETICS

Problems and Approaches

Fourth, Completely Revised Edition

With 343 Figures and 76 Tables

Springer

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In memory of Friedrich Vogel

The editors

To my wife, Irene, and our children,
Alexander and Julia.

Michael R. Speicher

To my parents, my wife, Grigoria, and
our children.

Stylianos E. Antonarakis

To the memory of my wife, Gretel, and
to my children.

Arno G. Motulsky

Preface

The first edition of *Human Genetics, Problems and Approaches*, was published in 1970 by human geneticists Friedrich Vogel and Arno Motulsky as sole authors. The aim was broad coverage and in-depth analysis of both medical and human genetics with an emphasis on problems and approaches with occasional historical comments. This point of view was fully explained in an introductory chapter of the three previous editions (1970, 1976, 1997). The book acquired an excellent reputation as an advanced text of human genetics and has been translated into Italian, Japanese, Russian, Chinese, and Portuguese. Our general aims for the fourth edition remain similar and together with novel developments are now set out in the Introduction of this new fourth edition.

Around 2004/2005, both Friedrich Vogel and Arno Motulsky, as well as the publishers, felt that the book should be continued with a new fourth edition in the same spirit and coverage as earlier editions, but should now include additional expert authors. After some delay and the death of Friedrich Vogel in the summer of 2006, a new editorial team consisting of Michael R. Speicher of the Medical University of Graz, Austria, Stylianos E. Antonarakis of the University of Geneva Medical School, and Arno G. Motulsky of the University of Washington School of Medicine, was constituted for the fourth edition of the Vogel/Motulsky book in the spirit of the original work.

An outline of the fourth edition's contents was developed and various internationally known geneticists, including the new editors, were selected to write the individual chapters. The resultant titles are listed in the table of contents. Most chapters are entirely new, while only three chapters (1, 5, and 6) utilize the third edition with appropriate, up-to-date revisions. Entirely new chapters include the description of the human genome, epigenetics, pharmacogenetics, genetic epidemiology, human evolution, genetics of mental retardation, autism, alcoholism and other addictions, consanguinity and related matters, gene therapy, cloning and genetic aspects of global health. Multiple chapters of various animal models used in the study of human and medical genetics are novel as are Databases and Genome Browsers as well as Databases Used in Human and Medical Genetics. The final content of the book was the result of many e-mails and conference calls. This new, updated, and totally revised edition does not contain some important and historically interesting chapters on certain topics. These can be found in the third edition of the book published in 1997, which was exclusively authored by F. Vogel and A.G. Motulsky. These topics were: enzymes in Chap. 7 (pp. 258–299); mutation rates in Chap. 9 (pp. 393–413); and mutation induction by ionizing radiation and chemicals in Chap. 11 (pp. 457–493).

The staff of Springer was most helpful in giving us extensive and firm aid in getting the book finished. We thank particularly Doris M. Binzenhöfer-Walker for her work during the early stage of the project and Isabella Athanassiou for her efficient help later. Andrea Pillman was a strict task mistress, who encouraged us to finish the book expeditiously.

The editors of the fourth edition want to express their intellectual indebtedness to Friedrich Vogel for having conceived of and played a major role in the first three editions of this book. Arno Motulsky particularly misses his discussions with Friedrich on human genetics and its role in science and medicine.

The cover illustration portrays a marble statue of Asclepius, the Greek god of healing, grasping a serpent-encircled staff as a symbol of medicine. The double helix of DNA joined to Asclepius symbolizes the applications of basic genetics to medicine.

4th edition
June 28, 2009

Michael R. Speicher, Graz
Stylianos E. Antonarakis, Geneva
Arno G. Motulsky, Seattle

Preface to the First Edition

Human genetics provides a theoretical framework for understanding the biology of the human species. It is a rapidly growing branch of science. New insights into the biochemical basis of heredity and the development of human cytogenetics in the 1950s heightened interest in this field. The number of research workers and clinicians who define themselves as full-time or part-time human and medical geneticists has increased sharply, and detailed well-founded knowledge has augmented exponentially. Many scientists and physicians are confronted with genetic problems and use concepts and methodology of human genetics in research and diagnosis. Methods developed in many different fields of the biologic, chemical, medical, and statistical sciences are being utilized toward the solution of genetic problems. The increasing number and sophistication of well-defined and elegantly solved problems helps to refine an extensive framework of genetic theory. These new conceptual insights in their turn lead to solutions of new questions. To mention only one example, the structure of hemoglobin genes has been elucidated using methods derived from protein chemistry and DNA technology. It is an exciting experience to participate in these developments!

Moreover, scientific progress in genetics has practical implications for human well-being. Improved knowledge of the genetic cause of an increasing number of human diseases helps to refine diagnosis, to find new therapeutic approaches, and above all, to prevent genetic diseases. So far, human genetics has had less of an impact on the behavioral and social sciences. It is possible that genetic differences involved in shaping personality structure, cognitive faculties, and possibly human social behavior may be at least as important as genetic variation affecting health and disease. The data, however, are less clear and more controversial. These problems are discussed in detail in the text. The rapid progress of human genetics in recent decades has attracted – and is still attracting – an increasing number of students and scientists from other fields. Various elementary textbooks, more advanced monographs of various branches of the field, and the original journal literature are the usual sources of introduction to human genetics. What seems to be lacking, however, is a fairly thorough and up-to-date treatise on the conceptual basis of the entire field of human genetics and its practical applications. Often, the absence of a broadly based background in the field leads to misunderstanding of its scope, unclear goals for research, improper selection of methods, and imbalanced theoretical discussions. Human genetics is based on a powerful theory, but this implicit conceptual foundation should be made explicit. This goal is the purpose of this book. It certainly is a formidable and possibly even too audacious task for two sole authors. However, both of us have been active in the field for more than 25 years.

We have worked on various problems and with a variety of methods. Since the early years of our careers, we have met occasionally, followed each other's writings, and were often surprised by the similarity of our opinions and judgments despite quite different early medical and scientific backgrounds. Moreover, our knowledge of the literature turned out to be in part overlapping and in part complementary. Since we are working in different continents, AGM had a better knowledge of concepts and results in the USA, while FV knew more of the continental European literature. Moreover, both of us have extensive experience as editors of journals in human genetics and one (FV) published a fairly comprehensive textbook in Germany some time ago (*Lehrbuch der allgemeinen Humangenetik*, Springer 1961), parts of which were still useful for the new book. We finally decided to take the risk, and, by writing an "advanced" text, to expose our deficiencies of knowledge, shortcomings of understanding, and biases of judgment.

A text endeavoring to expose the conceptual framework of human genetics cannot be dogmatic and has to be critical. Moreover, we could not confine ourselves to hard facts and well-proved statements. The cloud of conjectures and hypotheses surrounding a rapidly growing science had to be depicted. By doing so, we face the risk of being disproved by further results.

A number of colleagues helped by reading parts of the manuscript on which they had expert knowledge and by making useful suggestions: W. Buselmaier, U. Ehling, G. Flatz, W. Fuhrmann, S. Gartler, Eloise Giblett, P. Propping, Laureen Resnick, and Traute M. Schroeder. They should not be held responsible for possible errors. J. Krüger was of supreme help in the statistical parts. Our secretaries, Mrs. Adelheid Fengler and Mrs. Gabriele Bauer in Heidelberg, Mrs. Sylvia Waggoner in Seattle, and Mrs. Helena Smith in Stanford gave invaluable aid. The figures were drawn by Edda Schalt and Marianne Lebküchner. Miriam Gallaher and Susan Peters did an expert job of copy editing. The authors are especially grateful to Dr. Heinz Götze and Dr. Konrad F. Springer, of Springer Publishing Company, for the excellent production. The work could not have been achieved had the two authors not been invited to stay at the Center for Advanced Study in the Behavioral Sciences at Stanford (California) for the academic year of 1976/1977. The grant for AGM was kindly provided by the Kaiser Family Foundation, while the Spencer Foundation donated the grant for FV.

The cover of this book shows the mythical first human couple, Adam and Eve, as imagined by Albrecht Dürer (1504). They present themselves in the full beauty of their bodies, ennobled by the genius and skill of a great artist. The drawing should remind us of the uniqueness and dignity of the human individual. Human genetics can help us to understand humanity better and to make human life happier. This science is a cardinal example of Alexander Pope's statement. "The proper study of mankind is man."

Spring 1979

Friedrich Vogel, Heidelberg
Arno G. Motulsky, Seattle

Contents Overview

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Introduction

Human Genetics as Fundamental and Applied Science

Human genetics is both a fundamental and an applied science. As a fundamental science, it is part of genetics – the branch of science that examines the laws of storage, transmission, and realization of information for development and function of living organisms. Within this framework, human genetics concerns itself with the most interesting organism – the human being. This concern with our own species makes us scrutinize scientific results in human genetics not only for their theoretical significance but also for their practical value for human welfare. Thus, human genetics is also an applied science. Its value for human welfare is bound to have repercussions for theoretical research as well, since it influences the selection of problems by human geneticists, their training, and the financing of their research. Because of its continued theoretical and practical interest, human genetics offers fascination and human fulfillment unparalleled by work in fields that are either primarily theoretical or entirely practical in subject matter.

Science of Genetics

Genetics is based on a powerful and penetrating theory. The profundity of a theory depends on the depth of the problems that it sets out to solve and can be characterized by three attributes: the occurrence of high-level constructs, the presence of a mechanism, and high explanatory power [1]. In genetics, the high-level "construct" is the gene as a unit of storage, transmission, and realization of information. Since the rediscovery of Mendel's laws in 1900, genetic mechanisms have been worked out step by step to the molecular level – deciphering of the genetic code, analysis of transcription and translation, the function of gene-determined proteins, the fine structure of the genetic material, and DNA sequences outside of genes. The problems of regulation of gene activity in the development and function of organisms are currently a principal goal of fundamental research. So far, the explanatory power of the theory has not nearly been exhausted.

How Does a Science Develop?

Kuhn (1962) [10] described the historical development of a science as follows: In the early, protoscientific stage, there is substantial competition among various attempts at theoretical foundation and empirical verification. Basic observations suggest a set of problems that, however, is not yet visualized clearly. Then, one "paradigm" unifies a group within the scientific community in the pursuit of a common goal, at the same time bringing into sharper focus one or a few aspects of the problem field, and suggesting a way for their solution. If the paradigm turns out to be successful, it is accepted by an increasing part of the scientific community, which now works under its guidance, exploring its possibilities, extending its range of application, and developing it into a scientific theory.

This concept of a paradigm has three main connotations:

1. It points to a piece of scientific work that serves as an "exemplar," suggesting ways in which a certain problem should be approached.
2. It delimits a group of scientists who try to explore this approach, expand its applicability, deepen its

theoretical basis by exploration of basic mechanisms, and enhance its explanatory power.

3. Finally, while an elaborate theory must not – and, in most cases, does not – exist when a paradigm is initiated, its germ is already there, and a successful paradigm culminates in the elaboration of this theory.

This process of developing a science within the framework of a paradigm has been described by Kuhn as "normal science." The basic theory is taken increasingly for granted. It would be sterile at this stage to doubt and reexamine its very cornerstones; instead, it is applied to a variety of problems, expanded in a way that is comparable to puzzle solving. From time to time, however, results occur that, at first glance, defy explanation. First, this leads to attempts at accommodating such results within the theoretical framework by additional ad hoc hypotheses. These attempts are often successful; sometimes, however, they fail. If in such a situation an alternative paradigm is brought forward that explains most of the phenomena accounted for by the old theory as well as the new, hitherto unexplained phenomena, a scientific "revolution" may occur. The new paradigm gains support from an increasing majority of the scientific community, it soon develops into a new – more explanatory – theory, and the process of normal science begins anew.

This portrayal of scientific development has been criticized by some philosophers of science [11]. The concept of "normal" science as outlined above does not appeal to some theorists. Working within the framework of a given set of concepts has been denounced as dull, boring, and in any case not as science should be. According to these philosophers, scientists ought to live in a state of permanent revolution, constantly questioning the basic foundations of their field, always eager to put them to critical tests and, if possible, to refute them [15–18]. Many scientists actively involved in research, on the other hand, have readily accepted Kuhn's view; he has apparently helped them to recognize some important aspects in the development of their own fields.

Central Theory of Genetics Looked at as a Paradigm

While Kuhn's concepts were developed on the basis of the history of the physical sciences, his description well fits the development of genetics. Up to the second half of the nineteenth century, the phenomena of heredity eluded analysis. Obviously, children were sometimes – but by no means always – similar to their parents; some diseases were shown to run in families; it was possible to improve crops and domestic animals by selective breeding. Even low-level laws were discovered, for example Nasse's law that hemophilia affects only boys but is transmitted by their mothers and sisters (Chap. 5, Sect. 5.1.4). However, a convincing overall theory was missing, and attempts at developing such at theory were unsuccessful. In this situation, Mendel, in his work *Versuche über Pflanzenhybriden* (1865) [12] first improved a procedure; he complemented the breeding experiment by counting the offspring. He then interpreted the results in terms of the random combination of basic units; by assuming these basic units, he founded the gene concept – the nuclear concept underlying genetic theory (Chap. 1, Sect. 1.4).

Since the rediscovery of his work in 1900, Mendel's insight has served as a paradigm in all three connotations: it provided an exemplar as to how breeding experiments should be designed and evaluated, it resulted in the establishment of a scientific community of geneticists, and it led to the development of a deep and fertile scientific theory. A special problem that has not been answered satisfactorily, in our opinion, concerns the question of why acceptance of Mendel's paradigm had to wait for as long as 35 years after these experiments were published. It would be too simplistic to blame academic arrogance and shortsightedness of contemporary biologists who did not want to accept the work of a "nonacademic" outsider, even if this factor may indeed have been one of the components for this neglect. We believe rather that the many new biological discoveries in the 35 years following Mendel's discovery were of such a revolutionary nature as to qualify as a scientific crisis in the Kuhnian sense and therefore required a completely new approach.

Soon after the rediscovery of Mendel's laws in 1900, however, an initially small, but quickly growing group of scientists gathered who developed genetics in an interplay between theory and experiment and launched the major scientific revolution of the twentieth century in the field of biology.

Human Genetics and the Genetic Revolution

Meanwhile, the biological revolution of the nineteenth century – evolutionary theory – had been accepted by the scientific community. One major consequence was the realization that human beings had evolved from other, more "primitive" primates, that humans are part of the animal kingdom, and that the laws of heredity which had been found to apply for all other living beings are also valid for our species. Hence, Mendel's laws were soon applied to traits that were found in human pedigrees – primarily hereditary anomalies and diseases. Analyzing the mode of inheritance of alkaptonuria – a recessive disease – Garrod (1902) [5] clearly recognized the cardinal principle of gene action: genetic factors specify chemical reactions (Chap. 1, Sect. 1.5). This insight also required 30 years before being incorporated into the body of "normal" science.

Elucidation of inheritance in humans did not begin with Mendel's paradigm. Many relevant observations had been reported before, especially on various diseases. Moreover, another paradigm had been founded by F. Galton in his work on *Hereditary Talent and Character* (1865) [6] and in later works: to derive conclusions as to inheritance of certain traits such as high performance, intelligence, and stature, one should measure these traits as accurately as possible and then compare the measurements between individuals of known degree of relationship (for example, parents and children, sibs, or twins) using statistical methods. This approach did not contain the potential for elucidating the mechanisms of heredity. On the other hand, it seemed to be much more generally applicable to human characteristics than Mendelian analysis; pedigree analysis in terms of Mendel's laws was hampered by the fact that most human traits simply could not be classified as alternate characteristics, as could round and shrunken peas. Human characteristics are usually graded and show no alternative distribution in the population. Moreover, the phenotypes are obviously determined not only by the genetic constitution but by external, environmental influences as well – the result of an interaction between "nature and nurture" (Galton). Therefore, naive attempts at applying Mendel's laws to such traits were doomed to failure. For traits that are regarded as important, such as intelligence and personality, but also for many diseases and mental retardation, there was only the choice between research along the lines suggested by Galton or no research at all. Investigations on genetic mechanisms would have to await elucidations of the genetics of other, more accessible organisms. Under these circumstances, scientists chose to follow Galton. This choice had not only theoretical reasons; it was strongly influenced by the desire to help individuals and families by calculating risk figures for certain diseases, thereby creating a sound basis for genetic counseling. More important, however, was the concern of some scientists about the biological future of the human species, which they saw threatened by deterioration due to relaxation of natural selection. The motives for their research were largely eugenic: it seemed to provide a rational foundation for measures to curb reproduction of certain groups who were at high risk of being diseased or otherwise unfit.

History of Human Genetics: A Contest Between Two Paradigms

The two paradigms – Mendel's gene concept and Galton's biometric approach – have developed side by side from 1900 up to the present; many present-day controversies, especially in the field of behavior genetics but also those concerning strategies in the genetic elucidation of common diseases, are immediately understandable when the history of human genetics is conceived as a contest between these two paradigms. This does not mean that the two paradigms are mutually exclusive; in fact, correlations between relatives as demonstrated by biometric analysis were interpreted in terms of gene action by Fisher in 1918 [4]. Some human geneticists have worked during some part of their career within the framework of the one paradigm, and during another within the framework of the other paradigm. By and large, however, the two streams of research have few interconnections and may even become further polarized because of highly specialized training for each group, epitomized by the biochemical and molecular genetic laboratories for the one and the computer for the other group.

In the first decades of the last century the biometric paradigm of Galton appeared to be very successful. Genetic variability within the human population was believed to be established for normal traits such as stature or intelligence as well as for a wide variety of pathologic conditions such as mental deficiency and psychosis, epilepsy, and common diseases such as diabetes, allergies, and even tuberculosis. Mendelian analysis, on the other hand, seemed to be confined to rare hereditary diseases; the ever repeated attempts at expanding Mendelian explanation into the fields of normal, physical characteristics and common diseases were usually undertaken without critical assessment of the inescapable limitations of Mendelian analysis. The first major breakthrough of Mendelian genetics was the establishment of the three-allele hypothesis for the AB0 blood groups by Bernstein in the 1920s [2] (Chap. 5, Sect. 5.2.2). Further progress, however, had to await the development of genetic theory by work on other organisms such as *Drosophila,* bacteria, and viruses, especially bacteriophages.

The advent of molecular biology in the late 1940s and 1950s had a strong influence on human genetics and, indeed, brought the final breakthrough of Mendel's paradigm. A major landmark was the discovery by Pauling et al. in 1949 [14] that sickle cell anemia is caused by an abnormal hemoglobin molecule.

The foundation of human chromosome research in the late 1950s and early 1960s (Chap. 3, Sect. 3.1) came as a second, important step. At present, most investigations in human genetics have become a part of mainstream research within the framework of genetic theory. The human species, regarded by most early experimental geneticists as a poor tool for genetic research, is now displaying definite advantages for attacking basic problems. Some of these advantages are the large size of available populations, the great number and variety of known mutants and chromosome anomalies, and the unparalleled detailed knowledge of human physiology and biochemistry in health and disease. The improved understanding of human genome structure and its variability (Chap. 2) by the completion of the human genome project, by new sequencing and array technologies, and by efforts to identify all functional elements in the human genome sequence (Chap. 4), further facilitates both basic and applied research in human genetics.

One would expect that such breakthroughs have led to the establishment of Mendel's paradigm as the only leading paradigm in human genetics. This, however, is not the case. In spite of the fact that genetic theory is now pervading many fields that seemed to be closed to it, the paradigm of Galton – biometric analysis – has attained an unsurpassed level of formal sophistication over the past decades. The availability of software tools has greatly facilitated the development and application of biometric techniques. Moreover, in some fields, such as behavior genetics, the application of genetic theory – Mendel's paradigm – is still hampered by severe difficulties (Chap. 23), and here biometric methods have dominated for a long time. In the same field, however, they are most severely criticized and subject to controversial discussions about ethical issues and possible discrimination.

Progress in Human Genetics and Practical Application

The achievements of molecular biology and chromosome research have not only altered human genetics as a pure science, but have also brought marked progress in its application for human welfare. At the beginning, this progress did not appear very conspicuous; the diagnosis of hereditary diseases was improved, and many, hitherto unexplained malformations were accounted for by chromosome aberrations. The first practical success came in the early 1950s when the knowledge of enzyme defects in phenylketonuria (Chap. 1) and galactosemia led to successful preventive therapy by a specific diet. However, a breakthrough on a much larger scale was achieved when the methods of prenatal diagnosis for chromosome aberrations and for some metabolic defects were introduced in the late 1960s and early 1970s (Chap. 25, Sect. 25.2). Suddenly, genetic counseling could now be based not only on probability statements but, in an increasing number of cases, on certainty of individual diagnoses. This scientific development coincided with a growing awareness in large parts of the human population that unlimited human reproduction must not be accepted as a natural law but can – and should – be regulated in a rational way. Introduction of oral contraceptive agents signaled this awareness. The chance to avoid the births of severely handicapped children is now accepted by a rapidly increasing proportion of the population. At the same time, better knowledge of pathophysiological pathways is improving the chances for individual therapy of hereditary diseases, including the promise somatic gene therapy by introduction of genes into cells of functional tissues (see Chap. 27). Applications of human genetics as a practical tool to prevent suffering and disease have found wide resonance and have now one of the most rewarding approaches in preventive medicine. In many countries, the politically responsible bodies have already created, or are now creating the institutions for widespread application of the new tools.

Effects of Practical Applications on Research

These practical applications have led to a marked increase in the number of research workers and the amount of work within the past decades. From the beginning of the twentieth century up to the early 1950s, human genetics had been the interest of a mere handful of scientists for most of whom it was not even a full-time occupation. Many of the pioneers were trained and worked much of their lifetime as physicians in special fields of medicine, such as Waardenburg and Franceschetti in ophthalmology, and Siemens in dermatology. Others were interested in theoretical problems of population genetics and evolution and chose problems in human genetics as the field of application for their theoretical concepts, most notably J.B.S. Haldane and R.A. Fisher. Still others had their point of departure in physical anthropology. This heterogeneous group of scientists did not form a coherent scientific community. For a long time, there was almost no formal infrastructure for the development of a scientific specialty. There were almost no special departments, journals, and international conferences. This lack of focus resulted in a marked heterogeneity in quality and content of scientific contributions.

All this has changed. Departments and units of human and medical genetics are now the standard in many countries; universities and medical schools offer special curricula, many journals and other publications exist, and numerous congresses and conferences are being held. Human genetics is now an active and vigorous field which continues to grow exponentially.

Dangers of Widespread Practical Application for Scientific Development

This development, however, satisfactory as it is, has also a number of potentially undesirable consequences:

(a) Research is promoted primarily in the fields of immediate practical usefulness related to hereditary diseases; fields of less immediate practical importance may be neglected.

(b) Initially the contact with fundamental research in molecular genetics and cell biology was not intensive enough. This may have led to a slowdown in the transfer of scientific concepts and experimental approaches from these fields. Fortunately, this has changed with the advent of recombinant DNA techniques and many other methods. The speed with which results of basic research are being transferred into practical application has increased significantly.

(c) As in other sciences, certain topics may evolve to a mainstream research where vast human and financial resources are being invested, drawing it off other areas, which are then neglected in spite of their great importance. For example, at present the immense activities to unravel complex disorders by high-throughput assays have resulted in a decreased interest in studying monogenic Mendelian disorders although their detailed analyses may provide invaluable insights into consequences of mutations and their associated pathophysiology (see Chap. 4, Sect. 4.1).

(d) Much medical research applies established methods to answer straightforward questions. Many studies collect data with new techniques. Individual results are often not of great import, but the ensemble of such data are the essential building blocks for the future progress of normal science. Much of such work is being carried out in human and medical genetics and is quite essential for many medical and anthropological applications. However, there is continued need in human genetics to develop testable hypotheses and try to test their consequences from all viewpoints.

Human geneticists must not neglect the further development of genetic theory. Basic research is needed in fields in which the immediate practical application of results is not possible but might in the long run be at least as important for the future of the human species as current applications in diagnostic and preventive medicine.

Advantages of Practical Application for Research

The needs of medical diagnosis and counseling have also given strong incentives to basic research. Many phenomena that basic research tries to explain would simply be unknown had they not been uncovered by study of diseases. We would be ignorant regarding the role of sex chromosomes in sex determination had there not been patients with sex chromosomal anomalies. Phenomena such as spontaneously enhanced chromosome instability in Fanconi's anemia or Bloom's syndrome with all its consequences for somatic mutation and cancer formation (Chap. 3, Sect. 3.7) were discovered accidentally in the process of examining certain patients for diagnostic reasons. Genetic analysis of the "supergene" determining the major histocompatibility complex in humans contributes much to our fundamental understanding of how the genetic material above the level of a single gene locus is structured, and how the high genetic variability within the human population can be maintained (Chap. 6, Sect. 6.2.5). However, research in this field would certainly be much less active had there not been the incentive of improving the chances of organ transplantation.

Whether we like it or not, society pays increasing amounts of money for research in human genetics because we want to have practical benefits. Hence, to promote basic research, we must promote widespread practical applications. To guarantee progress in practical application for the future as well – and not only in the field of medicine – basic research needs to be supported. This is also the only way to attract good research workers and to maintain – or even improve – scientific standards. This paradox creates priority problems for all those concerned with research planning.

Human Genetics and the Sociology of Science

The discussion above should have demonstrated that human genetics – as all other sciences – has not developed in a sociological vacuum, following only the inherent logical laws of growth of theory and experimental testing. Human genetics is the work of social groups of human beings who are subject to the laws of group psychology and are influenced by the society at large in their attitudes toward research and their selection of problems. Unfortunately, sociological investigations of group formation and structure in human genetics have not been carried out. Another group active in the foundation of molecular biology, that which introduced the bacteriophages of *Escherichia coli* into the analysis of genetic information, has been studied extensively [3]. We know from this and from other examples that, during a phase in which a new paradigm is being founded, the group that shares this paradigm establishes close within-group contacts. The normal channels of information exchange such as scientific journals and congresses are superseded by more informal information transfer through telephone calls, e-mail communications, preprints, and personal visits. Within the group, influential personalities serve as intellectual and/or organizational leaders. Outside contacts, on the other hand, are often loose. When the acute phase of the scientific revolution is over, the bonds within the group are loosened, and information is again exchanged largely by normal channels of publication.

Similar developments can be observed in the field of human genetics. For example, in Chap. 6 (Sect. 6.2.5) we sketch the groups active in the elucidation of the major histocompatibility complex and in the assignment of gene loci to chromosome segments (Sect. 6.1).

Of similar influence on population genetics has been the first "big science" research project in human genetics – the Atomic Bomb Casualty Commission (ABCC, now the Radiation Effects Research Foundation, RERF; www.rerf.or.jp) project that was launched in the late 1940s in Japan by American and Japanese research workers to examine the genetic consequences of the atomic bombs in Hiroshima and Nagasaki (Chap. 10). In later years, this project led, for example, to comprehensive studies of the genetic effects of parental consanguinity. The second endeavor of this type is the "Human Genome Project" – the attempt at analyzing and sequencing the entire human genome by coordinated international cooperation (see Chap. 44). Today many research efforts are being conducted and can only be accomplished in large, international consortia, as for example the ENCyclopedia Of DNA Elements (ENCODE) project (www.genome.gov) or the Functional Annotation of the Mammalian Genome (FANTOM) project (fantom.gsc.riken.jp).

Many, if not most of the more interesting developments in the field were not initiated by investigators who would declare themselves human geneticists, or who worked in human genetics departments. They were launched by research workers from other fields such as general cytogenetics, cell biology, molecular biology, biochemistry, and immunology, but also from clinical specialties such as pediatrics, hematology, ophthalmology, and psychiatry. A common theme running through many recent developments has been the application of nongenetic techniques from many different fields such as biochemistry and immunology to genetic concepts. On the other hand, techniques originally developed for solving genetic problems, especially for molecular studies of DNA, are being introduced at a rapidly increasing rate into other fields of research, for example in both medical research and practical medicine. In fact, most recent progress in human genetics comes from such interdisciplinary approaches. The number of research workers in the field has increased rapidly. Most did not start as human geneticists but as molecular biologists, medical specialists, biochemists, statisticians, general cytogeneticists, etc. They were drawn into human genetics in the course of their research. This very variety of backgrounds makes discussions among human geneticists stimulating and is one of the intellectual assets of the present state of our field. However, such diversity is also a liability as it may lead to an overrating of one's small specialty at the expense of a loss of an overview of the whole field [8]. With increasing complexity of research methods, specialization within human genetics has become inevitable. However, this brings with it the danger that the outlook of the scientist narrows, whole fields are neglected, and promising research opportunities remain unexploited.

Human Genetics in Relation to Other Fields of Science and Medicine

The rapid development of human genetics during recent decades has created many interactions with other fields of science and medicine. Apart from general and molecular genetics and cytogenetics, these interactions are especially close with cell biology, biochemistry, immunology, and – with many clinical specialties. Until recently, on the other hand, there have been few if any connections with physiology. One reason for this failure to establish fruitful interactions may be a difference in the basic approach: genetic analysis attempts to trace the causes of a trait to its most elementary components. Geneticists know in principle that the phenotype is produced by a complex net of interactions between various genes, but they are interested more in the components than in the exact mechanism of such interactions. At present, genetic analysis has reached the level of gene structure and the genetic code; a final goal would be to explain the properties of this code in terms of quantum physics. A malevolent observer might compare the geneticist with a man who, to understand a book, burns it and analyzes the ashes chemically.

The physiologist, on the other hand, tries to read the book. However, he often presupposes that every copy of the book should be exactly identical; variation is regarded as a nuisance. To put it differently, physiology is concerned not with the elements themselves but with their mode of interaction in complicated functional systems (see Mohr [13]). Physiologists are more concerned with the integration of interacting systems than with the analysis of their components. The analysis of regulation of gene activities by feedback mechanisms, for example, the Jacob-Monod model in bacteria, and some approaches in developmental genetics of higher organisms have taught geneticists the usefulness of thinking in terms of systems. On the other hand, methods for molecular analysis of DNA have been introduced into physiology at an increasing scale. Genes for receptors and their components, for example for neurotransmitters, and genes for channel proteins are being localized in the genome and analyzed at the molecular level. Hence, the gulf between physiology and genetics is now being bridged. With the increasing interest of human geneticists in the genetic basis of common diseases and individual genetic variation in response to influences such as nutrition and stress, genetic concepts are increasingly influencing the many branches of medicine that, in the past, have profited relatively little from genetic theory. Molecular biology is developing increasingly into a common basis for many branches of science, and most biomedical scientists are nowadays becoming better acquainted with the principles of genetics. A field of molecular medicine is emerging.

Fields of Human and Medical Genetics

The field of human genetics is large, and its borders are indistinct. The development of different techniques and methods has led to the development of many fields of subspecialization. Many of these overlap and are not mutually exclusive. The field of *human molecular genetics* has its emphasis in the identification and analysis of genes at the DNA level. Methods such as DNA digestion by restriction endonucleases, Southern blotting, polymerase chain reaction (PCR), sequencing and many others are being applied. *Human biochemical genetics* deals with the biochemistry of nucleic acids, proteins, and enzymes in normal and mutant individuals. Laboratory methods of the biochemist are being used (e.g., chromatography; enzyme assays). *Human cytogenetics* deals with the study of human chromosomes in health and disease. *Immunogenetics* concerns itself largely with the genetics of blood groups, tissue antigens such as the HLA types, and other components of the immune system. *Formal genetics* studies segregation and linkage relationships of Mendelian genes and investigates more complex types of inheritance by statistical techniques.

Clinical genetics deals with diagnosis, prognosis, and to some extent treatment of various genetic diseases. Diagnosis requires knowledge of etiological heterogeneity and acquaintance with many disease syndromes. *Genetic counseling* is an important area of clinical genetics and requires skills in diagnosis, risk assessment, and interpersonal communication. *Population genetics* deals with the behavior of genes in large groups and concerns the evolutionary forces of drift, migration, mutation, and selection in human populations. The structure and gene pool of human populations are studied by considering gene frequencies of marker genes. In recent years population geneticists have become interested in the epidemiology of complex genetic disease that require biometric techniques for their studies. *Behavioral genetics* is a science that studies the hereditary factors underlying behavior in health and disease. Behavior geneticists attempt to work out the genetic factors determining personality and cognitive skills in human beings. The genetics of mental retardation and various psychiatric diseases are also considered. The field of sociobiology tries to explain social behavior by using biological and evolutionary concepts.

Somatic cell genetics is the branch of human genetics that studies the transmission of genes at the cellular level. Cell hybridization between different species is an important tool for the cartography of human genes. *Developmental genetics* studies genetic mechanisms of normal and abnormal development. This field employs to a large extent model organisms and has a strong emphasis on animal experimentation. *Reproductive genetics* is the branch of genetics that studies details of gamete and early embryo formation by genetic techniques. This area is closely related to reproductive physiology. Due to the growing application of assisted reproductive technologies in couples with infertility disorders this field has recently grown significantly. *Pharmacogenetics* deals with genetic factors governing the disposal and kinetics of drugs in the organism. Special interest in human pharmacogenetics relates to adverse drug reactions. *Ecogenetics* is an extension of pharmacogenetics and deals with the role of genetic variability affecting the response to environmental agents.

Clinical genetics has grown very rapidly in recent years because of the many practical applications of diagnosis and counseling, intrauterine diagnosis, and screening for genetic disease. Most research in human genetics is currently carried out in clinical genetics, cytogenetics, molecular and biochemical genetics, somatic cell genetics, and immunogenetics under medical auspices. Research in formal and population genetics has benefited enormously from the increasing knowledge about genome structure and its variation and the availability of new, cheaper high-throughput sequencing approaches.

Future of Human Genetics

Research methods in science are becoming ever more complicated and expensive, and human genetics is no exception. As a necessary consequence mastering of these methods increasingly requires specialization in a narrow field. Purchase of big instruments creates financial difficulties. Hence, the selection of research problems is often directed not by the intrinsic scientific interest in the problems or the conviction that they could, in principle, be solved, but by the availability of research methods, skilled coworkers, and instruments. Many research projects require large patient cohorts and complex, genome-wide analyses, tasks of a magnitude that can only be performed within international consortia. Such efforts are greatly facilitated by web-based databases (Sects. 29.1–29.3) which provide an easy means for distributing results to the genetic community. Furthermore, such databases ensure that new evolving information can easily be utilized by other persons in the field. For example, data on copy number variation in the human genome and possible consequences for the phenotype are now rapidly assessable in databases (Sects. 29.2 and 29.3) and are thus available for genetic counselors who can use this knowledge to provide their patients with detailed up-to-date information.

However, the tendency toward specialization will inevitably continue, and it is possible that, in this process, important parts of human genetics will be resolved into fields mainly defined by research methods, such as biochemistry, chromosome research, immunology, molecular biology [see 12], or into certain clinical areas. For example, hereditary metabolic diseases or syndromes associated with dysmorphic features and developmental delay are often studied and treated by pediatricians with little genetic training. Several departments of neurology have established their own neurogenetics branches, which are often independent from the respective department of human genetics. However, despite this tendency toward subspecialization, it is important to note that a laboratory performing genetic diagnostic procedures needs trained and experienced personnel, up-to-date equipment, and has to fulfill internationally defined quality standards, which are regulated by law in many countries. Therefore, it is probably not cost-effective to perform genetic diagnostics in small laboratories that offer only a few tests. Therefore, large laboratories performing all important human genetics diagnostic procedures may evolve to organizational structures in which human genetics remains united.

Survival of an established field of science has no value in itself. If a field dies because its concepts and accomplishments have been accepted and are being successfully integrated into other fields, little is lost. In human genetics, however, this state has not been reached yet and it may never get to this point. Many concepts of molecular biology, often in combination with "classical" methods such as linkage analysis, are now being applied to humans. A few decades ago human genetics was a medical field mainly dealing with rare syndromes and prenatal diagnostics. This picture has completely changed as the genetic contributions to common diseases are increasingly being unraveled. For example, genetic counseling is now an integral part of care in families with hereditary cancer diseases (Chap. 14) or neurologic disorders. In addition, data evolving from genome-wide association studies (GWAS) have identified numerous new loci in the genome that may change the susceptibility for diseases or phenotypic features. The effect of these loci may often be only moderate (Sect. 8.1), however, the evolving knowledge may further increase requests for genetic counseling. In future, genetic counseling provided by professionals in the field may have to compete with "direct-to-consumer genetic testing" over the Internet that is already offered by several companies. Such developments are accompanied by growing options for predictive genetic diagnosis, which require standardized procedures for both the counseling session and the molecular genetic testing and which often involve difficult ethical issues. Thus, the tasks in human genetics have changed tremendously over the past decades and new challenges are constantly arising in this rapidly evolving field. Newly evolving technologies, such as whole-genome sequencing (see below), will further expand the future of human genetics. In fact, it can be predicted that human genetics will change medicine, as it has the potential to identify persons with an increased risk for certain diseases and it may provide information about treatment options. These aspects are now often referred to as "personalized medicine" (Chap. 4, Sect. 4.4) and they will likely dominate medicine in upcoming years.

Unsolved and Intriguing Problems

With the rapid increase in knowledge over recent years new and often unexpected problems have arisen. At a time when hereditary traits were defined by their modes of inheritance, the relationship between genotype and phenotype appeared relatively simple. This straightforward relationship seemed correct when some hereditary diseases were shown to be caused by enzyme defects, and when hemoglobin variants turned out to be due to amino acid replacements caused by base substitutions. With increasing knowledge of the human genome, however, many hereditary traits with phenotypes that had been considered identical turned out to be heterogeneous. These were caused either by mutations in different genes or by different mutations within the same genes. However, even mutations that are identical by the strictest molecular criteria sometimes have striking phenotypic differences. Analysis of such genotype-phenotype relationships by the study of genetic and environmental modifiers poses intriguing future problems in human genetics.

The establishment of genotype–phenotype relationships was recently further complicated by two new findings. The first finding represents the unanticipated variation within the human genome (Chap. 2). Future research will have to elucidate how copy number variants (CNVs) contribute to human phenotypic diversity and disease susceptibility. CNVs are also of interest for a better understanding of the evolution of the genome, as they provide the raw material for gene duplication and gene family expansion. However, in addition to numerical variation there are extensive structural variations, such as inversions or insertions. Their impact on gene function remains to be elucidated. The second finding was the characterization of functional elements by the ENCODE consortium. To date, only 1% of the human genome has been analyzed by various high-throughput experimental and computational techniques; however, the findings revealed an unexpected number and complexity of the RNA transcripts that the genome produces. These findings have challenged traditional views about regulatory elements in the genome and added new insights into the complexity of human genetics, revealing that our understanding of the genome is still far from being complete. In order to address this, the National Human Genome Research Institute (NHGRI) launched two complementary programs in 2007: an expansion of the human ENCODE project to the whole genome (http://www.genome.gov/ENCODE) and the model organism ENCODE (modENCODE) project to generate a comprehensive annotation of the functional elements in the *Caenorhabditis elegans* and *Drosophila melanogaster* genomes (http://www.modencode.org; http://www.genome.gov/modENCODE). These efforts will likely contribute to a better understanding of genome complexity and gene regulation.

At present our understanding of somatic genome variability is very incomplete. Current concepts suggest that erroneous DNA repair and incomplete restoration of chromatin after damage may be resolved and may produce mutations and epimutations. Both mutations and epimutations have been shown to accumulate with age and such an increased burden of mutations and/or epimutations in aged tissues may increase cancer risk and adversely affect gene transcriptional regulation. This may in turn result in a progressive decline in organ function, a phenomenon frequently observed in aging. With the demographic trend of prolonged life expectancy, a better understanding of somatic genome variability and the stability of the genome may grow in importance.

Other problems may arise from new technologies, such as next-generation or third-generation whole-genome sequencing (Chap. 4, Sect. 4.4), which will make sequencing of entire genomes possible and affordable within in a short period of time. These possibilities will require new bioinformatic tools and interpretation of sequencing results will greatly depend on whether we understand better the aforementioned relevance of structural and copy number variation and whether we can make sense of the various transcriptionally active regions in the genome. If we succeed, there is no doubt that whole-genome sequencing will change human genetics tremendously. They will, for example, contribute to a better understanding of modifier genes in monogenic diseases and thus explain the frequently observed phenotypic variability. Furthermore, they will contribute significantly to further propel research on complex diseases. However, although the new possibilities of human genetics are fascinating they raise at the same time new ethical issues. For example, in prenatal diagnostic settings tests can now be offered not only for devastating diseases but also for common phenotypic traits. Thus, the consequences of the new technologies and new insights do not have consequences only for human geneticists but also for the entire society.

Possible Function of a Textbook

In his book on *The Structure of Scientific Revolutions,* Kuhn in 1962 [2] described the function of textbooks not very flatteringly: they are "pedagogic vehicles for the perpetuation of normal science" that create the impressions as if science would grow in a simple, cumulative manner. They tend to distort the true history of the field by only mentioning those contributions in the past that can be visualized as direct forerunners of present-day achievements. "They inevitably disguise not only the role but the very existence of . . . revolutions . . ."

Below we shall proceed in the same way: we shall describe present-day problems in human genetics as we see them. The result is a largely affirmative picture of normal science in a phase of rapid growth and success. Anomalies and discrepancies may exist, but we often do not identify them because we share the "blind spots" with most other members of our paradigm group. The "anticipation" phenomenon in diseases such as myotonic dystrophy is one example (Chap. 5, Sect. 5.1.7). This disease tends to manifest more severely and earlier in life with each generation. Obviously, this observation did not appear to be compatible with simple mendelism. Therefore, it was explained away by sophisticated statistical arguments which we cited in earlier editions of this book. In the meantime, however, anticipation has been shown to be a real phenomenon, caused by a novel molecular mechanism. What we can do is to alert the reader that human genetics, as all other branches of science, is by no way a completed and closed complex of theory and results that only needs to be supplemented in a straightforward way and without major changes in conceptualization. Our field has not developed – and will not develop in the future – as a self-contained system. Rather, human genetics, as all other sciences, is an undertaking of human beings – social groups and single outsiders – who are motivated by a mixture of goals such as search for truth, ambition, desire to be acknowledged by one's peer group, the urge to convince the society at large to allocate resources in their field – but also the wish to help people and to do something useful for human society.

Therefore, we shall emphasize the history and development of problems and approaches. Occasionally, we shall ask the reader to step back, reflecting with us as to why a certain development occurred at the time it did, why another development did not occur earlier, or why a certain branch of human genetics did not take the direction that one would have expected logically. Inevitably, this implies much more criticism than is usually found in textbooks. Such criticism will – at least partially – be subjective, reflecting the personal stance of the authors. Our goal is to convince the reader that a critical attitude improves one's grasp of the problems and their possible solutions – it is not our intention to convince him that we are always right.

We would have liked to give more information on the ways in which sociological conditions within the field and – still more important – the developments in the society at large have influenced the development of human genetics, and the ways in which thinking on these problems has in turn influenced the societies. The eugenics movement in the United States and the *Rassenhygiene* ideology in Germany have had a strong – and sometimes devastating – influence on human beings as well as on the social structure of society at large. Too little systematic research has been carried out, however, to justify a more extended discussion than that presented in Chap. 1 (Sect. 1.8) [17]. Much more historical research along these lines is all the more urgent, as many of the ethical problems – inherent, for example, in the sterilization laws of many countries during the first decades of the twentieth century – are now recurring with full force in connection with prenatal diagnosis, selective abortion and the possibility of germinal gene therapy (Chaps. 25 and 26). Scientists and physicians working in human genetics were actively involved in and sanctioned ethically abhorrent measures in the past such as killing severely malformed newborns and mentally defectives in Nazi Germany – and how will future generations judge our own activities? These are intriguing questions. They show the Janus face of human genetics: it is a fundamental science – guided by a fertile theory and full of fascinating problems. It is also an applied science, and its applications are bound to have a strong impact on society, leading to novel and difficult philosophical, social, and ethical problems.

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History of Human Genetics*

1

Arno G. Motulsky

Revised by A.G. Motulsky, S.E. Antonarakis, and M.R. Speicher

Abstract Theories and studies in human genetics have a long history. Observations on the inheritance of physical traits in humans can even be found in ancient Greek literature. In the eighteenth and nineteenth centuries observations were published on the inheritance of numerous diseases, including empirical rules on modes of inheritance. The history of human genetics as a theory-based science began in 1865, when Mendel published his Experiments on Plant Hybrids and Galton his studies on Hereditary Talent and Character. A very important step in the development of human genetics and its application to medicine came with Garrod's demonstration of a Mendelian mode of inheritance in alkaptonuria and other inborn errors of metabolism (1902). Further milestones were Pauling's elucidation of sickle cell anemia as a "molecular disease" (1949), the discovery of genetic enzyme defects as the causes of metabolic disease (1950s, 1960s), the determination that there are 46 chromosomes in humans (1956), the development of prenatal diagnosis by amniocentesis (1968–1969) for the detection of chromosomal defects such as Down syndrome, and the large-scale introduction of molecular methods during the last 25 years. Concepts appropriated from human genetics have often influenced social attitudes and introduced the eugenics movement. Abuses have occurred, such as legally mandated sterilization, initially in the United States and later more extensively in Nazi Germany, where the killing of mentally impaired patients was followed by the genocide of Jews and Romani (Gypsy) people.

Contents

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The history of human genetics is particularly interesting since, unlike in many other natural sciences, concepts of human genetics have often influenced social and political events. At the same time, the development of human genetics as a science has been influenced by various political forces. Human genetics because of its concern with the causes of human variability has found it difficult to either remain a pure science or one of strictly medical application. Concerns regarding the heritability of IQ and the existence of inherited patterns of behavior again have brought the field into public view. A consideration of the history of human genetics with some attention to the interaction of the field with societal forces is therefore of interest. We will concentrate our attention on historical events of particular interest for human genetics and refer to landmarks in general genetics only insofar as they are essential for the understanding of the evolution of human genetics.

Recently, an excellent history of medical genetics was published by the medical geneticist Peter Harper in 2008 [38]. This highly readable book with many photographs presents critical assessments of various developments in the field since its beginnings. Many tables document major discoveries and a detailed timeline of both human and medical genetics presents important developments ranging from early discoveries to recent findings. This book is currently the only major comprehensive text devoted to the history of human/medical genetics.

A 30-page "History of Medical Genetics" by Victor McKusick was published as Chap. 1 in Emery and Rimoin's *Principles and Practice of Medical Genetics*, 5th edition, 2007 [59]. This remarkably comprehensive chapter emphasizing clinical aspects starts with a brief description of pre-Mendelian concepts and ends with a broadly conceived assessment of current and future trends of medical genetics.

1.1 The Greeks (see Stubbe [83])

Prescientific knowledge regarding inherited differences between humans has probably existed since ancient times. Early Greek physicians and philosophers not only reported such observations but also developed some theoretical concepts and even proposed "eugenic" measures.

In the texts that are commonly ascribed to Hippocrates, the following sentence can be found:

> Of the semen, however, I assert that it is secreted by the whole body – by the solid as well as by the smooth parts, and by the entire humid matters of the body ... The semen is produced by the whole body, healthy by healthy parts, sick by sick parts. Hence, when as a rule, baldheaded beget baldheaded, blue-eyed beget blue-eyed, and squinting, squinting; and when for other maladies, the same law prevails, what should hinder that longheaded are begotten by longheaded?

This remarkable sentence not only contains observations on the inheritance of normal and pathological traits but also a theory that explains inheritance on the assumption that the information carrier, the semen, is produced by all parts of the body, healthy, and sick. This theory became known later as the "pangenesis" theory. Anaxagoras, the Athenian philosopher (500–428 B.C.), had similar views (see Capelle [15]).

A comprehensive theory of inheritance was developed by Aristotle (see [6]). He also believed in a qualitatively different contribution by the male and the female principles to procreation. The male gives the impulse to movement whereas the female contributes the matter, as the carpenter who constructs a bed out of wood. When the male impact is stronger, a son is born who, at the same time, is more like his father, when the female, a daughter, resembling the mother. This is the reason why sons are usually similar to their fathers and daughters are similar to their mothers.

Barthelmess (our translation) [6] writes: "Reading the texts from this culture, one gets the overall impression that the Greeks in their most mature minds came closer to the theoretical problems than to the phenomena of heredity." Aristotle's assertion even provides an early example of how observation can be misled by a preconceived theoretical concept. Sons are not more similar to their fathers, nor daughters to their mothers.

Plato, in the *Statesman (Politikos)* [71], explained in detail the task of carefully selecting spouses to produce children who will develop into bodily and ethically eminent personalities. He wrote:

> They do not act on any sound or self-consistent principle. See how they pursue the immediate satisfaction of their desire by hailing with delight those who are like themselves and by disliking those who are different. Thus they assign far too great an importance to their own likes and dislikes.
>
> The moderate natures look for a partner like themselves, and so far as they can, they choose their wives from women of this quiet type. When they have daughters to bestow in marriage, once again they look for this type of character in the prospective husband. The courageous class does just the same thing and looks for others of the same type. All this goes on, though both types should be doing exactly the opposite ...
>
> Because if a courageous character is reproduced for many generations without any admixture of the moderate type, the natural course of development is that at first it becomes superlatively powerful but in the end it breaks out into sheer fury and madness ...
>
> But the character which is too full of modest reticence and untinged by valor and audacity, if reproduced after its kind for many generations, becomes too dull to respond to the challenges of life and in the end becomes quite incapable of acting at all.

In the *Republic* [70], Plato not only requires for the "guards" (one of the highest categories in the social hierarchy of his utopia) that women should be common property; children, should be educated publicly but the "best" of both sexes should beget children who are to be educated with care. The children of the "inferior," on the other hand, are to be abandoned. Democritus, on the other hand, writes: "More people become able by exercise than by their natural predisposition." Here (as in other places), the nature–nurture problem appears already.

1.2 Scientists Before Mendel and Galton

The literature of the Middle Ages contains few allusions to heredity. The new attitude of looking at natural phenomena from an empirical point of view created modern science and distinguishes modern humans from those in earlier periods. This approach succeeded first in investigation of the inorganic world and only later in biology. In the work *De Morbis Hereditariis* by the Spanish physician Mercado (1605) [66], the influence of Aristotle is still overwhelming, but there are some hints of a beginning emancipation of reasoning. One example is his contention that both parents, not only the father, contribute a seed to the future child. Malpighi (1628–1694) [83, p 77] proposed the hypothesis of "preformation," which implies that in the ovum the whole organism is preformed in complete shape, only to grow later. Even after the discovery of sperm (Leeuwenhoek et al. 1677) [3, pp 72-73], the preformation hypothesis was not abandoned altogether, but it was believed by some that the individual is preformed in the sperm, only being nurtured by the mother. The long struggle between the "ovists" and the "spermatists" was brought to an end only when C.F. Wolff [99] attacked both sides and stressed the necessity of further empirical research. Shortly thereafter experimental research on heredity in plants was carried out by Gärtner (1772–1850) [33] and Kölreuter (1733–1806) [48]. Their work prepared the ground for Mendel's experiments [60].

The medical literature of the eighteenth and early nineteenth centuries contains reports showing that those capable of clear observation were able to recognize correctly some phenomena relating to the inheritance of diseases. Maupertuis [57], for example, published in 1753 an account of a family with polydactyly in four generations and demonstrated that the trait could be equally transmitted by father or by mother. He further showed, by probability calculation, that chance alone could not account for the familial concentration of the trait. Probably the most remarkable example, however, was Joseph Adams (1756–1818) (see [1,23,62,64]), a British apothecary who, in 1814, published a book with the title *A Treatise on the Supposed Hereditary Properties of Diseases* [1]. The following findings are remarkable:

(a) Adams differentiated clearly between "familial" (i.e., recessive) and "hereditary" (i.e., dominant) conditions.
(b) He knew that in familial diseases the parents are frequently near relatives.

(c) Hereditary diseases need not be present at birth; they may manifest themselves at various ages.

(d) Some disease predispositions lead to a manifest disease only under the additional influence of environmental factors. The progeny, however, is endangered even when the predisposed do not become ill themselves.

(e) Intrafamilial correlations as to age of onset of a disease can be used in genetic counseling.

(f) Clinically identical diseases may have different genetic bases.

(g) A higher frequency of familial diseases in isolated populations may be caused by inbreeding.

(h) Reproduction among persons with hereditary diseases is reduced. Hence, these diseases would disappear in the course of time, if they did not appear from time to time among children of healthy parents (i.e., new mutations!).

Adams' attitude toward "negative" eugenic measures was critical. He proposed the establishment of registries for families with inherited diseases. Weiss [96] recently pointed out that Adams in the same book also hinted at the existence of evolution stressing the concept of adaptive selection saying that environments such as climate put constraints on people: "By these means a race is gradually reared with constitutions best calculated for the climate" [1].

C.F. Nasse, a German professor of medicine, correctly recognized in 1820 one of the most important formal characteristics of the X-linked recessive mode of inheritance in hemophilia and presented a typical comprehensive pedigree [83, p 180]. He wrote (our translation):

> All reports on families, in which a hereditary tendency towards bleeding was found, are in agreement that the bleeders are persons of male sex only in every case. All are explicit on this point. The women from those families transmit this tendency from their fathers to their children, even when they are married to husbands from other families who are not afflicted with this tendency. This tendency never manifests itself in these women. …

Nasse also observed that some of the sons of these women remain completely free of the bleeding tendency.

The medical literature of the nineteenth century shows many more examples of observations, and attempts to generalize and to find rules for the influence of heredity on disease can be found. The once very influential concept of "degeneration" should be mentioned. Some features that older authors described as "signs of degeneration" in the external appearance of mentally deficient patients are now known to be characteristic of autosomal chromosomal aberrations or various types of mental retardation.

In the work of most of the nineteenth century authors, true facts and wrong concepts were inextricably mixed, and there were few if any criteria for getting at the truth. This state of affairs was typical for the plight of a science in its prescientific state. Human genetics had no dominant paradigm. The field as a science was to start with two paradigms in 1865: biometry, which was introduced by Galton, and Mendelism, introduced by Mendel with his pea experiments. The biometric paradigm was influential in the early decades of the twentieth century, and some examples and explanations in this book utilize its framework. With the advent of molecular biology and insight into gene action, the pure biometric approach in genetics is on the decline. Nevertheless, many new applications in behavioral or social genetics, where gene action cannot yet be studied, rely on this paradigm and its modern elaborations. The laws that Mendel derived from his experiments, on the other hand, have been of almost unlimited fruitfulness and analytic power. The gene concept emerging from these experiments has become the central concept of all of genetics, including human genetics. Its possibilities have not been exhausted.

1.3 Galton's Work

In 1865, F. Galton published two short papers with the title "Hereditary Talent and Character." He wrote [29]:

> The power of man over animal life, in producing whatever varieties of form he pleases, is enormously great. It would seem as though the physical structure of future generations was almost as plastic as clay, under the control of the breeder's will. It is my desire to show, more pointedly than – so far as I am aware – has been attempted before, that mental qualities are equally under control.
>
> A remarkable misapprehension appears to be current as to the fact of the transmission of talent by inheritance. It is commonly asserted that the children of eminent men are stupid; that, where great power of intellect seems to have been inherited, it has descended through the mother's side; and that one son commonly runs away with the talent of the whole family.

He then stresses how little we know about the laws of heredity in man and mentions some reasons, such as long generation time, that make this study very difficult. However, he considers the conclusion to be justified that physical features of humans are transmissible because resemblances between parents and offspring are obvious. Breeding experiments with animals, however, had not been carried out at that time, and direct proof of hereditary transmission was therefore lacking even in animals. In humans, "we have … good reason to believe that every special talent or character depends on a variety of obscure conditions, the analysis of which has never yet been seriously attempted." For these reasons, he concluded that single observations must be misleading, and only a statistical approach can be adequate.

Galton evaluated collections of biographies of outstanding men as to how frequently persons included in these works were related to each other. The figures were much higher than would be expected on the basis of random distribution.

Galton himself was fully aware of the obvious sources of error of such biological conclusions. He stressed that "when a parent has achieved great eminence, his son will be placed in a more favorable position for advancement, than if he had been the son of an ordinary person. Social position is an especially important aid to success in statesmanship and generalship … ."

"In order to test the value of hereditary influence with greater precision, we should therefore extract from our biographical list the names of those that have achieved distinction in the more open fields of science and literature." Here and in the law, which in his opinion was "the most open to fair competition," he found an equally high percentage of close relatives reaching eminence. This was especially obvious with Lord Chancellors, the most distinguished lawyers of Great Britain.

Galton concluded that high talent and eminent achievement are strongly influenced by heredity. Having stressed the social obstacles that inhibit marriage and reproduction of the talented and successful, he proceeded to describe a utopic society,

> In which a system of competitive examination for girls, as well as for youths, had been so developed as to embrace every important quality of mind and body, and where a considerable sum was yearly allotted. … to the endowment of such marriages as promised to yield children who would grow into eminent servants of the State. We may picture to ourselves an annual ceremony in that Utopia or Laputa, in which the Senior Trustee of the Endowment Fund would address ten deeply-blushing young men, all of twenty-five years old, in the following terms… .

In short, they were informed that the commission of the endowment fund had found them to be the best, had selected for each of them a suitable mate, would give them a substantial dowry, and promised to pay for the education of their children.

This short communication already shows human genetics as both a pure and an applied science: on the one hand, the introduction of statistical methods subjects general impressions to scientific scrutiny, thereby creating a new paradigm and turning prescience into science. Later, Galton and his student K. Pearson proceeded along these lines and founded biometric genetics. On the other hand, however, the philosophical motive of scientific work in this field is clearly shown: the object of research is an important aspect of human behavior. The prime motive is the age-old inscription on the Apollo temple at Delphi ("know yourself").

Hence, with Galton, research in human genetics began with strong eugenic intentions. Later, with increasing methodological precision and increasing analytic success, such investigations were removed from this prime philosophical motive. This motive helps to understand the second aspect of Galton's work: the utopian idea to improve the quality of the human species by conscious breeding. During the Nazi era in Germany (1933–1945) we saw how cruel the perverted consequences of such an idea may become (Sect. 1.8.2). The question first posed by Galton remains, even more than ever, of pressing importance: What will be the biological future of mankind?

1.4 Mendel's Work

The other leading paradigm was provided by Mendel in his work *Experiments in Plant Hybridization,* which was presented on 8 February and 8 March 1865 before the *Naturforschender Verein* (Natural Science Association) in Brünn (now Brno, Czech Republic) and subsequently published in its proceedings [60]. It has frequently been told how this work went largely unnoticed for 35 years and was rediscovered independently by Correns, Tschermak, and de Vries in 1900 (see [16, 84, 20]). From then on, Mendel's insights triggered the development of modern genetics, including human genetics. A book by Stern and Sherwood [82], which reprints these and a variety of other articles regarding Mendel's paper, is most helpful to assess the impact of this classic work.

Mendel was stimulated to carry out his experiments by observations on ornamental plants, in which he had tried to breed new color variants by artificial insemination. Here he had been struck by certain regularities. He selected the pea for further experimentation. He crossed varieties with differences in single characters such as color (yellow or green) or form of seed (round or angular wrinkled) and counted all alternate types in the offspring of the first generation crosses and of crosses in later generations. Based on combinatorial reasoning, he gave a theoretical interpretation: the results pointed to free combination of specific sorts of egg and pollen cells. In fact, this concept may have occurred to Mendel before he carried out his studies. He may have verified and illustrated his findings by his "best" results, since agreement between the published figures and their expectation from the theoretical segregation ratios is too perfect from a statistical point of view (Fisher [27]). The interpretation of this discrepancy remains controversial [82, 90]. In any case, there is no question that Mendel's findings were correct.

Mendel discovered three laws: the law of uniformity, which states that after crossing of two homozygotes of different alleles the progeny of the first filial generation (F_1) are all identical and heterozygous; the law of segregation, which postulated 1 : 2 : 1 segregation in intercrosses of heterozygotes and 1 : 1 segregation in backcrosses of heterozygotes with homozygotes; and the law of independence, which states that different segregating traits are transmitted independently.

What is so extraordinary in Mendel's contribution that sets it apart from numerous other attempts in the nineteenth century to solve the problem of heredity? Three points are most important:

1. He simplified the experimental approach by selecting characters with clear alternative distributions, examining them one by one, and proceeding only then to more complicated combinations.
2. Evaluating his results, he did not content himself with qualitative statements but counted the different types. This led him to the statistical law governing these phenomena.
3. He suggested the correct biological interpretation for this statistical law: The germ cells represent the constant forms that can be deduced from these experiments.

With this conclusion Mendel founded the concept of the gene, which has proved so fertile ever since. The history of genetics since 1900 is dominated by analysis of the gene. What had first been a formal concept derived from statistical evidence has emerged as the base pair sequence of DNA, which contains the information for protein synthesis and for life in all its forms.

1.5 Application to Humans: Garrod's Inborn Errors of Metabolism

The first step of this development is described in this historical introduction: A. Garrod's [30] paper on "The Incidence of Alkaptonuria: A Study in Chemical Individuality." There are two reasons for giving special attention to this paper. For the first time, Mendel's gene concept was applied to a human character, and Mendel's paradigm was introduced into research on humans. Additionally, this work contains many new ideas set out in a most lucid way. Garrod was a physician and in later life became the successor of Osler in the most prestigious chair of medicine at Oxford [8]. His seminal contribution to human genetics remained unappreciated during his lifetime. Biologists paid little attention to the work of a physician. Their interest was concentrated more on the formal aspects of genetics rather than on gene action. The medical world did not understand the importance of his observations for medicine. Garrod first mentioned the isolation of homogentisic acid from the urine of patients with alkaptonuria and stated the most important result of the investigations carried out so far:

> As far as our knowledge goes, an individual is either frankly alkaptonuric or conforms to the normal type, that is to say, excretes several grammes of homogentisic acid per diem or none at all. Its appearance in traces, or in gradually increasing or diminishing quantities, has never yet been observed. . . .

As a second important feature "the peculiarity is in the great majority of instances congenital. . . ." Thirdly: "The abnormality is apt to make its appearance in two or more brothers and sisters whose parents are normal and among whose forefathers there is no record of its having occurred." Fourthly, in six of ten reported families the parents were first cousins, whereas the incidence of first-cousin marriages in contemporary England was estimated to be not higher than 3%. On the other hand, however, children with alkaptonuria are observed in a very small fraction only of all first-cousin marriages.

> There is no reason to suppose that mere consanguinity of parents can originate such a condition as alkaptonuria in their offspring, and we must rather seek an explanation in some peculiarity of the parents, which may remain latent for generations, but which has the best chance of asserting itself in the offspring of the union of two members of a family in which it is transmitted.

Then, Garrod mentioned the law of heredity discovered by Mendel, which "offers a reasonable account of such phenomena" that are compatible with a recessive mode of inheritance as pointed out by Bateson [37]. He cited another remark of Bateson and Saunders (Report to the Evolution Committee of the Royal Society) [7] with whom he had discussed his data:

> We note that the mating of first cousins gives exactly the conditions most likely to enable a rare, and usually recessive, character to show itself. If the bearer of such a gamete mates with individuals not bearing it the character will hardly ever be seen; but first cousins will frequently be the bearers of similar gametes, which may in such unions meet each other and thus lead to the manifestation of the peculiar recessive characters in the zygote.

After having cited critically some opinions on the possible causes of alkaptonuria, Garrod proceeded:

> The view that alkaptonuria is a "sport" or an alternative mode of metabolism will obviously gain considerably in weight if it can be shown that it is not an isolated example of such a chemical abnormality, but that there are other conditions which may reasonably be placed in the same category.

Having mentioned albinism and cystinuria as possible examples, he went on: "May it not well be that there are other such chemical abnormalities which are attended by no obvious peculiarities [as the three mentioned above] and which could only be revealed by chemical analysis?" And further:

> If it be, indeed, the case that in alkaptonuria and the other conditions mentioned we are dealing with individualities of metabolism and not with the results of morbid processes the thought naturally presents itself that these are merely extreme examples of variations of chemical behavior which are probably everywhere present in minor degrees and that just as no two individuals of a species are absolutely identical in bodily structure neither are their chemical processes carried out on exactly the same lines.

He suggested that differential responses toward drugs and infective agents could be the result of such chemical individualities. The paper presents the following new insights:

(a) Whether a person has alkaptonuria or not is a matter of a clear alternative – there are no transitory forms. This is indeed a condition for straightforward recognition of simple modes of inheritance.

The condition is observed in some sibs and not in parents.

The unaffected parents are frequently first cousins.

This is explained by the hypothesis of a recessive mode of inheritance according to Mendel. The significance of first-cousin marriages is stressed especially for rare conditions; this may be a precursor to population genetics.

(b) Apart from alkaptonuria several other similar "sports" such as albinism and cystinuria may exist. This makes alkaptonuria the paradigm for the "inborn errors of metabolism." In 1909 Garrod published his classic monograph on this topic [31].
(c) These sports may be extreme and therefore conspicuous examples of a principle with *much more widespread applicability.* Lesser chemical differences between human beings are so frequent that no human being is identical chemically to anyone else.

From these concepts Garrod drew more far-reaching conclusions, which are often overlooked. In a book published in 1931 [32] and reprinted with a lengthy introduction by Scriver and Childs [80], Garrod suggested that hereditary susceptibilities or diatheses are a predisposing factor for most common diseases and not merely for the rare inborn errors of metabolism. These concepts were precursors of current work to delineate the specific genes involved in the etiology of common disease. A valuable biography of Garrod was published by A. Bearn [8], who was a pioneer of human biochemical genetics in the 1950s and later.

Throughout this book the principle of a genetically determined individuality will govern our discussions. Garrod's contribution may be contrasted with that of Adams [23, 62, 64]. Apart from the "familial" occurrence of some hereditary diseases, Adams observed a number of phenomena that were not noted by Garrod, such as the late onset of some diseases, the intrafamilial correlation of age of onset, and the genetic predisposition leading to manifest illness only under certain environmental conditions. However, Adams did not have Mendel's paradigm. Therefore, his efforts could not lead to the development of an explanatory theory and coherent field of science. Garrod did have this paradigm and used it, creating a new area of research: human biochemical genetics.

1.6 Visible Transmitters of Genetic Information: Early Work on Chromosomes

Galton's biometric analysis and Mendel's hybridization experiments both started with visible phenotypic differences between individuals. The gene concept was derived from the phenotypic outcome of certain crossings. At the time when Mendel carried out his experiments nothing was known about a possible substantial bearing of genetic information in the germ cells. During the decades to follow, however, up to the end of the nineteenth century, chromosomes were identified, and mitosis and meiosis were analyzed. These processes were found to be highly regular and so obviously suited for orderly distribution of genetic information that in 1900 the parallelism of Mendelian segregation and chromosomal distribution during meiosis was realized, and chromosomes were identified as bearers of the genetic information [18].

Many research workers contributed to the development of cytogenetics [5,6]. O. Hertwig [41] first observed animal fertilization and established the continuity of cell nuclei: *omnis nucleus e nucleo.* Flemming (1880–1882) discovered the separation of sister chromatids in mitosis [83, p 247]; van Beneden (1883) [85] established the equal and regular distribution of chromosomes to the daughter nuclei. Boveri (1888) [5] found evidence for the individuality of each pair of chromosomes. Waldeyer (1888) (see [18]) coined the term "chromosome."

Meanwhile, Naegeli (1885) [77] had developed the concept of "idioplasma," which contains – to use a modern term – the "information" for the development of the next generation [67]. W. Roux [77] seems to have been the first to set out by logical deduction which properties a carrier of genetic information was expected to have. He also concluded that the behavior of cell nuclei during division would perfectly fulfill these requirements. The most important specific property of meiotic divisions, the ordered reduction of genetic material, was first recognized by Weismann.

These results and speculations set the stage for the identification of chromosomes as carriers of the genetic information, which followed shortly after the rediscovery of Mendel's laws and apparently independently by different authors [16, 20, 84].

Chromosome studies and genetic analysis have remained intimately connected in cytogenetics ever since. Most basic facts were discovered and concepts developed using plants and insects as the principal experimental tools. The fruit fly *Drosophila* played a particularly important role.

The development of human cytogenetics was delayed until 1956 when the correct number of human chromosomes was established as 46 by use of rather simple methods. It should be stressed that this delay could not be explained by the introduction of new cytological methods at that time. In fact, this discovery could have been made many years earlier. The delay was probably related to the lack of interest in human genetics by most laboratory-oriented medical scientists. Human genetics did not exist as a scientific discipline in medical schools since the field was not felt to be a basic science fundamental to medicine. Hereditary diseases were considered as oddities that could not be studied by the methodology of medical science as exemplified by the techniques of anatomy, biochemistry, physiology, microbiology, pathology, and pharmacology. Thus, most geneticists worked in biology departments of universities, colleges, or in agricultural stations. They were usually not attuned to problems of human biology and pathology, and there was little interest to study the human chromosomes. The discovery of trisomy 21 as the cause of Down syndrome and the realization that many problems of sex differentiation owe their origin to sex chromosomal abnormalities established the central role of cytogenetics in medicine. Further details in the development of cytogenetics are described in Chap. 3.

1.7 Early Achievements in Human Genetics

1.7.1 AB0 and Rh Blood Groups

The discovery of the AB0 blood group system by Landsteiner in 1900 [50] and the proof that these blood types are inherited (von Dungern and Hirschfeld [87]) was an outstanding example of Mendelian inheritance applied to a human character. Bernstein in 1924 [11] demonstrated that A, B, and 0 blood group characters are due to multiple alleles at one locus. The combined efforts of Wiener, Levine, and Landsteiner 25–30 years later led to discovery of the Rh factor and established that hemolytic disease of the newborn owes its origin to immunological maternal–fetal incompatibility. The stage was set for the demonstration in the 1960s that Rh hemolytic disease of the newborn can be prevented by administration of anti-Rh antibodies to mothers at risk [73,100].

1.7.2 Hardy-Weinberg Law

Hardy [36], a British mathematici,an, and Weinberg [92], a German physician, at about the same time (1908) set out the fundamental theorem of population genetics, which explains why a dominant gene does not increase in frequency from generation to generation. Hardy published his contribution in the United States in *Science.* He felt that this work would be considered as too trivial by his mathematics colleagues to be published in the United Kingdom. Weinberg was a practicing physician who made many contributions to formal genetics. He developed a variety of methods in twin research [91] and first elaborated methods to correct for biased ascertainment in recessive inheritance [93].

1.7.3 Developments Between 1910 and 1930

The years between 1910 and 1930 saw no major new paradigmatic discoveries in human genetics. Most of the data in formal genetics (such as linkage, nondisjunction, mutation rate) as well as the mapping of chromosomes were achieved by study of the fruit fly, largely in the United States. Many scientists tried to apply the burgeoning insights of genetics to humans. British scientists exemplified by Haldane excelled in the elaboration of a variety of statistical techniques required to deal with biased human data. The same period saw the development of the basic principles of population genetics by Haldane, Fisher, and Penrose [69] in England and by Wright in the United States. This body of knowledge became the foundation of population genetics and is still used by workers in that field. In 1918, Fisher was able to resolve the bitter controversies in England between the Mendelians, on the one hand, and followers of Galton (such as Pearson) on the other, by pointing out that correlations between relatives in metric traits can be explained by the combined action of many individual genes [26]. Novel steps in the development of medical genetics during this period were the establishment of empirical risk figures for schizophrenia and affective disorders by the Munich school of psychiatric genetics.

1.8 Human Genetics, the Eugenics Movement, and Politics

1.8.1 United Kingdom and United States

The first decade of the century saw the development of eugenics in Europe and in the United States [2,19,21,45, 55,76]. Many biological scientists were impressed by their interpretation of an apparently all-pervasive influence of genetic factors on most normal physical and mental traits as well as on mental retardation, mental disease, alcoholism, criminality, and various other sociopathies. They became convinced that the human species should be concerned with encouragement of breeding between persons with desirable traits (positive eugenics) and discourage the sick, mentally retarded, and disabled from procreation (negative eugenics).

A recent reprint of Davenport's 1911 book, *Heredity in Relation to Eugenics*, is accompanied by thoughtful reflections from contemporary geneticists on Davenport's eugenic concepts and recommendations almost one hundred years later [98]. Various eugenic study units were established in the United States (Eugenics Record Office at Cold Spring Harbor) and the United Kingdom. Much of the scientific work published by these institutions was of poor quality. Particularly, many different kinds of human traits such as "violent temper" and "wandering trait" were forced into Mendelian straightjackets. Most serious geneticists became disenchanted and privately disassociated themselves from this work. For various reasons, including those of friendship and collegiality with the eugenicists, the scientific geneticists did not register their disagreement in public. Thus, the propagandists of eugenics continued their work with enthusiasm, and the field acquired a much better reputation among some of the public than it deserved. Thus, many college courses on eugenics were introduced in the United States.

These trends had several important political consequences. Eugenics sterilization laws were passed in many states in the United States, which made it possible to sterilize a variety of persons for traits such as criminality for which no good scientific basis of inheritance existed. The attitude that led to the introduction of these laws is epitomized by United States Supreme Court Justice Holmes' statement that "three generations of imbeciles are enough."

Eugenic influences also played an important role in the passing of restrictive immigration laws in the United States. Using a variety of arguments the proponents of eugenics claimed to show that Americans of northwestern European origin were more useful citizens than those of southern European origin or those from Asia. Since such differences were claimed to be genetic in origin, immigration from southern and eastern European countries and from Asia was sharply curtailed. Similar trends were also operative in the United Kingdom. While solid work in human genetics was carried out by a few statistical geneticists, there was also much eugenic propaganda, including that by the distinguished statistician Pearson, the successor to Galton's academic chair in London.

Kevles [46] has published a wide-ranging and insightful history of eugenics and human genetics in the Anglo-Saxon countries. His book is a most carefully researched and exhaustive study of the uses and abuses of eugenic concepts.

1.8.2 Germany

In Germany [9, 10, 34, 94, 95] eugenics took the name of *Rassenhygiene* from a book of that title published in 1895 by Ploetz [72]. The *Rassenhygiene* movement became associated with mystical concepts of race, Nordic superiority, and the fear of degeneration of the human race in general and that of the German *Volk* in particular by alcoholism, syphilis, and increased reproduction of the feebleminded or persons from the lower social strata. Often representatives of this movement became associated with a dangerous type of sociopolitical prejudice: antisemitism. They warned the public against contamination of German "blood" by Jewish influences. Most followers of the racial hygiene concept were nationalistic and opposed the development of an open society that allows individual freedom and democratic participation. They shared this attitude with a significant segment of the educated classes in Germany. General eugenic ideas divorced from racism and other nationalist notions were often espoused by intellectuals who were concerned about the biological future of mankind. Thus, socialists publicized such views in Germany [34]. In 1931, two years before Hitler's coming into power, the German Society of Racial Hygiene added eugenics to its name. However, all efforts in this area soon became identified with the Nazi ideology.

Prominent German human geneticists identified themselves with the use of human genetics in the service of the Nazi state. Recognized scientists, such as Fischer, F. Lenz, Rüdin, and von Verschuer, accepted Nazi leadership and Nazi philosophy. While most of the propaganda for the new racial hygiene was not formulated by scientists but by representatives of the Nazi party, men such as Fischer and von Verschuer [95] participated in spreading Nazi race ideology. Jews were declared foreign genetic material to be removed from the German *Volk*. A eugenic sterilization law was already passed in 1933 that made forced sterilization obligatory for a variety of illnesses thought to be genetic in origin [74]. Heredity courts were established to deal with interpretation of the sterilization law. This law was hailed by some eugenicists in the United States even at the end of the 1930s [47]. Sterilization laws for eugenic indications were also passed in some Scandinavian countries around the same time but allowed voluntary (in contrast to forced) sterilization [74].

The exact role of the German human geneticists in the increasing radicalization and excesses of the application of Nazi philosophy has been assessed [65, 74, 95]; von Verschuer's role in sponsoring twin and other genetic research by his former assistant Mengele in the Auschwitz concentration and extermination camp is clear. We have no record that any voices were raised by these men in protest against "mercy killings" of the mentally retarded and newborn children with severe congenital defects nor against the mass killings of Jews. Evidence suggests that von Verschuer must have had some idea of such events, since he had continued contact with Mengele when the mass killings at Auschwitz were at their height. The "final solution" to the "Jewish problem" resulted in the murder of about 6 million Jews in the early 1940s [75]. While there is no record that human geneticists favored this type of "solution," their provision of so-called "scientific" evidence for a justification of Nazi antisemitism helped to create a climate in which these mass murders became possible [88]. This episode is one of the most macabre and tragic chapters in the history of man's inhumanity to man in the name of pseudoscientific nationalism. Yet, despite their racist publications, several such "scientists" (including von Verschuer) were given academic positions in post-World War II West Germany.

1.8.3 Soviet Union/Russia (see Harper, Chap. 16 in [38])

Eugenics was initiated in the Soviet Union [21,34] in the 1920s by the establishment of eugenics departments, a eugenic society, and a eugenics journal. Eugenic ideals soon clashed with the official doctrine of Marxism-Leninism, however, and these efforts were abandoned by the late 1920s. Scientists who had become identified with eugenics left the field to work with plants and animals.

Remarkable work in early human cytogenetics was carried out between 1931 and 1936, such as using hypotonic solutions for spreading of chromosomes, analysis of cultured embryonic cells, chromosome analysis of human oocytes, and cytogenetic studies of leukemia and other cancers [3,4]. These studies were published in international journals and later taken up by American and European scientists some 20 years later. Would the critical chromosome-related discoveries of the 1950s have been made by Russian scientists if such work on human genetics had not been terminated by Soviet antigenetic policies? [38]

Interest in the medical application of human genetics nevertheless persisted. A large institute of medical genetics, with 200 physicians, was established in Moscow during the 1920s. Its director, the physician S.G. Levit, made notable contributions [54], but was executed in 1938 (Chap. 16 in [38]), and human genetics was officially declared a Nazi science. The later ascendance of Lysenko [45] stifled all work in genetics, including that of human genetics, and no work whatever was carried out in this field until the early 1960s, after Lysenko's domination ceased (pp. 435–450 in [38]). The reintroduction of human genetics into the Soviet Union occurred by way of medical genetics. A textbook of medical genetics was published by Efroimson in 1964 [22]. A new institute of medical genetics was established in 1969 under the directorship of the cytogeneticist Bochkov, who had been trained by the well-known *Drosophila* geneticist, Timofeeff-Ressovsky [38]. Work in many areas of medical genetics, similar to that carried out elsewhere, is now done in Russia.

1.8.4 Human Behavior Genetics

Vigorous discussion continues regarding the role of genetic determinants in behavior, IQ, and personality. Some observers entirely deny genetic influences on normal behavior or social characteristics such as personality and intellect. This attitude toward genetics is shared by some psychologists and social scientists and even a few geneticists who are concerned about the possible future political and social misuse of studies in human behavioral genetics that claim to show genetic determinants of intelligence and social behavior.

We do not agree with those who deny any genetic influence on behavior or social traits in humans. However, we also caution against a too ready acceptance of results from comparison of twins and other relatives, which claim high heritabilities for many of these traits. Genetic data and pseudodata may be seriously misused by political bodies. However, as biologists and physicians impressed by biological variation under genetic control, we would be surprised if the brain did not also show significant variation in structure and function. Such variation is expected to affect intellect, personality, and behavior, and usually will interact with environmental factors. The extent to which genetic variation contributes to such traits, and especially the biological nature of such variation, will have to await further studies.

1.9 Development of Medical Genetics (1950–the Present)

1.9.1 Genetic Epidemiology

In the 1940s and 1950s a number of institutions pioneered in research on epidemiology of genetic diseases. T. Kemp's institute in Copenhagen, J.V. Neel's department in Ann Arbor, Michigan, and A.C. Stevenson's in Northern Ireland and later in Oxford contributed much to our knowledge on prevalence, modes of inheritance, heterogeneity, and mutation rates of various hereditary diseases. Recent years have seen a renaissance in this area, with special attention to analysis of common complex diseases (see Chap. 8.1). Utilization of new laboratory methods, including DNA techniques, together with more powerful methods of association studies, and the search for rare mutations and structural chromosome changes, provide powerful new approaches in this area.

1.9.2 Biochemical Methods

The years after World War II brought a rapid expansion in the field of human genetics by the development of biochemical, molecular, and cytological methods. Human genetics, which had been the concern largely of statistically oriented scientists, now entered the mainstream of medical research. The demonstration by Pauling et al. [68] that sickle cell anemia is a molecular disease was a key event in this area. The hemoglobins allowed detailed study of the consequences of mutation. The genetic code was found to be valid for organisms as far apart as viruses and humans. Many detectable mutations were found to be single amino acid substitutions, but deletions of various sorts and frameshift mutations similar to those discovered in micro-organisms were discovered. The nucleotide sequences of the hemoglobin genes were worked out using techniques developed in biochemistry and molecular genetics. Many inborn errors of metabolism were shown to originate in various enzyme deficiencies, often caused by a genetic mutation that changes enzyme structure. Methemoglobinemia due to diaphorase deficiency and glycogen storage disease were the first enzyme defects to be demonstrated.

1.9.3 Genetic and Biochemical Individuality

Work on hemoglobin and variants of the enzyme glucose-6-phosphate-dehydrogenase and other enzymes helped to establish the concept of extensive mutational variation. Biochemical individuality explained some drug reactions and led to the development of the field of pharmacogenetics [61, 86, 63, 35]. Marked biochemical heterogeneity of human enzymes and proteins was shown [39]. The uniqueness of humans, which is apparent by the physiognomic singularity of each human being, was shown to apply at the biochemical and immunological level as well. Here, as in several other fields (such as the hemoglobin variants and the mechanism of sex determination), studies in humans led the way to generally valid biological rules. The significance of polymorphism for the population structure (including that of humans) is being widely studied by population geneticists. The hypothesis that some expressed polymorphisms are the genetic substrate against which the environment acts to determine susceptibility and resistance to common disease led to the development of the field of ecogenetics [13,17]. The histocompatibility gene complex has become an important paradigm for the understanding of why several genes with related function occur in closely linked clusters. This locus appears to be of great importance to understand susceptibility to autoimmune diseases. An enormous amount of apparently unexpressed genetic variation has been demonstrated at the DNA and chromosomal level.

1.9.4 Cytogenetics, Somatic Cell Genetics, Prenatal Diagnosis, Clinical Genetics

After cytogenetic techniques became available, they were applied to detect many types of birth defects and intersex states. A specific type of malignancy, chronic myelogenous leukemia, was shown to be caused by a unique chromosomal translocation [78]. Banding techniques developed by Caspersson in 1969 made it possible to visualize each human chromosome and gave cytogenetic methods added powers of resolution.

Soon, biochemical and cytogenetic techniques were combined in somatic cell genetics. Specific enzyme defects were identified in single cells grown in tissue cultures. The development of methods to hybridize human with mouse cells by Henry Harris and Watkins [40] and Ephrussi and Weiss [25] soon allowed the assignment of many genes to specific chromosomes and the construction of a human linkage map.

The developments in somatic cell genetics led to the introduction of prenatal diagnosis in the late 1960s, when amniocentesis at the beginning of the second trimester of pregnancy was developed. This allowed tissue cultures of amniotic cells of fetal origin, permitting both cytogenetic and biochemical characterization of fetal genotypes, assignment of sex, and the diagnosis of a variety of disorders in utero. In the early 1980s chorion villus biopsy – a procedure done during the first trimester of pregnancy – was introduced, and is being widely used. The discovery that neural tube defects are associated with increases in α-fetoprotein of the amniotic fluid permits intrauterine diagnosis of an important group of birth defects [14]. Ultrasound methods to visualize the placenta and to diagnose fetal abnormalities added to the diagnostic armamentarium. This noninvasive method allows phenotypic diagnosis of a variety of fetal defects more frequently.

Clinical Genetics. The field of clinical genetics was initiated in the 1970s [58] and has been growing rapidly. Many medical schools and hospitals are establishing special clinics in which genetic diseases can be diagnosed and genetic counseling provided. The heterogeneity of genetic disease has been increasingly recognized. Genetic counseling – often by specially trained genetic counselors – is now intensified to provide patients and their families with information on the natural history of the disease, recurrence risks, and reproductive options. Screening programs of the entire newborn population for diseases such as phenylketonuria are being introduced in many countries, and other screening programs such as those to detect carriers of Tay-Sachs disease and other conditions more common among Ashkenazi Jews have undergone extensive trials [81].

With the advent of novel biochemical and DNA techniques (Chap. 4), basic work in human genetics is now performed increasingly by biochemists, cell biologists, molecular biologists, and others, who do not necessarily have training in human genetics. However, human genetics is identified with medical genetics in many of its activities. The scientific developments of the past decades are thus being widely applied in practical medicine.

1.9.5 DNA Technology in Medical Genetics

Advances in molecular genetics and DNA technology are being applied rapidly to practical problems of medical genetics. Since understanding of the hemoglobin genes was more advanced than that of other genetic systems, the initial applications related to the diagnosis of hemoglobinopathies (Chap. 11). Several methods are now being utilized. Inherited variation in DNA sequence that is phenotypically silent was found to be common, supplying a vast number of DNA polymorphisms for study. Just as everyone's physiognomy is unique, each person (except for identical twins) has a unique DNA pattern. DNA variants are being used in family or association studies as genetic markers to detect the presence of closely linked genes causing diseases. Direct detection of genetic disease has been achieved by utilizing nucleotide probes that are homologous to the mutations that are searched for. The polymerase chain reaction, together with rapidly increasing knowledge on human DNA sequences, has opened up new opportunities for direct diagnosis at the DNA level. Occasionally, a specific restriction enzyme may detect the mutational lesion. Different DNA mutations at the same locus frequently cause an identical phenotypic disease. This finding makes direct DNA diagnosis without family study difficult unless the specific mutation that causes the disease is known.

Completion of the human gene map and human gene sequence was achieved at the beginning of this century. Several hundred DNA markers and SNPs that are spaced over all chromosomes provide the necessary landmarks for detection of the genes for monogenic diseases and are beginning to hint at the contribution of specific genes to common diseases.

Using normal DNA carried by innocuous viruses to treat patients with genetic diseases carried by defective DNA has been under study for the last 15 years (Chap. 26). Such gene transfer aims to repair affected somatic cells (somatic gene therapy). Human studies have been done but no definitive cures have been reported. However, acute leukemia developed in several children treated for hereditary antibody syndrome presumably due to activation of oncogenes. Germinal gene therapy, i.e., insertion of normal genes into defective germ cells (or fertilized eggs) for treatment of human genetic disease, has never been carried out and is not considered ready for safe study. Such an approach is highly controversial, and is even prohibited by law in some countries.

McKusick [59] described a variety of paradigm shifts in the study of human and medical genetics in recent years. These included an emphasis from structural to functional genomics, from map-based to sequence-based gene discovery, from monogenic disease diagnosis to detection of common disorder susceptibility, from the search for etiology to exploration of mechanisms, from an emphasis on single genes to approaches on systems pathways and gene families, from genomics to proteomics and from "old-fashioned" medical genetics to "genetic medicine," implying that genes may be involved in all diseases. McKusick (p. 28 in [59]) further pointed out that human genetics in recent years has been "medicalized," "subspecialized," "professionalized," "molecularized," "commercialized," and even "consumerized."

1.9.6 The "Industrialization" of Discoveries and Team Efforts

The technological advances, the enormous amount of data generated, the size of the genomes, the impressive variability of individual genomes, the necessary specialized expertise in several disciplines, and the revolution in communication technologies all resulted in the organization and execution of mega-projects related to human genetics in the last 15 years in order to achieve results freely available to the community that provide genome-wide answers to the objectives. These projects, mostly international and funded by different funding agents, often included more than 50 different laboratories and 200 scientists. This paradigm shift is similar to the evolution of experimentation in physics, and underscores the importance of international cooperation in genomic discoveries. In addition, it is remarkable that most of the funding was provided by public sources. The completion of the human genome sequence was the first example of such international projects [44,49]. Other examples include the sequence of the genomes of other organisms and comparative genome analysis [89], the identification of the common genomic variation in a number of human population groups (HapMap project [28,42,43]), the ENCODE project to identify the functional elements in the human genome and that of selected model organisms [12], and the genome-wide association studies to identify common risk variants for the common complex phenotypes [56,79,97] (Chap. 8.1). More recently, the 1000 Genomes Project (http://www.1000genomes.org) and other related efforts aim to identify all genetic variation in the genomes of individuals. The major challenge in the future is to provide causative links between genomic variants and phenotypic variation.

1.9.7 Unsolved Problems

Human genetics had been most successful by being able to guide work that was made possible by the development of techniques from various areas of biology using Mendelian concepts. Important basic frontiers that are still being extended concern problems of gene regulation, especially during embryonic development, control of the immune system and of brain function. Human genetics is likely to contribute to these problems by imaginative use of the study of genetic variation and disease applying novel concepts and techniques. In medical genetics, the problem of common diseases including many birth defects requires study of the specific genes and their interactions involved in such diseases. Insights into the mechanisms of gene action during the aging process remain to be elucidated.

As shown by the many advances in description of genomic anatomy (see Chap. 2) where function is not yet fully understood, there is much need for research in both basic and translational approaches in order to elucidate the role of genomic biology and post-genomic interactions in health and disease. The remarkable similarity of humans and other mammals (and even of more primitive organisms) in both gene number and gene function had not been entirely expected, demonstrating that both new concepts and technical methods will be required to understand and utilize our current and future knowledge for applications in prevention and treatment of disease.

At first glance, the history of human genetics over the past 50 years reads like a succession of victories. The reader could conclude that human geneticists of the last generation pursued noble science to the benefit of mankind. However, how will posterity judge current efforts to make use of our science for the benefit of mankind as we understand it? Will the ethical distinction between selective abortion of a fetus with Down syndrome and infanticide of severely malformed newborns be recognized by our descendants? Are we again moving down the "slippery slope?"

Issues such as selective termination of pregnancy due to disadvantageous genomic variation need to be re-discussed and re-debated due to the ability to diagnose genomic variants with low-penetrance phenotypic consequences. As the dividing line between "severe phenotype" alleles and "low burden" alleles becomes blurred and individualized, consensus criteria and compromised solutions are fluid and constantly revised. Genetic medicine gradually becomes a central preoccupation of health professionals, the patients and their families, and presymptomatic healthy clients.

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Human Genome Sequence and Variation 2

Stylianos E. Antonarakis

Abstract The knowledge of the content of the individual human genomes has become a *sine qua non* for the understanding of the relationship between genotypic and phenotypic variability. The genome sequence and the ongoing functional annotation require both comparative genome analysis among different species and experimental validation. Extensive common and rare genomic variability exists that strongly influences genome function among individuals, partially determining disease susceptibility.

Contents

2.1 The Human Genome

In order to be able to understand the biological importance of the genetic information in health and disease (assign a particular phenotype to a genome variant) we first needed to know the entire nucleotide sequence of the human genome. Thus an international collaborative project has been undertaken named "The Human Genome Project" to determine the nucleotide sequence of the human genome. The project was initiated on 1 October 1990 and was essentially completed in 2004. The potential medical benefits from the knowledge of the human genome sequence were the major rationale behind the funding of this international project. In addition, the involvement and contributions of the biotechnology company Celera may have provided the necessary competition for the timely completion of the project. The last (third) edition of this book was published in 1997 before the knowledge of the human genome sequence; thus, this fourth ("postgenome") edition of the book proudly begins with the discussion of "genome anatomy," as the genomic sequence was named by Victor McKusick.

The goals of the different phases of the Human Genome Project were to: (1) determine the linkage map of the human genome [1, 60]; (2) construct a physical map of the genome by means of cloning all fragments and arrange them in the correct order [32, 69]; (3) determine the nucleotide sequence of the genome; and (4) provide an initial exploration of the variation among human genomes.

As of October 2004 about 93% of the human genome (which corresponds to 99% of the euchromatic portion of the genome) had been sequenced to an accuracy of better than one error in 100,000 nucleotides

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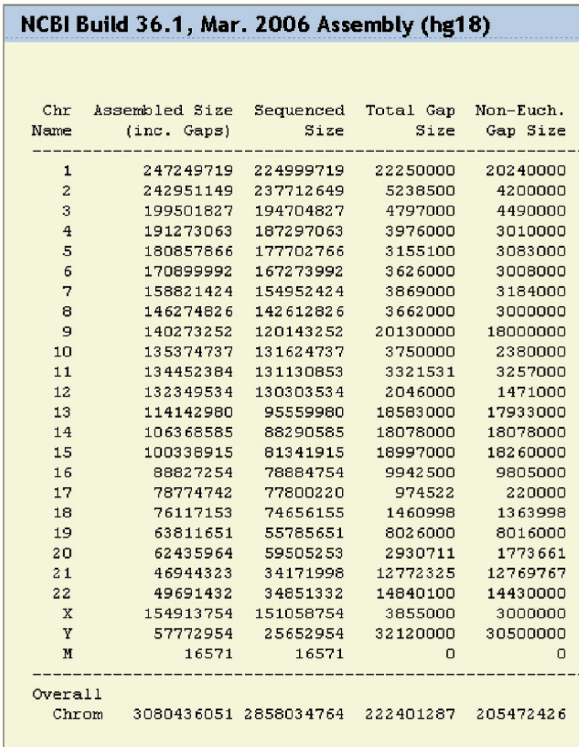
[3, 84, 137]. The DNA that was utilized for sequencing from the public effort came from a number of anonymous donors [84], while that from the industrial effort came from five subjects of which one is eponymous, Dr. J.C. Venter [85, 137]. The methodology used was also different between the two participants: the public effort sequenced cloned DNA fragments that had been previously mapped, while that of Celera sequenced both ends of unmapped cloned fragments and subsequently assembled them in continuous genomic sequences. Detailed descriptions of the genome content per chromosome have been published; the first "completed" chromosome published was chromosome 22 in 1999, chromosome 21 was published in 2000, and all other chromosomes followed in the next 6 years [38, 39, 45, 46, 55, 57, 62, 63, 66, 67, 70, 91, 97, 98, 101, 102, 111, 120, 121, 125, 131, 152, 153]. Figure 2.1 shows the parts of the genome (mainly the heterochromatic fraction) that have not yet been sequenced: the pericentromeric regions, the secondary constrictions of 1q, 9q, 16q, the short arms of acrocentric chromosomes (13p, 14p, 15p, 21p, 22p), and the distal Yq chromosome.

The total number of nucleotides of the finished sequence is 2,858,018,193 while the total estimated length that includes the current gaps is ~3,080,419,480 nucleotides (see Table 2.1, taken from the last hg18 assembly of the human genome http://genome.ucsc.edu/goldenPath/stats.html#hg18). The length of the human chromosomes ranges from ~46 Mb to ~247 Mb. The average GC content of the human genome is 41%. This varies considerably among the different chromosomes and within the different bands of each chromosome. Chromosomal bands positive for Giemsa staining have lower average GC content of 37%, while in Giemsa-negative bands the average GC content is 45%. Interestingly, Giemsa-negative bands are gene-rich regions of DNA (see Chap. 3, Sect. 3.2.4).

Table 2.1 Taken from http://genome.ucsc.edu/goldenPath/stats.html#hg18, showing the number of nucleotides per chromosome in the reference genome. Chromosome "M" is the DNA of the mitochondrial genome (see Sect. 2.1.3)



NCBI Build 36.1, Mar. 2006 Assembly (hg18)

Chr Name	Assembled Size (inc. Gaps)	Sequenced Size	Total Gap Size	Non-Euch. Gap Size
1	247249719	224999719	22250000	20240000
2	242951149	237712649	5238500	4200000
3	199501827	194704827	4797000	4490000
4	191273063	187297063	3976000	3010000
5	180857866	177702766	3155100	3083000
6	170899992	167273992	3626000	3008000
7	158821424	154952424	3869000	3184000
8	146274826	142612826	3662000	3000000
9	140273252	120143252	20130000	18000000
10	135374737	131624737	3750000	2380000
11	134452384	131130853	3321531	3257000
12	132349534	130303534	2046000	1471000
13	114142980	95559980	18583000	17933000
14	106368585	88290585	18078000	18078000
15	100338915	81341915	18997000	18260000
16	88827254	78884754	9942500	9805000
17	78774742	77800220	974522	220000
18	76117153	74656155	1460998	1363998
19	63811651	55785651	8026000	8016000
20	62435964	59505253	2930711	1773661
21	46944323	34171998	12772325	12769767
22	49691432	34851332	14840100	14430000
X	154913754	151058754	3855000	3000000
Y	57772954	25652954	32120000	30500000
M	16571	16571	0	0
Overall Chrom	3080436051	2858034764	222401287	205472426

Figure 2.2 shows the current status of the "completion" of the human genome sequence [3]. Red bars above the chromosomes represent the sequence gaps. The DNA content of the red blocks (heterochromatin) is still unknown. Heterochromatic regions of chromo-

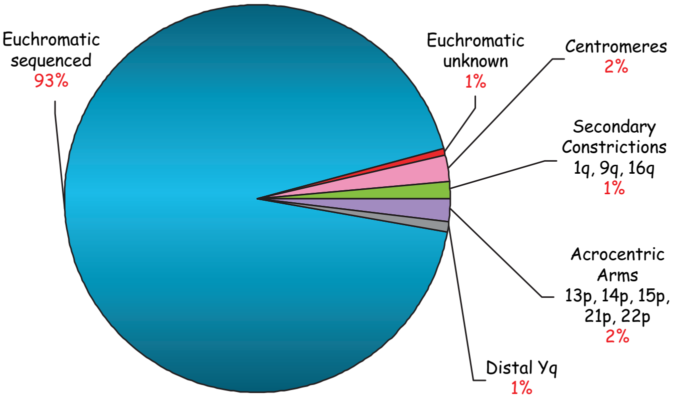


Fig. 2.1 Pie chart of the fractions of the genomes sequenced (*blue*) and not sequenced (*non-blue*)

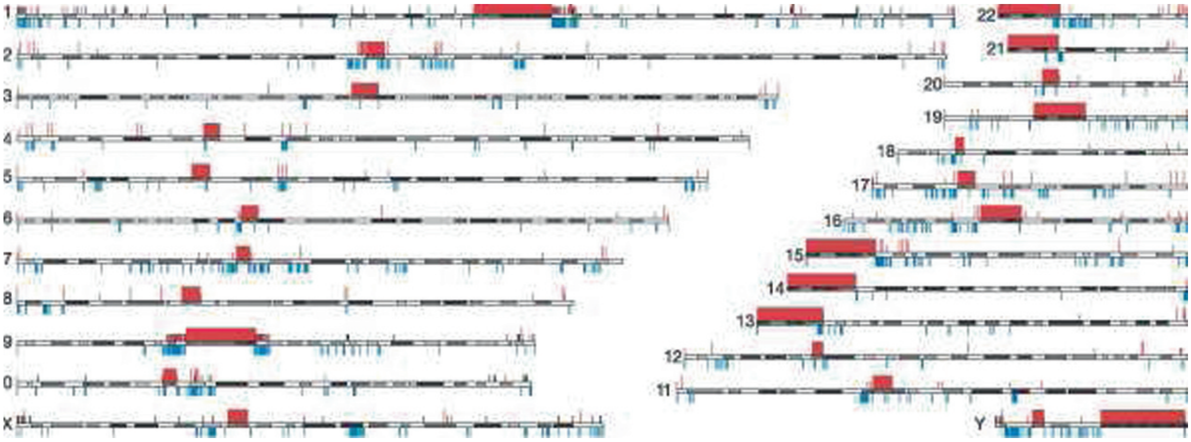


Fig. 2.2 Schematic representation of the completion of the human genome per chromosome. *Red regions* represent areas not sequenced; *blue regions below the chromosomal line* represent gaps in the sequences. The major blocks of unknown sequence include the short arms of acrocentric chromosomes, the pericentromeric sequences, and the large heterochromatic regions (From [3])

somes are those that remain highly condensed throughout the cell cycle (see Chap. 3, Sect. 3.2.1); it is thought that transcription is limited in these regions that contain a considerable number of repetitive elements that renders the assembly of their sequence almost impossible.

The sequence of the human genome is freely and publicly available on the following genome browsers, which also contain many additional annotations (see also Chap. 29):

(a) http://genome.ucsc.edu/
(b) http://www.ensembl.org/
(c) http://www.ncbi.nlm.nih.gov/genome/guide/human/

Representative pages of two of these browsers are shown in Fig. 2.3.

There is now a considerable effort internationally to identify all the functional elements of the human genome. A collaborative project called ENCODE (ENcyclopedia Of DNA Elements) is currently in progress with the ambitious objective to identify all functional elements of the human genome [2, 19].

The genome of modern humans, as a result of the evolutionary process, has similarities with the genomes of other species. The order of genomic elements has been conserved in patches within different species such that we could recognize today regions of synteny in different species, i.e., regions that contain orthologous genes and other conserved functional elements. Figure 2.4 shows a synteny map of conserved genomic segments in human and mouse.

The current classification of the functional elements of the genome contains:

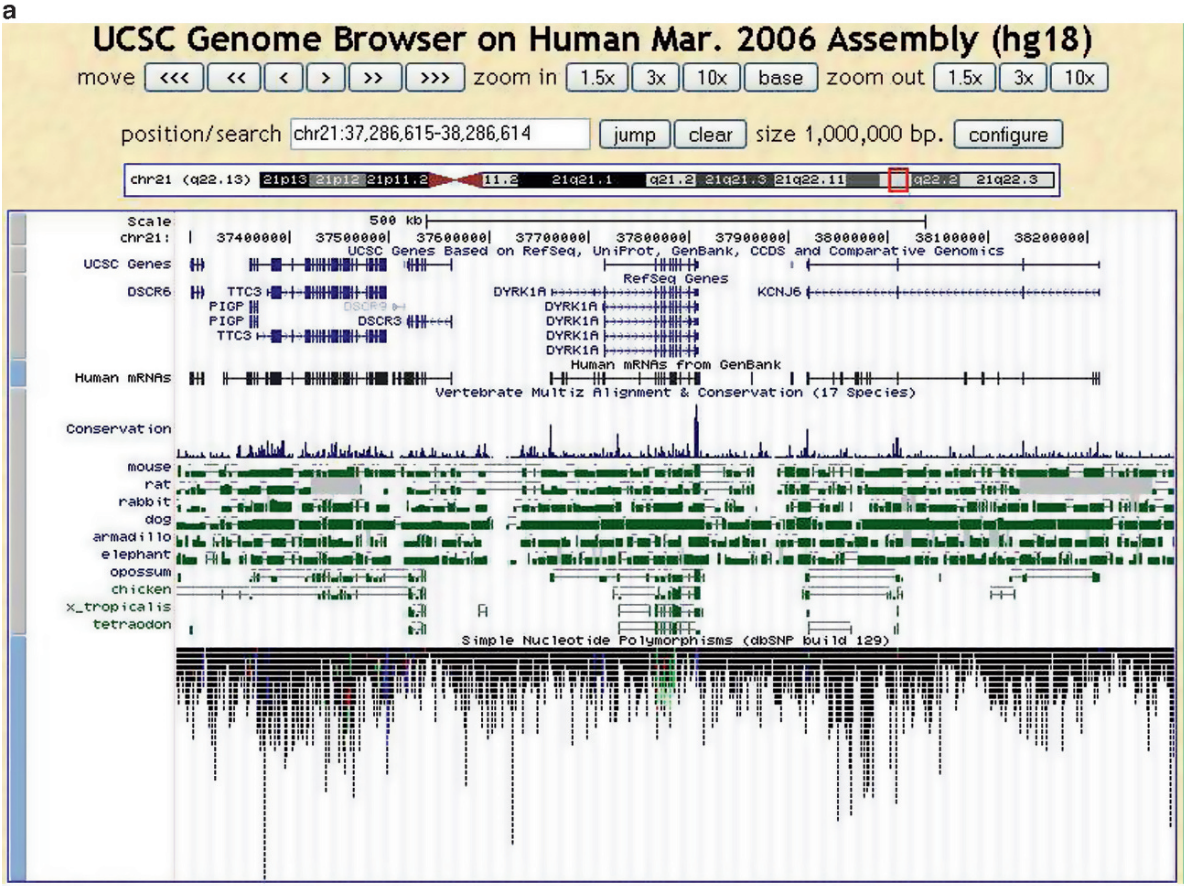
1. Protein-coding genes
2. Noncoding, RNA-only genes
3. Regions of transcription regulation
4. Conserved elements not included in the above categories

2.1.1 Functional Elements

2.1.1.1 Protein-Coding Genes

The total number of protein-coding genes is a moving target, since this number depends on the functional annotation of the genome, the comparative analysis with the genomes of other species, and the experimental validation. The so-called CCDS set (*c*onsensus *c*oding *s*equence) is built by consensus among the European Bioinformatics Institute (http://www.ebi.ac.uk/), the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/), the Wellcome Trust Sanger Institute (http://www.sanger.ac.uk/), and the University of California, Santa Cruz (UCSC; http://www.cbse.ucsc.edu/). At the last update (5 July 2009; genome build 36.3) CCDS contains 17,052 genes. This is the minimum set of protein-coding genes included in all genomic databases. The reference sequence (RefSeq) collection of genes of the NCBI contains 20,366 protein-coding gene entries (http://www.ncbi.nlm.nih.gov/

a



UCSC Genome Browser on Human Mar. 2006 Assembly (hg18)

position/search chr21:37,286,615-38,286,614

size 1,000,000 bp.

Fig. 2.3 (**a**) Screenshot of the UCSC genome browser (http://genome.ucsc.edu/) for a 1-Mb region of chromosome 21 (21: 37,286,615–38,286,614). Among the many features that could be displayed, the figure shows genes, sequence conservation in 17 species, and single nucleotide polymorphisms (SNPs) that map in this 1-Mb region. The tracks shown from *top to bottom* include: a scale for the genomic region, the exact location in nucleotides, schematic representation of genes included in the UCSC database, the mRNAs from GenBank, the conservation in the species shown, and the location of SNPs. The *color* of some SNPs corresponds to synonymous and nonsynonymous substitutions.

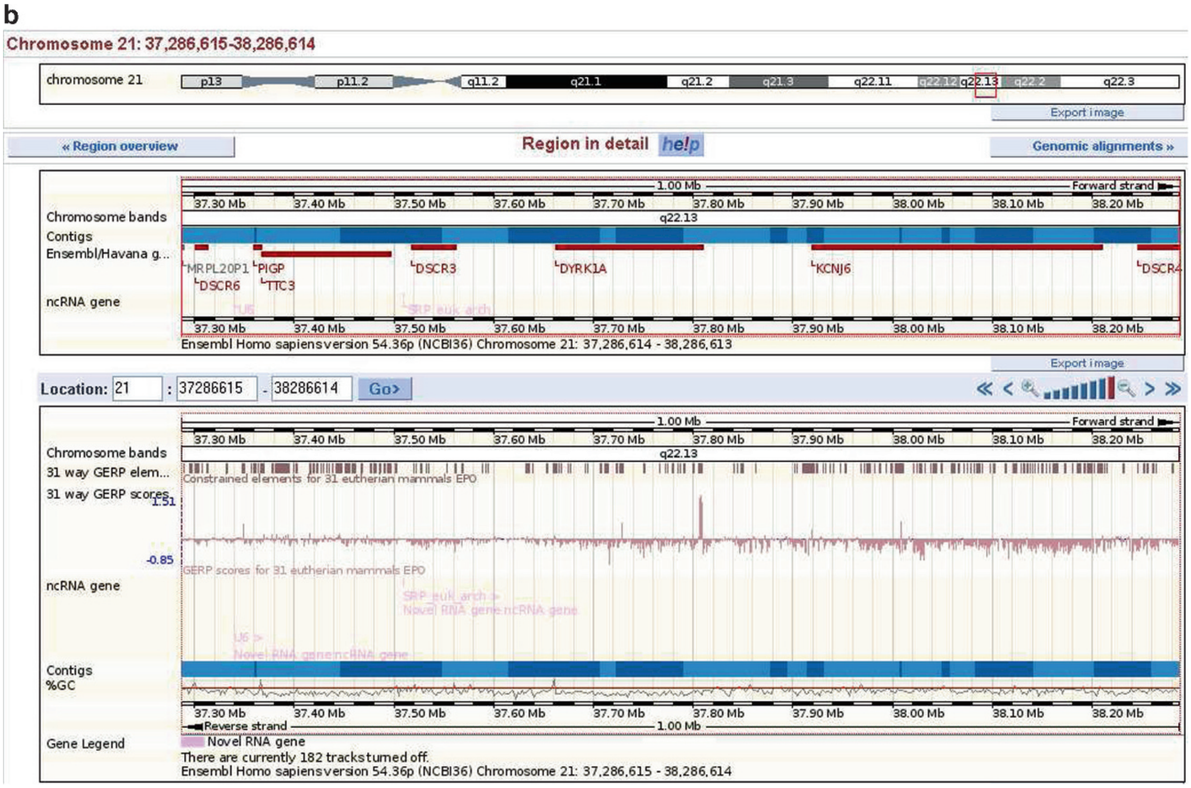
RefSeq/); the UCSC collection of genes contains 23,008 entries (http://genome.ucsc.edu/); the Ensembl browser contains 21,416 entries (23 June 2009; build 36; http://www.ensembl.org/Homo_sapiens/Info/StatsTable). The total number of annotated exons listed in the Ensembl database is 297,252 (23 June 2009; build 36). The discrepancy among the databases reflects the ongoing and unfinished annotation of the genome.

Table 2.2 lists the number of protein-coding and other genes in humans taken from different databases.

The human genes are not equally distributed in the chromosomes. In general, Giemsa pale bands are gene rich, and this results in unequal numbers of genes per size unit for the different chromosomes. Figure 2.5 from [84] displays the gene density per megabase for each chromosome and the correlation with CpG-rich islands.

Chromosomes 22, 17, and 19 are unusually gene-rich, while chromosomes 13, 18, and X are relatively gene-poor (interestingly, trisomies for chromosomes 13 and 18 are among the few human trisomies at birth). The average number of exons per gene is nine, and the average exon size is 122 nucleotides. Thus, the total number of annotated exons range from 210,000 to 300,000 (depending on the database), and the total exonic genome size is up to 78 Mb.

The mapping position of the genes can be seen in the genome browsers, and their names can be found in the gene nomenclature Web site, which contains 28,182 entries (http://www.genenames.org/; 30 June 2009).



Chromosome 21: 37,286,615-38,286,614

Location: 21 : 37286615 - 38286614

Ensembl Homo sapiensversion 54.36p (NCBI36) Chromosome 21: 37,286,614 - 38,286,613

There are currently 182 tracksturned off.

Ensembl Homo sapiensversion 54.36p (NCBI36) Chromosome 21: 37,286,615 - 38,286,614

Fig. 2.3 (continued) (**b**) Screenshot of the Ensembl genome browser (http://www.ensembl.org) for a 1-Mb region of chromosome 21 (21: 37,286,615–38,286,614). Among the many features that could be displayed, the figure shows genes (Ensembl/Havana gene track), noncoding RNAs (ncRNA gene track), sequence conservation in 31 species (31-way GERP track), and GC content in this 1-Mb region. The different browsers have similarities and differences, and some features could only be displayed in one browser (for details see Chaps. 29.1 and 29.2)

A single gene may have different isoforms due to alternative splicing of exons, alternative utilization of the first exon, and alternative 5′ and 3′ untranslated regions. There are on average 1.4–2.3 transcripts per gene according the different databases (Table 2.2); this is likely an underestimate since, in the pilot ENCODE 1% of the genome that has been extensively studied, there are 5.7 transcripts per gene [19, 61]. The average number of exons per gene, depending on the database, ranges from 7.7 to 10.9.

The size of genes and number of exons vary enormously. The average genomic size of genes (according to the current annotation) is 27 kb. There are, however, small genes that occupy less than 1 kb, and large genes that extend to more than 2,400 kb of genomic space. There are intronless genes (e.g., histones) and others with more than 360 introns (e.g., titin).

The initial results of the ENCODE and other similar projects provided evidence for additional exons to the annotated genes; these exons could be hundreds of kilobases away (usually 5′) to the annotated gene elements [19, 40, 44]. In addition, there is evidence for chimeric transcripts that join two “independent” genes [103]. The investigation of these complicated transcripts is ongoing, and the functional significance of them is unknown.

Protein-coding genes can be grouped in families according to their similarity with other genes. These families of genes are the result of the evolutionary processes that shaped up the genomes of the human and other species. The members of the gene families could be organized in a single cluster or multiple clusters, or could be dispersed in the genome. Examples of gene families include the globin, immunoglobulin, histones, and olfactory receptors gene families. Furthermore, genes encode proteins with diverse but recognizable domains. The database Pfam (http://pfam.sanger.ac.uk/, http://www.uniprot.org/) is a comprehensive collection of protein domains and families [48]; the current release

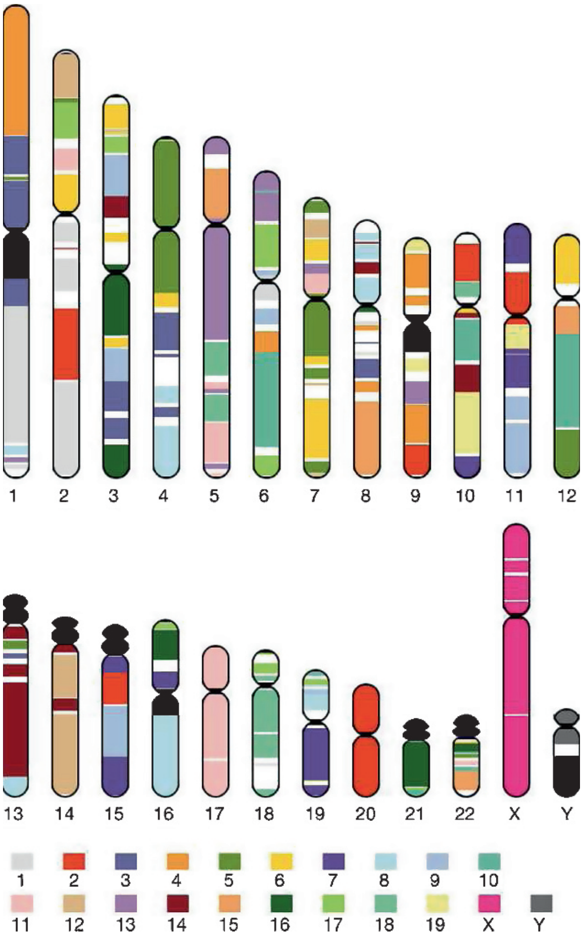


Fig. 2.4 Schematic representation of the observed genomic segments between the human and mouse genomes. The color code of the human chromosomes corresponds to the different mouse chromosomes shown on the bottom. For example, human chromosome 20 is all homologous to mouse chromosome 2; human chromosome 21 is homologous to mouse chromosomes 16, 17, and 10. Centromeric, and heterochromatic regions, and acrocentric p-arms are in *black*. (From [84])

of Pfam (23.0) contains 10,340 protein families. For example, the WD40 domain family (PF00400) includes 609 human genes, while the homeobox domain family (PF00046) has 430 genes. The identification of domains helps in the prediction of the function and structure of a protein.

Pseudogenes are "dead" nonfunctional genes. These sequences that could be transcribed and spliced contain mutations that render them inactive. Pseudogenes could be generated by several mechanisms that include:

1. Gene duplication events in which one of the duplicated copies accumulates inactivating mutations; alternatively, the duplicated genes may be truncated. These pseudogenes are also called nonprocessed pseudogenes.
2. Transposition events in which a copy of cDNA is reinserted into the genome. These pseudogenes, also called "processed," are not functional, usually because they lack regulatory elements that promote transcription. In addition, inactivating mutations also occur in processed pseudogenes.

The current estimated number of human pseudogenes (according to one of the databases http://www.pseudogene.org/human/index.php) [151] is 12,534 (~8,000 are processed and ~4,000 duplicated pseudogenes; build 36); while according to the Ensembl browser the number is 9,899 (build 36; 23 June 2009). These pseudogenes belong to 1,790 families; e.g., the immunoglobulin gene family has 1,151 genes and 335 pseudogenes, while the protein kinase gene family has 1,159 genes and 159 pseudogenes (http://pseudofam.pseudogene.org/pages/psfam/overview.jsf).

The total number of human genes is not dramatically different from that of other "less" complex organisms. Figure 2.6 depicts the current estimate of the protein-coding gene number for selected species.

2.1.1.2 Noncoding, RNA-Only Genes

Besides the protein-coding genes, there is a growing number of additional genes (transcripts) that produce an

Table 2.2 Human gene, exon, and transcript counts from various databases

Database (June 2009)	Protein-coding genes	RNA-only genes	Total genes	Total number of transcripts	Total number of exons	Average exons per gene	Average transcripts per gene
CCDS	17,052			45,428			2.7
Ensembl	21,416	5,732	27,148	62,877	297,252	10.9	2.3
UCSC	23,008	9,155	32,163	66,802	246,775	7.7	2.1
RefSeq	20,366	2,044	22,410	31,957	211,546	9.4	1.4

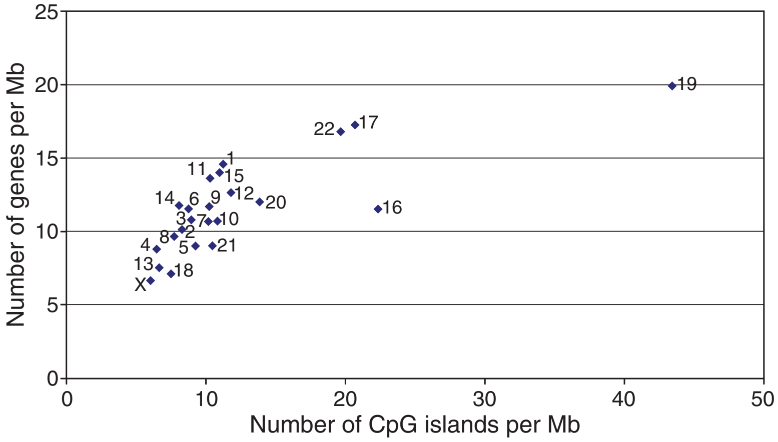


Fig. 2.5 Gene density per chromosome, and correlation with CpG-rich islands of the genome. Chromosome 19 for, example, has the highest gene content and the highest CpG island content. (From [84])

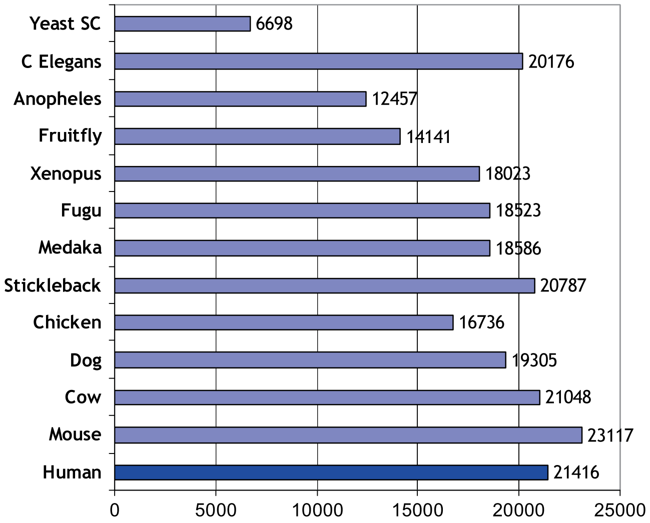


Fig. 2.6 Histogram of the current (5 July 2009) estimate of the number of protein-coding genes in different selected species from the Ensembl browser. These numbers are subject to change

RNA that is not translated to protein (see the databases http://biobases.ibch.poznan.pl/ncRNA/, http://www.ncrna.org/frnadb/search.html, http://www.sanger.ac.uk/Software/Rfam/ and [56]). Table 2.2 contains the current number of these genes, which ranges from 2,044 in RefSeq to 9,155 in the UCSC browser.

The different classes of RNA-only genes are briefly discussed below:

Ribosomal RNA (rRNA) Genes [53, 82, 84]: ~650–900. These are genes organized in tandemly arranged clusters in the short arms of the five acrocentric chromosomes (13, 14, 15, 21, and 22). The transcripts for 28 S, 5.8 S, and 18 S rRNAs are included in one transcription unit, repeated 30–50 times per chromosome. These tandemly arranged genes are continuously subjected to concerted evolution, which results in homogeneous sequences due to unequal homologous exchanges. The transcripts for the 5 S rRNAs are also tandemly arranged, and the majority map to chromosome 1qter. There exist also several pseudogenes for all classes. The total number of these genes is polymorphic in different individuals. The best estimates of the number of rRNA genes are:

28 S (components of the large cytoplasmic ribosomal subunit)	~150–200
5.8 S (components of the large cytoplasmic ribosomal subunit)	~150–200
5 S (components of the large cytoplasmic ribosomal subunit)	~200–300
18 S (components of the small cytoplasmic ribosomal subunit)	~150–200

Transfer RNA (tRNA): ~500 (49 Types). At the last count there are 497 transfer RNA genes (usually 74–95 nucleotides long) encoded by the nucleus and transcribed by RNA polymerase III (additional tRNAs are encoded by the mitochondria genome). There are also 324 tRNA pseudogenes [84]. The tRNA nuclear genes form 49 groups for the 61 different sense codons. Although the tRNA genes are dispersed throughout the genome, more than 50% of these map to either chromosomes 1 or 6; remarkably 25% of tRNAs map to a 4-Mb region of chromosome 6.

Small Nuclear RNA (snRNA) [84, 87, 105]: ~100. These are heterogeneous small RNAs. A notable fraction of these are the spliceosome [139] RNA genes many of which are uridine-rich; the U1 group contains 16 genes, while U2 contains six, U4 4, U6 44, and the other subclasses are represented by one member. Some of these genes are clustered, and there is also a large number of pseudogenes (more than 100 for the U6 class).

Small Nucleolar RNA (snoRNA): ~200. This is a large class of RNA genes that process and modify the tRNAs and snRNAs [135, 147]. There are two main families: C/D box snoRNAs that are involved in specific methylations of other RNAs; and H/ACA snoRNAs, mostly involved in site-specific pseudouridylations. Initially, there were 69 recognized in the first family and 15 in the second [84]; however, the total number is probably larger. A cluster of snoRNAs maps to chromosome 15q in the Prader–Willi syndrome region (at least 80 copies); deletions of which are involved in the pathogenesis of this syndrome [26, 117]. Another cluster of snoRNAs maps to chromosome 14q32 (~40 copies). The majority of snoRNAs map to introns of protein-coding genes and can be transcribed by RNA polymerase II or III.

Micro RNAs (miRNA): (706 Entries on 26 June 2009). These are single-stranded RNA molecules of about 21–23 nt in length that regulate the expression of other genes. miRNAs are encoded by RNA genes that are transcribed from DNA but not translated into protein; instead they are processed from primary transcripts known as pri-miRNA to short stem-loop structures called pre-miRNA and finally to functional miRNA. Mature miRNA molecules are complementary to regions in one or more messenger RNA (mRNA) molecules, which they target for degradation. A database of the known and putative miRNAs, and their potential targets, can be found in http://microrna.sanger.ac.uk/. miRNAs have been shown to be involved in human disorders.

Large Intervening Noncoding RNAs (LincRNAs): ~1,600. This new class has been recently identified using trimethylation of Lys4 of histone H3 as a genomic mark to observe RNA PolII transcripts at their promoter, and trimethylation of Lys36 of histone H3 marks along the length of the transcribed region [95] to identify the spectrum of PolII transcripts. Approximately 1,600 such LincRNA transcripts have been found across four mouse cell types (embryonic stem cells, embryonic fibroblasts, lung fibroblasts, and neural precursor cells) [59]. Among the "exons" of these LincRNAs, approximately half are conserved in mammalian genomes, and are thus present in human. Since this class was described in 2009, further work is needed for its characterization and validation, as well as the potential overlap of its members with the other classes.

Other Noncoding RNAs [7, 75, 113, 126, 136]: ~1,500. The field of noncoding RNA series is constantly expanding. Some of these RNA genes include molecules with known function such as the telomerase RNA, the 7SL signal recognition particle RNA, and the XIST long transcript involved on the X-inactivation [23]. There are also numerous antisense noncoding RNAs, and the current effort to annotate the genome suggests that a substantial fraction of the transcripts are noncoding RNAs.

2.1.1.3 Regions of Transcription Regulation

The genome certainly contains information for the regulation of transcription. The current list of these regulatory elements includes promoters, enhancers, silencers, and locus control regions [92]. These elements are usually found in *cis* to the transcriptional unit, but there is growing evidence that there is also *trans* regulation of transcription. The discovery of the regulatory elements, their functional interrelationship, and their spatiotemporal specificity provides a considerable challenge. A systematic effort during the pilot ENCODE project has provided initial experimental evidence for genomic regions with enriched binding of transcription factors [19, 80, 86, 133]. A total of 1,393 regulatory genomic clusters were, for example, identified in the pilot ENCODE regions; remarkably only ~25% of these map to previously known regulatory regions and only ~60% of these regions overlap with evolutionarily constrained regions. These results suggest that many novel regulatory regions will be recognized in the years to come, and also that there exist regions of transcriptional regulation that are not conserved and thus novel for different clades and species. The use of model organisms facilitates the experimental validation of regulatory elements, and there are systematic efforts underway for the exploration of conserved elements ([106] and http://enhancer.lbl.gov/).

2.1.1.4 Conserved Elements Not Included in the Above Categories

Since it is assumed that functional DNA elements are conserved while nonfunctional DNA diverges rapidly, it is expected that all other conserved elements are of interest and should be studied for potential pathogenic variability. How much of the human genome is evolutionarily conserved? The answer to this question depends on the species compared and the time of their common ancestor. Comparative genome analysis between human and mouse, for example, is particularly instructive, since the time of the common ancestor between these two species is estimated to be ~75 million years ago, and thus the conserved elements are likely to be functional. Approximately 5% of the human genome is conserved compared to mouse [145] (and to several other mammalian genomes). Of this, ~1–2% are the coding regions of protein-coding genes, and ~3% are conserved noncoding DNA sequences (CNCs; Fig. 2.7) [41, 42]. The function of the majority of CNCs is unknown. Please note that this 5% conserved fraction between human and mouse is an underestimate of the functional fraction of the human genome, which is likely to be bigger and to contain additional sequences not conserved with the mouse.

The ENCODE pilot project [19, 90], with data from 1% of the human genome and sequences from the orthologous genomic regions from 28 additional species, also estimated that the constrained portion of the human genome is at least ~4.9%; remarkably, 40% of this genomic space is unannotated and thus of unknown function (Fig. 2.8).

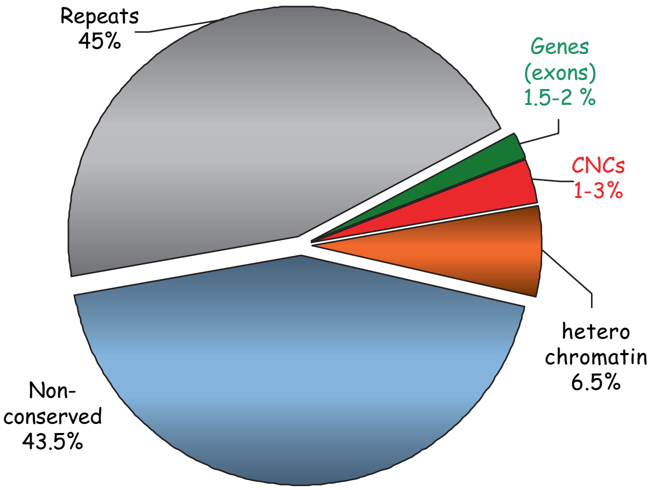


Fig. 2.7 The pie-chart depicts the different fractions of the genome. *CNC*s, conserved noncoding sequences

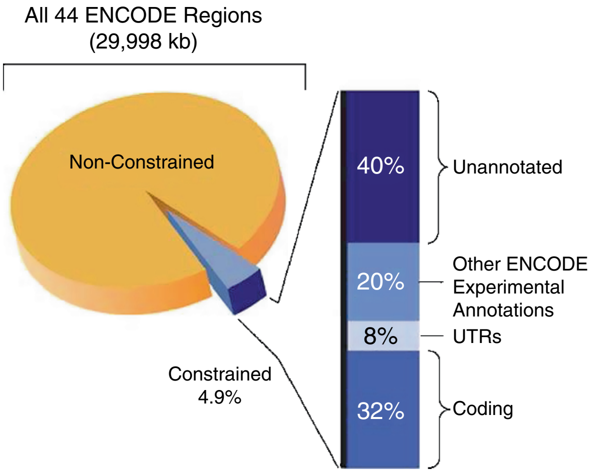


Fig. 2.8 The fractions of different genomic annotations among the 4.9% of constrained sequences in the human genome. Data from the pilot ENCODE project; figure taken from [19]. *UTR*, untranslated region; other ENCODE experimental annotations refers to the fraction of the genome that has been identified using a variety of experimental techniques for transcription, histone modifications, chromatin structure, sequence specific factors, and DNA replication. More information on these experiments is included in Table 1 of [19]

2.1.2 Repetitive Elements

The function of the majority of the human genome is unknown. Remarkably, ~45% of the genome is composed of repetitive elements, and another ~43% is not conserved and does not belong to the functional categories mentioned above. The different interspersed repeats of the human genome are shown in the Fig. 2.9 (from [84]):

- LINEs (long interspersed nuclear elements [76, 77]) are autonomous transposable elements, mostly truncated nonfunctional insertions (average size of 900 bp). More than 20% of the human genome is polluted by LINEs. Transposable elements are mobile DNA sequences which can migrate to different regions of the genome. Autonomous are those that are capable of transposing by themselves. A small fraction of LINEs (~100) are still capable of transposing. The full LINE element is 6.1 kb long, has an internal PolII promoter, and encodes two open reading frames, an endonuclease, and a reverse transcriptase. Upon insertion a target site duplication of 7–20 bp is formed. There are a few subclasses of LINEs according to their consensus sequence. The subfamily LINE1 is the only one capable of autonomous retrotransposition (copy itself and pasting copies back into the genome in multiple places). These LINEs enable transposition of SINEs (defined below), processed pseudogenes, and retrogenes [76, 77]. LINE retrotransposition has been implicated in human disorders [78]. LINEs are more abundant in G-dark bands of human chromosomes.
- SINEs (short interspersed nuclear elements [18]) mainly include the Alu repeats, which are the most abundant repeats in the human genome, occurring on average in every 3 kb. Thus, 13% of the genome is polluted by Alu sequences and other SINEs. They are inactive elements originated from copies of tRNA or from signal recognition particle (SRP; 7SL) RNA. The full-length element is about 280 nt long and consists of two tandem repeats each ~120 nt followed by polyA.

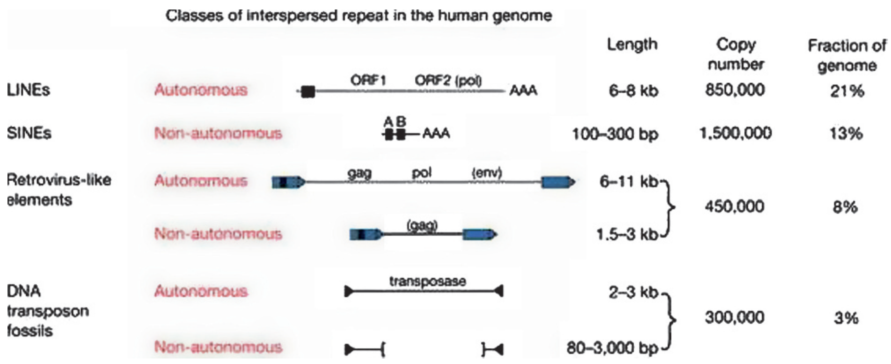


Fig. 2.9 Depicts some basic characteristics of the classes of interspersed repeats in the human genome. For more explanations, see text. (From [84])

Alu sequences are transcriptionally inactive, and are GC-rich. SINEs can retrotranspose in a non-autologous way, since they use the LINE machinery for transposition. Because of their abundance, they could mediate deletion events in the genome that result in human disorders [37]. SINEs are more abundant in G-light bands of human chromosomes (see Sect. 3.2.4).

- Retrovirus like (LTR transposons) are elements flanked by long terminal repeats. Those that contain all the essential genes are theoretically capable of transposition, but that has not happened in the last several million years. Collectively they account for 8% of the genome. Most are known as HERV (human endogenous retroviral sequences) and are transposition defective. Transcription from the HERV genes may modulate the transcriptional activity of nearby protein-coding genes [22].
- DNA transposon fossils [127] have terminal inverted repeats and are no longer active; they include two main families, MER1 and MER2, and comprise 3% of the genome.

More update information about repeats can be found in http://www.girinst.org/server/RepBase/.

2.1.2.1 Segmental Duplications

Approximately 5.2 % of the human genome consists of segmental duplications or duplicons, i.e., regions of more than 1 kb, with greater than 90% identify, that are present more than once in the genome. Segmental duplications are either intrachromosomal (on the same chromosome, 3.9%), or interchromosomal (on different chromosomes, 2.3%; Fig. 2.10). Most of the "duplicons" are in the pericentromeric regions.

Figure 2.11 shows the distribution of intrachromosomal duplicons in the human genome [16, 118]. These duplications are important in evolution and as risk factors for genomic rearrangements that cause human disorders because of unequal crossing-over in meiosis (pathogenic microdeletions and microduplications). Some examples of these include cases of α-thalassemia [65] on chromosome 16p, Charcot–Marie–Tooth syndrome [104] on chromosome 17p, and velo-cardiac-facial syndrome [96] on chromosome 22q, Williams–Beuren syndrome [107] on chromosome 7q, and Smith–Magenis syndrome [29] on chromosome 17p.

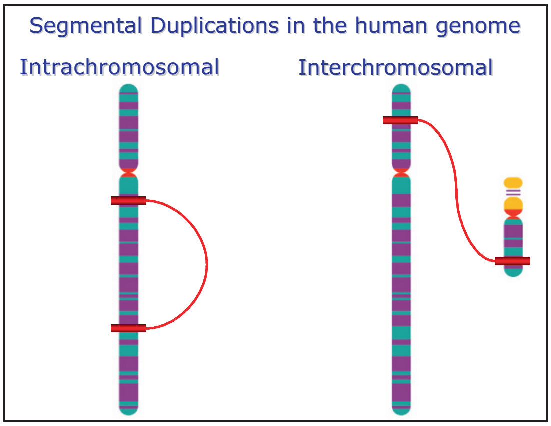


Fig. 2.10 Schematic representation of intra- and inter-chromosomal segmental duplications. The repeat element is shown in *red*, and there is a *connecting line* indicating the highly homologous sequences

2.1.2.2 Special Genomic Structures Containing Selected Repeats

2.1.2.2.1 Human Centromeres

Human centromeres consist of hundreds of kilobases of repetitive DNA, some chromosome specific and some nonspecific [114, 122, 124]. Actually, most of the remaining sequence gaps in the human genome are mapped near and around centromeres. The structure of human centromeres is unknown, but the major repeat component of human centromeric DNA is an α-satellite or alphoid sequence [30] (a tandem repeat unit of 171 bp that contains binding sites for CENP-B, a centromeric-binding protein; see also Chap. 3, Sect. 3.2.3). Figure 2.12 shows an example of the structure of two human centromeres [3].

2.1.2.2.2 Human Telomeres

Human telomeres [109] consist of tandem repeats of a sequence $(TTAGGG)_n$ that spans about 3–20 kb, beyond which at the centromeric side there are about 100–300 kb of subtelomeric-associated repeats [3] before any unique sequence is present.

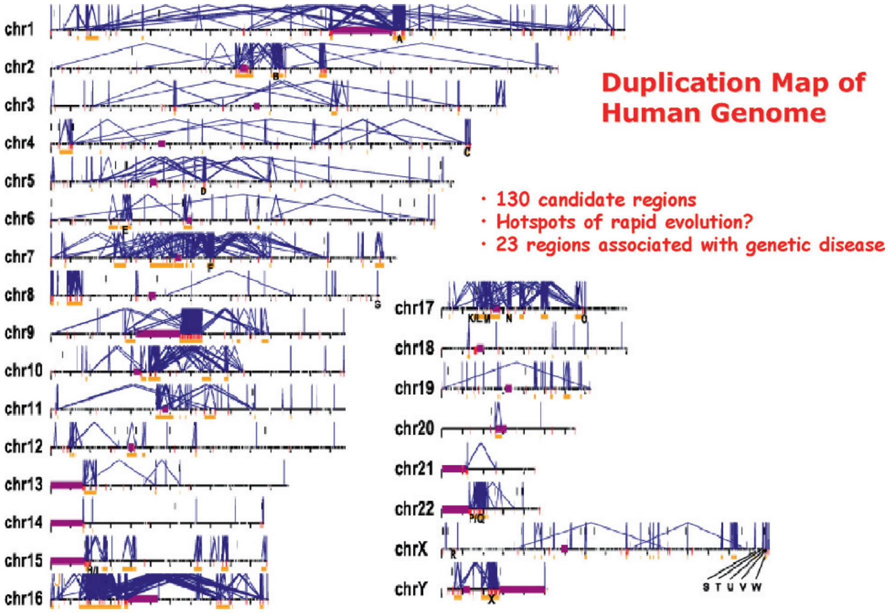


Fig. 2.11 Schematic representation of the intrachromosomal segmental duplications (from [16]). In each chromosome a *blue line* links a duplication pair. For example, on chromosome 21 there is only one duplicon shown; in contrast, on chromosome 22 there is a considerable number of duplications. *Richly blue areas* are considered susceptible to microduplication/microdeletion syndromes

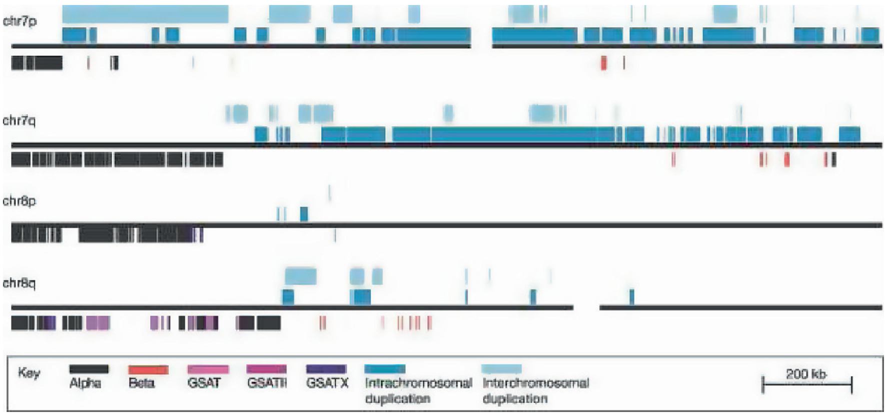


Fig. 2.12 Examples of sequence organization of two human centromeres (chromosomes 7 and 8, (from [3]). Alphoid repeats are the major component of this special chromosomal structure; in addition, several other repetitive elements border the alphoid sequences. The length of these regions is also polymorphic in different individuals

Figure 2.13 schematically shows the sequence organization of six human subtelomeric regions.

2.1.2.2.3 Short Arms of Human Acrocentric Chromosomes

The finished sequence of the human genome does not include the short arms of acrocentric chromosomes (13p, 14p, 15p, 21p, and 22p). Cytogenetic data show that the p arms contain large heterochromatic regions of polymorphic length [35, 138]. Molecular analysis revealed that they are composed mainly of satellite and other repeat families, including satellites I (AT-rich repeat of a monomer of 25–48 bp [73]), II (monomer repeat 5 bp [68]), III (monomer repeat also 5 bp [31]), β-satellite (a tandem repeat unit of 68 bp of the Sau3A family [94, 146]), and repeats ChAB4 [36], 724 [83], and D4Z4-like [89]. These repeats have a complex pattern and are often organized in subfamilies shared

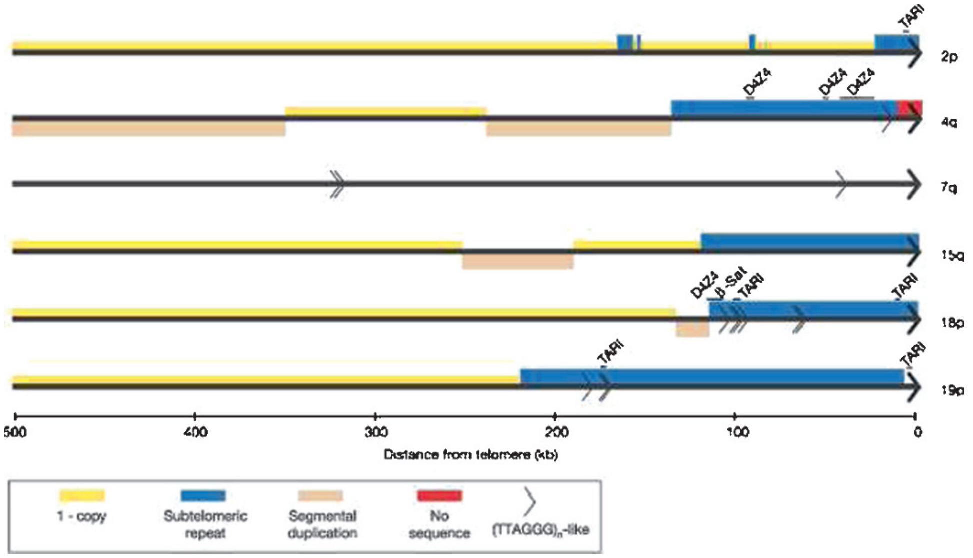


Fig. 2.13 Examples of the sequence organization of six human telomeres (chromosomes 2pter, 4qter, 7qter, 15qter, 18pter, and 19pter, taken from [3]). The *arrows* represent the TTAGGG repeat, while the *blue regions* depict the subtelomeric repeats that mainly consist of TAR1 (telomere associated repeat 1 family [24]), D4Z4 (a 3.3-kb tandem repeat, each copy of which contains two homeoboxes and two repetitive sequences, LSau and hhspm3 [64]) and β-satellite sequences (a tandem repeat unit of 68 bp of the Sau3A family [94])

between different acrocentric chromosomes. The p arms encode the ribosomal (RNR) gene [53, 82] but may also encode other genes [88, 130]. Currently there is an initiative to sequence the short arm of chromosome 21 and thus extrapolate on the structure of the additional p arms of the other acrocentrics [88].

The most common chromosomal rearrangements in humans are Robertsonian translocations (~1 in 1,000 births), which involve exchanges between acrocentric p arms. Three to five percent of these translocations are associated with phenotypic abnormalities [143].

2.1.3 *Mitochondrial Genome*

In human cells there is also the mitochondrial genome, which is 16,568 nucleotides long and encodes for 13 protein-coding genes, 22 tRNAs, one 23 S rRNA, and one 16 S rRNA ([140–142]; http://www.mitomap.org). The mitochondria genome-encoded genes are all essential for oxidative phosphorylation and energy generation in the cell. Each cell has hundreds of mitochondria and thousands (10^3–10^4) of mitochondria DNA (mtDNA) copies. Human mtDNA has a mutation rate ~20 times higher than nuclear DNA. The inheritance of mtDNA is exclusively maternal (the oocyte contains 10^5 mtDNA copies). Several human phenotypes are due to pathogenic mutations in the mitochondrial genome [140] (Fig. 2.14).

2.2 Genomic Variability

The human genome is polymorphic, i.e., there are many DNA sequence variants among different individuals. These variants are the molecular basis of the genetic individuality of each member of our species. In addition, this genetic variability is the molecular substrate of the evolutionary process. Finally, this variability causes disease phenotypes or predispositions to common complex or multifactorial phenotypes and traits.

2.2.1 *Single Nucleotide Polymorphisms*

The majority of the DNA variants are single nucleotide substitutions commonly known as SNPs (single nucleotide polymorphisms). The first SNPs were identified in 1978 in the laboratory of Y.W. Kan 3′ to the β-globin

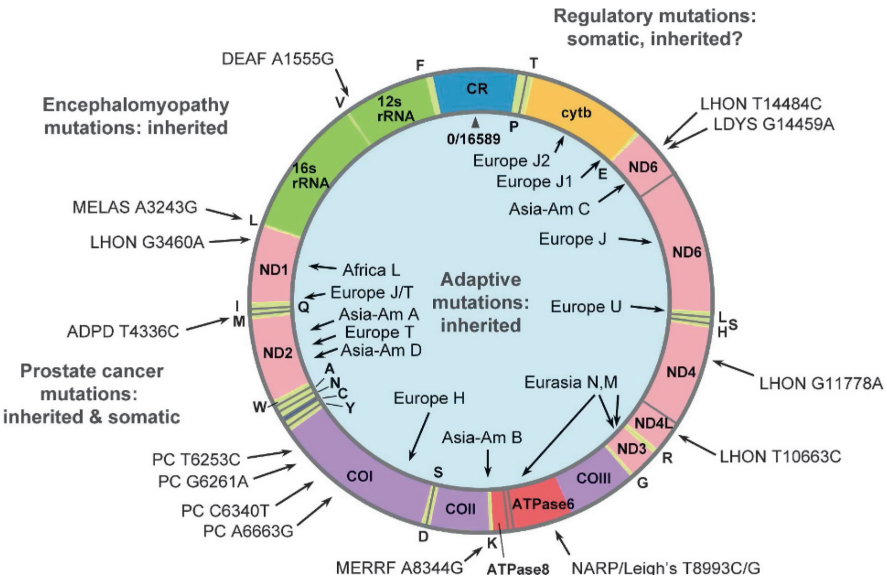


Fig. 2.14 Schematic representation of the circular mtDNA, its genes, its clinical relevant mutations, and certain polymorphic markers. *Letters within the ring* depict the genes encoded. *Letters on the outside* indicate amino acids of the tRNA genes. *CR*, the control of replication region that contains promoters for the heavy and light strands. *Arrows outside* show the location of pathogenic mutations. (From [142]

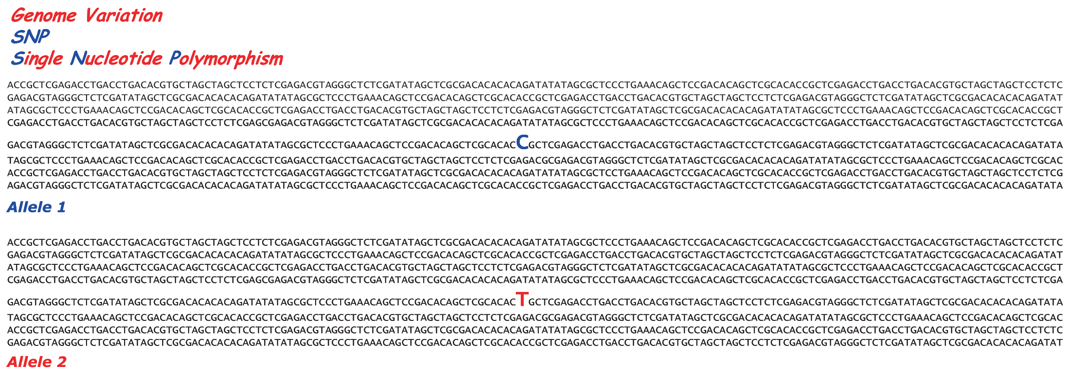
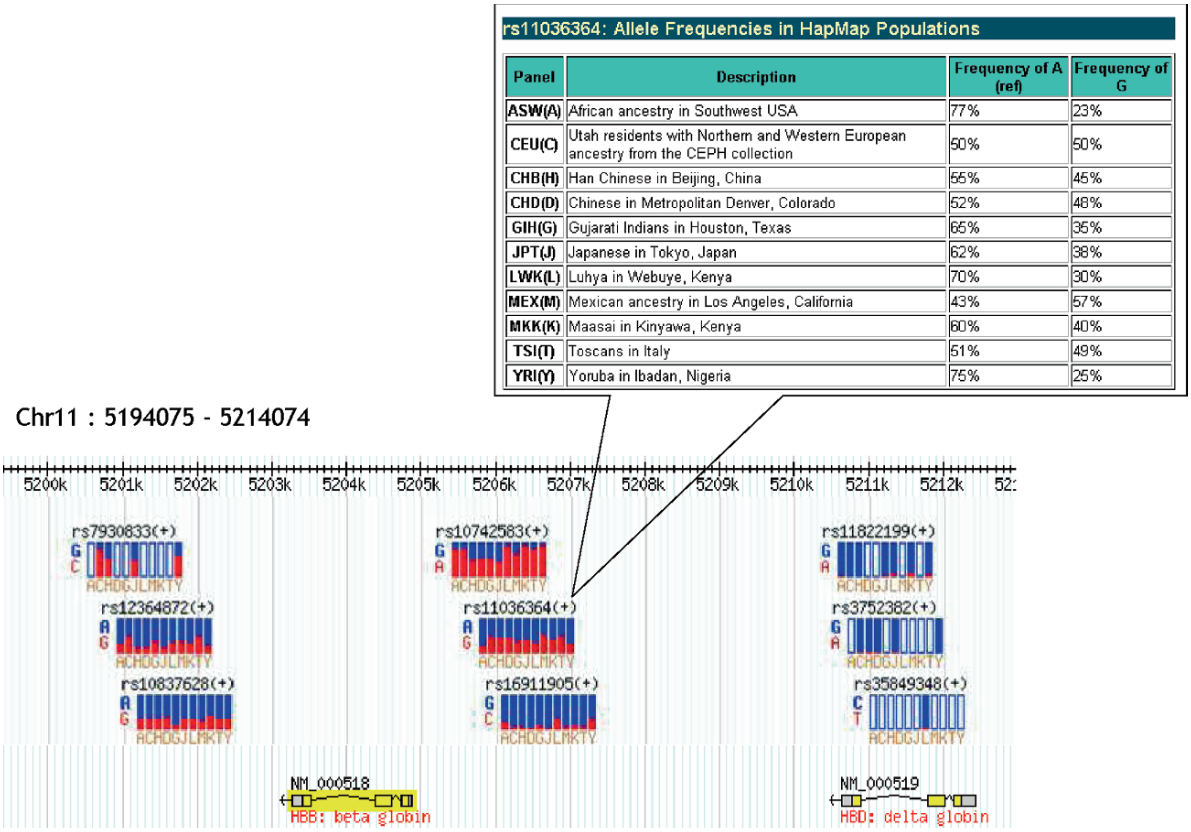


Fig. 2.15 Schematic representation of a single nucleotide polymorphism. Allele 1 has a C in the sequence, while allele 2 contains a T in the same position

gene [74] (at the time, these DNA polymorphisms were detected by restriction endonuclease digestion of DNAs and were called RFLPs, restriction fragment length polymorphisms). These polymorphic sites have two alternative alleles. In the example shown in Fig. 2.15, the depicted SNP has two alleles in the population: the blue C allele and the red T allele. The frequency of each allele could vary in different populations.

There is on average one SNP in ~1,000 nucleotides between two randomly chosen chromosomes in the population. Many of these SNPs are quite common. A common SNP is that in which the minor allele frequency (MAF) is more than 5%. On average two haploid genomes differ in ~3,000,000 SNPs. In addition, there is a large number of rare (MAF < 1%) or near-rare (MAF between 1% and 5%) SNP variants that could be identified by the genome sequencing of various individuals. The majority of heterozygous SNPs in the DNA of a given individual are relatively common in the population; on the other hand, most of the SNPs discovered in a population are more likely to be rare. The NCBI SNP database contains 25 million common and rare SNPs (http://www.ncbi.nlm.nih.gov/



rs11036364: Allele Frequencies in HapMap Populations

Panel	Description	Frequency of A (ref)	Frequency of G
ASW(A)	African ancestry in Southwest USA	77%	23%
CEU(C)	Utah residents with Northern and Western European ancestry from the CEPH collection	50%	50%
CHB(H)	Han Chinese in Beijing, China	55%	45%
CHD(D)	Chinese in Metropolitan Denver, Colorado	52%	48%
GIH(G)	Gujarati Indians in Houston, Texas	65%	35%
JPT(J)	Japanese in Tokyo, Japan	62%	38%
LWK(L)	Luhya in Webuye, Kenya	70%	30%
MEX(M)	Mexican ancestry in Los Angeles, California	43%	57%
MKK(K)	Maasai in Kinyawa, Kenya	60%	40%
TSI(T)	Toscans in Italy	51%	49%
YRI(Y)	Yoruba in Ibadan, Nigeria	75%	25%

Fig. 2.16 The genomic region of Chr11: 5,194,075–5,214,074 is shown. For each of the nine SNPs shown in the bottom, the frequency of the two alternative alleles is shown in different populations. For example, for SNP rs11036364 that maps between the HBB and HBD globin genes, the allele frequencies are shown in the *callout*. The four original populations of the HapMap project were EUR, YRI, JPT, and CHB, while the other populations were added in a later stage. Modified from http://www.hapmap.org/

SNP/snp_summary.cgi; version 130; July 2009; Fig. 2.16). Of those, ~301,000 are in the protein-coding regions of genes, and ~188,000 result in amino acid substitutions (nonsynonymous substitutions). An international project known as HapMap (http://www.hapmap.org/) [6, 34, 50] has completed the genotyping of ~4,000,000 common SNPs in individuals of different geo-ethnic origins (4,030,774 SNPs in 140 Europeans; 3,984,356 in 60 Yoruba Africans; 4,052,423 in 45 Japanese and 45 Chinese; http://www.hapmap.org/downloads/index.html.en). Additional samples from further populations have been added recently.

The information content of SNPs (and polymorphic variation in general) is usually measured by the number of heterozygotes in the population (homozygotes are individuals that contain the same variant in both alleles; heterozygotes are individuals that contain two different variants in their alleles). The number of heterozygotes is a function of MAF based on Hardy–Weinberg principles (see Chap. 10). The pattern of DNA polymorphisms in a single chromosome is called haplotype (a contraction of "haploid genotype"; allelic composition of an individual chromosome). In the example shown in Fig. 2.17 the haplotype of polymorphic sites for the paternal (blue) chromosome is CGAATC while for the maternally inherited red chromosome it is GACGAT.

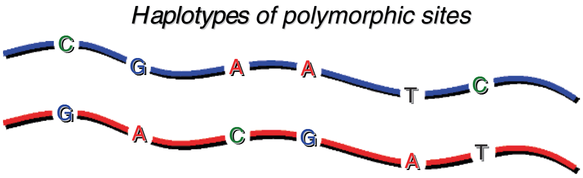


Fig. 2.17 Schematic representation of haplotype of polymorphic variants in a segment of the genome. The parental origin is shown as the *blue* (paternally-inherited) and *red* (maternally-inherited) *lines*. SNPs are shown as *letters interrupting the lines*. The haplotype is defined as the combination of SNP alleles per haploid genome

2.2.2 Short Sequence Repeats

Short sequence repeats (SSRs) are polymorphic variations due to a different number of short sequence repeat units, first described by Wyman and White [150] (then called VNTRs, variable number of tandem repeats[99]), and further elucidated by Jeffreys [72]. Most common are the dinucleotide repeats (described after the introduction of polymerase chain reaction amplification), but SSRs could be tri-, tetra-, or penta- repeats (often called microsatellites where the repeat unit $n = 1–15$ nucleotides). SSRs with longer repeat units ($n = 15–500$ nucleotides) are often termed minisatellites. These sequences comprise ~3% of the genome and there is ~1 SSR per 5 kb [84]. The most frequent dinucleotide SSR is the $(GT)_n$ with an occurrence in the genome of ~28 times per megabase, followed by the $(AT)_n$ SSR with ~19 times per megabase. The most common trinucleotide SSR is the $(TAA)_n$ that occurs approximately four times per megabase. The major advantage of SSRs (or microsatellites) is that there are more than two alleles per polymorphic site, and a large fraction of the human population is heterozygous for each SSR. Therefore, SSRs are extremely useful in linkage mapping and subsequent positional cloning for monogenic disorders [12, 17, 33] and other marking studies of the genome including the development of genomic linkage maps [43, 144]. In addition, SSRs are extensively used in forensic studies [15]. Figure 2.18 shows an example of an SSR with three alleles in the population.

2.2.3 Insertion/Deletion Polymorphisms (Indels)

This variation is due to the presence or absence of certain sequences. These sequences could be a few nucleotides, but they could also be transposons or interspersed repeats such as LINE or SINE elements [18, 112, 149]; alternatively, they could be pseudogenes [8] or other elements. Note that this category of variants is not completely separate from the next one; the arbitrary distinction is just the size of the variation in terms of base pairs. There are usually biallelic polymorphisms, which are not as common as SNPs but are useful for evolutionary studies and for the understanding of the dynamic structure of the human genome. In the example shown in Fig. 2.19, the blue sequence was inserted in the DNA and created a variant with two alleles: the blue allele 1 with insertion and the black allele 2 without.

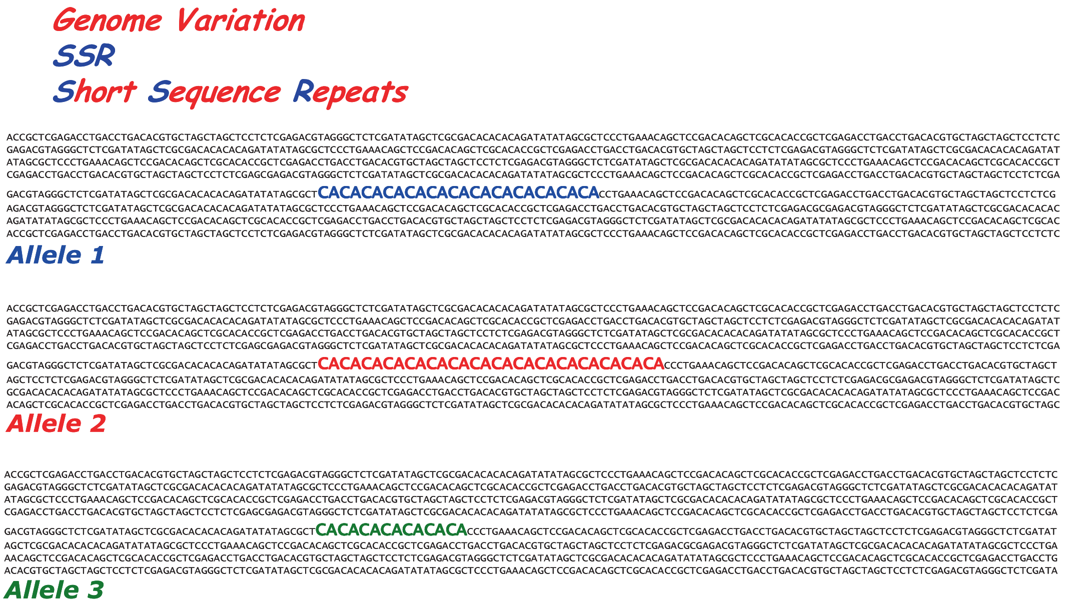


Fig. 2.18 An example of a dinucleotide SSR with three alleles in the population: the *blue* allele with $(CA)_{13}$ repeats, the *red* allele with $(CA)_{16}$, and the *green* allele with $(CA)_7$

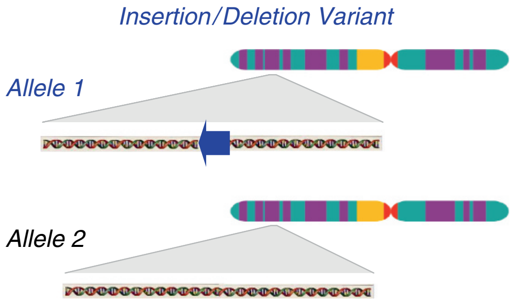


Fig. 2.19 Schematic representation of a polymorphic locus due to insertion deletion of a genomic element, shown as a *blue arrow*

2.2.4 Copy Number Variants

Copy number variant (CNV) refers to large-scale structural variation of our genome in which there are large tandem repeats of 50 kb to 5 Mb long that are present in a variable number of copies. This type of polymorphic variant includes large-scale duplications and deletions [123] (see also Chap. 3, Sect. 3.4.4). These have been known since studies of the α-globin genes in humans [54]. In the Fig. 2.20 example, allele 1 contains three copies and allele 2 five copies of a large repeat. The phenotypic consequences of some of these variants that may contain entire genes is unknown. A CNV map of the human genome in 270 individuals has revealed a total of 1,440 such CNV regions which cover some 360 Mb (~12% of the genome [79, 108]). More recent estimates using more accurate methods for precise mapping of the size of CNVs suggest that ~6% of the genome contains CNVs. A list of these variants can be found at http://projects.tcag.ca/variation/. The extent of CNV in the human genome is certainly underestimated since there are numerous additional CNVs of less than 50 kb. The current methodology for the detection of CNVs is using comparative genomic hybridization (CGH) on DNA microarrays [25]. A further improvement of this method will allow us to detect small CNVs. The most detailed currently available CNV map of the human genome was recently established by the Genome Structural Variation Consortium. This consortium conducted a CNV project to identify common CNVs greater than 500 bp in size in 20 female CEU (European ancestry) and 20 female YRI (African ancestry) samples of the HapMap project. By employing CGH arrays that tile across the assayable portion of the genome with ~42 million probes from the company NimbleGen, this consortium could map 8,599 copy number variant events. Parts of these data have been provisionally released to the scientific community and can be viewed at http://www.sanger.ac.uk/humgen/cnv/42mio/.

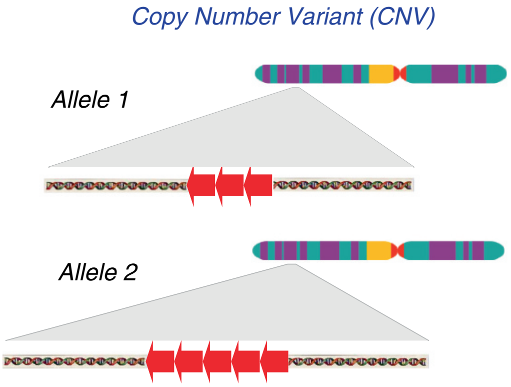


Fig. 2.20 Schematic representation of copy number variation in the human genome. For explanation, see text. Allele 1 in the population contains three copies of a sequence (*red arrowheads*), while allele 2 contains five copies

2.2.5 Inversions

Large DNA segments could have different orientation in the genomes of different individuals. These inversion polymorphisms (Fig. 2.21) predispose for additional genomic alterations [9]. An example of a common inversion polymorphism involves a 900-kb segment of chromosome 17q21.31, which is present in 20% of European alleles but it is almost absent or very rare in other populations [129]. These variants are difficult to identify and most of them have been detected by sequencing the ends of specific DNA fragments and comparing them with the reference sequence [79, 134].

2.2.6 Mixed Polymorphisms

There are combinations of repeat size variants and single nucleotide variants. Figure 2.22 depicts such an example; the repeat units of an SSR contain a SNP and, thus, even alleles with the same repeat number

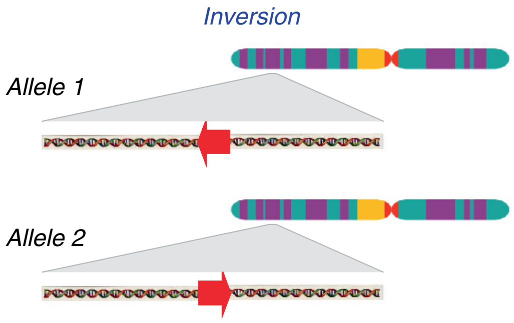


Fig. 2.21 Schematic representation of a polymorphic inversion shown as a *red arrowhead*

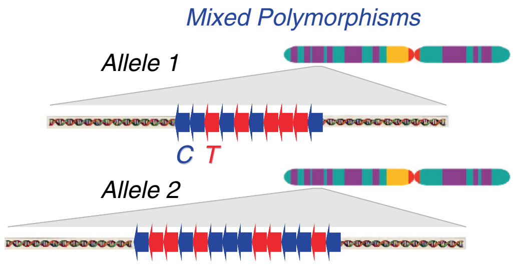


Fig. 2.22 Schematic representation of a highly polymorphic region of the genome with a mixed polymorphism that includes SNPs in the copies of CNVs or SSRs. The copies of the repeat are shown as *arrowheads*; the *blue/red* color of the repeats designates the SNP in them (*blue* for C and *red* for or T)

could be distinguished based on their exact DNA sequence [71]. These highly polymorphic systems could serve as "recognition barcodes" in humans.

2.2.7 Genome Variation as a Laboratory Tool to Understand the Genome

DNA variants, besides their functional importance in health and disease, are very useful in human genetics research because they serve as genomic markers for a variety of studies. Some of the uses of DNA variants are to:

1. Create linkage (genetic) maps of human chromosomes [1, 148]. This has allowed the initial mapping of the human genome and it was a prerequisite for the sequence assembly.
2. Map the genomic location of monogenic phenotypes to human chromosomes by linkage analysis [58, 81]. A large number of such phenotypes have been mapped to small genomic intervals because of the genotyping of members of affected families. Positional cloning of pathogenic mutations was subsequently possible.
3. Map the genomic location of polygenic phenotypes to human chromosomes by genomewide linkage and association studies [4, 20, 119].
4. Allow fetal diagnosis and carrier testing by linkage analysis of the cosegregation of a polymorphic marker and the phenotype of interest [10, 21].
5. Perform paternity and forensic studies [52]. A whole field was developed mainly with the use microsatellite SSR variants [49, 51].
6. Study genome evolution and origin of pathogenic mutations [115, 116].
7. Study the recombination rate and properties of the human genome [28, 93].
8. Study the instability of the genome in tumor tissues [5].
9. Identify loss-of-heterozygosity in human tumors [27, 47].
10. Study uniparental disomy and thus help with understanding genomic imprinting [100, 128].
11. Study parental and meiotic origin, and decipher the mechanisms of nondisjunction [11, 13, 14].
12. Study population history and substructure [110, 132].

The chapters that follow include further discussions on different aspects (including evolution, phenotypic consequences, and disease susceptibility) related to the most precious human genome variability.

Acknowledgments I thank the members of the laboratory, past and present, for discussions, ideas, debates, and data. I also thank my mentors and my students for the learning process.

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Chromosomes 3

Michael R. Speicher

Abstract The study of human chromosomes started in the late 19th century with first observations on cytological preparations and the area of cytogenetics evolved. Cytogenetics is the study of the structure, function and evolution of chromosomes. Cytogenetics is indispensable for both routine diagnostics in clinical genetics and research. To date cytogenetics is characterized by rapidly growing application of molecular biological techniques which have unraveled many mechanisms involved in fundamental steps during meiosis and mitosis. Furthermore, especially array-technologies allowed a spectacular increase in resolution, which has dramatically changed our view about genome plasticity. As a consequence of this new spectrum of methods cytogeneticists have now new options to improve diagnostics and to address basic research issues. Here basic principles of chromosome structure and biology, latest developments and future perspectives are described.

Contents

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3.1 History and Development of Human Cytogenetics

The chromosome theory of Mendelian inheritance was launched in 1902 by Sutton and Boveri. In the same year Garrod, establishing the autosomal-recessive mode of inheritance for alkaptonuria and commenting on metabolic individuality in general, created the paradigm of “inborn errors of metabolism.” Simple modes of inheritance were soon established for many other human disorders. A few years later, Bridges [33] examined in *Drosophila* the first case of a disturbance in chromosome distribution during meiosis and named it “nondisjunction.” Cytogenetics of animals and plants flourished during the first half of the twentieth century, and many important phenomena in the field of cytogenetics were discovered during this period. Moreover, cytogenetic methods helped to elucidate many basic laws of mutation.

However, the age of human cytogenetics did not begin until the 1950s, when Tjio and Levan [235] and Ford and Hamerton [79] established the diploid human chromosome number of 46. Lejeune et al. [142] discovered trisomy 21 in Down syndrome, while Ford et al. [80] and Jacobs and Strong [116] established that Turner and Klinefelter syndromes were caused by X-chromosomal anomalies.

The late arrival of human cytogenetics is usually ascribed to shortcomings in the methods used to prepare chromosomes. Indeed, the jumbled masses of chromosomes in old illustrations demonstrate the difficulties encountered by the pioneers who tried to count human chromosomes. Still it is hard to conceive that the development of more adequate methods would have been delayed for such a long period had the cytogeneticists realized there are human anomalies awaiting explanation, and few human geneticists considered the possibility that certain anomalies might be due to chromosomal aberrations.

3.1.1 First Observations on Human Mitotic Chromosomes

It could be said that research on human cytogenetics began with the work of Arnold [13] (Fig. 3.1), Flemming [78], and Hansemann [100], who were the first to examine human mitotic chromosomes. Owing to their affinity for certain stains, Waldeyer [244] dubbed the threadlike structures “chromosomes,” which is derived from the Greek words chroma (=color) and soma (=body) and means “colored body.”

A report with a strong impact lasting several decades was that of Painter in the 1920s [176]. Painter was the leading cytogeneticist of his time. When he examined chromosome preparations derived from testicles of three individuals in first meiotic divisions he was able to demonstrate that the sex was bivalent, consisting of the X and Y chromosomes, which at anaphase migrated to

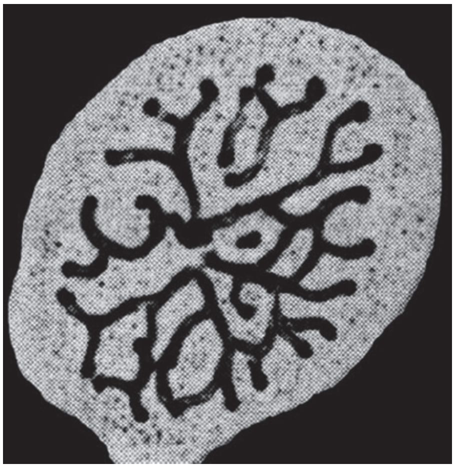


Fig. 3.1 One of the first images of human chromosomes made by the German pathologist J. Arnold in 1879. Arnold examined carcinoma and sarcoma cells because their voluminous nucleus facilitated analysis. The drawing shows a human sarcoma cell. From [13]

opposite poles. In a preliminary report he had described the chromosome number as 46 or 48, but in the definitive report [176] he had decided in favor of 48 chromosomes (Fig. 3.2). Surprisingly, in subsequent decades, a chromosome number of 48 in humans was supported in a number of reports [85]. The problem of the continuing incorrect chromosome count following Painter has been ascribed to a “preconception” [133]. The number was thought to be 48, so subsequent investigators did their utmost possible to make their counts 48.

However, two technical difficulties impeded further progress:

1. Sectioning by the usual histological techniques often disturbed mitoses.
2. The chromosomes tended to lie on top of each other and even to clump together.

These difficulties were ultimately overcome by:

(a) The use of suspensions of intact cells rather than of histological sections.

(b) The use of spindle poisons such as colchicine. Colchicine is an alkaloid derived from the autumn crocus, *Colchicum autumnale*. Because spindle formation is inhibited in cells during mitosis, chromosomes cannot separate during anaphase. The use of colchicines was introduced by Blakeslee and Avery [28] and Levan [145]. By arresting cells in metaphase, colchicine increases the number of metaphase spreads. Furthermore, colchicine increases chromosome condensation. Hence, varying the exposure time of cells to colchicine allows determination of the length of chromosomes.

(c) The subjection of cells to a brief treatment with a hypotonic solution, causing them to swell and burst, thus spreading out the chromosomes for better definition. The hypotonic treatment was described by a Russian group as long ago as in 1934 [262]. However, this discovery was abandoned and the treatment had to be rediscovered in 1952. The hypotonic shock technique paved the way for easy chromosome counting [110, 111]. Interestingly enough, even 30 years later Painter’s estimate of 48 was so strongly imprinted on investigators’ minds that in the first study on human chromosomes using the new technique the human chromosome number was reported as 48 [110].

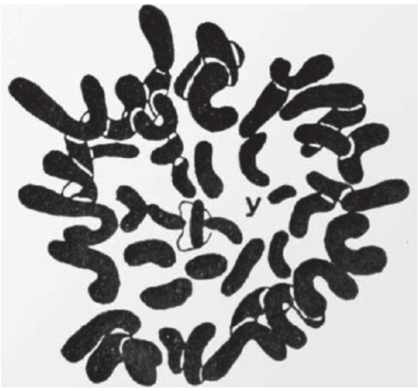


Fig. 3.2 Camera lucida drawing of a human spermatogonial metaphase made by Theopilus S. Painter. From this drawing Painter concluded that humans have 48 chromosomes in their cells. From Springer-Verlag 1979

3.1.2 An Old Error is Corrected and a New Era Begins

In the summer of 1955 Levan, a Swedish cytogeneticist visited Hsu in New York and learned the technique of squash preparation using hypotonic shock. He and Tjio then improved the technique by shortening the hypotonic treatment and by adding colchicine. They examined lung fibroblasts of four human embryos. To their surprise they found a chromosome number of 46 in most of 261 metaphases [235]. Figure 3.3 shows one example. This evidence was soon supplemented by Ford and Hamerton [18].

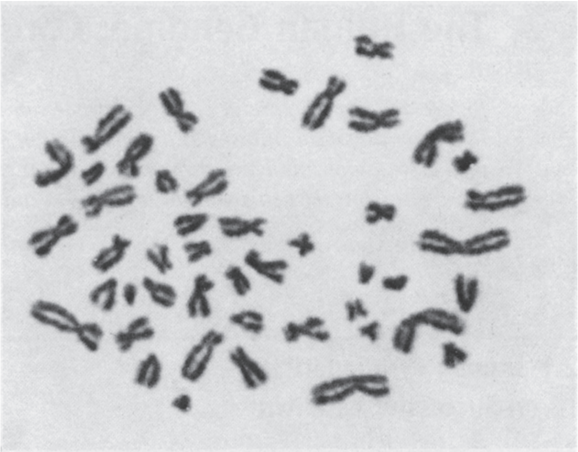


Fig. 3.3 A metaphase of a human embryonic lung fibroblast grown in vitro. The image is from the first report in which the human chromosome number was established as 46. From [235], with permission from Wiley-Blackwell, from the 3rd edition of this work

With these results the stage was set for the development of clinical cytogenetics. Still, it was almost another 3 years before the first abnormal karyotypes in humans were reported.

3.1.3 Birth of Human Cytogenetics 1956–1963

Lejeune et al. [142] reported chromosome studies from fibroblast cultures in nine children with Down syndrome. Fifty-seven diploid cells were regarded as technically perfect. In all of them the chromosomes numbered 47. The supernumerary chromosome was described as small and "telocentric." Meiotic nondisjunction was suggested as the most likely explanation for the additional chromosome. More aspects of Down syndrome are described in Sect. 3.6.1 and in Chap. 23.6.

As soon as in 1949 Barr and Bertram discovered the "X chromatin," an intranuclear body 0.8–1.1 µm in size, which is commonly located at the periphery of the interphase nuclei of females and is not present in males [22]. The discovery was incidental, since it originated in an investigation of the effects of fatigue on the central nervous system in cats. What first seemed to be a sex difference only in the neurons of cats turned out to be a normal finding characteristic of the nuclear inheritance of female mammals, including human females. Corresponding structures, the drumsticks, were found by Davidson and Smith [56] in polymorphonuclear neutrophil leukocytes. The obvious next step was the examination of X chromatin in cells of patients with disturbances in sexual development. In this sample, most male patients with the Klinefelter syndrome turned out to be X chromatin positive in spite of their predominantly male phenotype [171], whereas most female patients with the Turner syndrome were X chromatin negative – again in contrast to their female phenotype. If the X chromatin was directly related to the X chromosomes, these findings pointed to X chromosome anomalies in these two syndromes.

This suspicion was reinforced when the frequency of X-linked color vision defects in patients with Klinefelter syndrome was found to be lower than in normal males but much higher than would be expected in XX females [190].

This situation was explained when Jacobs and Strong [116], examining the chromosomes from bone marrow mitoses in Klinefelter patients, found 47 chromosomes, although both parents had a normal karyotype. The supernumerary chromosome belonged to the group of chromosomes including X chromosomes; the karyotype was tentatively identified as XXY.

Soon after this first report, the XXY karyotype in Klinefelter syndrome was confirmed in many more cases, and it is now known as the standard karyotype in this condition. At the same time, the result in the Klinefelter syndrome was complemented by chromosome examinations in another syndrome in which a discrepancy between phenotypic and nuclear sex seemed to exist: Turner syndrome. Ford et al. [80] showed that the karyotype had only 45 chromosomes, obviously with only one X and no Y chromosome. A third anomaly with 47 chromosomes and three X chromosomes was soon described in a slightly retarded woman with a dysfunction of the sexual organs [117]. Both Klinefelter and Turner syndromes will be discussed further in Sect. 3.6.3.3 and in Chap. 23.6.

Two autosomal trisomies, later identified as trisomies 13 and 18, were described by Patau et al. [178] and Edwards et al. [68]. Furthermore, in 1960 Peter Nowell [172] discovered that phytohemagglutinin stimulated white blood cells to divide, another tremendous stimulus to human cytogenetics. In the same year he and David Hungerford discovered a minute chromosome, later named the Philadelphia chromosome, which was regularly found in peripheral blood in human chronic myeloid leukemia [173]. The first deletion syndrome, cri-du-chat syndrome, caused by a deletion on the short arm of chromosome 5, was observed by Lejeune et al. [143].

3.1.4 Introduction of Banding Technologies from the Late 1960s to the Present

However, the exact identification of the homologous chromosome pairs or of structural rearrangements was still not possible. The only means of "identifying" chromosomes was by noting size differences and the position of the centromere, which is insufficient to distinguish chromosomes with a similar morphology. More detailed chromosome analyses had to await the discovery of chromosome banding. When fluorochromes coupled to an alkylating agent, such as quinacrine mustard, were used a highly characteristic fluorescence pattern for each individual chromosome could be achieved and a complete human karyotype could be presented [40, 41]. However, owing to its practical simplicity, Giemsa banding rapidly replaced quinacrine banding. To this day, Giemsa banding remains the most widely used banding procedure in routine chromosome analyses in most laboratories throughout the world. The improvement in the resolution of chromosome analyses achieved by banding analysis is reflected in the fact that 13 years after the first description of the Philadelphia chromosome, banding analysis revealed that the Philadelphia chromosome was the result not of a deletion in chromosome 22, but of a translocation between chromosome 22 and chromosome 9 [199].

3.1.5 The Birth of Molecular Cytogenetics in the Late 1960s

The first application of molecular techniques to chromosome cytology was based on the perception that sequences that were complementary to each other could anneal, or hybridize, and form much more stable complexes than noncomplementary sequences. The first in situ hybridization, done by Joe Gall and Mary Lou Pardue [83], applied DNA-RNA hybridization to locate the genes coding for ribosomal RNA. A similar in situ hybridization technique had been developed independently in the laboratory of Max Biernstiel [123]. However, these early in situ hybridizations depended on radioactive detection, which was prone to high background and was slow, the film exposure often taking several days or even weeks. Attempts to overcome these problems included the use of fluorescently labeled antibodies to recognize specific RNA-DNA hybrids [200].

3.1.6 Molecular Cytogenetics or Fluorescence In Situ Hybridization 1980 to Date

A more straightforward approach employed the chemical coupling of a fluorophore to an RNA probe for rapid and direct visualization. Such a "fluorescent *i*n *s*itu *h*ybridization" (FISH) was first realized in 1980 [24]. The coupling of fluorochrome to a DNA or RNA probe is often referred to as "direct labeling." In contrast, "indirect labeling" means the enzymatic or immunological detection of tags incorporated into a probe. The synthesis of modified nucleotide derivatives containing a biotin label, which could be incorporated by polymerases into probes, was instrumental for the development of indirect labeling techniques [140].

Nonetheless, the successful hybridization of complex probes was restricted by the presence of repetitive sequences, which occur ubiquitously in the genome and which are usually present in complex probes. These repetitive sequences were finally suppressed by the addition of an excess of unlabeled genomic DNA (in first experiments) or Cot-1 DNA to the hybridization mix [138]. With use of the suppression technique the painting of entire chromosomes rapidly became possible [52, 146, 187] and FISH became applicable in clinical cytogenetics. A distinct advantage of FISH is that DNA probes can be visualized in intact interphase nuclei, an approach referred to as "interphase cytogenetics" [51]. In the years since then, a clear aim of the continuing development of cytogenetic methods has been to increase the resolution at which chromosome rearrangements can be identified. This has been achieved by advances in the two crucial elements of cytogenetic analysis, i.e., the probe and the target. Target resolution has improved from metaphase chromosomes (resolution ~5 Mb), through interphase nuclei (50 kb to 2 Mb) and DNA fibers (5–500 kb) to the use of DNA microarrays offering resolutions to a single nucleotide. Simultaneously, probe development has also advanced, to best utilize the improvements in target resolution [222].

3.1.7 Array Technologies: New Dimensions in Resolution from 1997 to Date

The introduction of array technologies increased resolution – depending on the array platform – to the single nucleotide level and is definitively blurring the traditional distinction between cytogenetics and molecular genetics. Since the first array applications [188, 220], a multitude of various array platforms has been developed. One of the most significant findings elucidated by array techniques is the wide scope and prevalence of copy number changes (CNVs) in the human genome [196]. The unearthing of association of CNVs with biological function, recent human evolution, and common and complex human disease is at present one of the most fascinating areas in human genetics.

3.2 Organization of Genetic Material in Human Chromosomes

Chromosomes consist of a number of different building blocks, such as heterochromatin and euchromatin, centromeres, and telomeres. The main features of these building blocks are described below.

3.2.1 Heterochromatin and Euchromatin

Chromosomes are usually not visible in the nuclei of nondividing cells, because the chromatin in interphase nuclei is so densely packed that single chromatin threads are not directly detectable. However, some parts of chromatin may become visible after staining, giving rise to the distinction between "heterochromatin" and "euchromatin" [105]. Heterochromatin refers to chromosomal segments that remain compact and can be stained and thus remain visible during the entire cell cycle. In contrast, euchromatin decondenses in interphase, to the extent that it becomes invisible during late telophase and subsequent interphase. From a functional point of view, heterochromatin represents chromosomal segments with few active genes or none at all. These regions are dominated by repetitive DNA sequences. Our understanding of the establishment and maintenance of these functional domains has improved recently. This distinction is not only based on DNA sequence but also on epigenetic mechanisms, such as methylation patterns and histone modifications, as discussed below.

Heterochromatin can be more finely categorized as constitutive or facultative, depending on whether or not there is a consistent relationship between the DNA sequence involved and a compact organization across cell types and differentiation states. For example, usually all cells of an individual will package the same regions of DNA in *constitutive heterochromatin*. Constitutive heterochromatin can be observed in human cells close to the centromeres of chromosomes 1, 9, and 16. In addition, male cells have a large block of heterochromatin on the long arm of the Y chromosome. Constitutive heterochromatin is usually encountered around the chromosome centromere and near telomeres.

In contrast to constitutive heterochromatin, which is identical in all cells of a body, *facultative heterochromatin* refers to sequences that may be densely packaged in one cell, thus forming heterochromatin, but packaged in euchromatin in another cell. The best-known example of facultative heterochromatin is X-chromosome inactivation in female cells (see also Sect. 3.6.3.2): One X chromosome is packaged in facultative heterochromatin and thus to a large extent, but not completely, silenced. The other X chromosome is packaged in euchromatin. Such mechanisms allow heritable but reversible changes in gene expression without alterations in DNA sequence. Thus, epigenetic "on-off" transcriptional states are largely dependent on the position of a gene within an accessible (euchromatic) or an inaccessible (heterochromatic) chromatin environment (for further discussion see the next section).

In summary, while heterochromatin was first defined on morphological grounds [105], it is now defined in terms of a "histone code" of posttranslational modifications that influence transitions between chromatin states and the regulation of transcriptional activity (see below for further details).

3.2.2 From DNA Thread to Chromosome Structure

3.2.2.1 DNA Condensation

The human genome has a size of approximately 3.4×10^9 base pairs and harbors about 25,000 genes. The total length of DNA in the haploid chromosome complement in nondividing human cells is about 1 meter. However, the total length of the human haploid chromosome complement in metaphase cells is merely 115 μm.

As a consequence, chromosomes are visible as individual structures only during mitosis, with individual chromosomes ranging in size from 3 to 7 μm. These numbers illustrate that considerable packing and unpacking occurs during cell division. Furthermore, there is a direct relationship between higher order chromatin folding and transcriptional control. Hence,

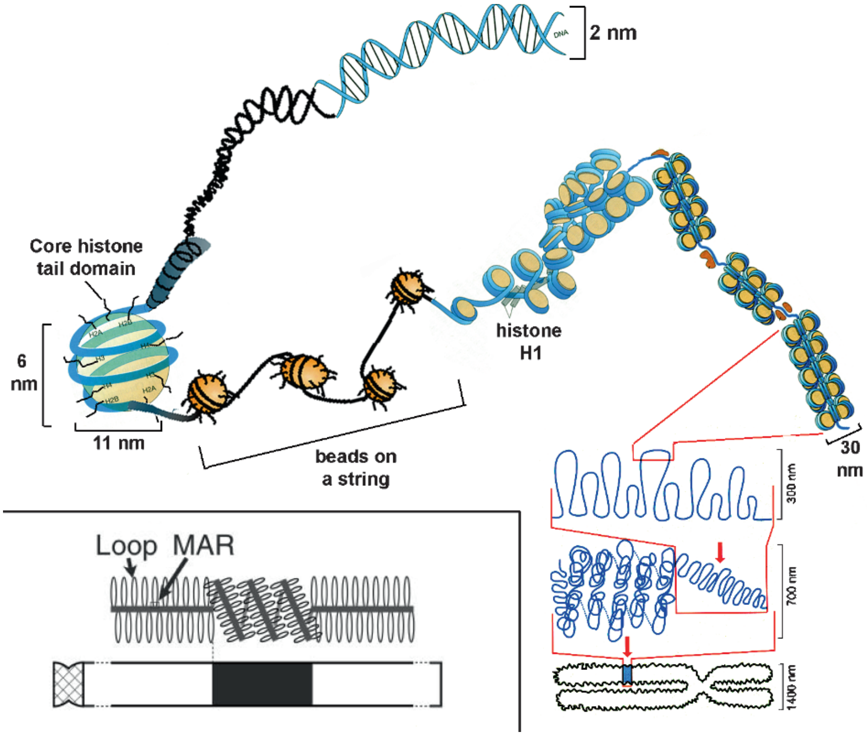


Fig. 3.4 Folding and packing of chromosomal DNA. Scheme of different levels of packing of DNA from the double helix to a metaphase chromosome. (Modified from [109]). *Inset:* Model of a metaphase chromatin structure. Adapted from [202]

the basic building blocks of chromatin determine the interplay between chromatin structure and transcription. Several levels of packing units of DNA can be distinguished. These various building blocks of chromatin are briefly reviewed here (Fig. 3.4).

The thickness of the DNA double helix is estimated at about 2 nm. The fundamental, basic subunit of chromosome structure is the nucleosome, which is made of DNA and histones and which divides the DNA into units of approximately 200 bp in length. At the molecular level, each chromosome is a repetition of nucleosomes and shorter segments of DNA that link the individual nucleosomes. Histones are proteins with a high proportion of positively charged amino acids, allowing them to attach themselves firmly to the negatively charged DNA double helix. A nucleosome consists of 147 bp of DNA wound 1.75 times around a core histone octamer. Such a core histone octamer consists of two copies of each histones H2A, H2B, H3, and H4. Every core histone contains two separate functional domains: a signature "histone-fold" motif sufficient for both histone-histone and histone-DNA contacts within the nucleosome and NH_2-terminal and COOH-terminal "tail" domains containing sites for posttranslational modifications (such as acetylation, methylation, phosphorylation, and ubiquitination). It is these posttranslational modifications, largely located in the N-terminal domains of the histone proteins, which encode most of the epigenetic information specifying chromatin structure and function. The histone octamer forms a cylinder 11 nm in diameter and 6 nm in height. Without histones the chromosome skeleton, surrounded by numerous threads corresponding to the DNA double helix, becomes visible under the electron microscope [180] (Fig. 3.5).

Nucleosomes are organized on a continuous DNA helix in linear strings separated by 10–60 bp of linker DNA. On electron-microscopic photographs they have a "string of beads" appearance. Between nucleosomes the DNA is bound to a fifth histone, histone H1, often referred to as linker histone, which binds DNA as it enters and exits the nucleosome to stabilize two complete turns of the DNA around the histone octamer. Histone tail-mediated nucleosome-nucleosome interaction leads to formation of the 30-nm fiber, which represents a secondary level of compaction [109].

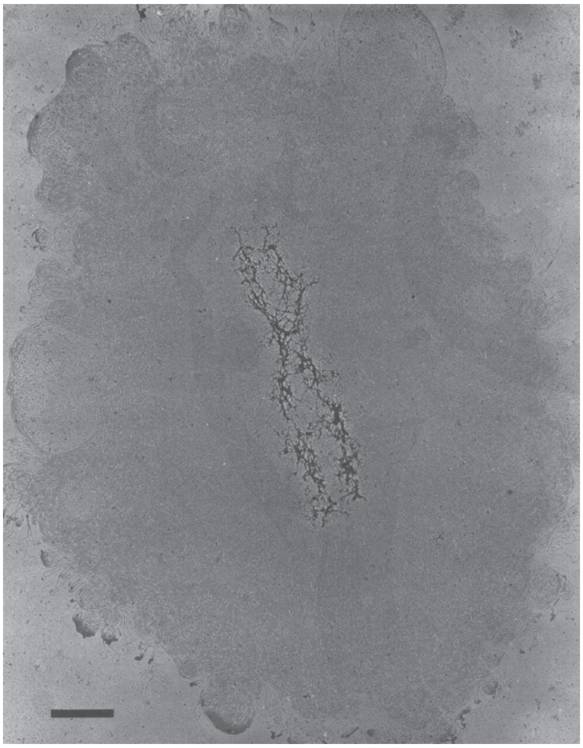


Fig. 3.5 Electron micrograph of a histone-depleted metaphase chromosome from HeLa. The depicted chromosome consists of a central, densely staining scaffold or core surrounded by a halo of DNA extending 6-9 μm outward from the scaffold. The low magnification makes it difficult to see the individual DNA strands except along the edge of the DNA halo. *Bar* 2 μm. From [120], reprinted by permission from Macmillan Publishers Ltd: *Nature Genetics*, copyright 2001

3.2.2.2 Histone Modifications Organize Chromosomal Subdomains

Besides being involved in nucleosome formation, histones are crucial for the functional organization of chromosomal subdomains, e.g., differentiation between euchromatin and heterochromatin. A "histone code" [225] hypothesis has been proposed. This predicts that modifications of histone N-termini, such as acetylation, phosporylation, or methylation, are fundamental mechanisms for the induction and stabilization of distinct chromosomal subdomains (reviewed in [121]). This is shown schematically in Fig. 3.6. In particular, the histone H3 lysine 9 methylation is highly characteristic for the pericentric heterochromatin and differs from the H3-K9 methylation present in other chromosomal regions [183, 184]. This will be discussed in further detail in the next section on centromeres.

Thus, histone modifications result in condensed chromatin, i.e., heterochromatin, or in extended chromatin representing euchromatin. The level of condensation also reflects the transcriptional activity of the corresponding DNA segment: DNA wound around a nucleosome is inactive and unreactive. Sequence-specific DNA-binding proteins are only found between nucleosomes. There are hundreds of sequence-specific DNA-binding proteins that recognize short DNA segments.

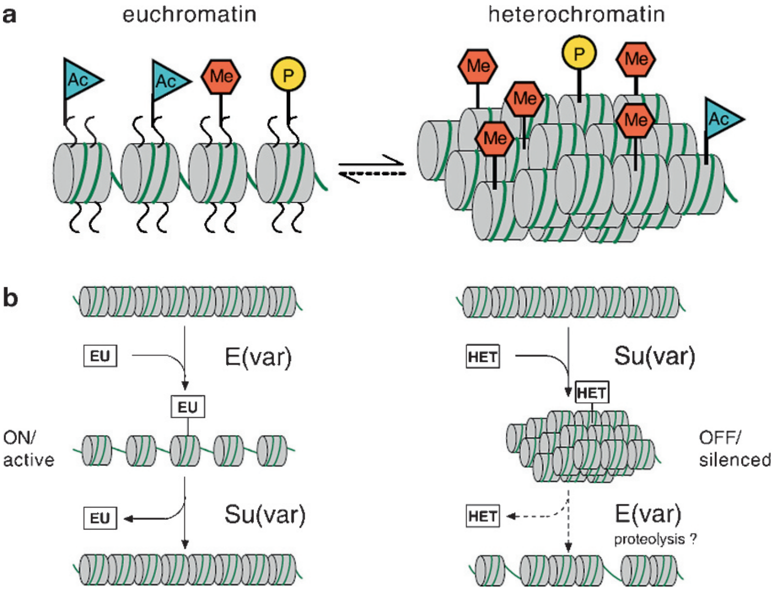


Fig. 3.6 (**a**, **b**) Models for euchromatin and heterochromatin histone tail modifications. (From [121]) (**a**) Schematic representation of euchromatin and heterochromatin as accessible or condensed nucleosome fibers containing acetylated (*Ac*), phosphorylated (*P*), and methylated (*Me*) histone NH_2 termini. (**b**) Model for adding euchromatic (*EU*) or heterochromatic (*HET*) modification marks onto a nucleosomal template. The position of a gene in an accessible (euchromatic) or an inaccessible (heterochromatic) chromatin environment has also been referred to as position-effect variegation (*PEV*). PEV modifiers can enhance variegation [*E(var)*] or suppress variegation [*Su(var)*]. The *left-hand side* depicts an example in which a region is made accessible, while the *right-hand side* shows the reverse, i.e., transfer into inaccessible heterochromatin. Both processes are reversible

The 30-nm fibers form part of a chromosome segment about 300 nm in diameter. A further packing on metaphase chromosomes is represented by a thickened segment, also called a condensed chromosomal segment, with a diameter of 700 nm. A multitude of these segments make up the chromatids of metaphase chromosomes (Fig. 3.4).

3.2.2.3 Chromatin Diseases

Mutations in genes encoding proteins that control the structure of chromatin have effects on the expression of a potentially large number of genes and may therefore cause diseases. An example are mutations in the gene *ATRX* on the X chromosome at Xq13, which cause in males a syndrome characterized by a mild form of alpha-thalassemia (HbH disease), mental retardation, facial dysmorphisms, and microcephaly. *ATRX* is a helicase that can unwind DNA double helices and is part of large multiprotein complexes controlling the local structure of chromatin. It is likely that *ATRX* is involved in establishing and maintaining repressive chromatin structures [89]. Another example for a chromatin disease is the neurological disorder Rett syndrome, caused by mutations in the human methyl-CpG-binding protein gene *MECP2*, which closely interacts with *ATRX* [164].

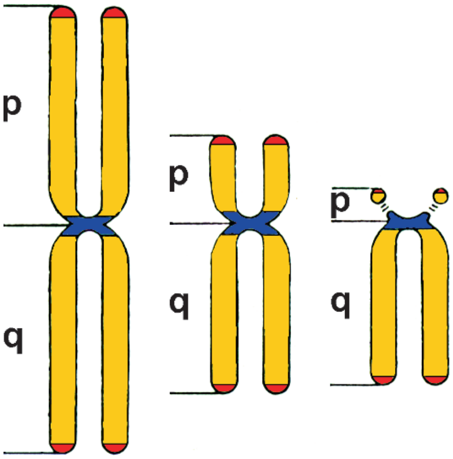


Fig. 3.7 Human metaphase chromosomes: metaphase chromosomes have two chromatids (also termed sister chromatids; *yellow*), which are held together at the centromere (*blue*). In metaphase chromosomes the centromere is usually readily visible as a constriction. The centromere divides each of the chromatids into two chromosome arms. The short arm is referred to as p-arm, and the long arm, as q-arm. Chromosomes with centromeres close to the middle as shown on the *left hand side* have arms of equal length and are known as metacentric chromosomes. If the centromere is not centrally located resulting in arms, which are unequal in length (as shown in the *middle*), the chromosome is termed submetacentric. A chromosome with a centromere very close to one end is called acrocentric. The p-arm of acrocentric chromosomes is often referred to as satellite. The regions at both ends of the chromosome are the telomeres (*red*)

3.2.3 Centromeres and Kinetochores

Each chromosome contains a specialized region known as its centromere. The position of each centromere divides the chromosome into two arms, and its location is characteristic for a given chromosome (Fig. 3.7).

3.2.3.1 Function of Centromeres

Centromeres are essential for normal segregation of chromosomes in both mitotic and meiotic cells. In mitosis, a proteinaceous structure, the kinetochore, assembles at the surface of the centromeres. The kinetochore serves as the attachment site for spindle microtubules and the site at which motors generate forces to power chromosome movement (Fig. 3.8a). The inner kinetochore forms the interface with chromatin, while the laminar outer kinetochore domain (frequently misnamed a "plate") forms the interaction surface for spindle microtubules [43]. Unattached kinetochores are also the signal generators for the mitotic checkpoint, which arrests mitosis until all kinetochores have correctly attached to spindle microtubules, thereby representing the major cell cycle control mechanism protecting against loss of a chromosome (aneuploidy). Thus, centromeres are active components in microtubule capture, stabilization, and empowerment of chromosome movements essential to proper segregation. More than that, they are the signaling elements for controlling cell cycle advance through mitosis.

3.2.3.2 Structure of Centromeres

Human centromeres have sizes approaching 10 Mb. At DNA level centromeres consist of so-called alpha satellite DNA, which is defined by a 171-base pair motif repeated in a tandem head-to-tail fashion [246]. Human

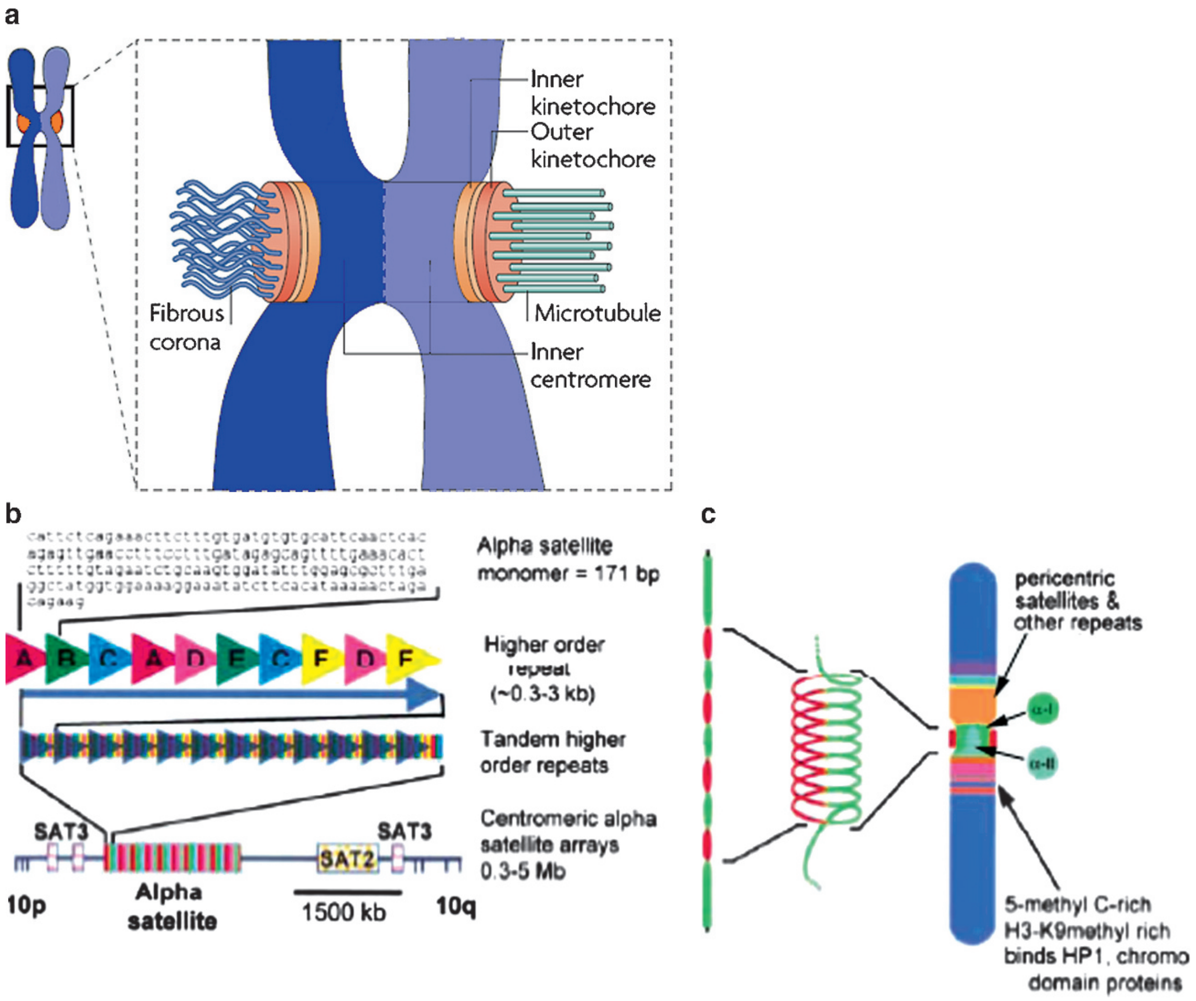

a
Inner kinetochore
Outer kinetochore
Fibrous corona
Microtubule
Inner centromere
b
cattctcagaaacttctttgtgatgtgtgcattcaactcac
agagttgaacctttcctttgatagagcagttttgaaacact
ctttttgtagaatctgcaagtggatatttggagcgctttga
ggcctatggtggaaaaggaaatatcttcacataaaaactaga
cagaag
Alpha satellite monomer = 171 bp
A B C A D E C F D F
Higher order repeat (~0.3-3 kb)
Tandem higher order repeats
SAT3
SAT3
SAT2
Centromeric alpha satellite arrays 0.3-5 Mb
10p
Alpha satellite
1500 kb
10q
c
pericentric satellites & other repeats
α-I
α-II
5-methyl C-rich H3-K9methyl rich binds HP1, chromo domain proteins

Fig. 3.8 (**a**) A schematic of a mitotic chromosome with paired sister chromatids and localization of centromere and kinetochore. The right chromatid is attached to microtubules and the chromatid on the *left* is unattached. Without microtubule attachment, the outer kinetochore shows a dense array of fibers, called the fibrous corona. (From [43], reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Molecular Cell Biology*, copyright 2008) (**b**) Organization of centromeric DNA. The hierarchic organization of an α satellite DNA is illustrated, with a 171-bp monomer sequence shown at the *top*. Monomer sequences are iterated with nucleotide sequence variations to form a higher order repeat (*colored arrows*), which itself is tandemly iterated with high (>99%) sequence conservation to form extensive arrays of higher order repeats. At the *bottom* is a diagram of the centromere region of chromosome 10, illustrating the ~2-Mb α-satellite array with surrounding pericentric satellite arrays (SAT2 and SAT3). (From [47], with permission from Elsevier) (**c**) Folding centromeric chromatin in mitotic chromosomes. CENP-A is interspersed with H3-containing nucleosomes. The centromeric chromatin fiber is folded or coiled to achieve the polarized distribution of CENP-A and H3 sites in mature mitotic kinetochores [47]. In this idealized centromere region CENP-A (*red*) assembles onto regular α-I satellite sequences (*green*). α-II satellite sequences (*teal blue*) are localized in the interior or central domain of the centromere. The centromere is flanked by pericentric satellite sequences (*orange*, *purple*). From [47], with permission from Elsevier

and great ape chromosomes contain alpha satellite organized hierarchically into higher order repeat arrays in which a defined number of alpha satellites have been homogenized as a unit to yield large chromosome-specific arrays that span several megabases [248]. The alpha satellite DNA is flanked by heterochromatin (Fig. 3.8b). Primate centromeres frequently have two major α-satellite families adjoining each other: a highly regular α-I and an α-II, which varies widely in monomer sequences and repeat structure.

The centromere challenges the classic view of a genetic locus. Usually a chromosomal locus is defined by its DNA sequence and its function is contained with the information content present in the primary sequence, e.g., recognition sites for DNA-binding proteins. However, the nature and specification of the centromere is epigenetically determined. As a consequence, chromatin in the centromere differs biochemically from the remainder of the genome in some very fundamental ways.

Chromatin is the key feature, and the centromere domain is built on a distinct type of nucleosome found nowhere else in the genome. Histone H3 is replaced by a divergent (50% identity) homolog usually referred to as CENP-A [218]. CENP-A is interspersed with H3-containing nucleosomes, and both domains are required for complete centromere function (Fig. 3.8b). H3-containing nucleosomes within centromeric chromatin are hypoacetylated, which is typical of heterochromatin, and enriched in dimethylated lysine 4, a modification typically associated with potentiated regions of chromatin [228]. Centromeric heterochromatin containing CENP-A is flanked by chromatin enriched in dimethylated lysine 9, which separates the centromeric heterochromatin from the pericentromeric constitutive heterochromatin. Constitutive heterochromatin is demarcated by enrichment in trimethylated lysine 9 [136].

CENP-A is bound primarily to α-I satellite sequences, at the surface of the chromosome (Fig. 3.8c). In contrast, α-II satellite is localized in the interior or central domain of the centromere, where components such as INCENP, aurora B, and cohesin are concentrated. A core domain built around CENP-A and centromere-specific chromatin-binding proteins establishes the kinetochore-forming component of the centromere, while flanking domains are enriched in proteins involved in chromatid cohesion.

3.2.3.3 Centromeres and Human Diseases

Centromeres are important for accurate chromosome segregation. Centromere impairment may significantly increase the number of aneuploid cells, which in turn is implicated in both aging and cancer. A paradigm for a centromere disease is the ICF (*i*mmunodeficiency, *c*entromeric instability and *f*acial anomalies) syndrome, which is a rare autosomal recessive disease caused by mutations in the DNA methyltransferase gene DNMT3B. These mutations cause demethylation of cytosine residues in classical satellites 2 and 3 at juxtacentromeric regions of these chromosomes, which causes centromeric instability visible during chromosome analysis as formation of radiated chromosomes, especially of chromosomes 1, 9, and 16 (see also Sect. 3.7.4).

3.2.4 Chromosome Bands

3.2.4.1 G- and R-bands

Giemsa staining is the banding technique used for identifying individual human chromosomes in most laboratories. G-Bands are obtained by digesting the chromosomes with the proteolytic enzyme trypsin (Fig. 3.9). This technique is commonly described as GTG (G-bands by trypsin using Giemsa). After Giemsa staining chromosomes display an alternating pattern of Giemsa-dark (G) and -light (R) bands.

In part, these bands reflect different higher order structures and functions at various levels and therefore differ in a number of respects. The light G-bands (R-bands) are rich in GC and replicate early. Furthermore, these bands are gene rich and contain most housekeeping genes and a large number of CpG islands. In addition, R-bands largely contain Alu- and SINE (short interspersed repetitive DNA sequences) repeats. In contrast, dark G-bands are rich in AT and replicate late. G-bands are gene-poor and preferentially contain tissue-specific genes. The most abundant family of repeats in G-bands is that of LINE (long interspersed repetitive sequences) repeats. Moreover, the distribution of G- and R-bands in interphase nuclei follows a specific pattern. G- and R-bands form separate domains, and G-bands are preferentially localized at the nuclear periphery, whereas R-bands are rather positioned in the interior of a nucleus (reviewed in [50]).

Chromatin DNA is composed of loops and matrix-associated regions (MARs), the regions of DNA attaching to nuclear scaffolds [202] (inset in Fig. 3.4). According to metaphase chromatin models, G-bands are regions where AT-strings are tightly folded, whereas R bands are regions where AT-strings are unfolded and located along a longitudinal axis of a chromatin (Fig. 3.4, inset). According to this model, MARs are frequently present in G-bands and sparsely in R-bands [202].

G-Banded chromosomes are used for a standardized and common description in human cytogenetics. At the Paris Conference in 1971 [177] a basic system for designation not only of individual chromosomes but also of chromosome regions and bands was proposed. A system of human cytogenetic nomenclature evolved from further subsequent meetings. Each of the autosomes is numbered, from 1 to 22. The sex chromosomes are X and Y. Within each chromosome, the short arm is the p-arm, and the long arm is the q-arm. Each

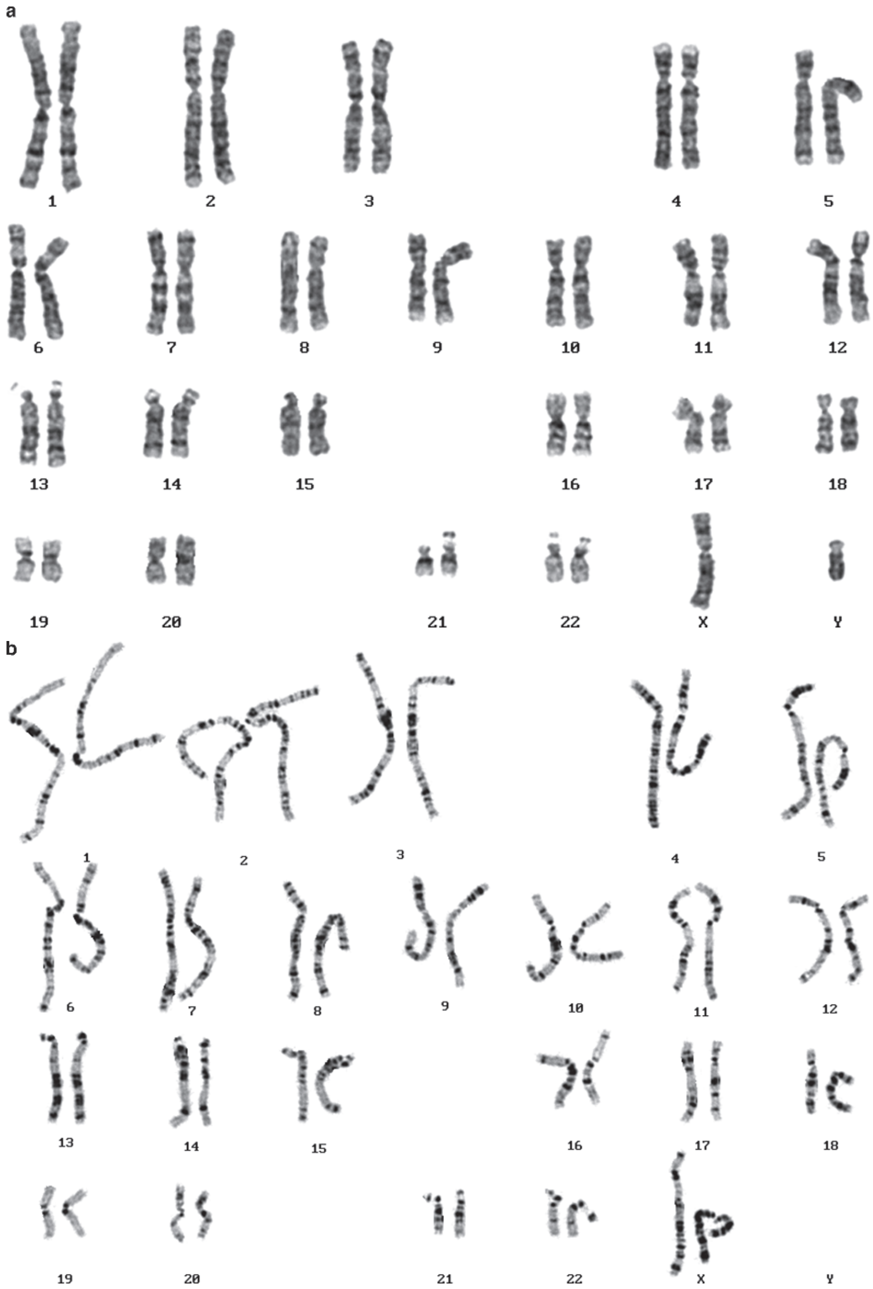


Fig. 3.9 (**a**, **b**) GTG-banded normal metaphase spreads with different resolution. (**a**) Normal male metaphase spread with approximately 450 bands; (**b**) normal female metaphase spread with approximately 600 bands

arm is divided into numbered regions. Within each region, the bands are designated by a number (Fig. 3.10). Such pictograms are in widespread use, in spite of some shortcomings deriving from the fact that the location and width of bands reflect the real chromosome morphology only in part. At present, pictograms created by Uta Francke, who used actual measurements and different gray values to mirror the staining intensities of G-dark bands, are used by most investigators [81] (Fig. 3.10).

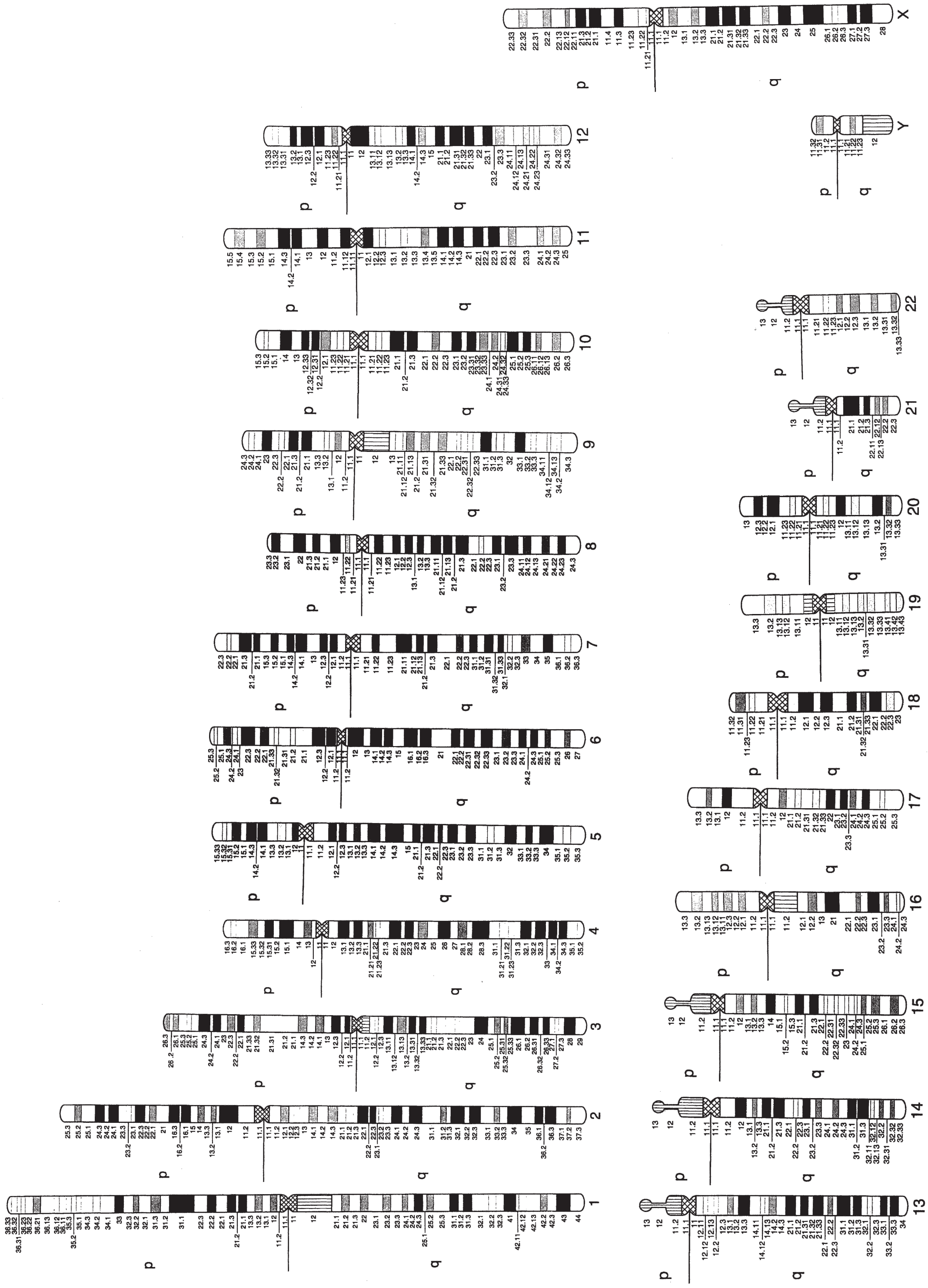


Fig. 3.10 An 850-band pictogram in which the relative widths of euchromatic bands are based on measurements and the staining intensities reflect GTG bands. (From [81], with permission from S. Karger AG, from the 3rd edition of this work)

3.2.4.2 In Silico-*Generated Bands*

More recently, new approaches have been used to achieve *in silico* chromosome staining. Using the DNA sequences of the draft human genome and sophisticated computer software which assigns gray values to chromosomal regions depending on the percentage of the GC content, it has proved possible to achieve successful reconstruction of bands resembling Giemsa bands [170] (Fig. 3.11). Such *in silico* approaches will probably improve with further refinements of draft human genome sequences and should result in the most reliable schematic representations of chromosomes.

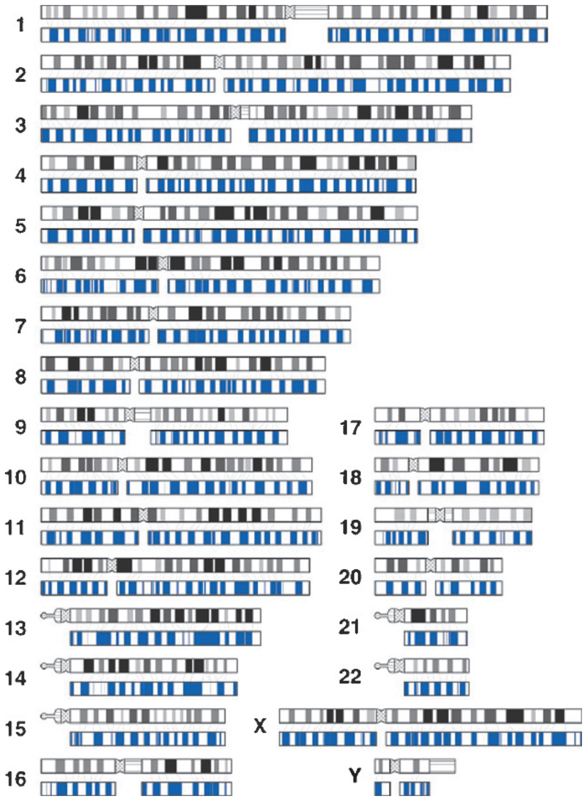


Fig. 3.11 Giemsa and *in silico* bands for all chromosomes. Short (p) and long (q) arms are positioned *left* and *right*, respectively. Giemsa bands obtained from Francke [81] are shown in *black pictograms*. The bands depicted in *black*, *gray*, and *white* represent euchromatins, and the darkness of each band reflects the shading. Pericentromeric heterochromatin and heteromorphic regions of chromosomes 1, 3, 9, 16, 19, and Y are depicted by *crosshatched* and *horizontal lines*, respectively. *In silico* bands constructed by using windows of 2.5 and 9.3 Mb are shown in *blue*. The *thin lines* between Giemsa and *in silico* bands denote aligned G-bands. From [170], copyright 2002, *National Academy of Sciences*, USA

In silico chromosome staining provided the solution to an old problem. As already mentioned, Giemsa-dark and -light bands are generally thought to correspond to GC-poor and GC-rich regions, respectively; however, several experiments have shown that the correspondence is quite poor. *In silico* banding clearly shows that Giemsa-dark bands are *locally* GC-poor regions compared with the flanking regions, but not compared with the entire genome. These findings are consistent with the model that MARs, which are known to be AT rich, are present more densely in Giemsa-dark bands than in Giemsa-light bands (as shown for example in the Fig. 3.4 inset). In fact, G-bands are the regions in which the GC content is only lower relative to the surrounding regions, and not relative to the entire genome [170].

3.2.5 *Telomeres*

The linear chromosomes of eukaryotes are "sealed" by a specialized region, the telomere, which stabilizes them at both ends. Telomeres protect chromosome ends from being recognized and processed as DNA double-strand breaks and, therefore, from triggering DNA-damage-induced responses and checkpoint activations. Furthermore, telomeres act to prevent the end-to-end fusion of chromosomal DNA molecules and, hence, to prevent the fusion of chromosomes with one another. The first evidence for the importance of telomeres in chromosomal integrity came from a cytogenetic analysis in the 1940s by McClintock on breakage and fusion of maize chromosomes, where loss of telomeric sequences renders DNA ends recombinogenic [155].

Telomeres are essential protein–DNA complexes. Two features characterize the telomere: telomeric DNA loop formation to stabilize the chromosome ends and telomerase activity to compensate for replication-related loss of nucleotides at the chromosome ends. Telomerase was discovered by Elizabeth H. Blackburn together with Carol W. Greider, who was a doctoral student in her laboratory at the time [96]. The first observation that telomeres form large duplex loops was made in the laboratory of Titia de Lange [98].

3.2.5.1 Structure of Telomeres (Loop and Proteins)

The telomere repeats consist of 250–1,500 G-rich tandem sequences (5′-TTAGGG-3′), which are highly conserved

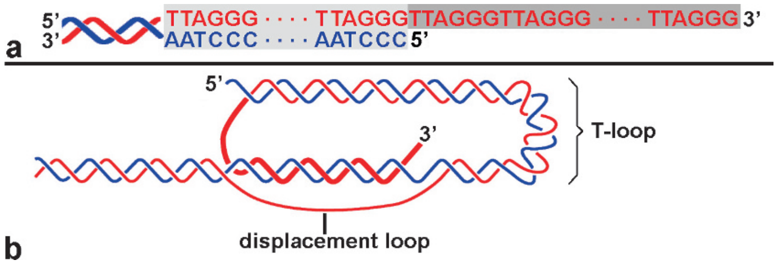


Fig. 3.12 (**a**) The G-rich strand of telomeric DNA (*red*) extends beyond the C-rich strand (*blue*). This creates a 3′ overhang (*dark gray shaded area*). This single stranded 3′ overhang may have a length of several hundred nucleotides. The double-stranded portion of telomeric DNA (*light gray shaded area*) has a length of 5–10 kb. (**b**) The single-stranded 3′ overhang is annealed to a small region of the C-rich strand, causing the formation of a displacement loop while the telomeric duplex DNA forms the T-loop. Adapted from [250]

among different species. Telomeric DNA of mammalian cells is formed from the repeating hexanucleotide sequence 5′-TTAGGG-3′ in one strand (the “G-rich” strand) and the complementary 5′-CCCTAA-3′ in the other (the “C-rich” strand). The G-rich strand is longer by one hundred to several hundred nucleotides, resulting in a long 3′ single-strand overhang (Fig. 3.12a), thus creating a “sticky end.” The end of the single-stranded overhanging region has to be put away in order to avoid initiation of the cellular repair machinery. The 3′ overhanging end of the G-rich strand is annealed to a small region of the C-rich strand, causing the formation of a displacement loop, while the telomeric duplex DNA forms a loop (T-loop) [98] (Fig. 3.12b). The T-loop together with the displacement loop helps to protect the ends of linear DNA molecules.

Both the relatively long double-stranded telomeric DNA and the short overhanging end are bound by specific proteins. These proteins participate in the loop formation and are essential for functional telomeres [91, 128]. In fact, these telomere-binding proteins and the telomeric DNA together form the nucleoprotein complexes referred to as “telomeres.”

3.2.5.2 The Telomere Replication Problem

The replication machinery has great difficulty in copying sequences at the very ends of linear DNA molecules, because DNA is synthesized in the 5′-to-3′ direction only. Therefore, the two templates of the parent molecule differ with respect to the continuity of synthesis. On the 3′-to-5′ template strand, copying is initiated by a primer and can proceed continuously as it occurs in the same direction as the fork movement (leading strand synthesis) (Fig. 3.13). In contrast, on the 5′-to-3′ template strand, synthesis occurs in the reverse direction relative to the fork movement (lagging strand synthesis; Fig. 3.13). Here, DNA is synthesized in short fragments of about 1,000–2,000 nucleotides in eukaryotes.

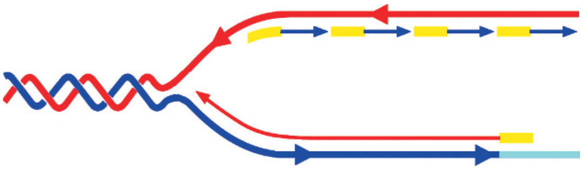


Fig. 3.13 During DNA replication, the parental DNA double helix is unwound. The replication process proceeds on the leading strand in leftward direction (leading strand synthesis) whereas, on the 5′ to 3′ template strand, synthesis occurs in the reverse direction relative to the fork movement (lagging strand synthesis). The synthesis of the new “lagging strand” occurs in short fragments of several hundred nucleotides involving short primers (Okazaki fragments, illustrated in *yellow*). The end of the leading strand template cannot be synthesized by DNA polymerase because the primer responsible for initiating leading strand synthesis may bind a significant number of nucleotides away from the 3′ end of the parental strand and the primer itself is lost when it is degraded after maturation of the recently synthesized DNA. The part of the parental strand, which will therefore be lost, is indicated in *light blue*. Adapted from [250]

The telomere replication problem is caused because the end of the leading strand template cannot be synthesized by DNA polymerase, since the required primer cannot be attached beyond the end of the template strand. In fact, the primer responsible for initiating leading strand synthesis may bind a significant number of nucleotides away from the 3′ end of the parental strand. Furthermore, the primer itself is lost when it is degraded after maturation of the recently synthesized DNA. Therefore, the leading strand synthesis usually results in underreplication of one of the parental strands of DNA. In addition to this underreplication of telomeric DNA ends, cellular exonucleases may contribute to further telomere erosion. Owing to the combined action of underreplication of the leading strand and these exonucleases, it is estimated that in normal human cells telomeres lose 50–100 base pairs of DNA during each cell generation [91].

Thus, in the absence of special telomere maintenance mechanisms, linear chromosomes shorten progressively with every round of DNA replication, eventually leading to cellular senescence or apoptosis. Telomere erosion is inexorably linked to cell division. Chromosomes with critically short telomeres trigger the activation of the p53 and Rb tumor suppressor pathways, providing the signal for replicative senescence [253]. These repetitive elements play a major role in senescence, as progressive shortening of the telomeres occurs with each cell division [3]. The limited replicative potential of primary human cells, also referred to as the Hayflick limit [103], is due to the progressive shortening with each successive round of cell division, leading to significant telomere attrition [91].

3.2.5.3 Telomerase

Because the length of telomeric DNA inexorably shortens during replication, there is a need for compensating mechanisms to preserve genome integrity and telomere functions. In many organisms a specialized reverse transcriptase named telomerase catalyzes the addition of short and simple repeats in a process that is tightly connected with replication.

Telomerase is a specialized polymerase that adds telomere repeats to the ends of chromosomes. It has two essential components: one subunit is a DNA polymerase, i.e., a reverse transcriptase to synthesize DNA from a RNA template. The telomerase holoenzyme provides its own RNA template, which is the second essential subunit. The polymerase is the catalytic component, named human telomerase reverse transcriptase (hTERT), while the "template provider" for nucleotide addition by hTERT, the RNA component is referred to as hTR. The addition of telomeric repeats onto the ends of the chromosome partly triggers the shortening that occurs during DNA replication (Fig. 3.14). In normal human cells telomerase activity is too low to prevent progressive telomere erosion. Substantial telomerase activity in normal cells is found in stem cells, in germ cells and, periodically, in lymphocytes when they become functionally activated. However, activation of telomerase is a common finding in many tumor cells [59].

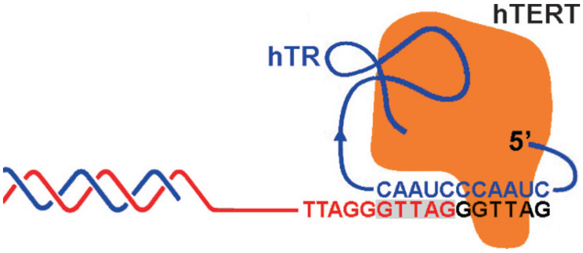


Fig. 3.14 Illustration of the two essential subunits of the human telomerase holoenzyme: the hTERT catalytic subunit (*orange*) and the associated hTR subunit (*blue*), which can elongate telomeres. The 3′ end of chromosomes (*red letters*) is extended by reverse-transcribing the template region of the telomerase RNA. Near the RNA 5′ end are sequences complementary to telomeric DNA repeat sequences (*blue letters*). A short nucleotide sequence of this RNA pairs with terminal DNA sequences (*gray shaded red letters*). The adjacent RNA nucleotides provide the template for adding nucleotides to the 3′ end of the chromosome (*black letters*). Repetition of this process in an iterative fashion makes it possible for telomeres to be elongated. Adapted from [250]

In cells expressing telomerase, telomeric DNA trimming still occurs but can be counterbalanced either partially or completely by the elongation of the G-rich strand and by its subsequent complementary replication.

In the absence of telomerase, telomeric DNA loss can also be compensated for by alternative lengthening of telomeres (ALT) mechanisms. The ALT mechanisms are still unclear and appear to rely on homologous recombination, rolling-circle replication, extrachromosomal circle integration, and break-induced replication. ALT-positive cells have a number of characteristics dissimilar from those in cells that use telomerase for telomere maintenance. Telomeres in ALT-positive cells are typically quite long and heterogeneously sized compared with the shorter, more homogeneous population of telomeres usually present in telomerase-positive cells. Based on differences in telomere structure, mechanisms that amplify subtelomeric repeats and mechanisms that lengthen the simple telomeric repeats alone can be distinguished. The ALT pathways can be considered as backups of telomere maintenance in organisms that normally exploit the telomerase system. For instance, whereas ALT is inhibited in normal human cells, some human tumors maintain their telomeres using ALT.

3.2.5.4 Telomeres and Human Diseases

Telomeres shorten with each cell division and ultimately activate a DNA damage response that leads to apoptosis or cell-cycle arrest. Telomere length thus limits the replicative capacity of tissues and has been implicated in age-related diseases. However, ectopic expression of hTERT can reconstitute functional telomerase activity [29]. In fact, in most epithelial tumors, telomere maintenance is accomplished by telomerase, which appears to be an important mechanism involved in the unlimited replicative potential of immortal cancer cells.

Mutations in the essential components of telomerase, i.e., hTR and hTERT, can cause dyskeratosis congenita, a rare hereditary disorder characterized by a triad of mucocutaneous manifestations, including skin hyperpigmentation, oral leukoplakia, and nail dystrophy. Heterozygous mutations underlie the defect in families with dominant inheritance, indicating that half the usual dose of telomerase is inadequate for telomere maintenance in tissues of high turn-over, such as the bone marrow [243]. Furthermore, telomerase mutations have been implicated in families with idiopathic pulmonary fibrosis [10].

3.3 Cell Cycle and Mitosis

3.3.1 Cell Cycle: Interphase G_1-G_2 and G_0

Almost all types of normal cells proliferate only if they receive appropriate signals to divide, which initiates a complex cycle of growth and division, referred to as the "cell cycle."

The mammalian cell cycle can be divided into four phases (Fig. 3.15). The mitotic phase (M) is a relatively short period which alternates with the much longer interphase where the cell prepares itself for the next cell division. Interphase is divided into three phases, G_1 (first gap), S (synthesis), and G_2 (second gap). During interphase cells remaining in the active growth-and-division cycle prepare for the next division. This preparation includes, for example, the duplication of macromolecular constituents which will later be equally distributed to the two daughter cells. The production of proteins and cytoplasmic organelles occurs during all three interphase stages (i.e., G_1, S, and G_2). For example, the centrosomes represent an important component, as they organize a microtubule meshwork throughout the cell cycle, thereby influencing both tissue architecture and the accuracy of chromosome segregation [169]. The centrosomes are duplicated in several steps, whereas chromosomes are replicated only during the S phase (Fig. 3.16). The centrosome cycle and the chromosome cycle have to be tightly coordinated. In the G_2 phase, every chromosome has doubled into two identical elements, called sister chromatids. As a consequence, the material of every chromosome is now present twice (2×2=4n). During or after replication,

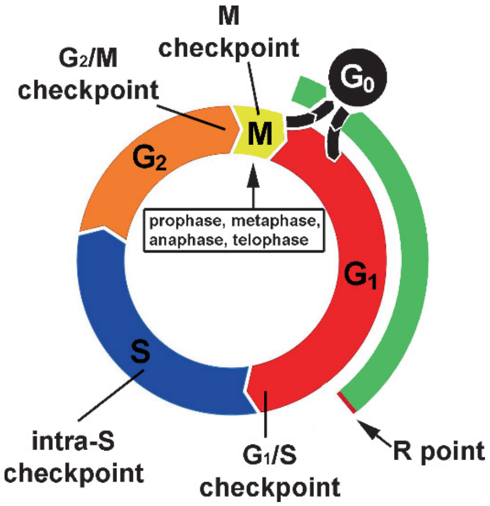


Fig. 3.15 Summary of the mammalian cell cycle and the cell cycle checkpoints: The mammalian cell cycle consists of four phases: G_1 (gap one), S (synthesis/replication of DNA), G_2 (gap two), and M (mitosis). The mitosis can be subdivided into prophase, metaphase, anaphase, and telophase. G_0 indicates a fifth, resting, nonproliferative state of cells, which have withdrawn from the active cell cycle. It is unclear when during G_1 exit into G_0 occurs. Cells may respond to extracellular mitogens and inhibitory factors only during a certain time period that begins at the onset of G_1 and ends before the end of G_1 (indicated in *green*). The end of this time window is designated the restriction (*R*) point. After this time point, the cell is committed to advance through the remainder of the cell cycle through M phase. A checkpoint is a regulatory pathway that controls the order and timing of cell-cycle transitions by checking at the beginning of each new step whether the previous one is completed. The image depicts various DNA-damage checkpoints: the G_1/S checkpoint blocks entrance into S-phase if the genome is damaged; the intra-S checkpoint halts replication in the case of any replication errors or other damages within the genome; the G_2/M checkpoint blocks entrance into M-phase if DNA replication is not completed; and the mitotic checkpoint prevents progress in mitosis if not all chromosomes are properly assembled on mitotic spindle

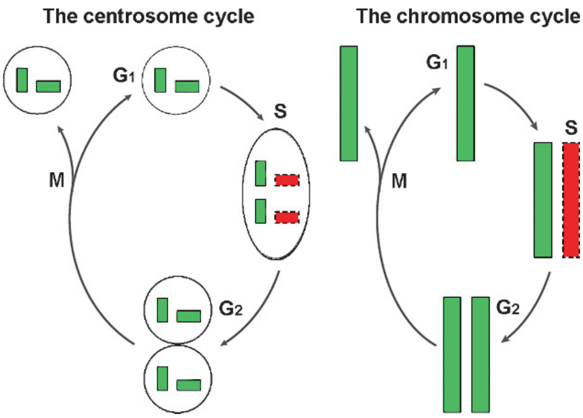


Fig. 3.16 Schematic drawing of the centrosome cycle and the chromosome cycle. Adapted from [169], reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Cancer*, copyright 2002

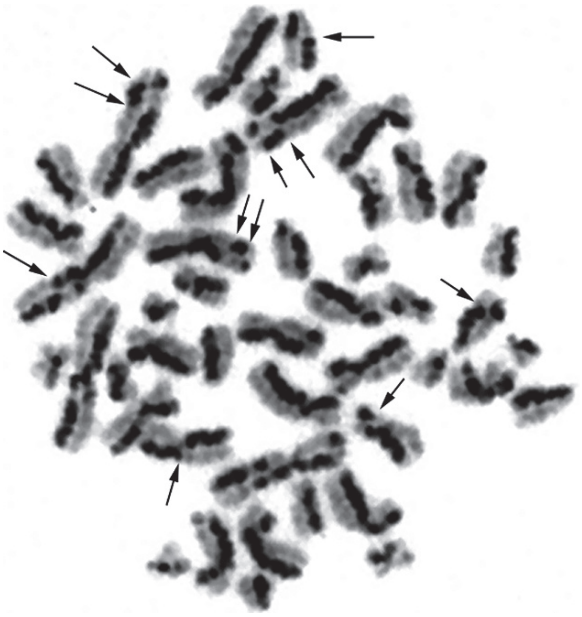


Fig. 3.17 Male metaphase spread after visualization of sister chromatid exchanges. The *arrows* point to some, but not all, locations of sister strand exchanges

the two sister chromatids exchange segments repeatedly, so that the two chromatid arms of a mitotic chromosome have parts of both chromatids. This process is called sister chromatid exchange (SCE) and can be made visible by using a specific staining technique after treatment with bromodeoxyuridine (Fig. 3.17). There are normally about 6–10 SCEs during each cell division. An increased SCE rate is observed in cases of defects in the homologous repair defect.

The duration of the different phases varies. G_1 lasts for 12–15 h, the S-phase 6–8 h, but may be much shorter in certain cell types, such as rapidly dividing embryonic cells or lymphocytes, and the G_2 phase may require about 3–5 h. The shortest phase is mitosis, which with its five subphases (prophase, prometaphase metaphase, anaphase, and telophase) takes about 1 h.

The decision on whether a cell will advance through another growth-and-division cycle is made in late G_1 at a transition called the restriction point or R point (Fig. 3.15). At this R point a cell commits itself to proceed beyond G_1 into S phase and then to complete the entire S, G_2, and M phases.

In the absence of mitogenic growth factors a cell can proceed from mitosis into the G_0 quiescent stage. This G_0 state can be reversible if the cell is again exposed to mitogenic growth factors. However, some cells leave the active cell cycle irreversibly without ever re-initiating active growth and division. Thus, some cells in a body have entered such a postmitotic, differentiated state from which they will never re-emerge and resume proliferation.

3.3.2 Cell Cycle: Mitosis

Mitosis is traditionally subdivided into five consecutive and morphologically distinct phases: prophase, prometaphase, metaphase, anaphase, and telophase (Fig. 3.18).

3.3.2.1 Mitosis and Cytokinesis

Mitosis starts with the condensation of chromatin and ends with the separation of the sister chromatids and their drawing to the opposite poles by the spindle fibers. It is followed by cytokinesis, which divides the nuclei, cytoplasm, organelles, and cell membrane into two daughter cells containing roughly equal shares of these cellular components. Both mitosis and cytokinesis represent the mitotic (M) phase of the cell cycle.

As mentioned above, mitosis is divided into five phases, i.e., prophase, prometaphase, metaphase, anaphase, and telophase.

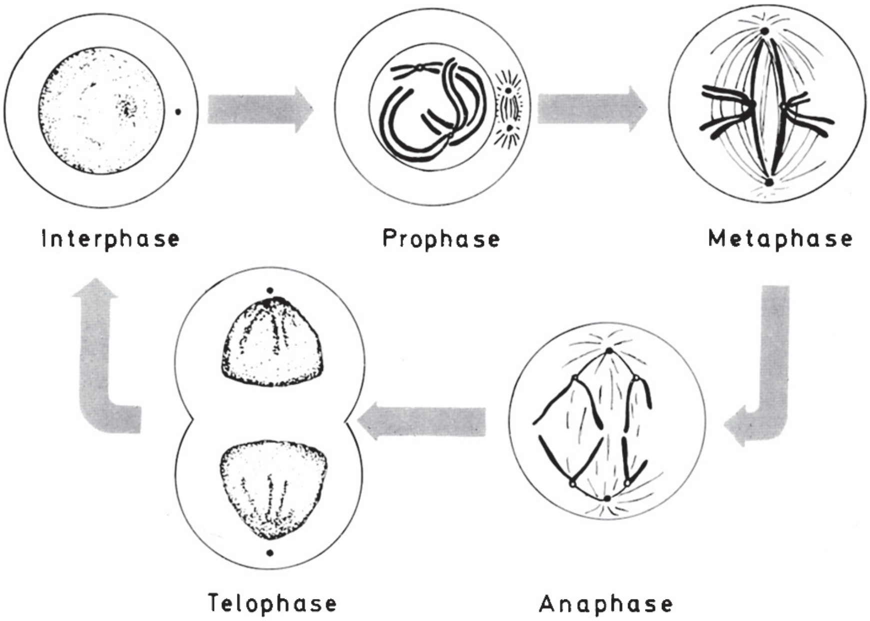


Fig. 3.18 Schematic representation of mitosis. Only 2 of the 46 chromosomes are drawn. For details see text. (Courtesy of Dr. W. Buselmaier, from the 3rd edition of this work)

3.3.2.2 Prophase

At the onset of prophase, chromosomes initiate condensation and become visible through a light microscope.

Each centrosome acts as a coordinating centre for the cell's microtubules. The two centrosomes nucleate microtubules (which may be thought of as cellular ropes or poles) by polymerizing soluble tubulin present in the cytoplasm. Molecular motor proteins create repulsive forces that will push the centrosomes to opposite side of the nucleus.

3.3.2.3 Prometaphase

The nuclear envelope and the interphase microtubule array disassemble and the fully compacted chromosomes spill into what was the cytoplasm to produce prometaphase.

The attachment of microtubules to kinetochores is a stochastic "search and capture" (Fig. 3.19a) [189]. The process begins with unattached sister kinetochores. Initial capture occurs frequently by the binding of one kinetochore of a duplicated chromosome pair along the side of a spindle microtubule allowing rapid (up to 1 µm/s) poleward translocation along that microtubule. This is followed by an attachment of additional microtubules, in humans up to 30. At the spindle pole, additional microtubules bind the captured kinetochore in an end-on fashion to create a microtubule fiber. A probing microtubule from the opposite pole then interacts with the remaining unattached kinetochore. Finally the sister chromatids gather around the center of the spindle, where the sister kinetochores achieve full microtubule occupancy.

The correct, bipolar or bioriented, attachment needed for correct chromosome segregation is called amphitelic (Fig. 3.19b). Here sister kinetochores are oriented to opposite poles and thus bind microtubules from the adjacent pole. However, correct and incorrect attachments can also occur during mitosis, resulting in several kinetochore-microtubule arrangements. Syntelic describes kinetochore-microtubule attachment in which both sister kinetochores face only one of the two poles and consequently attach only to microtubules from that pole (Fig. 3.19b). Monotelic refers to a situation in which both sister kinetochores face opposite poles but only one kinetochore is attached to microtubules. A merotelic kinetochore-microtubule attachment is present when both sister kinetochores face opposite poles but one kinetochore

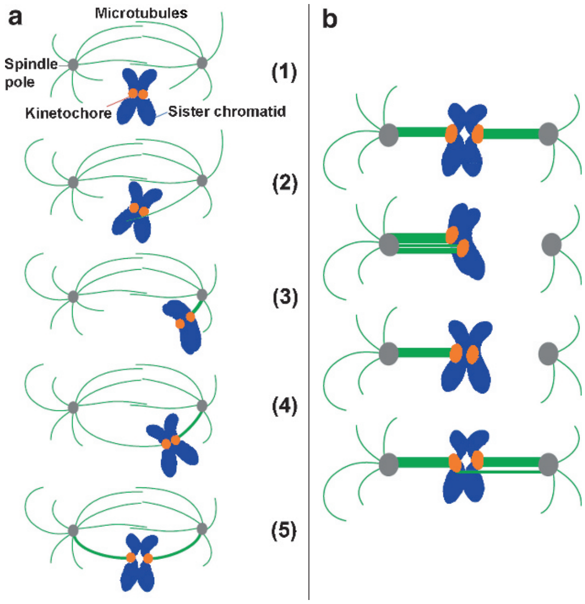


Fig. 3.19 (**a**, **b**) Kinetochore-microtubule attachments. (**a**) The dynamic probing of microtubules for kinetochore attachments represents a stochastic process, dubbed "search and capture." *1:* The *upper panel* shows unattached kinetochore. *2:* During the search and capture process single kinetochores may be captured by spindle microtubules. *3:* The sister chromatids are then pulled to the pole where more microtubules can bind. *4:* The sister kinetochore interacts with microtubules from the opposite pole. *5:* Finally microtubules from both poles have bound resulting in a correct, bipolar or bioriented attachment. (**b**) Types of kinetochore-microtubule attachments. From *above*: amphitelic, syntelic, monotelic, and merotelic. Adapted from [189], with permission from Elsevier

binds to microtubules from both poles. Chromosomes with syntelic, monotelic, or merotelic attachments have to be corrected, as otherwise they will segregate improperly.

Mechanisms exist to prevent incorrect chromosome inheritance, which would occur if improper attachment persisted until anaphase. Monotelic attachment is a normal condition during prometaphase before bi-orientation. In syntelic attachment both sisters in a pair connect to the same pole. Merotelic attachment occurs quite frequently. Both syntelic and merotelic attachments are corrected by the aurora-B kinase.

When proper bipolar attachments are formed, the poleward forces of the kinetochore microtubules are opposed by the cohesion between the sister chromatids, putting the chromosomes under tension. When all the chromosomes are aligned properly the cell is in metaphase.

3.3.2.4 Metaphase

The centromeres of the chromosomes convene along the *metaphase plate* or *equatorial plane*, a line usually equidistant from the two centrosome poles. This even alignment is due to the counterbalance of the pulling powers generated by the opposing kinetochores, analogous to a tug-of-war between equally strong people.

Proper chromosome separation requires every kinetochore to be attached to a bundle of microtubules (spindle fibers), and unattached kinetochores generate a signal to prevent premature progression to anaphase without all chromosomes being aligned. The signal creates the mitotic spindle checkpoint (for more details see Sect. 3.4.3.3).

3.3.2.5 Anaphase

The cell proceeds to anaphase when every kinetochore is attached to a cluster of microtubules and the chromosomes have lined up along the metaphase plate. Anaphase ensues about 20 min after the last kinetochore has attached itself to the spindle, and repeated detachment of a chromosome from a spindle by micromanipulation has delayed anaphase indefinitely [197].

The proteins that bind sister chromatids together, i.e., cohesin, are cleaved, allowing them to separate. This converts sister chromatids into sister chromosomes, which are pulled apart by shortening kinetochore microtubules and move towards the respective centrosomes to which they are attached. Subsequently, the nonkinetochore microtubules elongate, pushing the centrosomes (and the set of chromosomes to which they are attached) apart to opposite ends of the cell.

These two stages are sometimes called early and late anaphase. Early anaphase is usually defined as the separation of the sister chromatids, while late anaphase is the elongation of the microtubules and the microtubules being pulled farther apart. At the end of anaphase, the cell has succeeded in separating identical copies of the genetic material into two distinct populations.

Research of essential mitotic processes is ongoing and still reveals new, surprising findings. For example, it was recently demonstrated that alphoid centromeric DNA persists as thin threads connecting separating chromosomes even during anaphase. These findings were achieved by the identification of PICH (Plk1-interacting checkpoint helicase), which represents an essential component of the spindle assembly checkpoint and is localized to kinetochores (inner centromeres) [25]. Topoisomerase activity is required during anaphase for the resolution of these alphoid centromere PICH-positive threads, implying that the complete separation of sister chromatids occurs later than previously assumed [245].

3.3.2.6 Telophase

During telophase the cell continues to elongate owing to further lengthening of nonkinetochore microtubules, and sister chromosomes reach the opposite ends of the cell. A new nuclear envelope evolves around each set of separated sister chromosomes. Both sets of chromosomes, now surrounded by new nuclei, decondense and form chromosome territories in their respective nucleus.

3.3.2.7 Cytokinesis

Cell division is finally completed by cytokinesis. A cleavage furrow containing a contractile ring develops where the metaphase plate has been separating the two new nuclei.

3.3.3 Cell Cycle Checkpoints

Cells deploy a series of surveillance mechanisms that monitor each step in cell cycle progression. If certain steps in the execution of a process fail, these monitors rapidly stop further advance through the cell cycle until these problems have been successfully resolved. Another task of such monitors is to ensure that once a particular step of the cell cycle has been completed, it is not repeated until the cell passes through the next cell cycle. These monitoring mechanisms have been termed checkpoints or checkpoint controls [101]. Checkpoints impose quality control to ensure that a cell has properly completed all the requisite steps of one phase of the cell cycle before it is allowed to advance into the next phase.

3.3.3.1 Presently Known Checkpoints

The presently best defined checkpoints are in the G_1/S, intra-S, or G_2/M phases (Fig. 3.15). These checkpoints examine the genome integrity at specific points in the cell cycle and if they sense damage, mechanisms are activated that arrest cell-cycle progression to allow time for repair. If the damage is irreparable checkpoints can activate programmed cell death (apoptosis) or replicative senescence to eliminate the affected cell or prevent it from further replication. If the restoration has been successful, checkpoints re-initiate the continuation of the cell cycle.

3.3.3.2 The Spindle-Assembly Checkpoint in Detail

The molecular mechanisms involved in each checkpoint are beginning to emerge, and some basic plans of signaling cascades are fairly well characterized. Here, only the spindle-assembly checkpoint (SAC) during mitosis will be discussed in more detail. The SAC is a ubiquitous safety device ensuring the fidelity of chromosome segregation in mitosis. The SAC prevents chromosome mis-segregation and aneuploidy (Fig. 3.20), and its dysfunction is implicated in a constitutional disorder, termed mosaic variegated aneuploidy (MVA; further discussed below and in Sect. 3.7.6) and in tumorigenesis.

At the beginning of the M-phase the two sister chromatids of each chromosome are connected by cohesin, which needs to be removed from chromosomes to allow sister chromatid separation in mitosis (Fig. 3.20). Sister chromatid cohesion is essential for bi-orientation of sister kinetochores during mitosis and hence for propagation of the genome during cell proliferation. Cohesin is composed of four core subunits, called SMC1, SMC3, SCC1 (also known as MDC1 and RAD21), and SCC3 (also known as SA2 and STAG2) [148]. These proteins have been proposed to mediate cohesion by embracing sister chromatids as a ring [165]. During early mitosis the bulk of cohesin is already removed from the chromosome arms via a process that requires the WAPL (WAPAL) protein and involves the polo-like kinase PLK1 and Aurora B but does not require proteolytic cleavage of the cohesin subunit SCC1 [185]. However,

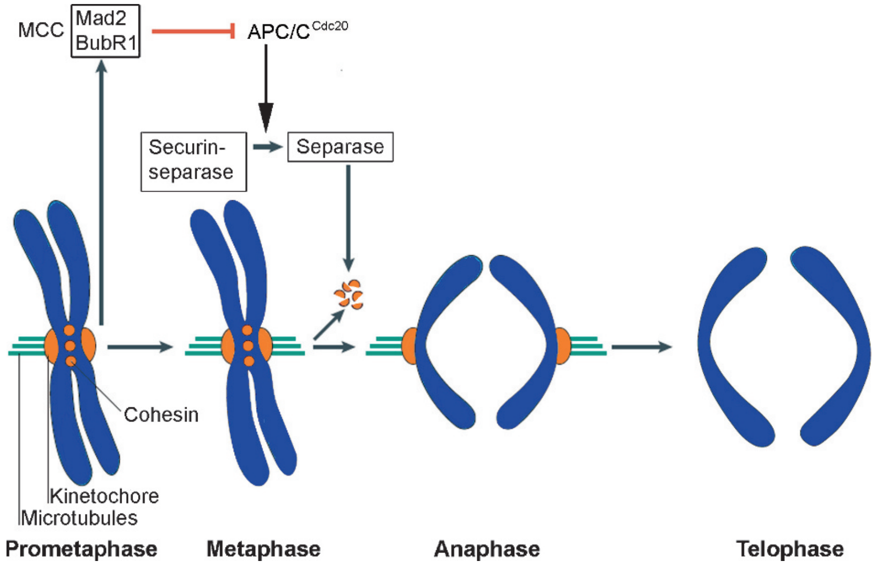


Fig. 3.20 At the beginning of metaphase, both sister chromatids are linked by cohesion. Sister-chromatid cohesion at the centromeres persists until the onset of anaphase. Removal of the centromeric cohesion is performed by a protease named separase. Therefore, separase is kept inactive prior to anaphase. This inactivation is done by binding of the protein securin. Unattached kinetochores contribute to the formation of the mitotic checkpoint complex (MCC), which consists of several kinases (i.e. BUBR1, BUB1) and the MAD1/MAD2 complex. The MCC inhibits CDC20 and thus prevents the activation of the APC/C. The attachment of all sister-kinetochore pairs to kinetochore microtubules and their bi-orientation results in release of CDC20, which can now activate the APC/C. This causes polyubiquitination of securin and therefore activation of separase, which can now remove the centromeric cohesion rings to separate the sister chromatids. Adapted from [185], reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Molecular Cell Biology*, copyright 2006

chromosomes remain connected to each other by centromeric cohesin until the onset of anaphase, because centromeric cohesion is protected by the shugoshin protein SGO1. Thus, for chromosome segregation to proceed it is essential that the centromeric cohesin is removed by a protease termed separase [185]. Separase is a protease whose activity is required to remove sister-chromatid cohesion at the metaphase-to-anaphase transition. However, separase should not accomplish this task before all kinetochores are connected to the mitotic spindle machinery and before all chromosomes are properly aligned in the metaphase plate. Hence, prior to anaphase, separase is kept inactive by the binding of a protein known as securin (Fig. 3.20).

Furthermore, signals are required to indicate whether kinetochores have attached to microtubules or not. Kinetochores that are not, or not fully, attached with microtubules are capable of binding and activating a collection of mitotic checkpoint components. These include kinases, such as BUB1 (*B*udding *u*ninhibited by *b*enomyl), BUBR1 (also referred to as BUB1B) and MAD2 (*m*itotic *a*rrest-*d*eficient homologue-2), which are part of the mitotic checkpoint complex (MCC), which creates a "wait anaphase" signal by inhibiting the ability of CDC20 to activate the anaphase-promoting complex/cyclosome (APC/C). The levels of MAD2 are high at unattached kinetochores and moderately high at attached kinetochores in a monotelic pair. Owing to the inactivated APC/C^{Cdc20} securin cannot be ubiquitylated, so that separase remains in an inactive state and thereby anaphase and mitotic exit are prevented.

After microtubule attachment, bi-orientation depletes MAD2 and BubR1 from kinetochores and promotes the acquisition of tension in the centromere area. As a consequence, checkpoint signaling is silenced and anaphase ensues after the decay of the previously made inhibitor.

The attachment of all sister-kinetochore pairs to kinetochore microtubules and their bi-orientation, which produces congression to the spindle equator, negatively regulates the SAC signal. This releases CDC20, which can now activate the APC/C. This results in the polyubiquitylation of securin and the subsequent proteolytic destruction. The degradation of securin results in the activation of separase, which targets the cohesin ring by proteolytic cleavage, especially the cohesin subunit SCC1 (RAD21, MCD1), which is holding the centromeric sister chromatids together, thus causing the loss of sister-chromatid cohesion and the separation of sister chromatids (reviewed in [162, 185]. When anaphase onset is delayed by the spindle-assembly checkpoint, the complete removal of cohesin from chromosome arms but not from centromeres generates typical X- or V-shaped chromosomes.

There is a continuing controversy as to whether the mitotic checkpoint is silenced by microtubule attachment or by the tension exerted between bioriented kinetochore pairs after attachment and whether activities of subsets of the known components are selectively silenced by one or the other [47].

Thus, the primary mission of this checkpoint is to prevent errors in chromosome segregation.

3.3.3.3 "Cohesinopathies," Cornelia de Lange and Robert Syndrome

Failure of components of the SAC may cause human diseases. Cornelia de Lange syndrome (CdLS) is characterized by growth and mental retardation, craniofacial anomalies, and microcephaly. This disease can be caused by mutations in a protein that is required to load cohesin onto DNA, called SCC2 (also known as NIPBL and delangin), or by mutations in SMC1 or SMC3. A related disease, Roberts/SC phocomelia syndrome (RBS/SC), has been linked to mutations in ESCO2, a protein implicated in the establishment of cohesion [62] (see also Sect. 3.7.5).

3.3.3.4 Possible Additional Checkpoints

In addition to the aforementioned checkpoints, there may be other checkpoints, which will not be explained in detail. For example, the existence of a topoisomerase II (decatenation) checkpoint has been proposed. DNA topoisomerase II is a highly conserved enzyme that is needed to remove catenations that form between sister DNA molecules during replication. As a consequence, sister chromatids are physically linked and these catenations have to be removed prior to mitosis. Thus, this putative topoisomerase II (decatenation) checkpoint operates during G_2 to prevent cells from entering mitosis with entangled DNA [45].

3.3.3.5 Checkpoint Failures and Human Diseases

Mutations in *BUB1B*, encoding the mitotic checkpoint protein BUBR1, were identified in individuals with mosaic variegated aneuploidy (MVA) [99]. MVA is a rare recessive disease characterized by growth retardation, microcephaly, childhood cancer, and constitutional mosaicism for whole chromosomal gains and losses. At present, MVA is the only human disease related to germline mutations in a spindle checkpoint gene.

On a somatic level mitotic checkpoint defects may promote aneuploidy and tumorigenesis, so that mitotic checkpoints might play a crucial role in cancer development [119]. However, mutations in spindle checkpoint genes are only rarely found in epithelial cancer, and therefore their real impact in tumorigenesis is still a matter of debate at present.

3.3.4 *Cell Cycle Coordinators*

As described in the previous section, progression into anaphase and beyond depends on the anaphase-promoting complex/cyclosome (APC/C). However, all other events of the eukaryotic cell-division cycle also require control and coordination. The central components of this system are the cyclin-dependent kinases (Cdks). Distinct cyclin-Cdk complexes form at specific cell-cycle stages and initiate the events of the S and M phases.

For example, cyclin B (cyclin B1, CCNB) is expressed predominantly in the G_2/M phase of cell division and is thought to be essential for the induction and coordination of M-phase events (Fig. 3.21). Cyclin B acts together with Cdk1. Therefore, the activated APC/C^{Cdc20} does not only ubiquitilate securing, but also cyclin B, thereby also inactivating Cdk1. Inactivation of cyclin B-Cdk1 enables exit from mitosis, and mitotic cyclin-Cdk complexes drive the events of early mitosis: chromosome condensation and resolution, nuclear envelope breakdown, and assembly of the mitotic spindle [229].

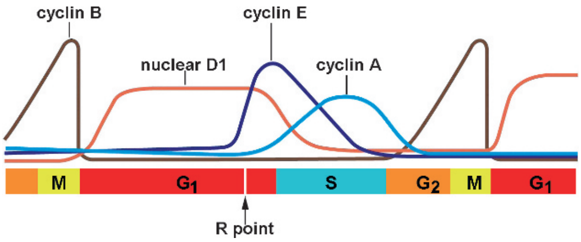


Fig. 3.21 The levels of most mammalian cyclins fluctuate in a tightly coordinated fashion as cells progress through the various phases of the cell cycle. Adapted from [250]

Similarly, levels of the majority of the cyclins fluctuate during the various phases of interphase (Fig. 3.21).

3.4 Chromosome Analysis Methods

A wide spectrum of chromosome analysis methods exists, ranging from traditional banding analyses to sophisticated, molecular array-based technologies. Detailed chromosome analysis has become possible both in metaphase spreads and in interphase nuclei. As the resolution of chromosome analyses has steadily increased, the traditional distinction between cytogenetics and molecular biology is continuously becoming blurred.

3.4.1 Banding Techniques

Banding techniques can generally be divided into differential and selective staining techniques. Differential staining techniques generate dark and light bands along the lengths of all chromosomes and therefore allow assessment of chromosome structure, morphology, and number for all chromosomes. During recent decades these techniques have been indispensable for the unequivocal identification of chromosomes and for standard routine diagnostics. The first differential staining technique introduced employed quinacrine mustard, resulting in bright and dull fluorescence gradations using ultraviolet light. This method was named the QFQ technique (Q-bands by fluorescence using quinacrine). Nowadays, the most commonly used differential staining techniques comprise the G- and the R-bands. In contrast, selective staining techniques do not aim at the staining of all chromosomal regions, but instead at the visualization of distinct chromosomal regions within the genome, such as centromeres or the p-arms of acrocentric chromosomes. These technologies are mainly used to clarify possible chromosomal polymorphisms. Among these selective staining techniques, the most important are the C-bands and AgNOR bands and DA/DAPI staining. In addition to the aforementioned approaches there are a number of other staining and banding technologies. Detailed explanations along with protocols are available [239].

3.4.1.1 Preparation of Mitotic Metaphase Chromosomes

In principle, chromosome preparations can be made from all tissues. For practical reasons, chromosome analysis from peripheral blood, which is usually easily available, is the material most commonly used. However, depending on the clinical question, other tissues may provide more information. For example, in patients with leukemia chromosome status is often assessed from a bone marrow culture. In patients with certain dysmorphic features it may be necessary to perform a fibroblast culture in addition to the blood culture, in order to establish the presence of a possible chromosomal mosaicism.

Mononuclear cells in the blood of healthy individuals usually do not divide; however, chromosomes are always prepared from dividing cells. Therefore, cell divisions must be stimulated artificially. This is usually accomplished by addition of phytohemagglutinin (PHA) to the cell culture. In routine cultures the first cell divisions appear at about 40 h of incubation at 37 °C after stimulation. Thus, a direct preparation of chromosomes immediately after blood is taken is not possible. Most laboratories have adopted a 70- to 76-h incubation protocol, since a higher number of cells entering the second mitosis yield chromosomes with better morphology than those of the first mitosis. To arrest as many cells as possible in prometaphase or metaphase, spindle formation is prevented by a drug with colchicine-like effect, preferably colcemid.

In order to obtain preparations in which the chromosomes are spread out in one plane, the cells are treated for a short period of time (10–30 min) with a hypotonic solution. The cells are then fixed with methanol and acetic acid; a drop of the cell suspension is subsequently placed on the slide, air-dried, and stained. As chromosomes are prepared from dividing cells in which the DNA has already been replicated the investigations consist in metaphase spread analysis of two identical sister chromatids, joined at the centromeres.

3.4.1.2 G-Bands

Giemsa bands (G-bands) can be achieved by different means. G-bands obtained by digesting the chromosomes with proteolytic enzyme trypsin are the most commonly used bands in clinical laboratories for routine chromosome analysis. The composition of G-bands is discussed in more detail in Sect. 3.2.4 (Chromosome Bands; see also Figs. 3.9 and 3.10).

3.4.1.3 R-Bands

The opposite of G-bands are the R-bands (reverse bands). To produce R-bands slides are treated at high temperatures in various buffers, followed by staining with either acridine orange or Giemsa [66]. The bands produced are the reverse of Q- and G-bands.

3.4.1.4 C-Bands

C-bands [15] are applied to stain, specifically, the constitutive heterochromatin in the centromeric regions (Fig. 3.22). Chromosomes 1, 9, and 16 have the largest heterochromatin blocks close to their centromeres, which results in an intensive staining of these regions (Fig. 3.22). In male karyotypes the distal half of the long arm of the Y-chromosome is also C-band positive. These regions often show polymorphisms visible as considerable size differences between the two homologous autosomes, or in the case of the Y-chromosome in significant size difference of the long arm between different males. These size differences are often difficult to interpret by G- or R-banding alone, so that C-bands often complement cytogenetic diagnostics. In fact, the most common chromosomal variant in the human race is the placement of 9q heterochromatin into 9p immediately adjacent to the centromere, which is usually best visible by C-banding.

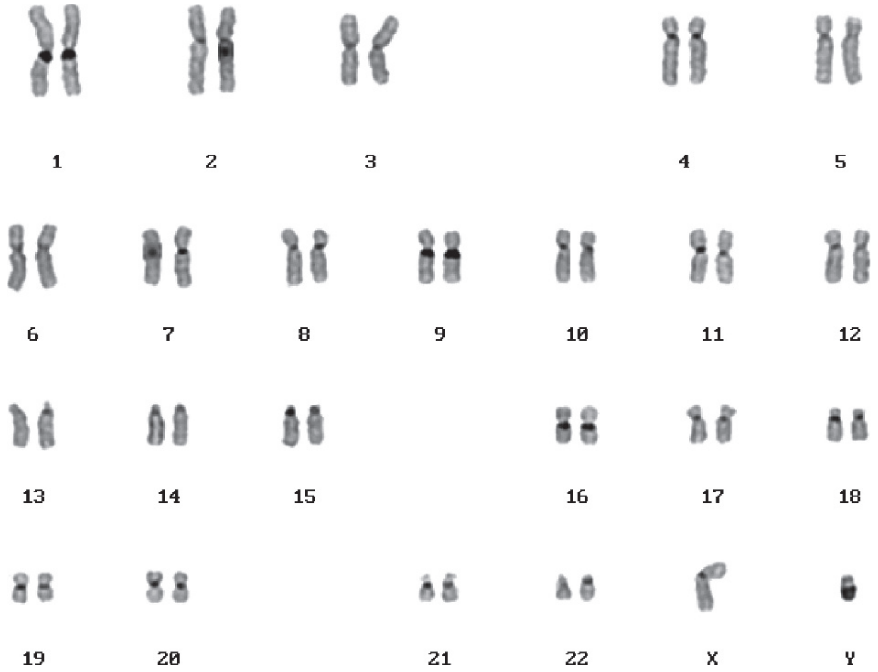


Fig. 3.22 C-Banded human male metaphase spread; C-banding produces selective staining of constitutive heterochromatin. C-Bands are located at the centromere of all chromosomes. They are best visible at chromosomes 1, 9, and 16 and at the long arm of the Y-chromosome

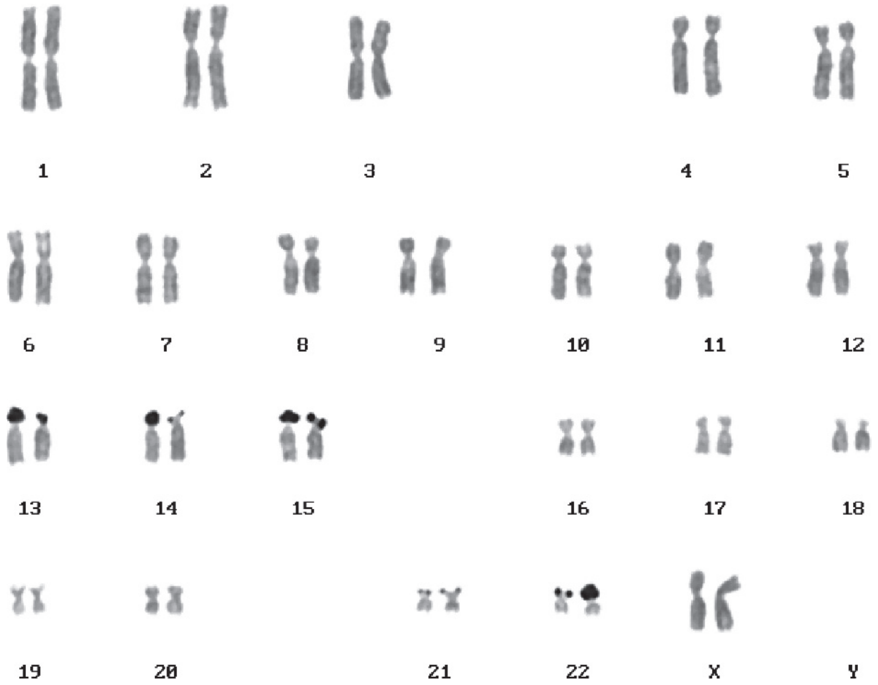


Fig. 3.23 AgNOR-banding of a normal female metaphase. Staining is usually visible at most but not at all p-arms of the acrocentric chromosomes

3.4.1.5 Ag-NOR Bands

Ag-NOR banding [154] aims at the specific staining of the chromosomal regions that form and maintain the nucleoli in interphase nuclei, the so-called nucleolus-organizing regions (NORs) (Fig. 3.23). These regions consist of multiple copies of DNA sequences or genes for ribosomal RNA and are located at the short arms of acrocentric chromosomes. The “Ag” indicates that the staining is done by silver impregnation. Ag-NOR staining reflects the transcriptional activity of the NORs. Thus, frequently not all p-arms of the acrocentric chromosomes show staining. Most individuals have four to seven active NORs per cell.

3.4.1.6 DA/DAPI Staining

DA/DAPI staining involves exposure to the nonfluorescent counterstain distamycin A (DA), followed by staining with fluorescent 4′-6-diamidino-2-phenylindole (DAPI) [209]. As a result, certain heterochromatic regions will appear brightly fluorescent.

3.4.2 Karyotype Description

3.4.2.1 Normal Human Karyotype in Mitotic Metaphase Chromosomes

Karyotypes are described by the number of chromosomes, the sex chromosome constitution, and – if present – any abnormalities (the description of numerical and structural chromosomal abnormalities is explained in detail in the next section). Locations on chromosomes are described according to the Paris Convention, whose basic principles were defined at a conference in 1971 [177]. Bands are numbered counting outwards from the centromere (see Fig. 3.10). Resolution of chromosome analysis depends on the chromosome condensation. In highly extended chromosomes bands split into sub-bands and sub-sub-bands. In routine chromosome analysis a resolution of approximately 500 bands per haploid chromosome set is achieved. At this resolution it is estimated that structural rearrangements with a size of up to 5–10 Mb can be detected. A resolution of less than 400 bands is considered to be inappropriate for the identification of structural chromosomal changes.

An autosome is any chromosome that is not the X or Y sex chromosome. Human cells have 22 pairs of autosomes, which are placed in a karyotype according to their size and position of the centromere. Traditionally, chromosomes in karyotypes are arranged in the following groups: A (chromosomes 1-3), B (4 and 5), C (6-12), D (13-15), E (16-18), F (19 and 20), and G (21 and 22).

3.4.2.2 Description of Normal Human Karyotypes and Karyotypes with Numerical and Structural Changes

Chromosomes are described according to the International System for Human Cytogenetic Nomenclature, abbreviated to ISCN. The latest version is the ISCN 2009 [115]. This publication combines and extends the now classic system of human cytogenetic nomenclature and provides the basic language for the description of chromosomes, especially of chromosomal disorders. For more details the reader should consult the ISCN 2009; here we only describe some basic, general principles for a karyotype description:

The first item to be recorded is the number of chromosomes, followed by a comma (,): the constitution of the sex chromosomes is given next. Thus, a normal female karyotype is designated as 46,XX and a normal male karyotype as 46,XY. Autosomes are only explicitly specified when an abnormality is present.

In the case of chromosome abnormalities sex chromosome aberrations are presented first, followed by abnormalities of the autosomes listed in numerical order irrespective of aberration type. Each abnormality is separated by a comma from the next. For each chromosome numerical abnormalities are listed before structural changes. There are a number of letter designations for the specification of rearranged (i.e., structurally altered) chromosomes. In single chromosome rearrangements the chromosome involved in the change is specified within parentheses immediately after the symbol identifying the type of rearrangement. For example, an inversion has the letter designation “inv,” an inversion on chromosome 9 is therefore designated as inv(9). If two or more chromosomes have been altered, a semicolon is used to separate their designations. For example, a translocation has the designation “t,” so that a translocation involving chromosomes 9 and 22 is described as t(9;22).

The following list summarizes some of the most important letter designations:

Add	Additional material of unknown origin
arrow (->)	From - to, in detailed system
brackets, square ([])	Surround the number of cells
cen	Centromere
colon, single (:)	Break, in detailed system
colon, double (::)	Break and reunion, in detailed system
comma (,)	Separates chromosome numbers, sex chromosomes and chromosome abnormalities
decimal point (.)	Denotes sub-bands
del	Deletion
de novo	Designates a chromosome abnormality which has not been inherited
der	Derivative chromosome
dic	Dicentric
dup	Duplication
fra	Fragile site
h	Heterochromatin, constitutive
hsr	Homogeneously staining region
i	Isochromosome
ins	Insertion
inv	Inversion
mar	Marker chromosome
mat	Maternal origin
minus sign (-)	Loss
p	Short arm of chromosome
parentheses	Surround structurally altered chromosomes and breakpoints
pat	Paternal origin
plus sign (+)	Gain
q	Long arm of chromosome
question mark (?)	Questionable identification of a chromosome or chromosome structure
r	Ring chromosome
rec	Recombinant chromosome
s	Satellite
sce	Sister chromatid exchange
semicolon (;)	Separates altered chromosomes and breakpoints in structural rearrangements involving more than one chromosome
slant line (/)	Separates clones
t	Translocation
ter	Terminal (end of chromsome)
upd	Uniparental disomy

The correct description of karyotypes according to the ISCN 2009 nomenclature represents an especial challenge in the presence of complex chromosomal rearrangements. It is impossible to summarize all

ISCN regulations here. Therefore, interested readers are referred to the ISCN manual [115].

3.4.3 Fragile Sites

Fragile sites represent heritable specific chromosome loci exhibiting an increased frequency of gaps, constrictions, or breaks when chromosomes are exposed to partial DNA replication inhibition. Chromosomal fragile sites are specific loci that preferentially exhibit gaps and breaks on metaphase chromosomes following partial inhibition of DNA synthesis. Fragile sites are present on all human chromosomes. They are named according to the chromosome band they are observed in, e.g., fra(X)(q27.3). In addition, the HUGO nomenclature committee assigns an official symbol. For example, the fra(X)(q27.3) site was called FRAXA (fragile site, X chromosome, A site), because this was the first fragile site detected on the X chromosome.

Fragile sites are classified as either common or rare, depending on their frequency in the population. Whereas common fragile sites are present in all individuals, rare fragile sites are observed in only a small proportion of the population, with a maximal frequency of 1/20, and are inherited in a Mendelian manner. To date, 31 rare and 87 common fragile sites have been described [57, 65, 231] (Tables 3.1, 3.2). A further subdivision is made based on the type of inducing chemicals. The majority of the common fragile sites are induced by aphidicolin, an inhibitor of DNA polymerase, while a smaller group of common fragile sites are induced by BrdU or 5-azacytidine, an inhibitor of DNA methylation. In contrast, the majority of the rare fragile sites are folate sensitive, i.e., they are expressed when cells are grown in folic acid-deficient medium.

Some rare fragile sites, such as FRAXA in the *FMR1* gene, are associated with human genetic disorders, and their study led to the identification of nucleotide-repeat expansion as a frequent mutational mechanism in humans. The FRAXA CGG repeat is located in the 5′ UTR of the fragile X mental retardation 1 (*FMR1*) gene [238]. The *FMR1* gene product (FMRP) is an RNA-binding protein with a high expression in neurons and gonads. However, in the case of so-called full mutations (i.e., >200 CGG repeats), the CpG island in the promotor of the *FMR1* gene is hypermethylated, causing transcriptional silencing of *FMR1* and preventing the synthesis of FMRP. This results in the fragile X syndrome, the most common form of inherited mental retardation [23].

Table 3.1 Classification of fragile sites (from [65])

Class	Number of loci	Sequence
Rare fragile sites	**31**	
Folate-sensitive	24	(CGG)n
Distimycin A	5	AT-rich repeat[a]
BrdU	2	AT-rich repeat
Common fragile sites	**87**	
Aphidicolin	76	AT-rich
BrdU	7	AT-rich?[b]
5-Azacytidine	4	?[b]

[a] Fra16B induced by both BrdU and distimycin A

[b] The BrdU- and 5-azacytidine–induced common fragile sites have not been characterized at molecular level

Table 3.2 Classification of rare fragile sites

Subgroup	Fragile site	Location
Folate sensitive (*n*=24)	FRA1M	1p21.3
	FRA2A	2q11.2
	FRA2B	2q13
	FRA2K	2q22.3
	FRA2L	2p11.2
	FRA5G	5q35
	FRA6A	6p23
	FRA7A	7p11.2
	FRA8A	8q22.3
	FRA9A	9p21
	FRA9B	9q32
	FRA10A	10q23.3
	FRA11A	11q13.3
	FRA11B	11q23.3
	FRA12A	12q13.1
	FRA12D	12q24.13
	FRA16A	16p13.11
	FRA18C	18q22.1
	FRA19B	19p13
	FRA20A	20p11.23
	FRA22A	22q13
	FRAXA	Xq27.3
	FRAXE	Xq28
	FRAXF	Xq28
Distamycin A-inducible (*n*=3)	FRA8E	8q24.1
	FRA11I	11p15.1
	FRA16E	16p12.1
Distamycin A/ BrdU-inducible (*n*=2)	FRA16B	16q22.1
	FRA17A	17p12
BrdU requiring (*n*=2)	FRA10B	10q25.2
	FRA12C	12q24.2

Other fragile sites that have been linked to mental retardation are FRAXE and FRA12A. FRA11B is associated with Jacobsen syndrome, a rare distal 11q deletion syndrome characterized by mental retardation, delayed growth, and specific malformations [57].

Furthermore, common fragile sites have taken on novel significance as regions of the genome that are particularly sensitive to replication stress and that are frequently rearranged in tumor cells. The most prominent example is FRA3B located at the chromosome band 3p14.2. This fragile site maps within the tumor suppressor gene *FHIT*, frequently deleted in cancers, including gastrointestinal tract, cervical, lung, and breast cancers [57].

3.4.3.1 Fluorescence In Situ Hybridization

A clear aim of the continuing development of cytogenetic methods has been to increase the resolution at which chromosome rearrangements can be identified. Crucial to this development was the area of molecular cytogenetics, which is often also referred to as fluorescence in situ hybridization (FISH). In this technique a labeled DNA probe is hybridized to cytological targets such as metaphase chromosomes, interphase nuclei, extended chromatin fibers or, in more recent developments, DNA microarrays (Fig. 3.24). The aim of improving resolution has been achieved by advances in the two crucial elements of cytogenetic analysis,

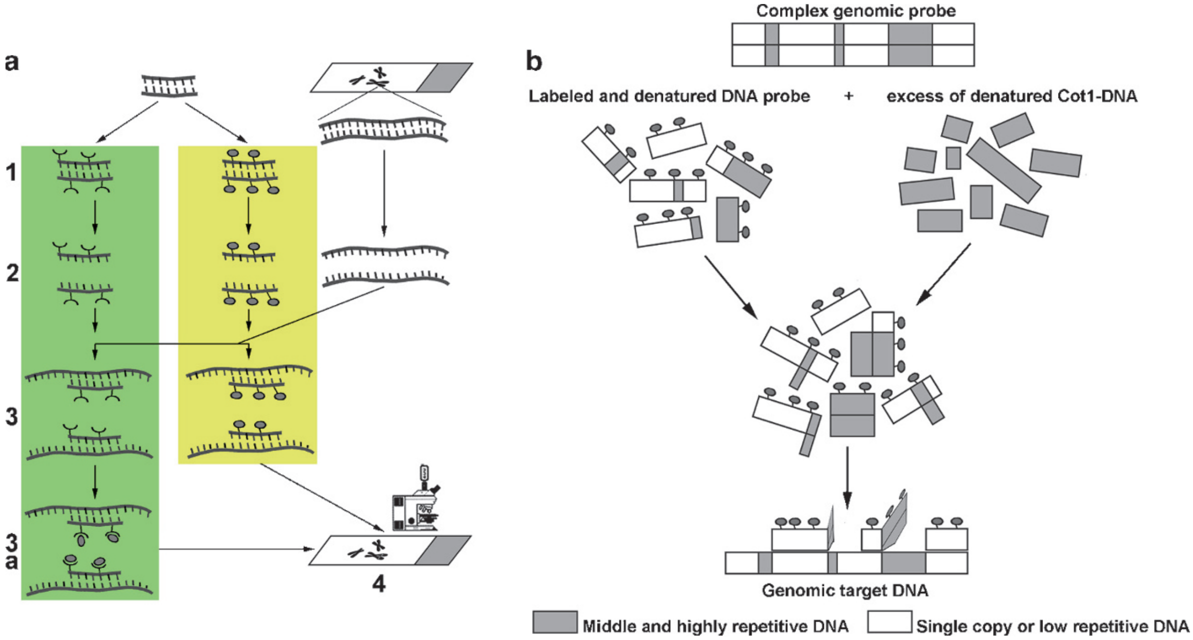

a
1
2
3
3
a
4
b
Complex genomic probe
Labeled and denatured DNA probe
+
excess of denatured Cot1-DNA
Genomic target DNA
▨ Middle and highly repetitive DNA
□ Single copy or low repetitive DNA

Fig. 3.24 (**a**) The basic elements of a FISH experiment involve a DNA probe and a target sequence to which the DNA probe is hybridized. Prior to the hybridization, the DNA probe is labeled (*step 1*) by various means such as nick translation, random primed labeling, and PCR. Two different labeling strategies are commonly employed, i.e. indirect labeling (*green box*) and direct labeling (*yellow box*). For indirect labeling, probes are labeled with modified nucleotides containing a hapten, while direct labeling employs incorporation of directly fluorophore-modified nucleotides. The labeled probe and the target DNA are then denatured to yield single-stranded DNA (*step 2*) and brought together allowing the hybridization of complementary DNA sequences (*step 3*). If indirect labeling of the probe has been employed, an additional step is required (*3a*) for visualization of the nonfluorescent hapten using an enzymatic or immunological detection system. While FISH is faster, with directly labeled probes, indirect labeling offers the advantage of signal amplification by using several layers of antibodies and may therefore produce a brighter signal above background. Finally (*step 4*), the signals are evaluated with a fluorescence microscope. Most fluorescence microscopes are equipped with camera systems allowing the imaging of fluorescence signals for digital image processing. (**b**) Complex DNA probes (*top panel*) contain repeat sequences, such as *Alu* and LINES, which are present throughout the genome. The direct use of these probes would result in hybridization signals across the genome and thus a high background level. Therefore, most FISH protocols include a pre-incubation of the denatured complex probe with excess unlabelled Cot-1 DNA. Cot-1 DNA is enriched for the highly repetitive DNA genomic sequences, such as the *Alu* and LINE-1 repeats. Labeled repeat sequences hybridize preferentially to the excess of unlabeled sequences in the Cot-1 DNA, become double stranded, and thus are unavailable for hybridization to the target

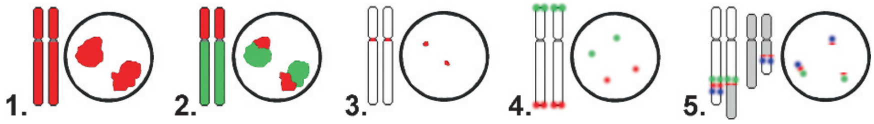


Fig. 3.25 A variety of different DNA probe types are available for hybridization to both metaphase chromosomes and interphase nuclei. The panel shows some examples: *1:* A painting probe stains the entire chromosome. In a normal diploid interphase nucleus two chromosome territories are visible. *2:* Microdissection allows the generation of probes specific for any region within the genome. Differentially labeled chromosome arm-specific probes are shown in this example. In interphase nuclei, the differently labeled chromosome arms within a chromosome territory can be distinguished. *3:* For almost all human chromosomes, probes specific for the centromere are available. Hybridization of these highly repetitive probes does not require suppression with Cot-1 DNA. Owing to their ease of use and high signal intensities, these probes are very popular for the counting of chromosome copy number in interphase nuclei. *4:* For almost any region within the genome, region-specific large insert clones are available. The example shows subtelomeric probes, which are often used to screen for cryptic translocations. Clones for other regions can easily be obtained from publicly available resources. *5:* For known structural rearrangements, special probe sets can be designed to facilitate diagnosis. In this example, the probe set includes a breakpoint-spanning probe and two breakpoint-flanking probes. Use of this probe set allows the structural rearrangement to be detected even in interphase nuclei

i.e., the probe and the target. Target resolution has advanced from metaphase chromosomes (resolution ~5 Mb), through interphase nuclei (50 kb to 2 Mb) and DNA fibers (5–500 kb) to the use of DNA microarrays offering resolutions to a single nucleotide. Probe development has also advanced simultaneously to best utilize the improvements in target resolution.

3.4.3.2 FISH to Metaphase Spreads

The analysis of metaphase spreads has largely benefited from greater probe availability and probe development (Fig. 3.25). As a result of the sequencing of the human genome in the public domain, mapped and sequenced large insert clones are now easily obtainable for virtually any region within the genome [44]. Probes are easily selected by the use of internet browsers such as Ensembl Cytoview (www.ensembl.org) [112], Map-Viewer (www.ncbi.nlm.nih.gov/mapview) [206], or the UCSC genome browser (http://genome.cse.ucsc.edu) [135]. Chromosome painting probes which stain an entire chromosome or a chromosome region are also now widely available.

Another important development is the increase in number of differentially labeled probes that can be hybridized and analyzed. The discrimination of many more targets than the number of available spectrally resolvable fluorochromes can be achieved by certain labeling strategies allowing the simultaneous visualization of all 24 human chromosomes, each in a different color in a single hybridization [73]. These 24-color karyotyping technologies are known as multiplex-FISH (M-FISH) [223] (Fig. 3.26), spectral karyotyping (SKY) [208], and combined binary ratio labeling (COBRA) [232] and have a wide range of uses [73]. The identification of intrachromosomal rearrangements may notably be facilitated by multicolor banding technologies [222].

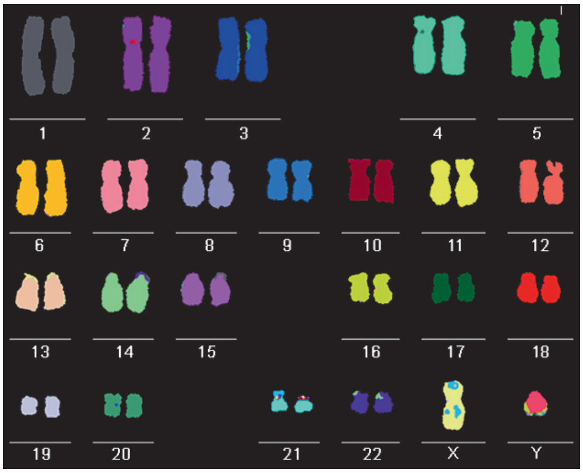


Fig. 3.26 Multicolor classified karyotype of a normal male metaphase, generated by an adaptive spectral classification approach for seven fluorochromes. The automated analysis is assisted by computer-generated pseudocolors. The *light blue colors* on the Y chromosome are caused by cross-hybridization of the Y chromosome to the X chromosome and represent the first pseudoautosomal region at chromosome Xp22.3 (2.6 Mb) and the XY-homologous region at chromosome Xq21.3 (4 Mb). The second pseudoautosomal region at the distal tip of the long arm of the X-chromosome is usually not visible as an extra color (cf. Fig. 3.52)

Together with probe technology there has been considerable improvement in both hardware and software used for the analysis of FISH images. Cooled charge-coupled device (CCD) cameras and more specific and efficient fluorescence filter sets have improved the sensitivity and resolution of imaging on the microscope and sophisticated software facilitates image acquisition and processing.

3.4.3.3 Interphase FISH (Interphase Cytogenetics)

One important feature of FISH-based assays is their ability to yield information about chromosomes or chromosomal subregions in intact interphase nuclei enabling a technology termed "interphase cytogenetics" [51]. Interphase cytogenetics is useful in diagnostic applications where metaphase spreads cannot be obtained, where only small cell numbers are available, or where large cell numbers are to be screened with a particular probe set. Interphase FISH enables rapid screening of large numbers of cells, such as the screening of tumor cell nuclei with centromere-specific probes to establish the presence of chromosomal instability [144] (Fig.3.27). With careful selection of probes even structural rearrangements, such as translocations and inversions, can be visualized in interphase nuclei [14, 236].

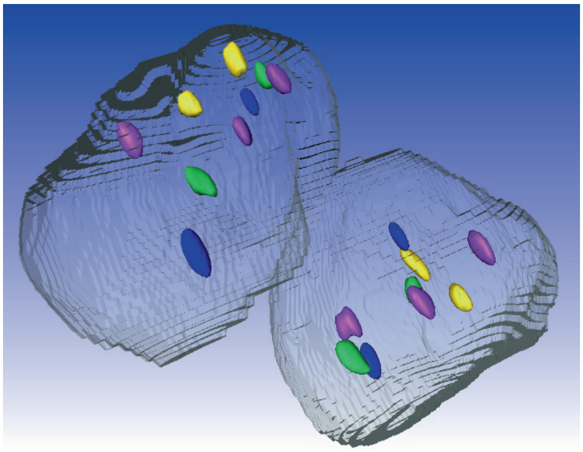


Fig. 3.27 Interphase FISH signals within two nuclei of colon adenoma cells after simultaneous hybridization of four differently labeled chromosome specific centromere probes for chromosomes 7 (*purple*), 8 (*blue*), 11 (*green*), and 17 (*yellow*). Serial optical section in *z*-direction were captured through these nuclei and subjected to 3D reconstruction programs. Each nucleus has three signals for the *purple* chromosome 7-specific probe, suggesting a trisomy 7. In contrast, all other probes yield the expected normal number of two signals

Interphase analysis has also found utility in basic research applications. Interphase FISH, both on fixed nuclei and in living cells, offers the opportunity to analyze how the genome is functionally organized and the dynamic interplay between the genome and its regulatory factors in gene regulation [137]. Furthermore, interphase FISH allows the study of higher order chromatin architecture, which represents an important part of the epigenetic control of gene expression patterns. The current view of higher order chromatin architecture in the cell nucleus is that the cell nucleus has a compartmentalized structure consisting of chromosome territories (CTs) and an interchromatin compartment (IC). The radial arrangement of chromosome territories may be dependent on gene density-related differences in many tissue types [50]. However, in fibroblasts the distribution of chromosome territories showed a probabilistic, highly nonrandom correlation with chromosome size, with small chromosomes being significantly closer to the center of the nucleus, while large chromosomes were located closer to the nuclear or rosette rim (Fig. 3.28) [31] Whether the spatial proximity of chromosomes in interphase nuclei determines the occurrence of possible translocations will be discussed in Sect. 3.6.2.1.

The development of new in vivo labeling techniques has allowed fluorescent labeling of DNA and proteins in living cells (reviewed in [53]). In order to study chromosome territories in living cells, labeling can be performed by incorporation of fluorescent nucleotides in DNA during S-phase. Clonal growth of labeled cells results in segregation of labeled and nonlabeled chromatids during subsequent mitoses.

3.4.3.4 Fiber FISH

While interphase chromatin is less condensed than metaphase chromatin, the highest resolution target for FISH studies is provided by the preparation of released chromatin fibers on microscope slides. This method, known as fiber FISH, provides ordering and structural resolution between 1 kb and 500 kb. A number of alternative techniques for the release of chromatin for fiber FISH have been developed, such as the use of an alkaline lysis buffer or high-salt treatment in an SDS-containing lysis buffer [249] to generate fibers. These approaches produce fibers of varying length and compaction and accordingly are not ideal for the quantification of signal length. For this purpose very uniform, evenly stretched DNA fibers

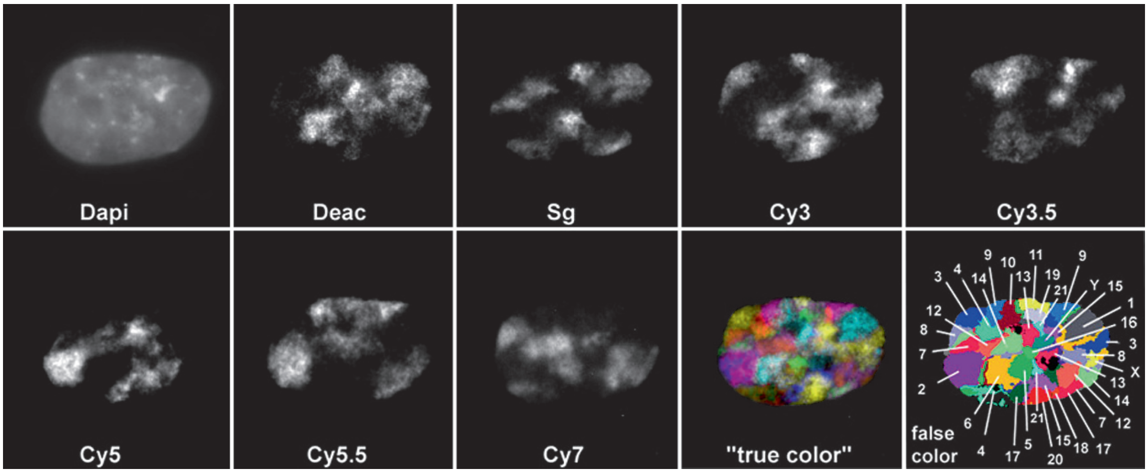


Fig. 3.28 All chromosome domains, each in a different color, can be simultaneously visualized in intact interphase nuclei to study the higher order 3D organization of the genome. The image illustrates a deconvoluted mid-plane nuclear section through a male human G_0 fibroblast nucleus. DAPI is applied to stain the nucleus. For the labeling of chromosome painting probes seven different fluorochromes (diethylaminocoumarin [*DEAC*], spectrum green [*SG*], and the cyanine dyes Cy3, Cy3.5, Cy5, Cy5.5, and Cy7) were used. Here, each channel represents the painting of a subset of chromosome painting probes with the respective fluorochrome. A RGB image of the 24 differently labeled chromosomes (1-22, X, and Y) was produced by superposition of the seven channels. As in 24-color karyotyping, each chromosome has a combinatorial label of a unique fluorochrome combination. Thus, each chromosome territory is amenable to an automated classification so that appropriate software assigns the corresponding chromosome number to a territory. If a stack of these images is collected through a nucleus, a simultaneous 3D reconstruction of all chromosomes is possible. For further details see [31]

are required, as provided by the process of molecular combing [158]. In molecular combing, DNA in solution is stretched at the meniscus as a glass slide is removed at a constant rate, generating fields of very even DNA fibers all parallel to each other.

3.4.3.5 Detection of Copy Number Changes in the Genome

3.4.3.5.1 Conventional CGH on Metaphase Chromosomes

The preparation of high-quality metaphase spreads from clinical and tumor cell samples, and in particular from solid tumors, is often difficult. To overcome this problem comparative genomic hybridization (CGH) was developed [63, 127]. In CGH, DNA is extracted directly from the test sample, e.g., DNA from a patient or a tumor, thus avoiding any culturing artifacts. In a second step the test DNA and normal reference DNA are labeled differentially, for example the test DNA with a green and the reference DNA with a red fluorochrome, and co-hybridized to normal metaphase spreads (Fig. 3.29). The two DNAs compete for the hybridization sites on the target metaphases in such a way that if a region is amplified in the tumor the corresponding region on the normal metaphases becomes predominantly green, and if a region is deleted in the test the corresponding chromosome region becomes red (Fig. 3.29). The actual test to reference DNA fluorescence ratios along all chromosomes is quantified by using digital image analysis systems. DNA gains and amplifications in the test DNA are seen as chromosomal regions with an increased fluorescence ratio, while losses and deletions result in a reduced ratio. However, CGH is limited for rearrangements which do not involve genomic imbalance, such as balanced chromosome translocations and inversions and whole-genome copy number changes (ploidy), which cannot be detected by CGH. Furthermore, CGH does not provide information on the way in which chromosome segments involved in gains and losses are arranged.

In conventional CGH using DNA extracted from hundreds or thousands of cells, chromosomal imbalances are only detected if they are present in most of

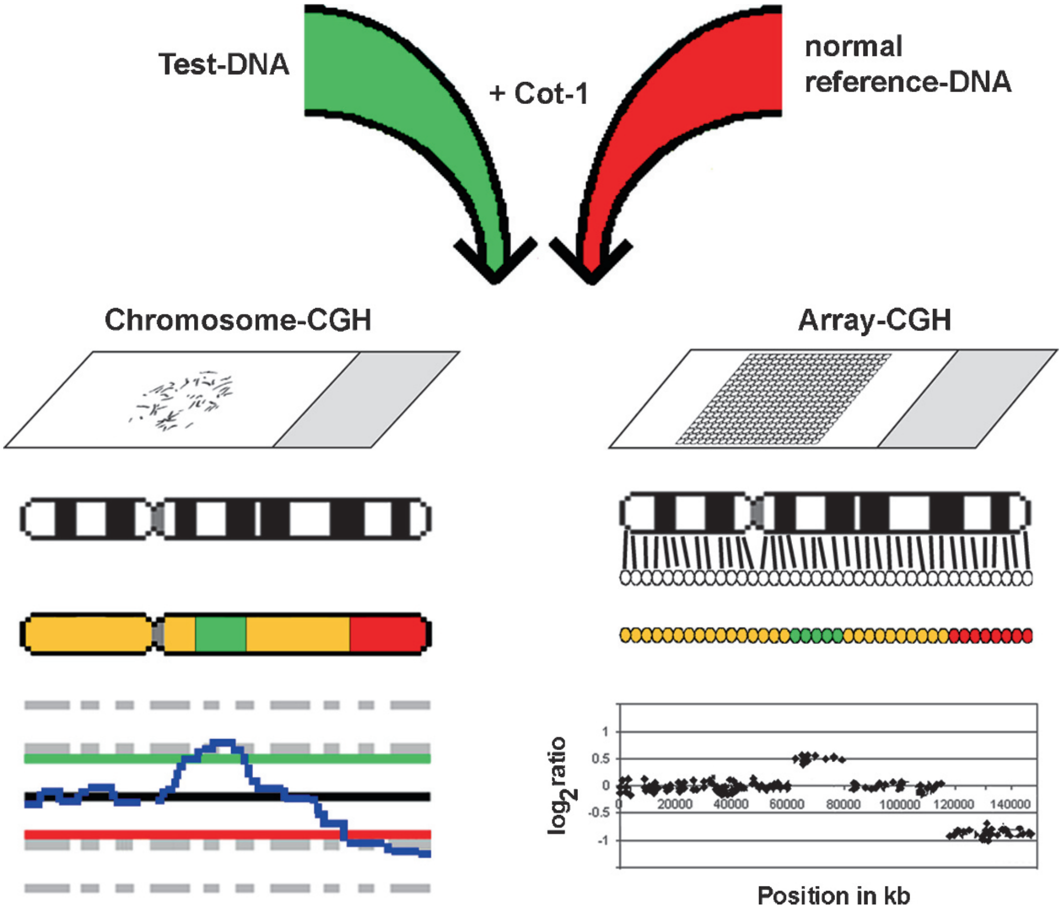


Fig. 3.29 Outline of comparative genomic hybridization (CGH) experiments. CGH maps readily regions of gains and losses in a test-DNA, which is in most cases retrieved from a patient or from a tumor sample. In a CGH analysis, test DNA and normal reference DNA are labeled with different fluorochromes. Here, a test DNA is labeled with a *green* fluorochrome, whereas the reference DNA is labeled with a *red* fluorochrome. In conventional CGH a mixture of these two DNA samples is together with an excess of unlabeled Cot-1 DNA hybridized to normal chromosome spreads (*left-hand side*). In array-CGH/matrix-CGH the hybridization target can consist of large insert clones or oligonucleotides arrayed onto glass slides (*right-hand side*)

the cells in the specimen. Thus, information about heterogeneity may get lost. Furthermore, rare cell events, such as minimal residual disease, where only a single cell or a few cells are available for study, initially could not be analyzed by conventional CGH. However, protocols were developed for the analysis of single cells by CGH. A prerequisite for single-cell CGH analyses are methods for unbiased single-cell DNA amplification yielding amplification products that retain the copy number differences of the original genome [129, 241, 252]. Such approaches have been applied to prenatal diagnostics or for the analyses of minimal residual disease.

3.4.3.5.2 Other Approaches for Evaluation of Copy Number Changes

Copy number alterations of specific genomic sequences can also be measured without the need for chromosomes using multiplex amplifiable probe hybridization (MAPH) [11] and multiplex ligation-dependent probe amplification (MLPA) [207]. MAPH is based on the hybridization of specific probes with uniform linkers to denatured genomic DNA immobilized to a nylon membrane. Probes for different regions vary in length and hybridize to the immobilized DNA in proportion to the copy number of the corresponding sequence in the genome. After hybridization and stringent washing the probes bound to the membrane are released and amplified by PCR using a radioactively or fluorescently labeled primer pair that recognizes the linker sequence. After size separation on a gel the relative intensity of the peaks between a test and reference sample is compared to determine the copy number of the target DNA sequence [11]. MPLA is similar to MAPH, but hybridization and amplification take place in solution with no need to immobilize the DNA onto a nylon membrane (Fig. 3.30). Each region is represented by two adjacent tailed probes that are joined by a ligation reaction on the target DNA. Subsequent amplification can then only take place from ligated probes as target and not from any other sequence [207]. These methods are very rapid and cost-effective and have found favor for specific diagnostic procedures, such as exon deletion screening, but are not easily scaled for whole-genome scanning.

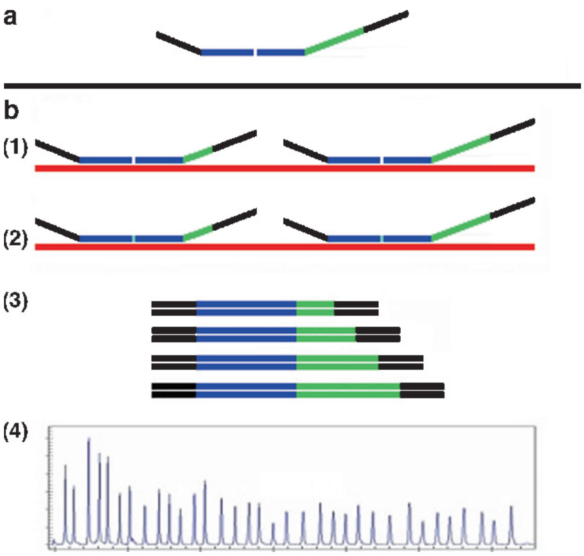


Fig. 3.30 (**a**, **b**) The multiplex ligation-dependent probe amplification (MLPA) procedure. (**a**) Design of a MLPA probe: MLPA probes consist of two separate oligonucleotides, each containing the target sequence (shown in *blue*), a "stuffer sequence" (*green*), and the PCR primer sequences (*black*). The PCR primer sequences are identical for all probes, so that all probes can be later amplified with the same primer set. The stuffer sequence has a different size for each MLPA probe, which allows multiplexing, i.e., multiple probes can be hybridized simultaneously. Different probes are then distinguished by their size. (**b**) The MLPA procedure consists of several steps: *1:* In a first step the DNA to be analyzed is denatured and MLPA probes are hybridized to the DNA. Here, two different MLPA probes are hybridized; the right probe has a larger stuffer sequence than the left probe. *2:* In the second step the ligation reaction takes place. The two probe oligonucleotides hybridize to immediately adjacent target sequences and only then can they be ligated during the ligation reaction. *3:* The ligated probes are amplified by PCR. Probe oligonucleotides that are not ligated only contain one primer sequence and cannot be amplified exponentially. *4:* In the next step amplification products are separated by electrophoresis; and the data can be analyzed. (Adapted from www.mlpa.com)

3.4.3.5.3 Array-CGH

The replacement of the metaphase chromosomes with large numbers of clones spotted onto a standard glass slide as the target for CGH has significantly increased the resolution of screening for genomic imbalance. In array-CGH, the test and normal reference genomes are labeled with different fluorochromes, as in conventional CGH. However, as opposed to hybridizing the DNA probes to metaphase spreads, they are hybridized to a microarray platform. The array is then imaged and the relative fluorescence intensities calculated for each mapped clone, where the resulting intensity ratio is proportional to the DNA copy number difference. The resolution of the analysis is only restricted by the clone size and clone density on the array. An additional advantage is the ease with which array-CGH can be automated for high-throughput applications.

The first descriptions of array-CGH using large insert clones were published in the late 1990s [188, 220] and were rapidly followed by the development of whole-genome arrays with one clone every megabasepair (e.g., [75, 219]). The density of clones on the slide has continued to increase, and the highest resolution for array-CGH is now provided by spotted and synthesized oligonucleotide arrays containing as many as 500,000 or even more elements. The inherent noise of hybridizations of these arrays requires statistical analysis for the confident identification of small gains and losses.

Thus, the main advantages of array-CGH over conventional array comprise the increase in resolution, which also includes the capability of breakpoint mapping (Fig. 3.31). At present a number of different array platforms are available for both whole-genome analysis and detailed investigation of selected regions (Fig. 3.32). Array-CGH is extensively used for the analysis of gains and losses in tumors, but is also applied to the analysis of patients with constitutional rearrangements.

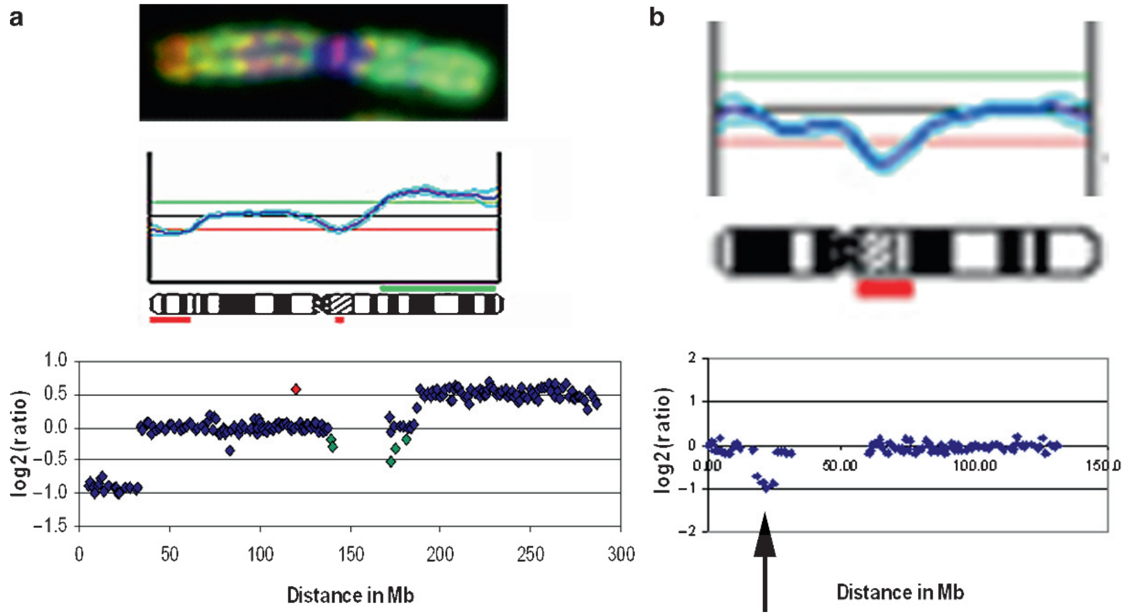


Fig. 3.31 (**a**, **b**) Comparison of the different resolution limits of conventional and array-CGH. In this example, DNA from a primary renal cell adenocarcinoma line (769P, ATCC No. CRL-1933) was hybridized to both metaphase spreads and a large-insert clone, i.e., BAC array. (**a**) The *top panel* illustrates the hybridization pattern on chromosome 1. The distal tip of the p-arm (*left*) appears *red*, which indicates a loss of this region. In contrast, the q-arm (*right*) is extensively stained with *green*, which suggested overrepresentation of this region. The center of chromosome 1 has very low fluorescence signals as this region represents the large chromosome 1 heterochromatin block. As this region consists only of repetitive sequences, hybridization is blocked by the addition of an excess of unlabeled Cot-1 DNA to the hybridization mix. Interpretation of CGH-hybridization patterns does not depend on visual inspection. Instead computer programs are employed, which calculate the intensities of both the *green* and the *red* fluorochrome and the ratio values between these fluorochromes. The result is displayed in a graph, shown in the *center*. The ratio profiles are usually calculated as a mean value of several metaphase spreads. The *three horizontal lines* above the chromosome 1 pictogram represent different values of the fluorescence intensities between the tumor and the reference DNA. The *black line* represents balanced fluorescence intensities, while the *right line* is the threshold for loss and the *green line*, the threshold for a gain of DNA material. The *lower panel* shows the same result obtained on a BAC array. The respective gains and losses are identified with ease. In contrast to conventional CGH, the breakpoints of lost and gained regions can be accurately mapped as they appear as sharp transitions in the ratio profile. (**b**) Cell line 769P also has a small single-copy deletion on chromosome 9p of about 6.3 Mb. The resolution limits for the detection of deletions or duplications with conventional CGH were estimated to be in the range of about 10 Mb. Consequently, this deletion is not identified with conventional CGH. The "deletion" shown in the *upper panel* is caused by the large heterochromatic block on chromosome 9, which owing to the suppression conditions has no or only very low fluorescence intensities. Such chromosomal regions, rich in repetitive sequences, are prone to resulting in artifacts in chromosome CGH. In array-CGH this heterochromatin block is visible as a large gap around chromosome position 50 Mb because these regions are not represented on arrays. However, the 6.3-Mb deletion is readily visible in array-CGH (*arrow*)

3.4.3.5.4 Copy Number Changes in Healthy Individuals

After completion of the Human Genome Project it was considered that the DNA sequence of essentially every human being was known, as the genomes of healthy individuals were 99.9% identical. The major genetic differences believed to exist between individuals were in the form of scattered single-base pair changes, i.e., single nucleotide polymorphisms (SNPs), accounting for 0.1% of the genome. However, when array-CGH was applied to the genomes of unrelated, healthy individuals hundreds of genomic regions that varied were identified [114, 210]. Many of these copy number polymorphisms are common in gene-rich regions of the genome and are associated with segmental duplication. Using an *in silico* sequence comparison method for identifying not only copy number polymorphism but other structural variants, such as inversions, multiple sites of putative structural variation involving inser-

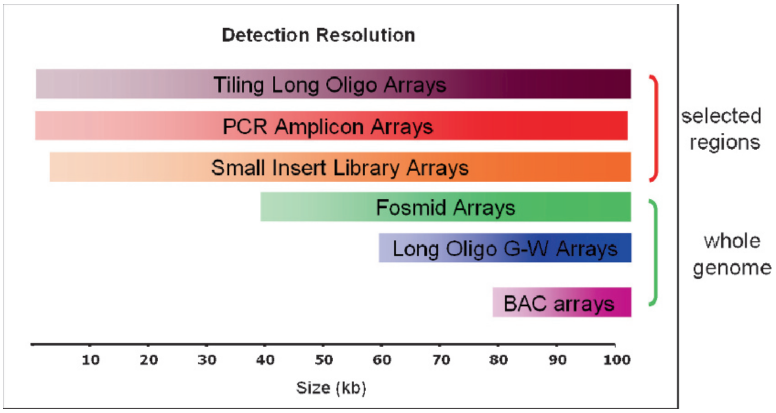


Fig. 3.32 There is now a multitude of different array-platforms for CGH available. In principle, these arrays can be distinguished between arrays to scan the entire genome for gains and losses or in arrays for selected regions. For example, at present many laboratories employ in diagnostic setting for the analysis of DNA samples derived from patients with unknown syndrome long oligo arrays. For more detailed analyses of individual chromosomes there exist chromosome specific tiling long oligo arrays, which provide a resolution in the low kb range

tions, deletions, and inversion breakpoints with the rearrangement ranging in size from 8 kb to as much as 0.9 Mb, were identified [237]. Thus, a surprising and unexpected finding yielded by these high-resolution approaches was that even the genome of normal, healthy individuals has a high number of copy number changes. When several individuals were screened for CNVs, a total of 1,447 copy number variable regions (CNVRs) covering 360 Mb (12% of the genome) were identified. These CNVRs contained hundreds of genes, disease loci, functional elements, and segmental duplications. Notably, the CNVRs encompassed more nucleotide content per genome than SNPs, underscoring the importance of CNVs in genetic diversity and evolution. The data obtained delineate linkage disequilibrium patterns for many CNVs and reveal marked variation in copy number among populations [196].

Submicroscopic CNVs are both intriguing and of particular concern to clinical cytogeneticists, because they can no longer rely on a “standardized” genome – represented at the cytogenetic level as the human karyotype – to identify “abnormal” chromosomal alterations that can be implicated in the etiology of a disease or disorder. In fact, with array-CGH a new terminology was introduced (e.g., structural variants, structural abnormality, CNV, copy-number polymorphism, segmental duplication, low-copy repeat):

Structural variants are operationally defined as genomic alterations that involve segments of DNA larger than 1 kb and can be microscopic or submicroscopic. There is no implication of their frequency, their association with disease or phenotype, or lack thereof. Other alterations that can be considered structural variants include heteromorphisms, fragile sites, ring and marker chromosomes, isochromosomes, double minutes, and gene-conversion products.

The term *structural abnormality* is often used if a structural variant is thought to be disease causing or is discovered as part of a disease study. Here we generally refer to smaller (<1 kb) variations or polymorphisms involving the CNV of a segment of DNA as insertions or deletions (indels).

A *copy-number variant (CNV)* is a segment of DNA that is 1 kb or larger and is present in a variable copy number in comparison with a reference genome. Classes of CNVs include insertions, deletions, and duplications. This definition also includes large-scale CNVs, which are variants involving segments of DNA ≥50 kb in size, allowing them to be detected by clone-based array comparative genome hybridization (array-CGH).

Copy-number polymorphism is the term used for a CNV that occurs in more than 1% of the population. Originally, this definition was used to refer to all CNVs [74].

Segmental duplication or low-copy repeat means a segment of DNA >1 kb in size that occurs in two or more copies per haploid genome, with the different copies sharing >90% sequence identity. They often vary in copy number and can therefore also be CNVs [74].

It is difficult to determine whether a CNV might contribute to phenotypic effects. In general, larger CNVs are probably more likely to cause developmen-

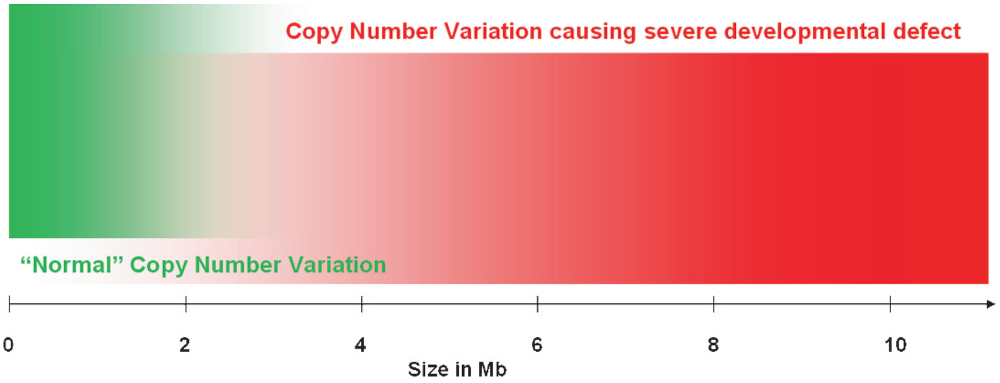


Fig. 3.33 Relationship between CNV size and possible phenotypic effects

tal defects; however, there is no strict threshold to distinguish between "normal" CNVs and disease-causing CNVs (Fig. 3.33).

Association analyses of expression levels of 14,925 transcripts with SNPs and CNVs in individuals who are part of the International HapMap project were performed in order to determine the overall contribution of CNVs to complex phenotypes. SNPs and CNVs captured 83.6 and 17.7% of the total detected genetic variation in gene expression, respectively, but the signals from the two types of variation had little overlap [226].

Interrogation of the genome for CNVs is also an effective way to elucidate the causes of complex phenotypes and disease in humans. In fact, CNVs have already been linked to a variety of different diseases and disease susceptibility, including autism, Crohn disease, AIDS susceptibility, glomerulonephritis, and many others.

Using array-CGH, it was even possible to identify an example of positive selection on a copy number-variable gene, because the copy number of the salivary amylase gene (*AMY1*) is positively correlated with salivary amylase protein level and individuals from populations with high-starch diets have, on average, more AMY1 copies than those with traditionally low-starch diets [181]. This provides a striking example of the role of CNVs in adaptive evolution and of diet in producing selective pressures.

In addition, it even proved possible to identify a novel autosomal-dominantly inherited syndrome associated with a CNV on 4p. This syndrome is characterized by microtia, eye coloboma, and imperforation of the nasolacrimal ducts associated with a CNV on 4p, and represents the first example of an amplified CNV associated with a Mendelian disorder [20].

Furthermore, CNVs may be helpful in population genetics because genome-wide patterns of variation across individuals provide a powerful source of data for uncovering the history of migration, range expansion, and adaptation of the human species [118].

3.4.3.5.5 Array-CGH Identifies Causes of Known Syndromes

Using a 1-Mb resolution array-CGH a deleted region of ~5 Mb at 8q13 was identified in a patient with CHARGE (coloboma of the eye, heart defects, atresia of the choanae, retarded growth and developmental anomalies, genital hypoplasia and/or urinary tract anomalies, and ear anomalies and/or hearing loss) syndrome [240]. Subsequent use of a tiling clone array of chromosome 8 narrowed the CHARGE region to 2.3 Mb containing nine annotated genes. Sequencing of these genes identified mutations in the *CHD7* gene, which were later also found in other patients with CHARGE. Thus, in this case array-CGH pinpointed the region of the disease-causing gene in this disease entity.

Another example was the discovery by array-CGH that patients with Pitt-Hopkins syndrome, a rare syndromic mental disorder, are associated with a microdeletion on 18q21.2 resulting in haploinsufficiency of *TCF4*. Further analysis revealed that Pitt-Hopkins syndrome may also be caused by autosomal dominant mutations in TCF4 [4, 35, 260].

Array-CGH also identified a deletion in chromosome 1q21.1 in patients with TAR (*t*hrombocytopenia-*a*bsent adius) syndrome, which is characterized by hypomegakaryocytic thrombocytopenia and bilateral radial aplasia in the presence of both thumbs.

Interestingly, presence of the deletion on chromosome 1q21.1 alone does not appear to be a sufficient cause of the TAR syndrome, but requires an additional, as yet unknown, modifier (mTAR). Thus, in this case array-CGH identified one component of an apparently complex inheritance pattern [130].

3.4.3.5.6 Analysis of Patients with Unexplained Developmental Delay and Dysmorphic Features

Array-CGH is increasingly being used for diagnosis of constitutional genomic imbalance. It has rapidly evolved to become an indispensable tool for the work-up of patients with unexplained developmental delay and dysmorphic features. Multiple studies related to this group of patients have already been published, suggesting that in up to 20% of cases of mental retardation and multiple congenital anomalies a deletion or duplication that is causative for the phenotype may be found [157]. At present, our knowledge of the phenotypic effects of most gains and losses is minimal. Accordingly, it may be difficult to distinguish between a disease-causing copy number change, a genomic imbalance of unknown clinical significance, and a polymorphism. In general, an imbalance occurring de novo, i.e., which is not present in one of the parents, is more likely to be causative for a conspicuous phenotype. Another indication for a possible causative role is the size of a deletion or duplication: the larger it is the more likely are phenotypic consequences. Owing to the difficulties in establishing whether a copy number is disease associated or not, there are international efforts for a systematic collection of CNVs and their association with specific phenotypes in publicly accessible databases, such as DECIPHER (*D*atabas*e* of *C*hromosomal *I*mbalance and *P*henotype in *H*umans using *E*nsembl *R*esources) (www.sanger.ac.uk/PostGenomics/decipher/) [76] or ECARUCA (*E*uropean *C*ytogeneticists *A*ssociation *R*egister of *U*nbalanced *C*hromosome *A*berrations) (www.ecaruca.net).

3.4.3.5.7 Identification of New Microdeletion Syndromes

The vast majority of imbalances are "private" deletions or duplications, i.e., they are not recurrent and are observed just in individual patients. However, array-CGH has already identified some new microdeletion syndromes, e.g., in chromosomal regions 17q21.3 [132, 211, 213], 16p11.2-p12.2 [21], and 15q13.3 [212].

3.4.3.6 Other Array-Based Methods

3.4.3.6.1 Array Painting: Structural Aberrations at High Resolution

Array-CGH is of little use for the analysis of patients with rearrangements not involving copy number gains or losses, such as inversions or balanced translocations. For balanced translocations, array painting, a modification of the array-CGH method, has been developed which uses flow sorting to separate the derivative chromosomes from the rest of the genome for hybridization to the array [75]. In a balanced translocation, differential labeling of the two derivatives results in the sequences proximal to the breakpoint on a chromosome being labeled in one color while sequences distal to the breakpoint are labeled in the other color. When the two derivatives are hybridized to the array, the measured ratio for the chromosomes involved in the rearrangement switches from high or low (or vice versa) at the breakpoint. If a clone on the array spans the it will report an intermediate value. Array painting will also identify other rearrangements associated with the translocation such as inversion and deletion within the derivative chromosomes [75] and the involvement of additional chromosome regions in the rearrangement [97]. The combined use of array-CGH and array painting provides a comprehensive analysis of genomic rearrangements in patients with apparently balanced translocation and has uncovered a surprisingly high frequency of complexity in these cases [97].

3.4.3.6.2 ChIP on CHIP: Analysis of Chromatin Structure

It is now well established that chromatin organization and modification of associated proteins and complexes have a major functional role in such fundamental processes as transcription, recombination, replication, and

DNA repair. Cytogenetic methods, particularly when combined with chromatin fractionation are proving to be useful tools in the study of chromatin structure and function. Gilbert et al. [90] separated compact and open chromatin structures using sucrose gradients and analyzed the genomic distribution of the differing chromatin states by hybridization of the enriched DNA onto metaphase chromosomes and by array-CGH. They found that regions of open chromatin correlated with high gene density but not necessarily with gene expression, as inactive genes were found in regions of open chromatin and active genes in regions of closed chromatin. Perhaps the most powerful approaches to the study of chromatin structure and function use DNA fractionation and enrichment by chromatin immunoprecipitation (ChIP). In ChIP, chromatin associated with protein-DNA interactions or modifications is specifically enriched by precipitation with an antibody directed against the protein of interest. The enriched DNA sequences can then be mapped and quantified using, in particular, DNA microarrays which provide the resolution required. This combined methodology has been termed ChIP on CHIP. For example, ChIP against phosporylated H2AX, a marker of double-strand breaks in DNA, was used to demonstrate that DNA damage occurring at chromosome ends as a consequence of telomere shortening is associated with cellular senescence [55].

3.5 Meiosis

3.5.1 Biological Function of Meiosis

The somatic cell is diploid, containing both members of a pair of homologous chromosomes (2 n), whereas the germ cell is haploid, containing only one of each pair (n). Thus, in the usual type of somatic cell division, or mitosis, the number of chromosomes in daughter cells remains constant. In contrast, the meiotic process is designed to reduce the number of chromosomes from the diploid number (46 in humans) to one half of this number (23 in humans). Fertilization of two germ cells, each with the haploid number, reconstitutes the diploid number of 46 in the zygote and in all of its descendant cells. Furthermore, meiosis generates genetic diversity, because chance alone determines which of two homologous chromosomes ends up in a given germ cell. Beside this independent assortment of chromosomes, physical exchange of chromosomal regions by homologous recombination during prophase I results in new genetic combinations within chromosomes.

3.5.2 Meiotic Divisions

Complete meiosis consists of two cell divisions, meiosis I and II. Meiosis I is a reductional cellular division involving the segregation of homologous chromosomes. Meiosis II is an equational division with the segregation of sister chromatids. Perhaps the most relevant events occur during the first – and most complicated – phase of meiosis I. In this phase homologous chromosomes pair, become intimately associated (synapsis), and exchange genetic material (crossing over or meiotic recombination; Fig. 3.34).

Meiotic Division I. Prophase I: The complex series of prophase events is subdivided into five stages (leptotene, zygotene, pachytene, diplotene, and diakinesis) reflecting the progression of synapsis and recombination.

Leptotene: Long chromosome threads become visible, but the two sister chromatids are still tightly bound, making them indistinguishable from one another. The chromosomes show a specific arrangement with telomeres oriented towards the nuclear membrane. This stage is called the "bouquet stage."

Zygotene: During this stage homologous chromosomes pair, frequently starting at the chromosome ends. The paired homologous chromosomes are referred to as bivalent, or also as tetrad as they consist of four sister chromatids. The paired homologous chromosomes are connected by the so-called synaptonemal complex, in a process known as synapsis. The synaptonemal complex is a tripartite protein structure between homologous chromosomes, which is essential for meiotic recombination to take place.

Pachytene: After completion of pairing, the chromosomes become shorter through contraction. At this stage, nonsister chromatids of homologous chromosomes may randomly exchange segments of genetic information over regions of homology. This exchange occurs at sites where recombination nodules or chiasmata have formed. Sex chromosomes exchange occurs only over

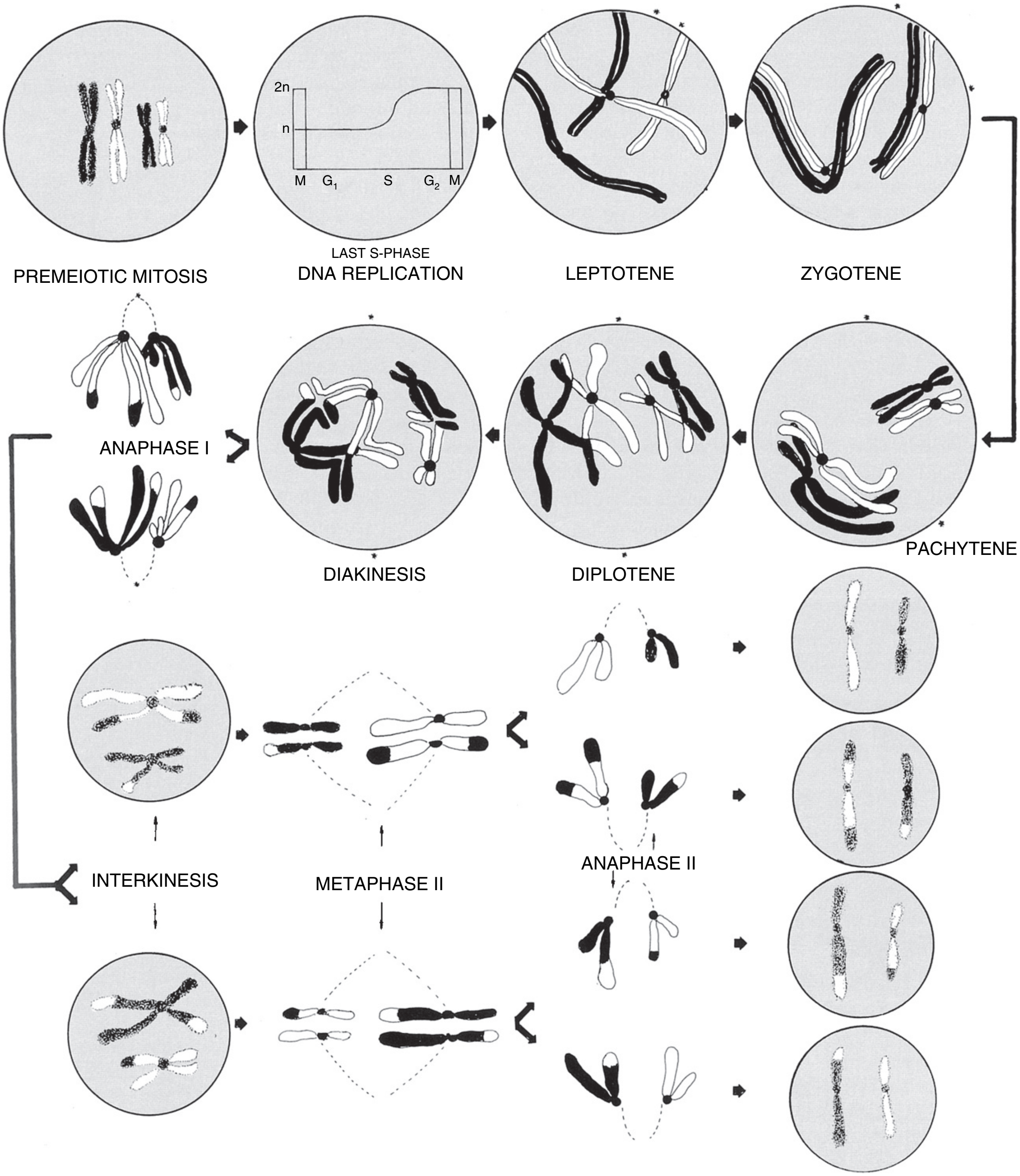


Fig. 3.34 The stages of meiosis. *Black* paternal, *white* maternal chromosomes. Male meiosis is depicted; in female meiosis, polar body formation occurs. (From the 3rd edition of this work)

a small region of homology, the “pseudo-autosomal region” (explained in Sect. 3.6.3.2).

Diplotene: A longitudinal cleft in each pair of chromosomes becomes visible as the synaptonemal complex degrades. This allows some separation of the homologous chromosomes; nonsister chromatids are separated, while sister chromatids remain paired. However, the homologous chromosomes of each bivalent remain connected to each other at the chiasmata – the regions where crossing-over occurred – and become visible between nonsister chromatids. The chiasmata persist until anaphase I. In mammalian females meiosis stops at a modified diplotene stage (dictyate) and can remain in this state for decades.

Diakinesis: During this phase the four chromatids of each kind become visible and appear side by side. The chiasmata are now clearly visible. Much of this stage resembles prometaphase of mitosis as the nucleoli disappear, the nuclear membrane disintegrates and the meiotic spindle begins to form.

Metaphase I: The chromosomes are ordered in the metaphase plane as the centromeres are drawn to the poles. The homologous chromosomes are drawn somewhat apart but are still kept together by the chiasmata.

Anaphase I: As microtubules shorten, pulling homologous chromosomes apart, the chiasmata are resolved. As a consequence, whole chromosomes are pulled toward opposing poles, forming two haploid sets.

Telophase I: The centromeres arrive at the poles and each daughter cell now has half the number of chromosomes with each chromosome consisting of a pair of chromatids. The microtubules disappear and a new nuclear membrane surrounds each haploid set. The chromosomes uncoil back into chromatin. Cytokinesis completes the creation of two daughter cells.

Meiotic Division II: This is in principle a mitotic division of the replicated haploid set of chromosomes. As such, four phases can be distinguished (prophase II, metaphase II, anaphase II, and telophase II). As noted above, meiosis begins after replication. The genetic material which during division I has become fourfold (2 × 2 homologous chromosomes) is, at the completion of division II, ordinarily distributed to four cells.

A second important aspect in meiosis is the random distribution of nonhomologous chromosomes, which leads to a very large number of possible combinations in germ cells. In humans with 23 chromosome pairs, the number of possible combinations in one germ cell is $2^{23} = 8{,}388{,}608$. The number of possible combinations of chromosomes in an offspring of a given pair of parents is $2^{23} \times 2^{23}$ and is further enhanced by crossing over during pairing of homologous chromosomes. The morphological counterpart of crossing over is chiasma formation. Every chiasma corresponds to one crossing over event involving two nonsister chromatids.

3.5.3 Meiotic Recombination Hotspots

Each chiasma corresponds to a locus in which crossing over has occurred. The result of crossing over is an exchange between two chromatids of homologous chromosomes. Any one pair of homologous chromosomes normally experiences at least one crossing-over event during the first meiotic division in order to assure proper chromosomal segregation. A single meiotic crossover event produces two chromosomes, each divided into one portion of grandmaternal and one portion of grandpaternal origin.

Recombination by crossing over also defines the genetic distance between two loci. The *physical distance* is the number of nucleotide base pairs between DNA loci. In contrast, the *genetic distance* between two DNA loci on a chromosome is given as the amount of crossing over between these two markers. The unit of the genetic distance is morgan. Thus, one morgan, M, is the unit of map distance between linked genes; this unit measures recombination frequencies. One centimorgan (cM) means 1% recombination and equals the crossover frequencies between genes.

The distribution of crossing-over events within a chromosome was originally thought to be uniform. However, newer technologies, such as sperm-typing technologies, i.e., screening sperm for recombinant DNA molecules [120], linkage studies, mapping crossovers in large pedigrees by high-density SNP typing [48], and computational inferences from population genetic data have provided detailed high-resolution maps of the sites of crossing over. Together the data suggest that the sites of recombination are not randomly distributed along the chromosomes, and this is likely due to the presence of numerous hotspots and coldspots of recombination [186]. Thus, recombination occurs preferentially in hotspots. Linkage disequilibrium (LD) data, e.g., from the International HapMap Project, can be used to infer the recombination history. Similarities between LD maps of different populations presumably reflect common localizations of recombination hotspots, and these hotspots are frequently referred to as "historical" hotspots. Computational approaches have inferred more than 25,000 historical hotspots, and it is estimated that as many as 50,000 hotspots may exist [163]. Historical hotspots have a median width of 4 kb. Thus, there are many narrow and intense hotspots throughout the genome. It is estimated that 80% of any recombination occurs in only 10–20% of the sequence [12, 163].

3.5.4 Differences Between Male and Female Meiosis

There are two principal aspects by which meiosis differs in males and females:

1. In males all four division products develop into mature germ cells, whereas in females only one of them becomes a mature oocyte, while the others are lost.
2. In males, meiosis immediately follows a long series of mitotic divisions; it is completed when spermatids start developing into mature sperms. In females, meiosis begins at a very early stage of development, immediately after a much smaller series of mitotic divisions. It is then arrested for many years and is only finished after fertilization.

These sex differences are important in human genetics. The fact that only one of the four division products develops into a mature oocyte, and the three polar bodies contain little or no cytoplasm, enables this oocyte to transmit to the new zygote a full set of cytoplasmic constituents, such as mitochondria and messenger RNA. These differences in cell kinetics are probably responsible for sex differences in mutations rates for trisomies, on the one hand, and point mutations, on the other.

3.5.4.1 Meiosis in the Human Male

From the beginning of puberty human spermatocytes continuously undergo meiosis. After the second meiotic division, DNA is densely packed during sperm development and the sperm acquires the ability to move actively.

Genes located on a common segment of the X and Y chromosome can freely recombine with each other, and this region has therefore been dubbed the "pseudoautosomal region" to indicate that it behaves as autosomes do in terms of recombination.

3.5.4.2 Meiosis in the Human Female

In all mammals, oogenesis differs substantially from spermatogenesis. Meiosis is initiated in the human fetal ovary at 11–12 weeks of gestation. On completion of recombination, the oocyte progresses to diplotene of prophase and enters a protracted arrest stage known as dictyate. Around the time of arrest, oocytes become surrounded by somatic cells (pregranulosa cells), forming primordial follicles. Many oocytes are lost during follicle formation, and the newborn ovary contains only a fraction of the total oocytes that entered meiosis in the fetal ovary.

In the sexually mature female one oocyte, on average, completes growth each month and is ovulated in response to a midcycle surge of luteinizing hormone (LH). In response to the LH surge, the oocyte resumes meiosis, as outlined above. Whereas one group of chromosomes remains in the oocyte, the other is segregated to the first polar body. Meiosis I (MI) is immediately followed by meiosis II. The metaphase II-arrested cell is known as an egg, and it remains in arrest until it is fertilized or degenerates. Fusion of the sperm and egg plasma membranes at fertilization triggers the resumption and completion of MII so that sister chromatids segregate, with one group of chromosomes remaining in the egg and the other segregated into a second polar body. After the division, separate nuclear envelopes form around the remaining egg chromosomes and the chromosomes contributed by the sperm, forming a zygote. The chromosomes in the male and female pronuclei undergo DNA replication and condense in preparation for the first mitotic cleavage division.

Thus, in females only one of the four meiotic products develops into an oocyte, while the others become polar bodies that are not fertilized.

3.5.4.3 A Dynamic Oocyte Pool?

The view that the pool of oocytes in the ovary is established during fetal development and all eggs ovulated by the adult female initiate meiosis in the fetal ovary was recently challenged [124, 125]. Evidence for the existence of germline stem cells capable of giving rise to new oocytes in the adult was provided. At present there is a controversial debate about whether such “adult oocytes” with the capability to mature and ovulate exist beside “fetal oocytes.”

3.5.4.4 Maternal Age and Aneuploidy

Chromosome anomalies are extraordinarily common in human gametes, with approximately 21% of oocytes and 9% of spermatozoa abnormal. The types of abnormalities are quite different, since most abnormal oocytes are aneuploid, whereas the majority of abnormalities in spermatozoa are structural. Chromosomes 21 and 22 (the smallest chromosomes) are overrepresented in aneuploid gametes in both oocytes and sperm. Chromosome 16 is also frequently observed in aneuploid oocytes, whereas the sex chromosomes are particularly predisposed to nondisjunction in human sperm. Maternal age is clearly the most significant factor in the etiology of aneuploidy, and most aneuploidy derives from errors in maternal meiosis I (Table 3.3). Paternal age does not have a dramatic effect on the frequency of aneuploid sperm; there is some evidence for a modest increase in the frequency of sex chromosomal aneuploidy. Meiotic recombination has a significant effect on the genesis of aneuploidy in both females and males. New techniques allowing the analysis of recombination along the synaptonemal complex have yielded interesting new information in healthy and infertile individuals, establishing a link between infertility and the genesis of chromosome abnormalities. Future studies will unravel more of the underlying causal factors.

At least 7–10% of clinically recognized pregnancies start with an abnormal set of chromosomes [102]. The incidence of chromosomal errors increases exponentially with advancing maternal age. Among women in their early twenties, the risk of trisomy in a clinically recognized pregnancy is 2–3%, but among women in their forties the risk increases to 30–35%. Studies of the origin of human trisomies demonstrated that age influences the likelihood of errors at both meiosis I (MI) and meiosis II (MII) [257]. However, despite considerable research effort, the effect of maternal age remains the “black box” of human aneuploidy. To date, no single hypothesis has provided a satisfactory explanation for this phenomenon. Instead of rather simple terms of cause and effect a more complex picture has emerged, suggesting that female fertility is influenced by a series of events occurring at different stages of egg development. Three vulnerable stages of oogenesis can be distinguished: first, the meiotic prophase events of synapsis and recombination occurring in the fetal ovary; secondly, follicle formation during the second trimester of fetal development; thirdly, oocyte growth in the adult ovary [113]. In fact, eggs may be vulnerable at each of these stages, and environmental influences are likely to have an additional impact [113]. Thus, future research efforts aiming at a better understanding of each of these phases

Table 3.3 Summary of studies of the origin of human trisomies (from [102])

		Maternal		Paternal		
Trisomy	*N*	MI (%)	MII (%)	MI (%)	MII (%)	PZM (%)
Acrocentrics						
13	74	56.6	33.9	2.7	5.4	1.4
14	26	36.5	36.5	0.0	19.2	7.7
15	34	76.3	9.0	0.0	14.7	0.0
21	782	69.6	23.6	1.7	2.3	2.7
22	130	86.4	10.0	1.8	0.0	1.8
Nonacrocentrics						
2	18	53.4	13.3	27.8	0.0	5.6
7	14	17.2	25.7	0.0	0.0	57.1
8	12	50.0	50.0	0.0	0.0	50.0
16	104	100	0.0	0.0	0.0	0.0
18	150	33.3	58.7	0.0	0.0	8.0
XXX	46	63.0	17.4	0.0	0.0	19.6
XXY	224	25.4	15.2	50.9	0.0	8.5

MI meiosis I, *MII* meiosis II, *PZM* postzygotic mitotic may unravel factors involved in the close relationship between maternal age and increasing aneuploidy.

3.5.5 Nonallelic Homologous Recombination During Meiosis Can Cause Microdeletion/Microduplication Syndromes

DNA rearrangements during meiosis may occur owing to homologous recombination involving region-specific, low copy repeats. This is also referred to as "nonallelic homologous recombination" (NAHR) between similar sequences (repeats) present at more than one site in the genome. Nonallelic homologous crossover was first described by Sturtevant [227] and Bridges [34] in the bar locus in *Drosophila*. The NAHR mechanism was described and documented in alpha thalassemias, beta thalassemia Lepore, and the growth hormone cluster (see Sect. 3.2.7 on unequal crossing over). More recently, genomic rearrangements attributable to NAHR have been referred to as genomic disorder [150], because they are caused by special conditions that result from genome architecture.

NAHR occurs during meiotic crossing over between homologous chromosomes each carrying two repeats and separated by interrepeat DNA. Recombination between direct repeats results in deletion and/or duplication, whereas recombination between inverted repeats results in an inversion (Fig. 3.35). NAHR can also occur between repeats on the same chromosome or between repeats on different chromosomes, and the specific genetic outcome of NAHR (duplication, deletion, inversion, or chromosomal translocation) depends on the chromosomal location of the repeats and whether the repeats are oriented head to tail, head to head, or tail to tail with respect to one another (reviewed in [224]). Large literature exists showing that many human diseases and syndromes arise by NAHR (reviewed in [224]). Variations in DNA sequence copy

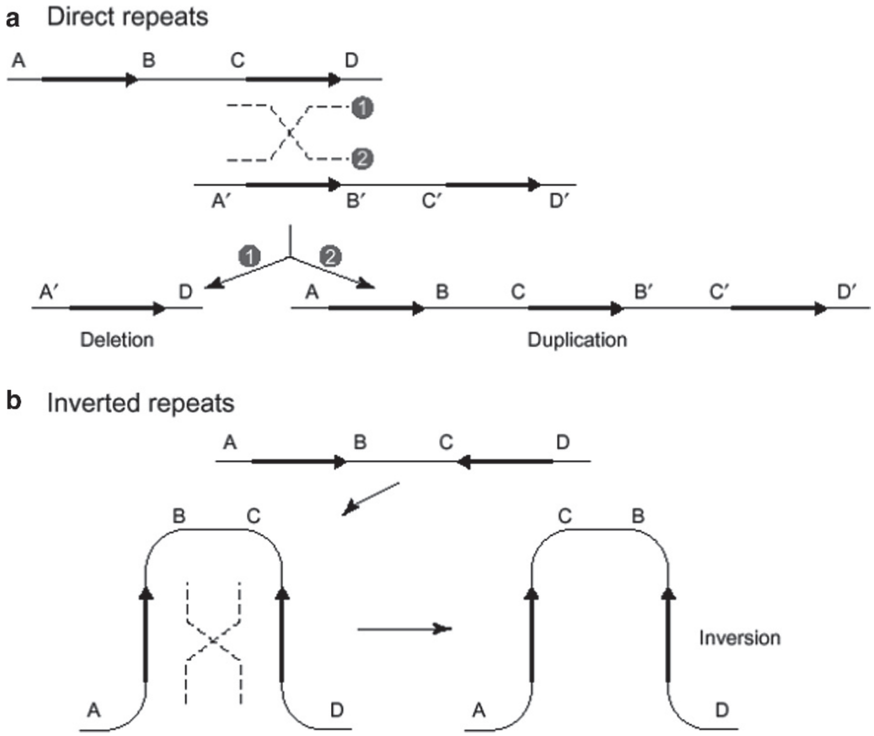


Fig. 3.35 Duplication and deletion formation by NAHR. The *black arrows* represent repeated sequences, and the orientation of these repeats is indicated by the direction of the *arrowhead.* The inter-repeat DNA is depicted as *thin lines*; *capital letters* point to the flanking unique sequences and illustrate consequences of genomic rearrangements. Direct repeats (*a*) result in deletion and/or duplication, whereas inverted repeats (*b*) result in an inversion. From [150], with permission from Elsevier

number in the normal population may also arise by NAHR (see also Sect. 3.5.5).

3.5.6 Molecular Mechanisms Involved in Meiosis

During meiosis spindle formation and chromosome segregation have to proceed in a highly reliable fashion. The spindle assembly checkpoint (SAC) (see Sect. 3.3.3) monitors attachment to microtubules and tension on chromosomes not only in mitosis but also in meiosis. Thus, many components involved in mitosis are also important during meiosis. At the same time differences exist. It would be beyond the scope of this chapter to review the entire current knowledge about the signaling cascade of the SAC for normal chromosome segregation and meiosis here, but some important components will be summarized.

During meiosis two consecutive rounds of nuclear division are required to first segregate homologous chromosomes (at anaphase I) followed by the segregation of sister chromatids (at anaphase II) for the formation of a haploid gamete.

Meiotic prophase I is an especially long and complex phase, because homologous recombination occurs between homologous chromosomes. Formation of chiasmata, which hold homologous chromosomes together until the metaphase I to anaphase I transition, is critical for proper chromosome segregation. Sites of double-stranded DNA breaks (DSBs) have to be generated that are thought to be the starting points of homologous recombination. Processing of these sites of DSBs requires sophisticated repair mechanisms involving the function of RecA homologs, such as RAD51, DMC1, and others. Failure to repair these meiotic DSBs results in abnormal chromosomal alternations, leading to disrupted meiosis.

To pull homologous chromosomes to opposite spindle poles during meiosis I, both sister kinetochores of a homolog must establish attachment to the same pole (syntelic attachment). Sister chromatids of meiotic chromosomes are held together all along the chromosome arms and centromeres by the meiosis-specific cohesin complexes containing REC8. The kinase PIK1 marks REC8 at chromatid arms by phosphorylation for degradation by separase. After silencing of the SAC, at the metaphase I-to-anaphase I transition, separase cleaves REC8 to release cohesin between sister chromatid arms while centromeric cohesin are kept unphosporylated by activity of the phosphatase PP2A. A complex of Shugoshin (SGO) and PP2A is recruited by BUB1 to centromeres at meiosis I to protect Rec8 from phosphorylation by PLK1. The spindle assembly checkpoint (SAC) is constitutively active during prometaphase of meiosis I when sister kinetochores of homologous chromosomes are either co-oriented to the same pole or not fully attached to spindle microtubules and under tension from kinetochore microtubules.

The SAC is also reactivated and halts cell cycle progression when attachment to the spindle is lost at metaphase I by inhibiting the APC/C, as is also characteristic for mitosis. Upon chromosome congression, when chromosomes are under full tension from spindle fibers at metaphase I, the SAC is inactivated and cyclin B and securin become degraded after ubiquination by APC/C. Separase cleaves meiotic cohesin REC8 marked by phosphorylation by PLK1 along chromosome arms.

MPF becomes active again at prometaphase II. However, in the presence of CSF, the mammalian oocyte arrests at metaphase II in spite of aligned chromosomes until fertilization triggers progression into anaphase II for completion of meiosis (reviewed in [182]).

3.6 Human Chromosome Pathology in Postnatal Diagnostics

3.6.1 Syndromes Attributable to Numeric Anomalies of Autosomes

3.6.1.1 Mechanisms Creating Anomalies in Chromosome Numbers (Numerical Chromosome Mutations)

Anomalies in chromosome numbers may be caused by various mechanisms:

(a) The most important mechanism is nondisjunction. Chromosomes that should normally be separated during cell division stick together and are transported in anaphase to one pole. This may occur at mitotic division and during meiosis. Meiotic nondisjunction was discovered by Bridges in 1916 in *Drosophila* [33].). For every gamete with one additional chromosome, another one is formed with one chromosome fewer (Fig. 3.36). After fertilization with a normal gamete the zygote is either trisomic or monosomic. Somatic nondisjunction in mitotic cell division during early development may lead to mosaics with normal, trisomic, and monosomic cells.

(b) A second mechanism leading to numerical abnormalities is loss or gain of single chromosomes. Such alterations in whole chromosomes may be facilitated by errors in the mitotic spindle checkpoints. Chromosome losses can become visible as "anaphase lagging" when one chromosome may lag behind the others. Chromosome loss leads to mosaics with one euploid and one monosomic cell population. Data from preimplantation aneuploidy screening studies suggest that the pronucleus stage and the first cleavage stages are especially susceptible to chromosome segregation errors.

(c) A third mechanism is polyploidization. Here all chromosomes are present more than twice in every cell. For example, in triploid cells the chromosome number is 3n = 69. In prenatal diagnosis, triploidy may be observed in early pregnancy (1–3% of recognized pregnancies). However, the vast majority (more than 99%) are lost as first-trimester miscar-

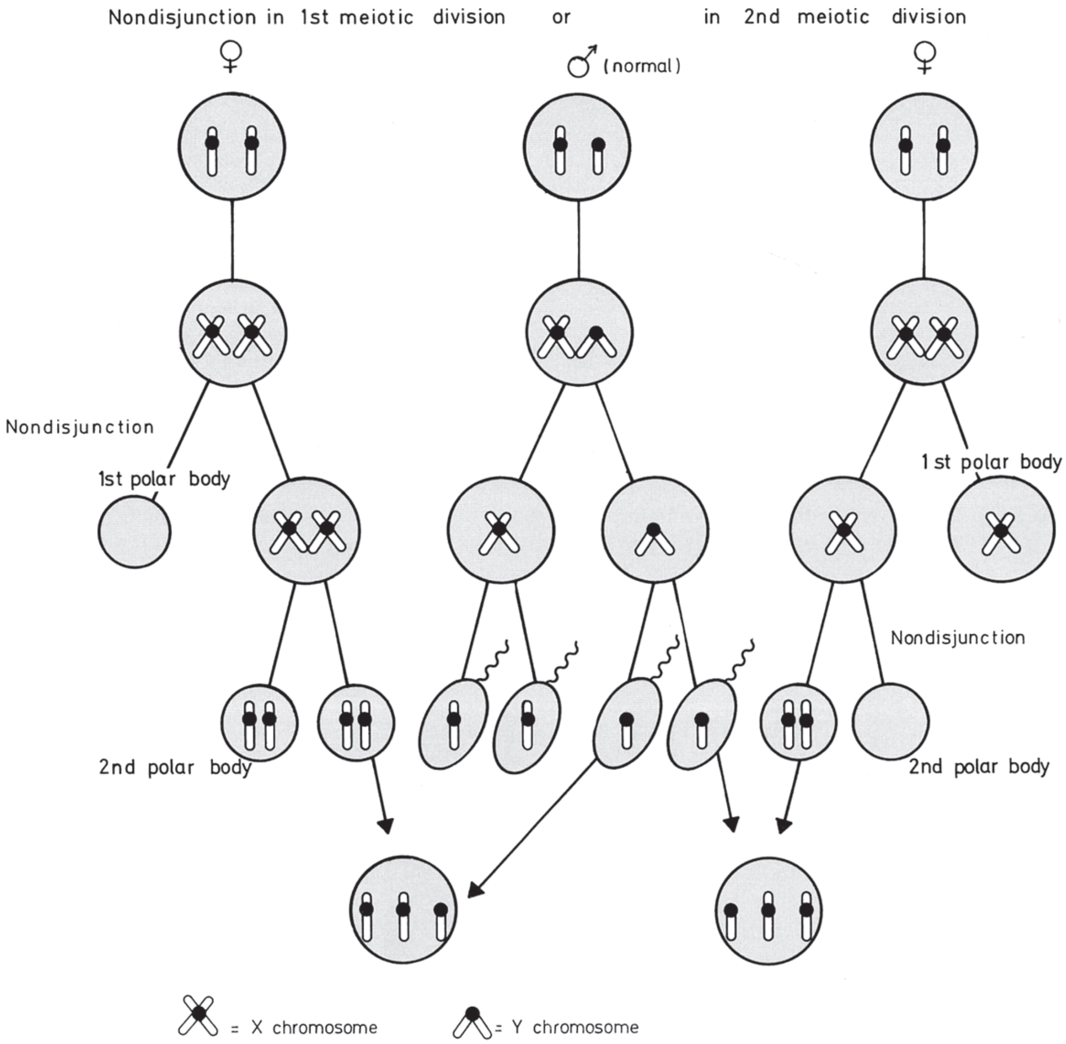


Fig. 3.36 Nondisjunction of the X chromosome in the first (*left*) and second (*right*) meiotic division in a woman. Fertlization by a normal Y sperm. An XXY individual can result from either first or second meiotic division nondisjunction. (From the 3rd edition of this work)

riage or second-trimester fetal death in utero. In some human somatic cell types, such as liver cells, heart muscle cells, or megakaryocytes, polyploidization may occur physiologically. Furthermore, polyploidization is also often observed in tumor cells.

An abnormal number of chromosomes in a cell (aneuploidy) increases the risk of further irregularities. Almost all conditions that result from chromosome imbalances affect multiple systems and produce both structural and functional defects.

There are only three autosomal trisomy syndromes which can be present in all somatic cells and result in the live birth of children, and these are the trisomies 21 (Down syndrome), 13 (Patau syndrome), and 18 (Edwards syndrome). All other autosomal trisomies are assumed to be invariably lethal, and they can be observed by applying chromosome studies in spontaneous abortion material. If autosomal trisomies other than 13, 18, or 21 are observed postnatally, they are usually present only as mosaics, i.e., together with a normal cell line in live newborns. One reason why chromosomes 13, 18, or 21 may result in a live birth is likely their low gene density. For example, chromosomes 18 and 19 have about the same size. However, chromosome 18 has approximately 5.3 genes/Mb, whereas chromosome 19 has approximately 25 genes/Mb. Thus, chromosome 18 is one of the gene-poorest human chromosomes and similar considerations are also true for chromosomes 13 and 21.

In contrast, there is no autosomal monosomy which can result – again with the exception of possible mosaic constellations – in a live birth.

3.6.1.2 Down Syndrome

With an incidence at birth of 1–2/1,000, Down syndrome is the most frequent chromosome aberration syndrome in humans, and a common condition encountered in genetic counseling services. Down syndrome was named after the British physician J. Langdon Down, who recognized the phenotype as a clinical entity in 1866, i.e., long before trisomy 21 was discovered as the genetic basis. In fact, the association between trisomy 21 and Down syndrome was only established in 1959 by Lejeune [142].

Paradigm for Meiotic Nondisjunction: In the majority of cases trisomy 21 is caused by meiotic maternal nondisjunction, which is related to the age of the mother. This nondisjunction is possibly associated with errors in recombination and age-dependent loss of cohesion of meiotic chromosomes [7, 8, 102, 247]. In fact, about 68% of nondisjunctions leading to the conception and birth of children with Down syndrome occur during maternal meiosis I, about 20% during maternal meiosis II, about 4% during paternal meiosis II, and about 3% during paternal meiosis I. The rest (approximately 5%) are postzygotic events, i.e., represent mitotic chromosome segregation errors [7, 8, 102, 247].

Standard Karyotype in Down Syndrome: Trisomy of the entire chromosome is present in about 95% of cases, whereas in a small fraction of cases trisomy of only a part of chromosome 21 leads to Down syndrome. A standard karyotype of a human with Down syndrome is illustrated in Fig. 3.37. Such a free translocation will be found in the vast majority (about 92.5%) of humans with Down syndrome. In about 5% of cases the additional chromosome 21 will participate in formation of a Robertsonian translocation with another acrocentric chromosome, with chromosomes 14 and 21 being the most frequent translocation partners. Very occasionally (<1%) chromosome 21 is translocated to another chromosome. In about 3–5% chromosome 21 is found in mosaic constellations, these cases are typically the results of postzygotic segregation errors.

Phenotypic Variability of Down Syndrome: Some aspects of the phenotype appear to occur in every person with Down syndrome, whereas other traits are more highly variable [9]. However, even for phenotypes that occur in every individual there is variability in expression. Cognitive impairment is apparent in all persons with Down syndrome, but ranges from mild to moderate. By contrast, congenital heart defects occur in only 40%, and its severity also varies. In spite of this appreciable variability of signs, the clinical diagnosis is rarely in doubt for experienced clinicians. The clinical diagnosis can be made at birth based on the presence of marked hypotonia and several minor dysmorphic features, which are especially visible in the craniofacies, hands, and feet (Fig. 3.38). Importantly, there is a temporal dimension to the evolution of the phenotype, which changes with age [6] and which is summarized in Table 3.4. Life expectancy of Down syndrome individuals is reduced. However, and especially because of more efficient treatment of congenital heart diseases by surgery and by compensating the compromised immune system by immuniza-

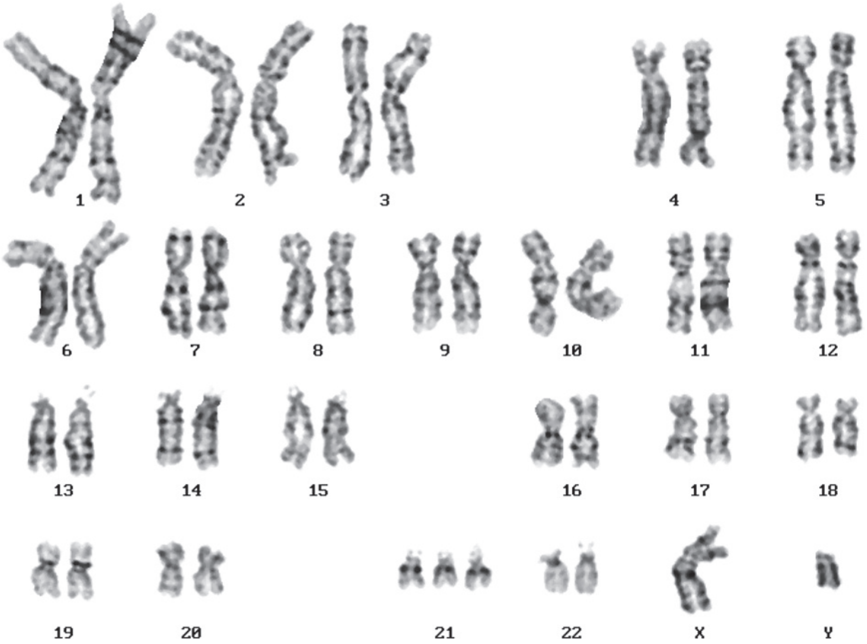


Fig. 3.37 G-Banded chromosome with a free trisomy 21

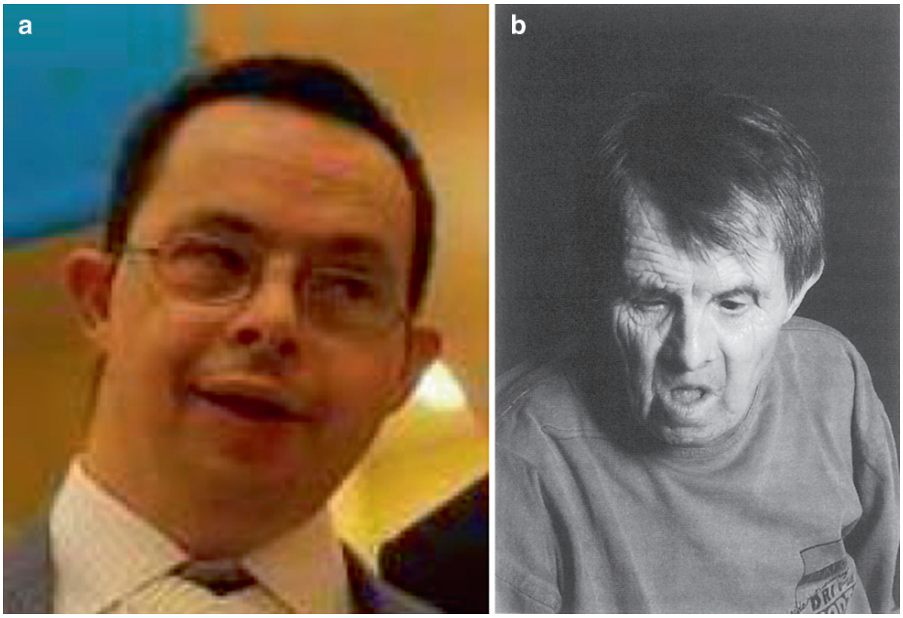


Fig. 3.38 Adults with Down syndrome. (**a**) This person was one of the original cases described by Lejeune [142]. (From [6], with permission from Elsevier) (**b**) Man with Down syndrome at the age of 64. (Courtesy of Dr. G. Tariverdian, from the 3rd edition of this work)

tions and antibiotics, the life expectancy has been rapidly increasing over the last 20 years.

Males with Down syndrome usually have no children and are infertile, likely as a result of defective spermatogenesis. However, there are a few case reports of confirmed paternity in offspring of males with apparently nonmosaic trisomy 21 [192, 215]. These occasional reports are likely very rare exceptions from

Table 3.4 Characteristics of Down syndrome (from [6], with permission from Elsevier)

At birth	Infancy and childhood	Adulthood
Structural		
Dysmorphic features	Growth retardation and obesity	
Congenital heart disease		
Duodenal stenosis or atresia		
Imperforate anus		
Hirschsprung disease		
Central nervous system		
Hypotonia	Developmental and mental retardation	Decrease in cognitive function
	Decreased sensitivity to pain	Alzheimer disease
Immune and hematopoietic systems		
Transient myeloproliferative disorder	Leukemia	
	Immune defects and/or infection	
Other	Thyroid dysfunction	Male sterility
		Reduced longevity

the rule. By contrast, several women with this syndrome have been reported to have offspring. The subfertility in Down syndrome females can likely be attributed to the reduced number of ovarian follicles and the increased rate of atresia. A review summarizing 29 pregnancies in 26 nonmosaic trisomy 21 females reported that 10 offspring also had Down syndrome, 18 offspring were chromosomally normal but 2 of these were mentally retarded, and 4 had other congenital abnormalities. The other pregnancies ended in spontaneous abortions or premature birth followed by death of the babies [215].

Down Syndrome as a Multigene Disorder: The length of the long arm (21q) of human chromosome 21 is 33.6 Mb and represents about 1% of the total euchromatic genome sequences. Current estimates are that 21q harbors 253 protein-coding genes (www.ensembl.org; Ensembl release 52, December 2008); however, this number may be amended as our knowledge of chromosome 21 progresses. Current research is directed at the question of whether only a few of the chromosome 21 genes with a major phenotypic effect are involved in determining the phenotype of Down syndrome or whether the phenotype results from the interaction of several genes of modest effect [6]. At present, based on studies in animal models, the former option may be correct, suggesting that the number of genes involved in Down syndrome is likely considerably lower than their total number on the chromosome. Hence, Down syndrome is likely a disorder of gene dosage caused by increased amounts of the products of the genes on chromosome 21. An example for such a gene-dosage hypothesis is the amyloid precursor protein (*APP*) gene, whose increased dosage is likely involved in the development of Alzheimer disease, which is invariably present in humans with Down syndrome from 35 years of age onward. In addition to protein-coding genes, functional non-protein-coding DNA elements may contribute to phenotypic features, but the exploration of their roles is in their early infancy.

Down Syndrome and Malignant Diseases: Infants with Down syndrome have a 20-fold increased risk of developing acute leukemia, most commonly acute megakaryoblastic (M7) leukemia (AMKL). This is often preceded by a neonatal leukemoid reaction (transient myeloproliferative disorder), which might be a form of transient leukemia. This observation gave rise to the hypothesis that humans with Down syndrome may have a higher risk of aneuploidy owing to mitotic disturbances in blood stem cells. However, and in contrast to such a hypothesis that cells with an autosomal trisomy may be more prone to chromosome segregation errors, Down syndrome individuals are less prone to developing solid tumors [205, 256]. Thus, trisomy 21 appears to increase the risk for a special form of leukemia, while it apparently has a protective effect against other malignancies. Gene dosage alterations caused by the trisomy 21 alone appear not to be sufficient to cause AMKL, as current transformation models suggest that in addition somatic mutations in an X-chromosomal gene, i.e., *GATA1* (GATA-binding protein 1), which encodes an essential transcriptional regulator of normal megakaryocytic differentiation, are required [106]. This unique nonactivating mutation in the *GATA1* gene is associated with both the leukemia and the leukemoid reaction. To explain the reduced incidence of many cancer types in individuals with Down's syndrome, innovative combinations of mouse models were used and these models revealed that the expression level of only one gene, *Ets2*, correlated directly with its copy number and inversely with tumor number in a model for colon cancer [230]. Furthermore, another gene, Down's syndrome candidate region-1 (*DSCR1*, also known as *RCAN1*), which encodes a protein that suppresses vascular endothelial growth factor (VEGF)-mediated angiogenic signaling by the calcineurin pathway, was shown to be increased in Down syndrome tissues. In a mouse model a modest increase in expression afforded by a single extra transgenic copy of *Dscr1* was sufficient to confer significant suppression of tumour growth in mice. This resistance may be a consequence of a deficit in tumor angiogenesis arising from suppression of the calcineurin pathway. Thus, increased expression of *DSCR1* could be another explanation for the reduced cancer risk [17].

3.6.1.3 Other Autosomal Trisomies

Patau et al. [178] described the first case of an autosomal trisomy, i.e., trisomy 13, other than trisomy 21. At about the same time, trisomy 18 was also discovered by Edwards et al. [68]. These trisomies are much less frequent than Down syndrome (about 1:3,000 and 1:5,000, respectively), and both show a maternal age effect. Both syndromes are clinically severe conditions; about 85–90% of liveborns do not survive beyond 1 year of life. For both syndromes there are some case reports describing long-term survival. The main signs and symptoms of both trisomies are shown in Figs. 3.39 and 3.40.

3.6.1.4 Triploidy

In triploidy (3n = 69) there is a double (2n) chromosomal contribution to the conceptus from one parent. Diandry may be due to either two sperms simultane-

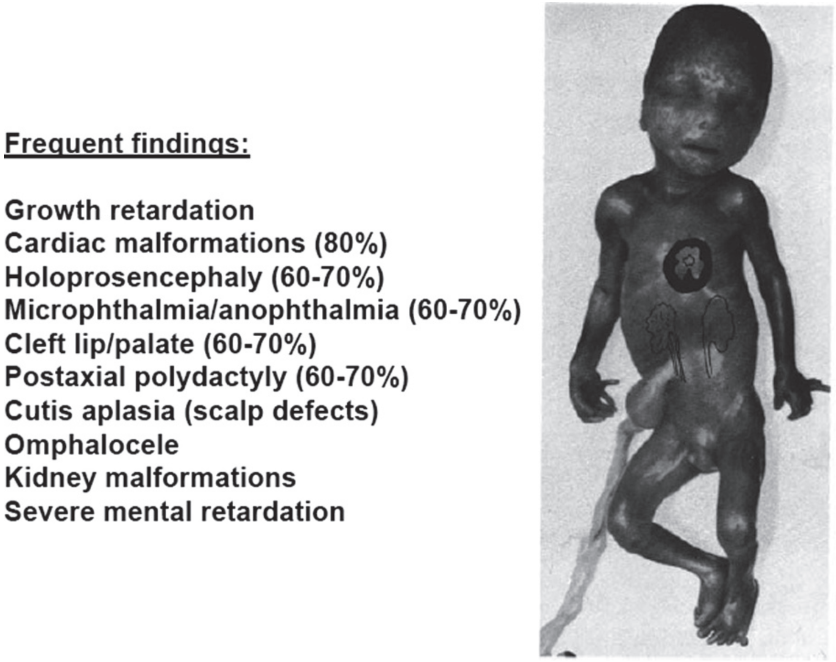
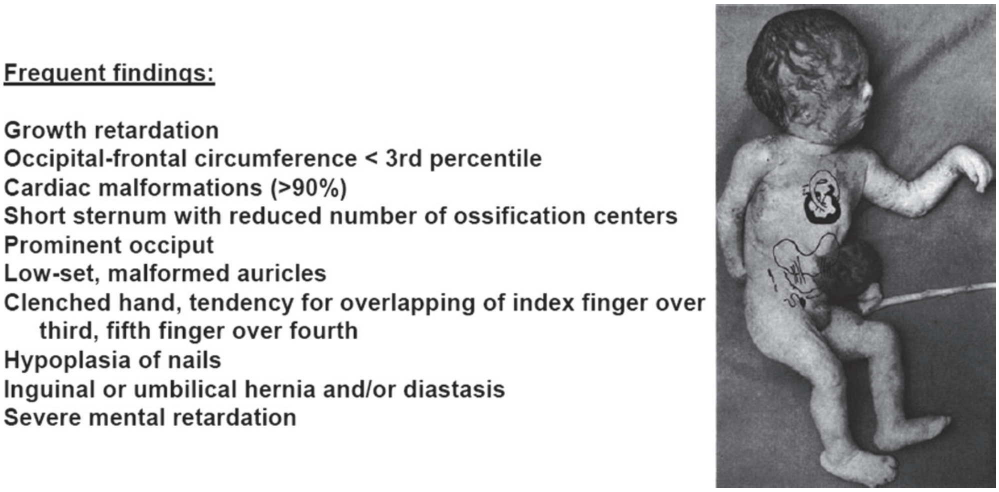

Frequent findings:
Growth retardation
Cardiac malformations (80%)
Holoprosencephaly (60-70%)
Microphthalmia/anophthalmia (60-70%)
Cleft lip/palate (60-70%)
Postaxial polydactyly (60-70%)
Cutis aplasia (scalp defects)
Omphalocele
Kidney malformations
Severe mental retardation

Fig. 3.39 Main clinical findings of trisomy 13. (From the 3rd edition of this work)



Frequent findings:

Growth retardation
Occipital-frontal circumference < 3rd percentile
Cardiac malformations (>90%)
Short sternum with reduced number of ossification centers
Prominent occiput
Low-set, malformed auricles
Clenched hand, tendency for overlapping of index finger over third, fifth finger over fourth
Hypoplasia of nails
Inguinal or umbilical hernia and/or diastasis
Severe mental retardation

Fig. 3.40 Main clinical findings of trisomy 18. (From the 3rd edition of this work)

ously fertilizing the ovum or of a diploid sperm from a complete nondisjunction in spermatogenesis. Digyny may be the consequence of a complete nondisjunction at either the first or the second meiotic division in oogenesis, of retention of a polar body, or of the fertilization of an ovulated primary oocyte. More than 99% of pregnancies starting with a triploid chromosome set are lost during the first or second trimester. There are only a few anecdotal case reports of live births of triploid newborns, and one exceptional report of a triploid infant who survived for 10.5 months [214].

3.6.1.5 Mosaics

Individuals with two or more genetically different cell populations are referred to as mosaics. This is found relatively often in numerical chromosome aberrations of the sex chromosomes, but also in autosomal aberrations. A mosaic may be formed either by mitotic nondisjunction or by loss of single chromosomes caused by anaphase lagging.

An individual with an aneuploid cell line in only some of her or his tissues is likely to have a less severe phenotype than someone with a nonmosaic aneuploidy. For example, mosaic Down syndrome (i.e., the coexistence of both 47,N,+21 and 46,N cell lines) can result in a less obvious phenotype and with a lesser compromise of intellectual function than in standard trisomy 21. Most aneuploidies of autosomes can only exist in mosaic state because the nonmosaic forms are lethal in utero. If the distribution of the aneuploid cell line is asymmetric, the body shape may be asymmetric in consequence. In sex chromosome mosaicism, fertility can exist when otherwise infertility is the rule, which often represents a likely explanation for fertility reported in individuals with Turner syndrome (45,X/46,XX mosaic instead of 45,X) or with Klinefelter syndrome (47,XXY/46,XY instead of 47,XXY). The diagnosis of mosaics is often difficult, and those with a more obvious phenotypic defect are more likely to be detected. Furthermore, diagnosis is hampered by the obvious fact that many tissue sources are not readily available for a detailed chromosomal analysis.

3.6.2 Syndromes Attributable to Structural Anomalies of Autosomes

3.6.2.1 Karyotypes and Clinical Syndromes

3.6.2.1.1 Breaks, Gaps, and Rearrangements

Chromosomes must first be broken to form any kind of rearrangement. A chromosome may break at any stage of the cell cycle. If a chromosome breaks during G_1 and remains unrepaired through S-phase, the result will be visible in both chromatids as a chromosome break in the next metaphase (Fig. 3.41). If the fragment is not displaced, a gap may become visible. A break not affecting the centromere produces a shorter chromosome with a centromere and an acentric fragment. This fragment may or may not form a small ring (Fig. 3.41) but, lacking a centromere, it runs a high risk of being lost during the subsequent mitosis. Hence, chromosome breakage often leaves behind a cell deficient in a chromosome segment. In most cases, such a loss will be lethal for the respective cell.

When a break occurs during G_2, it usually involves only one of the two chromatids and is therefore called a chromatid break. A single break yields a deleted chromatid and an acentric fragment. Chromatid breaks in two chromosomes can lead to chromatid exchanges and result in quadriradial configurations, which may give rise to interchromosomal rearrangements, which are discussed below.

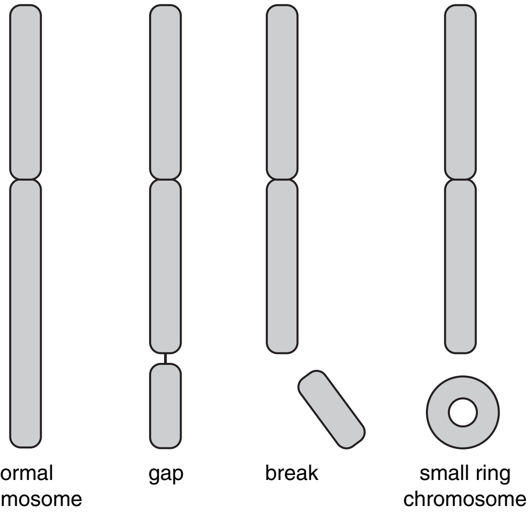


Fig. 3.41 Possible results of G_1 breaks in one chromosome: from *left* to *right*: normal chromosome, gap, break visible as small acentric fragment, and formation of a small ring

3.6.2.1.2 Interchromosomal Rearrangements (Interchanges)

In many cases, joining occurs between different chromosomes, homologous or nonhomologous. If breakage occurs in the G_1 phase, joining follows in the G_1 (or early S)-phase before DNA replication. If each of the resulting chromosomes happens to have one centromere, the translocation chromosomes may pass through the next mitosis without difficulties. If one of the resulting chromosomes happens to get two centromeres, a dicentric chromosome is formed. Depending on the exact mode of replication, it may be able to pass the next mitosis, under the following conditions: (a) the two centromeres of the dicentric chromosome migrate to the same pole and (b) replication and sister chromatid exchange between the two centromeres has not led to intertwining of the two chromatids (Fig. 3.42a). However, if the centromeres migrate to opposite poles, anaphase bridges are formed, and the chromosomes may break (Fig. 3.42b, c).

If two breaks occur during G_2, with the subsequent formation of a quadriradial configuration, there are two types of segregation: one in which alternate chromatids will segregate to opposite poles and one in which adjacent chromatids will segregate to opposite poles (Fig. 3.42d). The mitotic anaphase proceeds without further difficulties if the two centromeres happen to be located on different elements (Fig. 3.42d; classes I, III, and V). If the centromeres are located on the same configuration, the resulting daughter cell is in any case aneuploid. Either the centromeres migrate to different poles, in which case an "anaphase bridge" is formed, and the chromosome finally breaks again, or the two centromeres migrate to the same pole, which can only happen with nonhomologous reunions (Fig. 3.42d; classes V, VI, and VII). In the latter case the problem is postponed to the next mitosis, in which the chromosome appears to be dicentric. It may or may not survive this mitosis. In any case, under the conditions mentioned above interchanges cause a great deal of cell loss owing to aneuploidy or mitotic disturbance.

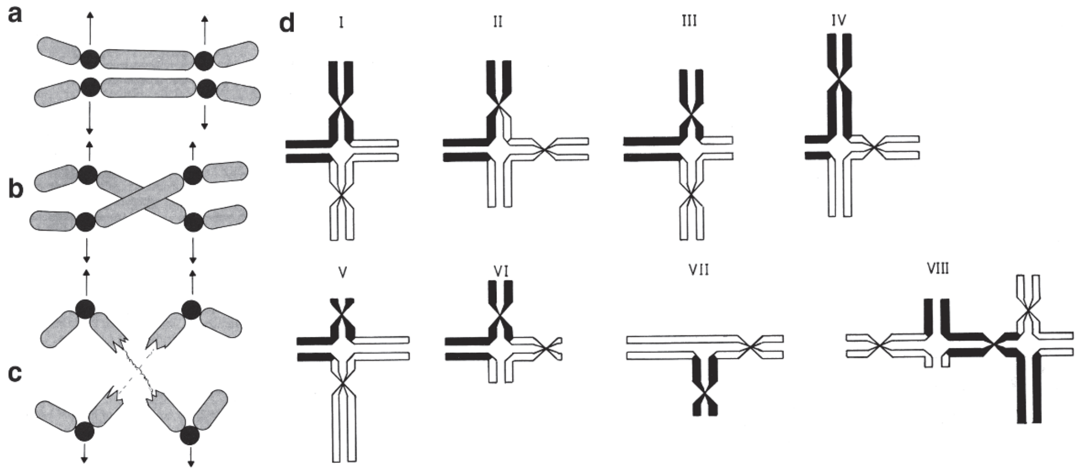


Fig. 3.42 (**a–d**) Mitotic anaphase of a dicentric chromosome (**a–c**) and classes of interchanges found after translocation during G_2-phase (**d**). (**a**) If both centromeres migrate to the same pole the dicentric chromosomes will remain intact. (**b**) If the centromeres are pulled to opposite poles anaphase bridges are formed. (**c**) The chromosomes are broken. (**d**) Illustrations of possible involvements of two homologous chromosomes: *I* Alternate position of the centromeres; exchange of fragments of equal length. *II* Adjacent position of the centromeres; exchange of fragments of equal length. *III* Alternate position of the centromeres; exchange of fragments of different length. *IV* Adjacent position of the centromeres; exchange of fragments of unequal length. Involvement of two non-homologous chromosomes. *V* Alternate position of the centromeres. *VI* Triradial configuration (loss of fragments required). Complex of non-homologous chromosomes (more than two). *VIII* One example of a figure with three chromosomes involved. (From the 3rd edition of this work)

3.6.2.1.3 The Impact of Spatial Proximity on Translocations

Genomes are nonrandomly arranged within the cell nucleus. In higher eukaryotes, each chromosome occupies a distinct, spatially limited space within the interphase nucleus, referred to as "chromosome territory." The position of each territory within the nuclear space is nonrandom. In human cells, the location of chromosomes has been linked to their gene density, with gene-rich chromosomes preferentially located toward the interior of the nucleus, whereas gene-poor chromosomes accumulate at the nuclear periphery. Similar preferential localization patterns have been detected for single genes [50, 54, 221].

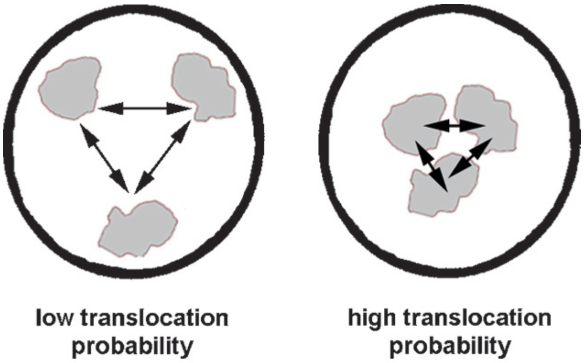


Fig. 3.43 The concept of spatial proximity as a contributor to translocation frequency: chromosome and genome regions that are preferentially located in close spatial proximity owing to the nonrandom organization of the genome in the cell nucleus have a higher probability of undergoing translocations with each other than chromosomes that are located distantly from each other. Adapted from [221], by permission of Oxford University Press

A role for spatial proximity in the formation of chromosomal translocations seems obvious considering that the generation of a chromosomal translocation requires the physical interaction of the two translocation partners. Given the nonrandom spatial positioning of genes and chromosomes within the nucleus, the closer two genome regions are on average to each other, the higher is their probability of translocating with each other (Fig. 3.43). Thus, there is evidence for a role of spatial proximity in formation of translocations. Although chromosomes clearly occupy distinct territories, their edges intermingle and chromatin loops from one chromosome invade the territory of its neighbors, bringing genome regions from distinct chromosomes in intimate spatial proximity. The intermingling chromatin loops may be more susceptible to DSBs owing to their decondensed nature, and the zone between chromosome territories may be a preferential site of formation of chromosomal translocations among neighboring chromosomes [32].

Two fundamentally different models for how chromosomal translocations form have been developed (Fig. 3.44). In the contact-first model, translocations can only occur between DSB that are located in close spatial proximity at the time of breakage. In this model, physical association of translocating regions occurs before DSB formation. On the other hand, in a breakage-first model, translocations occur between DSBs located far apart. In this model, broken chromosome ends undergo large-scale motions to relocalize within the nucleus in search of suitable translocation partners and

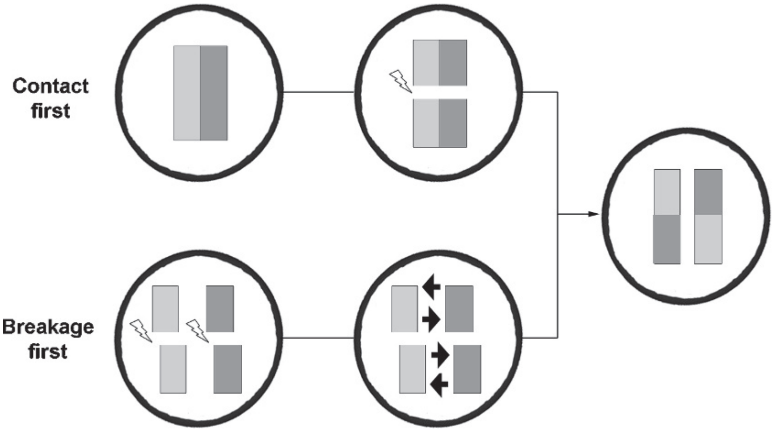


Fig. 3.44 The two models for formation of chromosome translocations: In the contact-first model, translocations occur among proximally positioned DSBs. In the breakage-first model, DSBs roam the cell nucleus in search of potential translocation partners. Adapted from [221], by permission of Oxford University Press

physical association between broken chromosome ends on different chromosomes occurs only after chromosomes suffer DSBs. At present there are experimental data supporting both models [221].

3.6.2.1.4 Segregation and Prenatal Selection of Translocations

The majority of constitutional balanced translocations have no consequences for the carrier. However, a translocation carrier may have an increased risk for a child with mental or physical abnormalities caused by segmental aneuploidy. Such a segmental aneuploidy consists typically of a segment of one of the participating translocation chromosomes, which is duplicated, and a segment of the other translocation chromosome, which is deleted. This results in both a partial trisomy and a partial monosomy. The reason is that at meiosis I, the four chromosomes with common segments form a special configuration, a "quadrivalent," which is best visible during pachytene stage (Fig. 3.45). Depending on which spindle attaches to which centromere, the distribution of the four homologous chromosomes to the daughter cells may vary. In fact, in theory 16 possible chromosomal combinations could be produced in the gametes of a translocation carrier (Fig. 3.45). The various modes of chromosome segregation can be described by the number of chromosomes, which go to the respective cells (i.e., a 2:2 segregation: two chromosomes go to one cell at a time; 3:1 segregation: three chromosomes are moved to one cell and one to the other; 4:0 segregation: four chromosomes go to one and none to the other cell). In addition, the terms "alternate" or "adjacent" describe which centromeres are moved to a daughter cell. "Alternate" describes the situation where these centromeres go alternately to one or the other pole. In contrast, "adjacent" signifies that centromeres that are next to each other travel together. In adjacent-1 segregation chromosomes with nonhomologous centromeres go to the same daughter cell, whereas in adjacent-2 segregation homologous centromeres are pulled to the same daughter cell.

As depicted in Fig. 3.45, only the alternate 2:2 segregation results in balanced daughter cells. In all other cases the gametes are in an imbalance, with the aforementioned double segmental aneuploidy. This may result in a miscarriage or in a child with mental retardation and dysmorphic features. A few translocations, especially those with small translocated chromosomal segments, are associated with a high risk of having an abnormal child.

3.6.2.1.5 Intrachromosomal Rearrangements (Intrachanges)

A single chromosome may break at two different sites, and the intermediate part may rejoin upside down. Inversions can be diagnosed by use of banding methods and/or in situ hybridization. A shift in centromeric position readily identifies pericentric inversions, whereas inversions in which the centromere is not included result in paracentric inversions (Fig. 3.46). Inversion heterozygotes are not particularly rare in human populations. There may be difficulties in chromosome pairing at meiosis, because crossing-over follows the reversed loop model, which allows optimal alignment and pairing of matching segments (Fig. 3.47). If in the case of a paracentric inversion a recombinant gamete is formed after a crossing over in the inverted segment, the chromosome would be either acentric (without centromere) or dicentric (two centromeres) (Fig. 3.47, left panel). Thus, in the case of a paracentric inversion, a recombination within the inverted segment cannot usually produce a viable unbalanced progeny. Few exceptions have been reported. For example, if the crossing-over within the inversion loop reverses upon itself, a "U-loop" is formed, which may result in either a duplication or a deletion. In contrast, a crossing over in the inversion loop of a pericentric inversion may lead to the production of two complementary recombinant chromosomes (Fig. 3.47, right panel).

Another type of intrachange is the ring chromosome. Here two telomeres are usually lost as fragments, and the open ends rejoin. Rings of almost every chromosome have been reported. Ring chromosomes often give rise to new variants. For example, a sister chromatid exchange may lead to a continuous double ring with one centromere. When the centromere divides in anaphase, the daughter centromeres may go to the same pole, with the result that one daughter cell has the double-sized ring while the other daughter cell has no ring. In contrast, if the centromeres move to opposite poles the ring may break randomly, and if the broken ends rejoin the daughter cells have rings of unequal size.

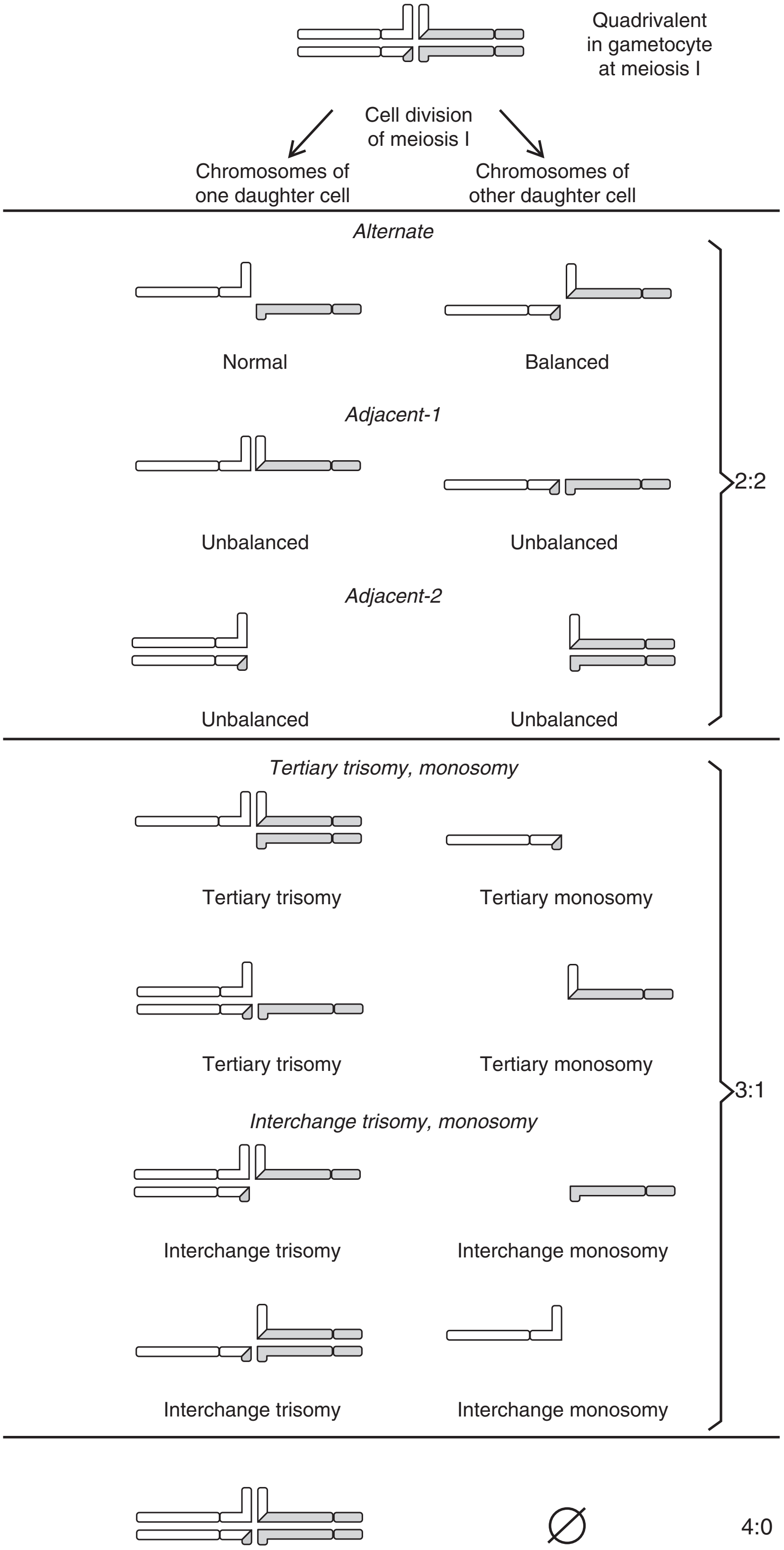


Fig. 3.45 At meiosis I, the four chromosomes with common segments form a quadrivalent. The distribution of the four homologous chromosomes is determined by which spindle attaches to which centromere. This determines different modes of segregation, such as 2:2 segregation (two chromosomes go to one cell at a time); 3:1 segregation (three chromosomes are moved to one cell and one to the other), or 4:0 segregation (four chromosomes go to one and none to the other cell). Adapted from [84]. By permission of Oxford University Press

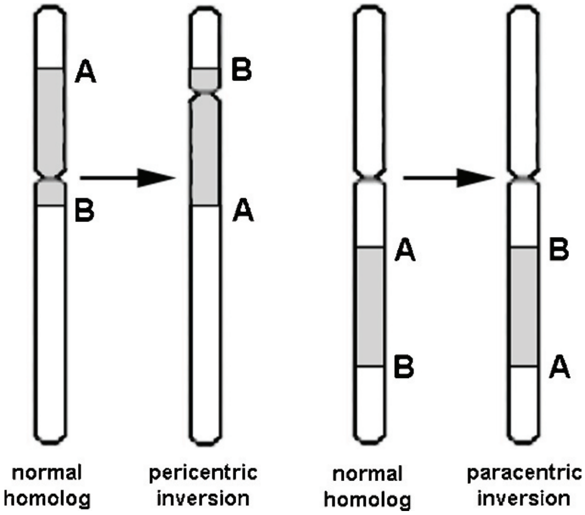


Fig. 3.46 Structure of pericentric (*left*) and paracentric (*right*) inversions

Therefore, ring chromosomes are often unstable in both mitosis and meiosis and are frequently lost. Furthermore, rings can generate mosaics in which different cells contain different derivatives of the original ring.

3.6.2.1.6 Deletion Syndromes

An individual who is heterozygous for a deletion is monosomic for a part of the chromosome. The term "deletion syndrome" usually refers to chromosomal losses that are large enough to be seen in standard banding analysis. In contrast, "microdeletions," as discussed in Sect. 3.5.5, are beyond the resolution limits of banding analysis and can therefore only be detected by other means, e.g., FISH or array-CGH.

De Grouchy et al. [58] were apparently the first to publish a case with del 18p-, in 1963. The first deletion syndrome was established by Lejeune et al. [143], also in 1963. They described three children with a deletion of the short arm of chromosome 5 (del 5 p-). In addition to the usual signs of autosomal chromosome aberration, such as developmental retardation and low birth weight, the children showed a moonlike face with hypertelorism. Their appearance was not extraordinarily peculiar, but they had a striking cry that resembled that of a cat (cri du chat=cat cry), which resulted in the name cri-du-chat syndrome.

There are various mechanisms by which a deletion may be formed: (a) true terminal deletion, (b) interstitial

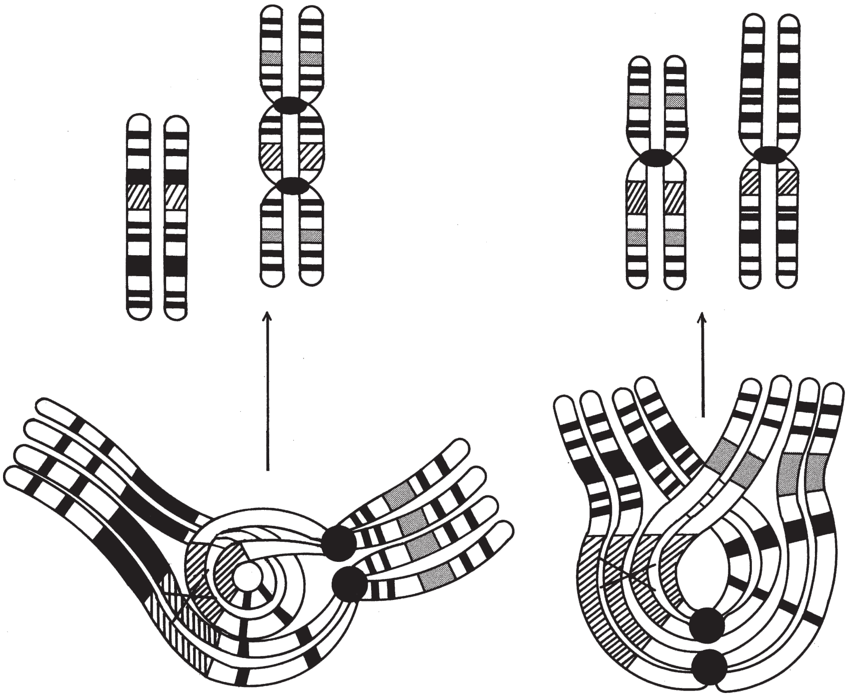


Fig. 3.47 Theoretical recombinant products from classical crossing-over in paracentric (*left*) and pericentric (*right*) inversions. In the two figures, crossing-over is assumed in the segments marked by an *X*. As a consequence, abnormal chromosomes are produced that lead to aneuploidy of zygotes in the next generation. (From the 3rd edition of this work)

deletion, and (c) unbalanced translocation. Array-CGH revealed that in patients with only 5p deletions different deleted regions exist, each with differing effect on retardation. Depending on size and location of the deletion, the level of mental retardation may range from moderate to profound [261].

There are a number of well-defined deletion syndromes, such as De Grouchy I (18p-), De Grouchy II (18q-), Wolf-Hirschhorn (4p-), Jacobsen syndrome (11q-), 1p36 deletion syndrome, 9q subtelomere syndrome, and so on.

3.6.2.1.7 Isochromosomes

Chromosomes consisting of two identical arms are occasionally found. Such chromosomes are known as isochromosomes and presumably originate by abnormal division of metaphase chromosomes, as shown in Fig. 3.48. Isochromosomes for the short arm or isochromosomes for the long arm may result.

Isochromosomes are observed relatively frequently for the X chromosome; an isochromosome of the long arm of the X, i(Xq) leads to the Turner syndrome, since this chromosome is always inactivated and only the normal X is active (Sect. 3.6.3.3).

3.6.2.1.8 Centric Fusions (Robertsonian Translocations)

Centric fusion is the most frequent type of chromosome rearrangement in human populations. The first reported cases of a translocation Down syndrome were due to centric fusion between the long arm of chromosome 21 and one chromosome of the group 13–15 or 21–22 (D or G group). Similar cases have since been observed repeatedly.

Thus, a Robertsonian translocation chromosome comprises the long arm elements of two different acrocentric chromosomes. These translocations are named after the American insect cytogeneticist Robertson, who first described translocations of chromosomes resulting from the fusion of two acrocentrics in his study of insect speciation in 1916.

Only the five acrocentric pairs undergo centric fusion. In the interphase nucleus the short arms and centromeric regions of these chromosomes are located close to the nucleolus, the short arms containing the nucleolus organizers, which carry genes for rRNA. This constellation appears to make acrocentric chromosomes prone to fusions. In fact, Robertsonian translocations are among the most common balanced structural rearrangements, with a frequency in newborn surveys of about 1 in 1,000. The great majority of Robertsonian translocations

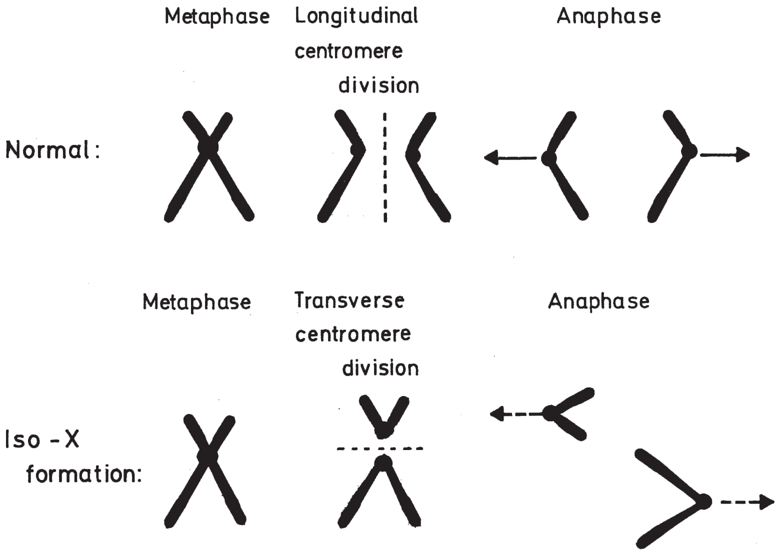

Metaphase
Longitudinal centromere division
Anaphase
Normal:
Metaphase
Transverse centromere division
Anaphase
Iso -X formation:

Fig. 3.48 Formation of an isochromosome by abnormal cleavage of the centromere. (From the 3rd edition of this work)

involve two different chromosomes (a heterologous or nonhomologous translocation). Translocations involving the fusion of homologs are very rare. The rob(13q14q) and the rob(14q21q) are predominant.

3.6.2.2 Small Deletions, Structural Rearrangements, and Monogenic Disorders: Genomic Disorders and Contiguous Gene Syndromes

3.6.2.2.1 Genomic Disorders

As pointed out in Sect. 3.5.5, the term "genomic disorder" was coined by Jim Lupski [150] and is typically used to describe a gain (duplication) or loss (deletion) of a specific chromosomal region, associated with a clinical genetic syndrome that may present with congenital anomalies, or with impairment in neurological and cognitive function (Fig. 3.35). A synonym for genomic disorders is "partial aneuploidies," which may be a more descriptive name [9]. Genomic regions associated with known human deletion and duplication syndromes are characterized by the presence of chromosome-specific, low copy repeats, or segmental duplications. Many of these segmental duplications flank the genomic regions that are prone to deletions and duplications and are the underlying basis for genomic disorders. Segmental duplications share a high level of sequence identity, predisposing the regions they occupy to nonallelic homologous recombination (NAHR), which may result in deletions, duplications, and inversions. NAHR may even allow intrachromosomal recombination events, which may result, for example, in translocations [150].

This mechanism explains why identical recurrent deletions/duplications may be observed in various, unrelated individuals and why these rearrangements often have the same size. Therefore phenotypic consequences of genomic disorders are usually very similar, often enabling clinical geneticists to suspect the presence of a specific microdeletion or microduplication based on the observed spectrum of clinical symptoms.

3.6.2.2.2 Contiguous Gene Syndromes

Contiguous gene syndromes refer traditionally to any deletions, i.e., deletions based on the aforementioned NAHR mechanism or deletions occurring randomly somewhere in the genome. Often these are interstitial chromosomal deletions, which contain several genes. The resulting phenotype may be the consequence of the combined haploinsufficiency of all genes in the deleted region, or of haploinsufficiency of only a few deleted genes, or even just of one of the deleted genes. It may be an especial challenge to assign deleted genes to specific phenotypic features. The extent and nature of phenotypic consequences depend on the genes encompassed by the deletion. Often, but not always, the main clinical features of microdeletions are mental retardation, growth retardation, craniofacial dysmorphy, and various congenital defects. Such a combination of symptoms should be clarified by high-resolution approaches, such as microarray analyses.

3.6.2.2.3 22q11.2 Deletion Syndrome

The 22q11.2 deletion syndrome has been characterized as one of the most frequent of the genomic disorders. Estimates about the prevalence of live birth are in the range of 1 in 2,000–4,000 [131]. Low copy repeats mediate genomic instability on chromosome 22 and result in recurrent deletion endpoints. While most patients have a 3-Mb heterozygous deletion, some rare patients have a nested 1.5- or 2-Mb deletion [70] (Fig. 3.49).

The 22q11.2 deletion can cause velo-cardio-facial syndrome (VCFS; MIM: 192430) [216] / DiGeorge syndrome (DGS; MIM: 188400) [61]. VCFS/DGS is characterized by multiple developmental anomalies occurring with varying severity, including craniofacial (cleft palate, velo-pharyngeal insufficiency; 69–100%), thymic, and parathyroid defects, including hypocalcemia (17–60%) and also cardiovascular malformations (70%) [131]. The disorder is fully penetrant, and everyone harboring the deletion is affected with some of the main clinical findings. However, variability is considerable and phenotypes may vary from life-threatening cardiovascular anomalies to mild craniofacial defects and learning disabilities. In some deletion carriers minor symptoms may be detected only after a careful physical examination. In addition, there is overwhelming evidence that children and adults with the 22q11.2 deletion have a characteristic behavioral phenotype, which is discussed in Chap. 23.6.

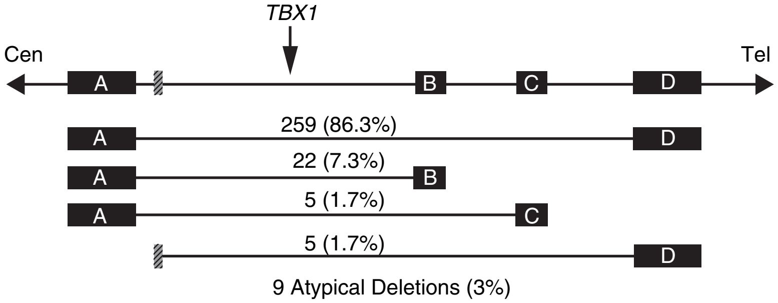


Fig. 3.49 Diagrammatic representation of the deletion region on 22q11.2 and deletion size in 300 patients. In this region there are four low copy repeats (*LCRs*), designated as *A*, *B*, *C*, and *D*, which are illustrated as *filled boxes*. The figure summarizes results from 300 patients. In 86.3% of these 300 individuals the deletions span the same 3-Mb region from LCR A-D. About 7.3% had a smaller 1.5-Mb deletion (between LCRs A and B), while 1.7% had a 2-Mb deletion (between LCRs A and C). In 3% atypical deletions were found. Adapted from [70]; data from [64]. Reprinted with permission of John Wiley & Sons, Inc.

All the defects in VCFS/DGS derive from the pharyngeal apparatus, which is a temporary embryological structure lateral to the developing head that contributes to diverse tissues of the head, neck, and thorax. Many birth defects, including a large fraction of those in congenital heart disease, derive from developmental problems of the pharyngeal system. Thus, the research interest in this syndrome is driven not only by the obvious clinical significance of the disease but also by a broader biological importance.

Because the 22q11.2 deletion is a contiguous gene syndrome, research is directed at elucidating the contribution of genes within the deleted region to the phenotype. A candidate gene within the deletion region, which may have the highest impact in the etiology of VCFS/DGS, is *TBX1*. This gene is a member of the T-box family of transcription factors, expressed in the pharyngeal apparatus. Inactivation of *Tbx1* in the mouse results in a phenocopy of the syndrome [122]. Furthermore, inactivating mutations in *TBX1* have been found in some patients with VCFS/DGS who were not carriers of the 22q11.2 deletion [255]. However, given the variable expressivity seen in the syndrome, it is obvious that modifying loci for VCFS/DGS must exist. Stochastic, environmental, and genetic factors likely modify the phenotype, and current research efforts are directed at the attempt to elucidate such genetic modifiers. Some potential modifier candidates were identified in the genetic pathway of *TBX1* or in pathways required for the development of the face, thymus, and parathyroid glands and heart [2].

3.6.2.2.4 22q11.2 Duplication Syndrome

The same mechanism as results in the 22q11.2 deletion syndrome, i.e., meiotic homologous recombination after misalignment of LCRs, should not only result in one deleted chromosome, but also in one chromosome containing a duplication of the deleted sequence (cf. Fig. 3.35). Accordingly, 22q11.2 deletions and duplications might be expected to occur with approximately the same frequency in the population. However, although individuals with 22q11 duplications have been identified, they seem to be fewer than anticipated on the basis of the prevalence of the deletion. One explanation for the relative lack of recognized 22q11.2 duplications compared with deletions may be the technical difficulties involved in detecting duplications by the standard cytogenetic analysis for 22q11.2 deletions (metaphase FISH). Similar to the various deletion sizes there is also a typical common approximately 3-Mb microduplication, approximately 1.5-Mb nested duplication, and smaller microduplications within and distal to the DiGeorge/velo-cardio-facial syndrome region.

The first report of a 22q11 duplication described a 4-year-old girl with failure to thrive, marked hypotonia, sleep apnea, and seizure-like episodes in infancy, who later showed delay of gross motor development with poor fine-motor skills, velo-pharyngeal insufficiency, and a significant delay in language skills. Her facial features were mildly dysmorphic, with a narrow face and down-slanting palpebral fissures. Hearing and vision were normal, and there were no detectable cardiac abnormalities [67].

In general, the phenotypes seen in these individuals are usually mild and highly variable, familial transmission is frequently observed. For example, in one family eight individuals over three generations who carried a 3-Mb duplication were identified. The duplication carriers showed intrafamilial phenotypic variation including heart defect, submucous cleft, intellectual disability, speech delay, behavior problems, and brachydactyly [259]. In another family, two duplication carriers were completely normal with high intellect [49]. Thus, it was noted that the delineation of a 22q11.2 microduplication syndrome may be due to ascertainment bias when seeking microdeletions of this region, and the authors of this study suggested that 22q11.2 microduplication could be either a nonpathogenic polymorphism or a syndrome with reduced penetrance [49].

3.6.2.2.5 Other Microdeletion/Microduplication Syndromes

Given the NAHR mechanism, which may cause recurrent deletions and duplications, the obvious question is how many regions there are in the human genome that may be similar to the aforementioned 22q11.2 region prone to being lost or duplicated during meiosis. Methods were developed to analyze *in silico* the public sequence database at the clone level for overrepresentation within a whole-genome shotgun sequence. This test had the ability to detect duplications larger than 15 kb irrespective of copy number, location, or high sequence similarity. This *in silico* approach mapped 169 large regions flanked by highly similar duplications [19]. When this report was published, i.e., in August 2002, only 24 of these hotspots of genomic instability had been associated with genetic disease.

The known number of microdeletion syndromes has dramatically changed over the past few years, because array-CGH has revolutionized the cytogenetic testing. Screening large patient cohorts with mental retardation and some dysmorphic features by array-CGH has recently led to the characterization of many novel microdeletion and microduplication syndromes. Several new recurrent microdeletion/microduplication syndromes were identified, which are flanked by LCRs and whose existence was predicted by the landmark study of Bailey et al. [19]. Such new recurrent microdeletion syndromes were identified for example for chromosomal regions 17q21.31, 15q13.3, 15q24, 1q41-1q42, 16p11-12.1, 2p15-16.1, and 9q22.3[217]. In addition, there are a steadily growing number of other microdeletion and microduplication syndromes, which are apparently not associated with LCRs. As a consequence, the breakpoints of these copy number changes are not recurrent, i.e., they are observed only in single individuals, or if similar regions are deleted in unrelated persons they usually do not have identical breakpoints. Thus, many aberrations are novel or extremely rare, making clinical interpretation problematic and genotype-phenotype correlations uncertain.

Therefore, a new challenge in human genetics is the generation of an appropriate infrastructure facilitating the utilization of the immense wealth of information generated by array-CGH. Aims of such efforts should be the identification of patients sharing a genomic rearrangement and with phenotypic features in common, which may lead to greater certainty in the pathogenic nature of the rearrangement, and the definition of new syndromes. To facilitate the analysis of these rare chromosomal events, interactive web-based databases, such as DECIPHER or ECARUCA, were developed. These databases are explained in detail in chapter 29.

3.6.3 Sex Chromosomes

3.6.3.1 First Observations

3.6.3.1.1 Nondisjunction of Sex Chromosomes and Sex Determination in Drosophila

Meiotic nondisjunction was discovered by Bridges [33] in the sex chromosome of *Drosophila melanogaster.* Morgan [159] had earlier described the X-linked mode of inheritance, and at the same time had elucidated the X–Y mechanism of sex determination in *Drosophila.* In his experiments a few exceptions had occurred that did not conform to the predictions of X-linkage. Bridges explained them by an anomaly in the mechanism of meiosis.

Drosophila has four chromosome pairs, three pairs of autosomes, and two sex chromosomes. Just as in humans, the males have the complement XY, the females

XX. Hence, each normal male germ cell has either one X or one Y chromosome; all female germ cells have an X. In crosses between an affected homozygote for the X-linked recessive trait white and a wild type or normal male, all male offspring would be expected to have white eyes as their mothers do. All daughters should be heterozygous and have normal red eyes. As a rule, this expectation was fulfilled. In exceptional cases, however, male offspring had normal red eyes, and some females were white-eyed. This was shown by Bridges to be due to nondisjunction of the maternal X chromosome, leading to an oocyte with either two X chromosomes or none. Fertilization with sperm from a wild type male was expected to lead to four different types of zygotes: XXX, XXY, XO, and YO. YO was not observed at all; apparently, zygotes without an X chromosome cannot survive. The other three types were observed and gave evidence regarding the mechanism of sex determination: sex chromosome constellations XXX and XXY both resulted in a female phenotype, while XO resulted in a male, sterile phenotype. Hence, the phenotypic sex in this fruit fly depends on the number of X chromosomes. One X chromosome makes a male, while more than one X chromosome makes a female. The Y is also involved in sex determination, as XO males are sterile.

3.6.3.1.2 XO Type in the Mouse

The X-linked mutation scurfy (sf) appeared first by spontaneous mutation, and affected animals have scurfy skin. The *scurfy* mutation causes loss of function of the *FoxP3* gene, which is essential for development and maintenance of naturally occurring regulatory CD4$^+$CD25$^+$ T cells. The hemizygous males are sterile; therefore, the strain can be maintained only by crossing heterozygotes (X^{sf}/X$^+$) with normal males (X$^+$/Y). From this mating, scurfy and normal males are expected in a segregation ratio of 1:1; all females should be normal. From time to time, however, an exceptional sf female is observed. As with male hemizygotes, they are sterile. However, their ovaries can be transplanted to normal females, which have been mated with wild-type males. The sons are all sf; the daughters are all normal but fall into two groups, those transmitting sf and those not transmitting it. Further analysis showed that these daughters have two different karyotypes, X$^+$/O and X$^+$/X^{sf}; the first group does not transmit sf, but the second does. This experiment showed that, contrary to the findings in *Drosophila,* XO is a fertile female in the mouse [201]. Hence, in this animal, the Y and not the X chromosome is decisive for the phenotypic sex. Subsequently, the XO types of the mouse have been found to be fairly frequent. In most cases the condition is caused not by meiotic nondisjunction but by chromosome loss after fertilization. Not long after the XO type, the XXY type was also discovered in the mouse. It is a sterile male, in contrast to *Drosophila*, where the XXY type was female.

3.6.3.1.3 First X Chromosomal Aneuploidies in Humans

XXY, XO, XXX. Jacobs and Strong [116] studied a 42-year-old man with the typical features of Klinefelter syndrome (Fig. 3.50), including gynecomastia, small testicles, and hyalinized testicular tissue. X Chromatin was found in cells of buccal smears and drumsticks, in granulocytes. Chromosome examination from bone marrow revealed an additional, submetacentric chromosome “in the medium size range.” The authors felt that the patient very probably had the constitution XXY. However, “The possibility can not be excluded … that the additional chromosome is an autosome carrying feminizing genes.” The patient’s parents both had nor-

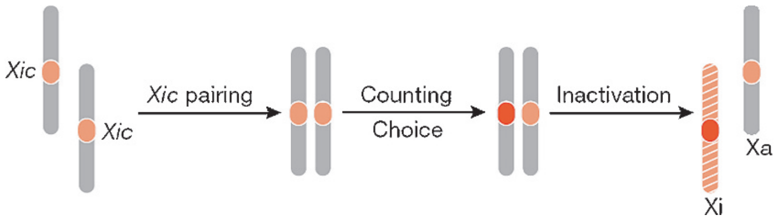


Fig. 3.50 Proposed scheme of X chromosome inactivation. After establishment of an X chromosome-to-autosome ratio one X chromosome is selected to become inactivated. This involves pairing of the two XICs. This may activate Xist transcription on one chromosome which is the one committed to become silenced. The other X chromosome remains active. From [166], reprinted by permission from Macmillan Publishers Ltd: *EMBO Reports*, copyright 2007

mal karyotypes with 46 chromosomes; hence, nondisjunction had occurred in one of their germ cells. Shortly afterward, the XXY status for Klinefelter syndrome was confirmed in many other cases.

At the same time, the XO type was discovered by Ford et al. [80]. Their patient, a 14-year-old girl, presented clinically as a Turner syndrome patient (Fig. 3.53) and was X chromatin negative. The modal number of chromosomes in bone marrow cells was 45; there were only 15 "medium length metacentric chromosomes," as in normal males. The evidence strongly suggested a chromosome constitution XO. The authors, comparing this result with that known from *Drosophila*, concluded that, in contrast to the fly, the XO type in humans may lead to an "agonadal" individual with female phenotype.

3.6.3.2 Dosage Compensation for Mammalian X Chromosomes

3.6.3.2.1 X Inactivation as the Mechanism of Gene Dosage Compensation: Lyon's Hypothesis

Because of their different sex chromosome composition (XX in female cells and XY in male cells), female and male cells have different numbers of genes, which could result in large-scale genetic imbalances between the sexes. Therefore, mechanisms are needed to equalize gene dosage between XX and XY cells. In 1949, Barr and Bertram described a unique nuclear structure present only in XX female cat neurons [22]. However, they did not associate this structure with an X chromosome or a dosage compensation mechanism. This realization came later with the publication of Mary Lyon's hypothesis of X inactivation [151]. Lyon made the step from morphological evidence to function, concluding that the heteropyknotic X chromosome may be either paternal or maternal in origin and is functionally inactive [151]. With this, she formulated one of the most fertile hypotheses in mammalian genetics. Lyon also tentatively explained an observation on a human X-linked disease in the same way: In X-linked ocular albinism, male hemizygotes lack retinal epithelial pigment and have a pale eye fundus. Heterozygous females have irregular retinal pigmentation, with patches of pigment and patches lacking pigment, so that the fundus has a stippled appearance. Lyon also predicted that mosaicism would be demonstrable in other X-linked genes, among them the variants of the enzyme glucose-6-phosphate dehydrogenase (G6PD). However, another human geneticist, Beutler, who should be given credit for having formulated the X-inactivation hypothesis independently of Lyon, using human G6PD as his experimental material [27].

Since then, the phenomenon of X-chromosome inactivation (XCI) has been analyzed in detail, and many features distinguishing the active X (Xa) from the inactive X (Xi) have been described.

In eutherians, X-chromosome inactivation (XCI) affects the paternal or maternal X chromosome randomly during early development. Once silenced, epigenetic mechanisms ensure the maintenance of silencing in future cell divisions, so that the inactive state is then stably inherited, giving rise to adults that are mosaics for two cell types, expressing one or the other X chromosome. Random XCI is achieved in three genetically separable events (Fig. 3.51). First, the X chromosome-to-autosome ratio is counted to ensure that one X chromosome is inactivated per female diploid nucleus. Next, one X chromosome is "chosen" in a mutually exclusive fashion to be the future inactive X (Xi). Lastly, silencing is initiated. The initiation of X inactivation is controlled in mammals by a region on the X chromosome, called the X-inactivation center (Xic), which regulates the different steps of XCI. The Xic produces the noncoding *Xist* transcript responsible for triggering silencing in *cis*. This is achieved by coating of the future Xi by the noncoding Xist RNA, recruitment of silencing factors, and condensation of the X-chromatin.

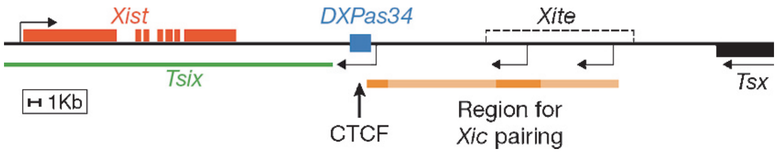


Fig. 3.51 Map of the regulatory elements of the mouse X-inactivation center. These regulatory elements are associated with X-chromosome counting and inactivation choice. From [166], reprinted by permission from Macmillan Publishers Ltd: *EMBO Reports*, copyright 2007

The *Xic* is comprised of several genetic elements that make long noncoding RNAs (ncRNAs), including *Xist*, *Tsix*, *Xite*, *DXPas34*, and *Jpx/Enox* (Fig. 3.51). As explained in detail below, the ncRNAs *Xist* and *Tsix* are choice regulators, which are critically involved in designating the Xa and Xi, respectively. *Tsix* is regulated by enhancers contained in the *Xite* and *DXPas34* elements. Differential methylation of *Xite* and the CCCTC-binding factor (CTCF)-binding sites on *DXPas34* correlate with X chromosome choice in mice [166].

Xist is negatively regulated by its antisense gene partner, *Tsix*. *Tsix* is like *Xist* a choice regulator, which overlaps with the *Xist* gene and is transcribed in the antisense direction. *Tsix* is initially expressed on both X chromosomes and is down-regulated on the Xi before inactivation; conversely, *Tsix* expression persists longer on the Xa. On the X chromosome, which was selected to become inactivated (the future Xi), loss of *Tsix* permits up-regulation of *Xist* and silencing of the *cis* chromosome. In parallel on the other X chromosome, which will be active (Xa), persistence of *Tsix* expression prevents up-regulation of *Xist* and thereby prevents silencing on that chromosome. *Tsix* is in turn regulated by *Xite*, a gene located ~10 kb upstream of *Tsix* and which bears a *Tsix*-specific enhancer. As a consequence, *Xist* is expressed exclusively from the Xi. The *Xist* RNA physically associates with the X chromatin and nuclear matrix around the X and coats the Xi [37] (reviewed in [71, 166]). The observation of Xist spreading along the Xi and maintaining the inactive status instigated the idea of "way stations" or "boosters" at intervals along its length. Mary Lyon proposed long interspersed repeat elements, i.e. line-1 (L1) as a candidate for way stations [152]. In fact there is evidence that L1 sequences are enriched near inactivated genes, which supports the proposed role of L1 elements as way stations. However, the search of sequence features which may be involved in the spread of silencing is still ongoing.

However, XCI includes another challenge because the two X chromosomes in the female must adopt mutually exclusive fates of Xa and Xi, and the cell must ensure that neither both nor neither X be inappropriately inactivated. Recent reports have demonstrated evidence that this task may be accomplished by physical interactions in trans of the Xic loci. In fact, the *Xic* region of the two X chromosomes appear to touch or "pair" just prior to the onset of XCI [16, 254] (Fig. 3.51). According to this pairing model, the two X chromosomes are epigenetically equivalent before the onset of XCI. The pairing of the Xci loci is then critically involved in the asymmetric localization of factors upon separation of the two X chromosomes. Relatively small and diverse 1- to 2-kb DNA elements of very low complexity which lie within *Tsix* and *Xite,* such as the 34mer repeat of *DXPas34* or CTCF, are sufficient to establish ectopic pairing. In addition, ncRNA might also be involved.

In their early studies, Barr and Bertram had already noted that the unique nuclear structure present in female cells, which later became known as the Barr body, often resides near the nucleolus [22]. In fact, the Xi co-localizes with the perinucleolar compartment during mid-late S-phase at a time when the Xi is undergoing DNA replication. This compartment is enriched for Snf2h, a chromatin-remodeling factor known to be required for replication of heterochromatic sequences [261]. Furthermore, XCI involves significant chromosomal reorganization of the Xi, because the core of the Xi consists of silenced nongenic sequences involving centromeric and other repetitive DNA elements, whereas genes that escape X inactivation lie outside or at the edge of the Xi core [42, 46].

Once the silent state of the Xi is created, the repressed status is maintained throughout subsequent cell divisions. *Xist* only has a minor role in the maintenance of the Xi, whereas multiple epigenetic marks, including DNA methylation, late replication and hypoacetylation of histone H4, act synergistically in the maintenance of X inactivation. To achieve this Polycomb group (PcG), proteins are recruited to the Xi to establish specific epigenetic marks at the histones H3 and H2 [104].

3.6.3.2.2 Many Genes Escape Silencing on the Human Xi

Not all, but most, genes on the Xi are transcriptionally silenced. However, approximately 15% of human X-linked genes escape inactivation and are therefore expressed from both the Xa and Xi [39]. Genes expressed from the Xi rarely express to the extent of the Xa. In fact, gene expression data performed on microarrays identified only a small number of X-linked genes with overexpression in females relative to males, so that the X chromosome dosage compensation is virtually complete. The genes that escape inactivation are nonrandomly distributed along the X chromosome, with the majority clustered on the short arm of the X chromosome.

3.6.3.2.3 Pseudoautosomal Regions (PAR1 and PAR2)

Between the X and the Y chromosome there are two limited regions of identical sequence, which are located at the tips of the short and long arms of the X and Y chromosomes. During meiosis, pairing and crossover in men takes place in these two regions, and they have therefore been termed "pseudoautosomal regions" or "PARs." The region at the distal Xp arm is the first pseudoautosomal region (PAR1), with a physical length of approximately 2.7 Mb, while the second pseudoautosomal region (PAR2) is located at the distal Xq arm and has a size of about 0.33 Mb (Fig. 3.52). In men, the PAR1 region exhibits the highest recombination frequencies of the genome. Crossover activity in PAR1 is much higher in men than in women, and also higher than for each of the autosomes. It is estimated that during male meiosis on average at least one crossover occurs. The rate of recombination in PAR2 is much lower than in PAR1, but still higher than the average rate of the remainder of the X chromosome. To date, 24 genes have been reported in PAR1 and 5 genes in PAR2. Possible connections with clinical disorders such as short stature, asthma, psychiatric disorders, and leukemia have been suggested in the past, but only one pseudoautosomal gene, *SHOX* (short stature homeobox), has been unambiguously associated with various short stature conditions and disturbed bone development [77]. Loss of the *SHOX* gene is likely associated with phenotypic features in women with Turner syndrome (Sect. 3.6.3.3).

3.6.3.2.4 Skewed X Chromosome Inactivation

Because inactivation of one X in early development generally occurs in a random manner, most females will have some cells with a maternally derived X and some with a paternally derived X inactivated. However, owing to the limited number of embryo precursor cells at the time of marking or commitment of a particular X to inactivation, most females have a detectable bias in terms of the proportion of cells that have inactivated one or the other X chromosome. An extremely skewed X-chromosome inactivation, which is defined using an arbitrary cutoff of ≥90% of cells with the same X inactivated, is less common. Such a skewed X chromosome inactivation may be a consequence of various factors, such as (1) biases in the choice of which X to inactivate, (2) X-chromosome mutations or rearrangements, which affect the viability of cells with one or the other X active, or (3) stochastic factors [36]. The frequency of extremely skewed X chromosome inactivation in unselected female persons of reproductive age is approximately 7% when measured using methylation-based assays and DNA derived from whole blood [26]. Females with X chromosome structural abnormalities usually selectively inactivate their abnormal X to avoid the occurrence of genetic imbalance that could result in an abnormal phenotype. Furthermore, X;autosome

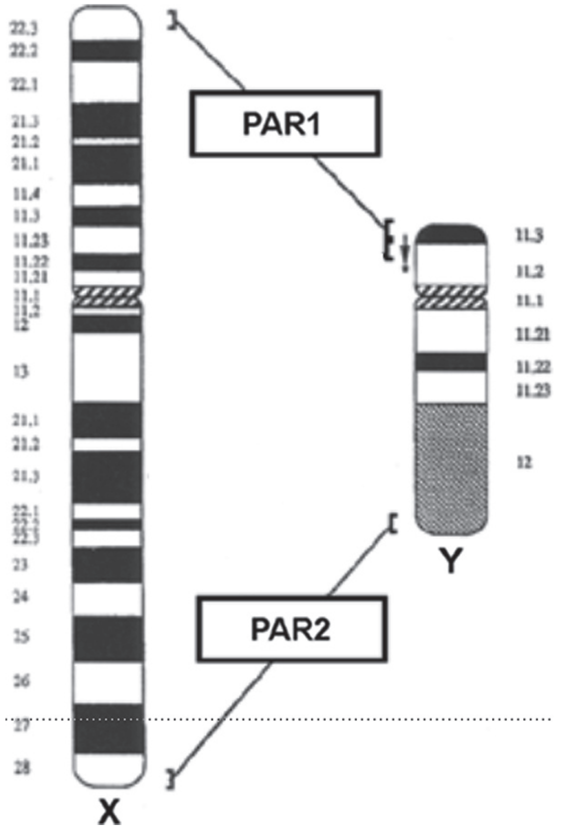


Fig. 3.52 Scheme showing the locations of the pseudoautosomal regions 1 (PAR1) and 2 (PAR2)

translocations also usually result in skewed X chromosome inactivation. When the Xic is translocated onto autosomes, spreading of the silent chromatin structure into autosomal chromatin can occur, but is frequently attenuated and incomplete [191].

3.6.3.3 X Chromosomal Aneuploidies in Humans

3.6.3.3.1 Difference Between X Chromosomal and Autosomal Aneuploidies

Soon after these first discoveries a great number of other aneuploidies of sex chromosomes were described. As a group, they show some remarkable differences from the autosomal aneuploidies discussed before.

(a) Mean intelligence can be reduced, but the extent of mental retardation is not nearly as pronounced as in the autosomal conditions; many probands have normal intelligence, and in some it is even above average (Chap. 23.6).

(b) The phenotypic disturbances most severely affect development of the sexual organs and sex-hormone-dependent growth. Other malformations do occur – mainly in Turner syndrome, but except for the small stature of Turner patients they usually are less frequent and less severe.

In brief, X chromosomal aneuploidy does not disturb embryonic development nearly as much as does autosomal aneuploidy. The reason is that normal women have two, normal men only one, X chromosome. This difference led to the development in evolution of a powerful mechanism of gene dosage compensation that happened to benefit carriers of X aneuploidies (Sect. 3.6.3.2).

Clinical Classification of X Chromosomal Aneuploidies: Mosaics. In general, the number of additional X chromosomes enhances the severity of mental retardation. The number of X chromatin bodies is one less than the number of X chromosomes.

Theoretically, zygotes with 45,X should be somewhat more frequent than any other types, since they can be produced by nondisjunction in both sexes and both meiotic divisions. This expectation does not fit in with the observed data, as all the karyotypes together that lead to Turner syndrome are much rarer than XXX or XXY. This finding points to strong selection against germ cells without the X chromosome and/or to strong intrauterine selection against zygotes with 45,X. The latter expectation is corroborated by observations on aborted fetuses, among which the 45,X chromosome constellation is, indeed, frequent. Another line of evidence points in the same direction: the risk of nondisjunction in general increases with the age of the mother. For XXY and XXX karyotypes this increase can be clearly demonstrated; but not for the 45,X karyotypes. Hence, it is assumed that surviving 45,X zygotes are the result not of meiotic, but of mitotic, nondisjunction or of early chromosome loss. The relatively greater proportion of mosaics in this group compared with XXX and XXY fits this hypothesis.

XYY zygotes, on the other hand, can be formed only by nondisjunction during the second meiotic division in males. Nevertheless, they are about as frequent as XXY zygotes. Therefore, the probability of nondisjunction of Y chromosomes appears to be much higher than the combined probabilities for X chromosome nondisjunction. Mosaics have been observed for all types.

3.6.3.3.2 Intersexes

Sex determination depends on the sex-chromosome complement of the embryo and multiple molecular events involved in the development of germ cells and their migration to the urogenital ridge. In the presence of the Y chromosome (46,XY) testes are formed, whereas the absence of a Y chromosome together with the presence of a second X chromosome (46,XX) results in the formation of ovaries. Many genes have been identified that contribute to the process of sex determination and differentiation. The best-defined gene involved in gonadal differentiation is *SRY,* which is located on the short arm of the Y chromosome and induces the bipotential gonad to differentiate into a testis. *SRY* induces the *SOX9* gene, and both genes have major roles in this process, together with steroidogenic factor 1 (SF-1) and opposition from *DAX1*. Other genes, such as *WT1*, have smaller contributions [153]. Mutations in these genes, which are involved in sex determination and development, may result in intersex anomalies, independently of the constitution of the sex chromosomes.

For example, true hermaphroditism is characterized by ambiguous genitalia, and both ovarian and testicular tissue is present either in the same or in a contralateral gonad. The karyotype is predominantly 46,XX, although testes, ovaries, and ovotestes may be present in various combinations. The molecular events have not been elucidated, but some cases were associated with translocation of a fragment containing the *SRY* gene to the X chromosome [153].

Furthermore, failure to produce testosterone or mutations in the testosterone receptor can result in 46,XY phenotypic females or phenotypic males with various degrees of diminished masculinization (male pseudohermaphroditism). On the other hand, patients with congenital adrenal hyperplasia produce an excess of adrenal androgens, which can cause female pseudohermaphroditism in 46,XX patients. A number of specific mutations have been identified, which are associated with such intersex anomalies. Examples are mutations in *SRD5A2*, located on chromosome 5p15 or *CYP17* (10q24-25), which may cause male pseudohermaphroditism, or mutations in *CYP21* (6q21.3), which may result in female pseudohermaphroditism. There are many other genes that are associated with intersex anomalies [153].

3.6.3.3.3 Turner Syndrome

Since the description of Turner syndrome by Henry H. Turner in 1938, a wealth of information has been added and improved our current understanding of the syndrome.

For a girl or woman to be diagnosed with Turner syndrome, she must be missing all or part of one copy of the second sex chromosome, as confirmed by a chromosome analysis. In addition, mosaicism with two or more cell lines may be present. The first cases described had the “classic” karyotype 45,X. However, this classic karyotype only accounts for 50% of cases; the remaining cases comprise mosaic karyotypes (i.e., cells with 45,X and cells with 46,XX), karyotypes with an isochromosome of X – for example i(Xq) or i(Xp) – or karyotypes with an entire or part of a Y chromosome [18]. All individuals who have Turner syndrome have a female phenotype. Approximately 1 in 2,500 live female births is affected by Turner syndrome.

The Turner syndrome physical phenotype is characterized by abnormalities in three basic systems: the skeletal, the lymphatic, and the reproductive systems. Short stature is the cardinal finding in girls with Turner syndrome, affecting 95–99% of them. Other skeletal defects include cubitus valgus or an unusual carrying angle of the elbows and arms (see Fig. 3.53), as well as a short 4th metacarpal, micrognathia, and a high-arched palate. Without treatment, females with Turner syndrome are typically more than 2 standard deviations below their peers in height and achieve a final height of about 140 cm. Since the advent of growth hormone therapy, as well as of other hormones to augment growth, heights within the bottom end of the normal range can be achieved. Because their facial abnormalities can cause an abnormal orientation of the ear canal, children with Turner syndrome are at high risk of ear infections [107, 198].

The lymphatic system defect is caused by abnormal lymphatic clearance. The consequences are frequently visible in utero as brain hygroma during ultrasound examination. While permanent neck webbing can result after the hygroma recedes, in many cases the hygroma is so severe as to cause fetal demise. Many neonates present with severe edema, which is often the reason for the diagnosis of Turner syndrome. Most postnatal diagnoses are made at birth (15%), during teenage years (26%), and in adulthood (38%), with the remainder being diagnosed during childhood, and

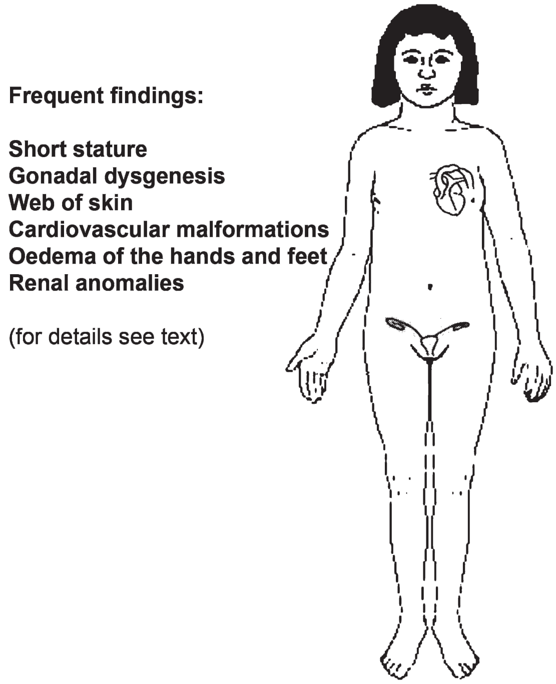


Fig. 3.53 Main clinical findings in Turner syndrome

therefore there is considerable delay in diagnosing girls and adolescents. The key to diagnosis was lymphedema in 97% during infancy, and short stature in 82% during childhood and adolescence [107, 198].

Regarding reproductive system defects, most females with Turner syndrome have ovarian dysgenesis owing to streak ovaries containing no ova. As a result, they lack endogenous estrogen and have reduced androgen production. Unless they receive hormonal replacement therapy during adolescence, they remain sexually infantile throughout life. Although the majority of females with Turner syndrome are infertile, a few individuals do spontaneously produce estrogen and undergo normal pubertal development. There are also a handful of women with karyotypes other than 45,X who have successfully reproduced.

In addition, individuals with Turner syndrome are at risk for cardiac abnormalities owing to coarctation of the aorta. Turner syndrome is associated with mainly left-sided CV malformations, such as elongated transverse arch of the aorta seen in 50% of the women, bicuspid aortic valves in 13–43 vs 1–2% in the general population, and coarctation of the aorta (4–14% in Turner syndrome). Less commonly, right-sided malformations such as persistent left vena cava superior and partial anomalous venous return are seen. Rarely atrial and ventricular septal defects, persistent ductus arteriosus, aortic and mitral stenosis and insufficiencies, and hypoplastic left heart syndrome appear [108]. Less frequent other symptoms include renal abnormalities from horseshoe kidneys, multiple pigmented nevi, and nail dysplasia. In addition to the congenital structural cardiac malformations such as bicuspid aortic valve and coarctation of the aorta, hypertension is also thought to be a major factor shown by the relatively high incidence of aortic dissection and rupture of 40 per 100,000 Turner syndrome-years versus 6 per 100,000 general population-years. It also, strikingly, affects Turner syndrome patients at a median age of 35 years, as opposed to 71 years in the general population [95]. Aortic dilation normally precedes dissection, and rupture is seen in 3–42% of randomly selected Turner syndrome women, where aortic diameter correlates significantly to systolic blood pressure but, surprisingly, is not associated with vascular atherosclerotic indices such as aortic stiffness or plasma lipids. An intrinsic arterial defect is therefore likely, as part of the generalized vasculopathy in Turner syndrome. Morbidity and mortality are increased, which is due especially to the risk of dissection of the aorta and other cardiovascular diseases, as well as the risk of type 2 diabetes, osteoporosis, and thyroid disease [107, 198].

Despite the consistency of these physical features, there is wide variability among affected individuals, and few if any have every abnormality. Generally, a more severe presentation is associated with complete loss of a single X chromosome or the ring X condition, while the least severe presentation is associated with a mosaic karyotype involving a normal 46,XX cell line. Deletions, rearrangements, or translocations of the X chromosome represent intermediary conditions. Part of the explanation of the reduced final height relates to the action of the *SHOX* gene located in the PAR1 region of the X and Y chromosome. Haploinsufficiency of the *SHOX* gene explains the reduction in final height, changes in bone morphology, sensorineural deafness, and other features [194]. However, additional genes are thought to be involved in the pathogenesis of Turner syndrome, but await discovery.

The treatment of Turner syndrome entails biosynthetic recombinant human growth hormone to increase height, estrogen to initiate puberty and maintain normal female functioning, and androgens to advance linear bone growth.

Ideally, the timing of endocrine therapy should allow onset of puberty at the same time as the peers of the patient to avoid social problems at school because of delayed physical and psychological development. This would also allow optimal bone mineralization to take place. Estrogen therapy should be coordinated with the use of growth hormone. This should be individualized for each patient, so as to optimize both growth and pubertal development.

During adulthood, infertility is rated as the most prominent problem of the syndrome. Oocyte donation is an option in many countries. The most recent studies show good results comparable with those of oocyte donation in other groups of patients, although better preparation of the uterus for implantation (uterine size and endometrial thickness) with prolonged treatment with high daily doses of estradiol may improve results [107].

The average intellectual performance is within the normal range. Behavioral aspects of Turner syndrome are described in Chap. 23.6.

3.6.3.3.4 Klinefelter Syndrome

Klinefelter syndrome is the most common sex-chromosome disorder and the most common numerical chromosomal aberration among men, with an estimated frequency of 1:500–1:1,000 of live deliveries [139].

Klinefelter syndrome is characterized by X chromosome polysomy with X disomy being the most common variant (47,XXY). Ninety percent of men with Klinefelter syndrome have nonmosaic X chromosome polysomy [139]. The 47,XXY karyotype of Klinefelter syndrome arises spontaneously by nondisjunction in stage I or II of meiosis. While most human trisomies originate from errors at maternal meiosis I, Klinefelter syndrome is a notable exception, as nearly one-half of all cases derive from paternal nondisjunction [234]. Postfertilization nondisjunction is responsible for mosaicism, which is seen in approximately 10% of Klinefelter syndrome patients. Men with mosaicism are less affected and often are not diagnosed. Advanced maternal age and possibly paternal age have been linked to an elevated risk of Klinefelter syndrome [149]. In fact, increased paternal age may be responsible for an increasing prevalence of Klinefelter syndrome [160].

The X chromosome carries genes that have roles in many body systems, including testis function, brain development, and growth. The "prototypic" man with Klinefelter syndrome has traditionally been described as tall, with narrow shoulders, broad hips (i.e., tall eunuchoid body proportions), sparse body hair, gynecomastia, small hard testicles, micropenis, androgen deficiency, sterility, azoospermia, and decreased verbal intelligence. It is now well known that this original description is not accurate and that men with Klinefelter syndrome represent a broad spectrum of phenotypes, professions, incomes, and socioeconomic status [139]. The classic descriptions of men with Klinefelter syndrome, as shown for example in Fig. 3.54, are based on the most severe cases of phenotypic abnormalities. Klinefelter syndrome is an underdiagnosed condition; only 25% of the expected number of patients are diagnosed, and of these only a minority are diagnosed before puberty.

Frequent findings:

Tend to be rather tall
Increased risk for transient gynecomastia
Hypergonadotrophic hypogonadism
Small testes
Infertility
Osteoporosis
Modest decrement in intelligence quotient compared to siblings

(for details see text)

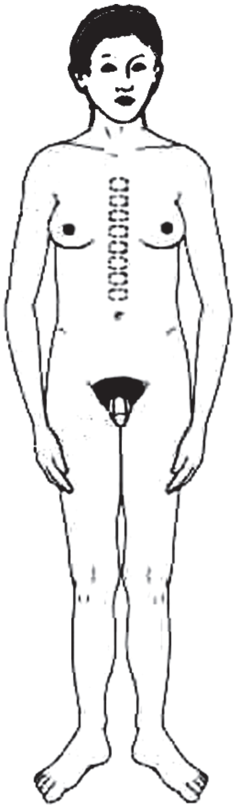


Fig. 3.54 Main clinical findings in Klinefelter syndrome

Most commonly, men with Klinefelter syndrome will present to their urologist with infertility: azoospermia or severe oligospermia, low testosterone, and complications of low testosterone, such as erectile dysfunction and poor libido. Boys will present with concerns about genital and pubertal development [139]. Spermatogenic and steroidogenic dysfunction are cardinal and the most prevalent signs of Klinefelter syndrome. A typical patient with Klinefelter syndrome will present with low serum testosterone, high LH and FSH levels, and often elevated estradiol; however, the decline in testosterone production is progressive over the life span, and not all men suffer from hypogonadism.

Patients with Klinefelter syndrome should be treated with lifelong testosterone supplementation that begins at puberty, to secure proper masculine development of sexual characteristics, muscle bulk, and bone structure and to prevent the long-term deleterious consequences of hypogonadism.

The fact that sperm can be found in the testes of men with Klinefelter syndrome has challenged the previous assumption that men with Klinefelter syndrome are always sterile. This has raised the mechanistic questions of whether children with Klinefelter syndrome are born with a severely depleted number of spermatogonia and whether there is a period in life when the spermatogonia undergo massive apoptosis that results in depletion of the spermatogonial population and subsequent azoospermia [30, 175]. Based on the current data, it is reasonable to assume that most men with Klinefelter syndrome are born with spermatogonia. However, during early puberty – most likely after initiation of the first wave of spermatogenesis – the spermatogonia appear to undergo massive apoptosis.

Sperm found in testes of men with Klinefelter syndrome have only a slightly increased frequency of sex chromosome polysomies, and most boys born of fathers with Klinefelter syndrome have a normal karyotype. These findings indicate that during early stem cell proliferation or meiotic division, the checkpoint mechanisms are able to overcome X chromosome polysomy resulting in sperm with a single X or Y chromosome [30, 175].

In the last decade, developments in microsurgical techniques and advances in artificial reproductive technologies allowed over 50% of men with Klinefelter syndrome to have their own children through the combination of microsurgical testicular sperm extraction and use of freshly retrieved sperm for in vitro fertilization.

Men with Klinefelter syndrome are at a higher risk of autoimmune diseases; diabetes mellitus, leg ulcers, osteopenia and osteoporosis, and tumors (breast and germ cells), and historically they have an elevated mortality rate. It is not known whether the morbidity associated with Klinefelter syndrome is a result of hypogonadism and hyperestrogenism or rather due to abnormal function of X chromosome-linked genes [30, 175].

Severe intellectual deficits are rare. Often, the auditory processing delay and language dysfunction seen in men with Klinefelter syndrome are misdiagnosed as cognitive deficits [203]. More behavioral aspects will be discussed in Chap. 23.6.

3.6.3.3.5 Triple-X Syndrome

The triple-X syndrome (47,XXX) was first described by Jacobs et al. [117] in a woman of average intelligence who had secondary amenorrhea. In fact, this finding was described as "Evidence for the existence of the human 'super female'". The triple-X syndrome is one of the most frequent aneuploid variations in female infants, occurring in about 1 per 1,000 newborn females [168]. The incidence increases with maternal age, and nondisjunction appears to occur mainly during maternal meiosis.

To date, several hundred 47,XXX women have been reported. Much of the data is based on isolated case studies of women ascertained through the presence of another condition. Perhaps more unbiased information can be achieved through chromosomal screening of newborns and following them longitudinally. Such studies have revealed that 47,XXX girls have no distinguishing physical features and their pubertal development and reproductive capacity are normal [72]. Most 47,XXX girls present with a normal phenotype at birth [147], but their average birth weight may be slightly lower than that of girls with normal chromosomes. The final height of triple X syndrome females usually exceeds that of controls, and the proportion of their leg length to their overall height increases to a significant level [194]. Bone age is below normal in early childhood, but is within normal limits by 7–10 years of age. Triple X females usually have normal fertility, and almost all their children have normal karyotypes.

Head circumference in 47,XXX infants is often smaller than in control girls. In fact, the presence of an extra X chromosome may cause a reduction in craniofacial growth and reflects on the overall length of the calvaria, the anterior and posterior cranial bases, and the facial complex [134].

The average intelligence of triple X girls seems to be slightly lower than that of siblings or a population control group [167], and an interesting correlation was found between head circumference at birth and IQ at 7 years of age [195]. However, IQ distribution has the potential to improve to within normal limits. Such an improvement has been attributed to providing the parents with appropriate information and counseling, which increases the chance that resources for stimulation of the children, including both parental resources and kindergarten, school, and social system resources, will be available [167]. These observations reinforce the notion that parents who have a child with a sex chromosome abnormality usually need information, counseling, and assistance. The type and magnitude of this assistance depend on the individual child, the specific sex chromosome anomaly, and the parents' own resources, psychologically, socially, and otherwise.

3.6.3.4 Chromosomal Aneuploidies in Humans

3.6.3.4.1 47,XYY Karyotype

The first description of a 47,XYY karyotype was made in 1961 [204]. 47,XYY has an estimated incidence of 1 per 1,000 live births [1]. The clinical features of patients with 47,XYY karyotype can be subtle and variable. Generally, the patients are physically normal in infancy, with phenotypic characteristics becoming increasingly apparent over time. Patients are usually tall and thin and may have delayed speech, lower cognitive function, hyperactivity, learning disabilities, and other central nervous system (CNS) abnormalities (such as intentional tremor and hypotonia). However, several patients with an XYY constitution and a normal phenotype have also been described. Possible behavorial aspects, such as a potential association with an increased risk of criminal and antisocial behaviour, will be discussed in detail in Chap. 23.6.

Males with an extra Y chromosome are mostly fertile and have normal gonadal function. The premeiotic loss of the extra Y chromosome, frequently observed in XYY human males, permits the achievement of spermatogenesis and normal sperm production [82].

3.6.3.4.2 Other Y Polysomies

While the 47,XYY karyotype is relatively frequent, cases with trisomy and tetrasomy Y are rare, having been reported in the literature 20 and 10 times, respectively. No pentasomy Y has been reported. While the small case numbers do not allow reliable descriptions of the phenotype, psychomotor delay ranging from speech delay to severe mental retardation, skeletal abnormalities, and facial dysmorphism have been reported in every case [60].

3.6.3.4.3 XXYY Syndrome

XXYY syndrome was first described in 1960 [161] and occurs in approximately 1:18,000 to 1:40,000 males. Approximately 100 cases have been reported to date. Initially the XXYY syndrome was described as a variant of 47,XXY Klinefelter syndrome because of a shared physical and endocrinological phenotype, such as tall stature, hypergonadotropic hypogonadism, and infertility. However, XXYY differs in its medical, neurodevelopmental and behavioral characteristics.

A review of 95 males with XXYY syndrome reported that the mean age at diagnosis was 7.7 years. Developmental delays and behavioral problems were the most common primary indication for genetic testing (68.4%). Across all age groups physical and facial features such as hypertelorism, clinodactyly, pes planus, and dental problems were common. In both adolescents and adults tall stature was a prominent feature. The mean adult stature was in the range of 192 cm. Frequent medical problems included allergies and asthma (>50%), congenital heart defects (19.4%), radioulnar synostosis (17.2%), inguinal hernia and/or cryptorchidism (16.1%), and seizures (15%). In adulthood, such medical problems as hypogonadism (100%), intention tremor (71%), and deep venous thrombosis and type II diabetes (each 18.2%) were encountered. Brain MRI, done in 35 cases, showed white matter abnormalities in 45.7% of patients and enlarged ventricles in 22.8% [233]. Behavioral patterns of the XXYY syndrome will be discussed in Chap. 23.6.

3.6.4 Chromosome Aberrations and Spontaneous Miscarriage

3.6.4.1 Incidence of Prenatal Zygote Loss in Humans

About 15% of all pregnancies in humans end in recognizable spontaneous abortion, defined as pregnancy loss before the 22nd week (body weight of the embryo: 500 g or less). However, there is good evidence in both humans and other mammals that many more zygotes are lost at an earlier stage of development; they are often severely malformed. It appears that almost 50% of all conceptuses may be lost within the first 2 weeks of development, before the pregnancies are recognized. In humans this early zygote loss usually goes unnoticed.

3.6.4.2 Aneuploidy in Oocytes and Embryos

The high prevalence of aneuploidy in human oocytes and embryos has long been recognized, and the developmental impact of these anomalies, especially those of meiotic origin, is well documented. Present data based on molecular cytogenetic techniques suggest that the incidence of aneuploidy in first trimester spontaneous miscarriages may be as high as 65% [156]. There is a dramatic increase in chromosomally abnormal pregnancies with advancing maternal age. These data are mirrored by findings from direct cytogenetic analysis of human oocytes, which demonstrates oocyte aneuploidy rates in excess of 50% for many women over 40 years of age [102].

Studies applying CGH to the analysis of embryos have provided fascinating data on the variety and frequency of chromosome abnormalities in human embryos and confirmed that aneuploidy can affect any chromosome during human preimplantation development, including the largest chromosomes. In fact, published CGH data reveals that 20–40% of embryos carry chromosome abnormalities [242, 251].

The great majority of these chromosomally abnormal pregnancies end in abortion between 8 and 16 weeks of gestation. A few may remain beyond this time and be lost as a later abortion (which may present as intrauterine fetal death). As among liveborn babies only 1 in 200–250 has an unbalanced chromosome abnormality, there must be very effective natural selection against those large numbers of gametes and conceptions that were abnormal.

3.7 Chromosome Instability/Breakage Syndromes

There are several syndromes that are associated with chromosomal instability. The instability refers to the predisposition of the chromosomes to undergo rearrangement or to display other abnormal cytogenetic patterns. Often multiple random, nonclonal chromosomal aberrations can be observed, which result in a high cell-to-cell variability. Thus, diagnosis often requires analyses of multiple metaphase spreads.

Defects of DNA repair often underlie such syndromes, which frequently exhibit characteristic clinical features and may cause serious clinical results. Although genetic defects in each repair or checkpoint pathway are associated with clinically distinct entities, as a group they are characterized by developmental abnormalities, cancer predisposition, and accelerated aging.

“Classic” chromosomal instability syndromes are Fanconi anemia, Bloom syndrome, and ataxia-telangiectasia. Rarer chromosomal instability syndromes include Nijmegen breakage syndrome, Werner syndrome, Rothmund-Thomson syndrome (RTS), ICF syndrome, Roberts syndrome, mosaic variegated aneuploidy (MVA).

3.7.1 Fanconi Anemia

Fanconi anaemia (FA) is a rare autosomal recessive disease with a prevalence of 1–5 per million. FA is characterized by diverse clinical features that often include developmental anomalies affecting the skeleton (absent or abnormal thumbs and radii), kidneys, heart, or any other major organ system. The life expectancy of FA patients is reduced to an average of 20 years (range 0–50 years), primarily because these individuals develop life-threatening bone marrow failure and are susceptible to developing malignancies, especially acute myeloid leukemia and, to a lesser extent, solid tumors, in particular squamous cell carcinomas.

FA is caused by biallelic mutations in at least 13 different genes (*FANCA, B, C, D1/BRCA2, D2, E, F, G, I, J/BRIP1, L, M,* and *N/PALB2*) interacting with others (e.g., *ATM, RAD51, NBS1*) in cellular DNA damage recognition and repair network. Eight of the FA proteins form a nuclear core complex with a catalytic function involving ubiquitination of the central FANCD2 (Fanconi anemia complementation group D2) protein that interacts with BRCA1 (breast cancer 1, early onset) in microscopically visible subnuclear foci. At present, FA patients are assigned to one of seven complementation groups (FA-A to FA-G; on FA nomenclature), and most (60–80%) fall into group A.

At the cellular level, manifestations of genetic instability include chromosomal breakage, cell cycle disturbance, and increased somatic mutation rates. Because of the extreme variability of the clinical features of this disease, as well as variations in the level of spontaneous chromosome aberrations, a cytogenetic test that quantifies crosslinker-induced chromosomal breakage has become the gold standard for diagnosing

FA [126, 179]. Therefore, genetic testing for FA is routinely based on conventional chromosome breakage analyses. Mainly chromatid breaks and radial figures are counted and compared with those of normal control cells.

3.7.2 Instabilities Caused by Mutations of Proteins of the RecQ Family of Helicases: Bloom syndrome, Werner syndrome, and Rothmund-Thomson syndrome

Homologous recombination (HR) is a process that repairs DNA double-strand breaks and restores productive DNA synthesis following disruption of replication forks. Although HR is indispensable for maintaining genome integrity, it must be tightly regulated to avoid harmful outcomes, because excessive HR can generate DNA damage. For example, the genomes of higher eukaryotes contain many tandem and dispersed repeat sequence DNA. Genetic exchange between nonhomologous copies of these repeat sequences (illegitimate recombination) can generate translocations, deletions, or inversions. Illegitimate recombination events are more likely to occur when the cell is replicating its DNA because the DNA is exposed. If replication forks have been stalled, for example because the polymerase has encountered a damaged base, special structures can be formed, which could be converted into DSBs by up-regulated HR.

To oppose these potentially adverse effects of HR, the cell holds the recombination machinery in check with proteins that have antirecombination activity. The specialized DNA helicases of the RecQ family are intimately involved in the regulation of HR and can inhibit HR through the stabilization of stalled replication forks [174].

The human RecQ helicases have been a major source of interest in the DNA repair field because of their links to human disease. Humans possess five distinct RecQ helicases – RECQL1, BLM, WRN, RECQL4, and RECQL5. Mutations in three of these helicases, *BLM*, *WRN,* and *RECQL4*, result in the genomic instability disorders Bloom syndrome (BS), Werner syndrome (WS), and Rothmund–Thomson syndrome (RTS), respectively. Cells derived from persons with these syndromes display varying types of genomic instability, as evidenced by the presence of different kinds of chromosomal abnormalities and different sensitivities to DNA-damaging agents. Persons with these syndromes exhibit a variety of developmental defects and are predisposed to a wide range of cancers. WS and RTS are further characterized by premature aging. Although these three human disorders share the general characteristics of genomic instability and cancer predisposition syndromes, each syndrome possesses unique clinical, cellular, genetic, and biochemical features that point to nonoverlapping roles in the maintenance of genome integrity [174].

3.7.2.1 Bloom Syndrome

BS is a rare autosomal recessive disorder caused by bi-allelic loss-of-function mutations in the *BLM* gene. Clinically it is characterized by proportionately short stature, sun-sensitive facial erythema, hypo- and hyperpigmented skin lesions, immune deficiency, infertility in males and subfertility in females, lack of subcutaneous fat, and susceptibility to type 2 diabetes. A prominent feature of BS is a marked predisposition to all types of cancers [88], which is notable for its high incidence, broad spectrum (including leukemia, lymphomas, and carcinomas), early diagnosis relative to the same cancer in the general population, and the development of multiple cancers in single individuals [86].

Analysis of metaphases from cultured cells from persons with BS reveals striking chromosome instability, visible as chromatid breaks and gaps, dicentric and ring chromosomes, acentric fragments, pulverized metaphases, telomere associations, and anaphase bridges [87]. BS cells exhibit special cytogenetic signs of dys-regulated HR, which are pathognomic for BS. The first of these abnormalities is the classic quadriradial configuration, which is a symmetric, four-armed arrangement composed of a pair of homologous chromosomes that have apparently undergone somatic crossing over. Such quadriradials are very rarely observed in metaphases from normal lymphocytes (<1 per 1,000) but in BS they are relatively common (1–2 per 100). Another diagnostic cytogenetic finding in BS is a markedly increased level of spontaneous sister chromatid exchange (SCE), which is an exchange event between the sister chromatids. In metaphases prepared from normal persons, the average number of SCEs ranges from 5 to 10 per 46 chromosomes, whereas in BS it is more than 50 per cell.

At the molecular level, the consequences of this excessive HR are increased rates of loss of heterozygosity, unequal SCEs, chromosome deletions and rearrangements – all of which are seen in BS cells – along with an elevated mutation rate. BS cells are hypersensitive to various genotoxic agents, such as ultraviolet light, mitomycin C, and topoisomerase inhibitors, especially when these and other agents are administered to cells synchronized in S-phase [174].

Werner Syndrome: WS is a rare autosomal recessive disorder caused by bi-allelic loss-of-function mutations in the *WRN* gene [258]. In this disorder premature development with features resembling aging, often already shortly after adolescence, is typically observed [93]. Premature aging features include the development of alopecia and graying hair, arteriosclerosis, atherosclerosis, osteoporosis, hypogonadism, cataracts, and type II diabetes. Persons with WS are generally smaller than average. Furthermore, there is an increased cancer predisposition, leading primarily to rare cancers of mesenchymal origin [94].

WS cells exhibit a distinctive cytogenetic abnormality referred to as “variegated translocation mosaicism.” Lymphocytes show frequent, nonclonal translocations, and fragile sites appear to constitute preferred sites for chromosomal translocations. Fibroblast cultures are frequently pseudodiploid and exhibit clonal expansions of cells containing different structural rearrangements. In addition, fibroblast cultures also enter premature replicative senescence, implicating WRN in telomere function.

At the molecular level, WS cells accumulate mutations at a higher rate, consisting predominantly of large spontaneous deletions. Cells are hypersensitive to a number of DNA damaging agents [174].

3.7.2.2 Rothmund–Thomson Syndrome

RTS is a rare autosomal recessive genetic disorder characterized by chromosome instability and clinical heterogeneity with growth deficiency, skin and bone defects, premature aging symptoms, and cancer susceptibility, and patients present clinically heterogeneous symptoms. A subset of RTS patients presents mutations of the *RECQL4* gene. Two forms of RTS have been defined based on clinical and molecular analysis: Type I RTS is associated with a characteristic poikiloderma and type II RTS with poikiloderma and an increased risk of osteosarcoma related to deleterious mutations in the *RECQL4* gene [38].

3.7.3 The Ataxia-Telangiectasia Group

In this group of chromosomal instability syndromes the basic pathogenetic process represents a failure in one of the DNA damage monitoring and repair systems. The group comprises ataxia-telangiectasia (AT), Nijmegen breakage syndrome (NBS), and AT-like disorder (ATLD). The genes for AT (*ATM*), NBS (*NBS1*), and ATLD (*MRE11*) encode proteins, which belong to a complex which senses aberrant DNA structures and monitors postreplication DNA repair.

3.7.3.1 Ataxia-Telangiectasia

Ataxia-telangiectasia (AT) is a rare autosomal recessive genetic disorder characterized by progressive neurodegeneration, a high risk of cancer, and immunodeficiency. Humans with AT are also hypersensitive to radiation. No curative strategy for this disease exists.

The gene product defective in this syndrome, ATM (ataxia-telangiectasia mutated), normally recognizes DNA damage. ATM is a protein kinase, which is activated immediately after a DNA double-strand break (DSB) occurs, and the resulting signal cascade generated in response to cellular DSBs is regulated by post-translational protein modifications, such as phosphorylation and acetylation. ATM signals to the DNA repair machinery and the cell cycle checkpoints to minimize the risk of genetic damage.

ATM also responds to physiological breaks in DNA during the development and differentiation of B and T cells and in the resolution of DNA DSBs that are generated during V(D)J recombination. ATM is involved in supporting efficient Vα–Jα coding. Consistent with this, increased accumulation of unrepaired coding ends during different steps of antigen receptor-gene assembly, for both immunoglobulin and T-cell receptor loci, has been reported in ATM-deficient B and T lymphocytes [141].

The involvement in V(D)J recombination likely explains the cytogenetic hallmarks of AT, which include frequent nonrandom rearrangements of chromosomes 7 and 14 in T lymphocytes, nonspecific chromosomal breaks in fibroblasts, and normal chromosomes. The breakpoints in the lymphocyte rearrangements are at 7p14, 7q35, 14q12, and 14q32, involving the T-cell receptor and immunoglobulin heavy chain genes. In addition, ataxia-telangiectasia homozygotes have markedly increased rates of nonspecific spontaneous translocations.

3.7.3.2 Nijmegen Breakage Syndrome

Nijmegen breakage syndrome (NBS) is a rare recessive genetic disorder. Characteristics include bird-like facial appearance, congenital microcephaly, early growth retardation, immunodeficiency, and high frequency of malignancies.

Upon chromosome analysis NBS cells display frequently spontaneous chromosomal aberrations and are hypersensitive to DNA double-strand break-inducing agents, such as ionizing radiations. NBS shares with AT cytogenetic features because preferentially chromosomes 7 and 14 are involved in rearrangements. The gene underlying the disease, *NBS1*, interacts with the *ATM* gene. NBS1 forms a multimeric complex with MRE11/RAD50 nuclease at the C terminus and retains or recruits them in the vicinity of sites of DNA damage by direct binding to histone H2AX, which is phosphorylated in response to DNA damage. Thereafter, the NBS1 complex proceeds to rejoin double-strand breaks predominantly by homologous recombination repair in vertebrates. NBS cells also show to be defective in the activation of intra-S-phase checkpoint [5].

3.7.4 Immunodeficiency, Centromeric Region Instability, and Facial Anomalies Syndrome

The immunodeficiency, centromeric region instability, and facial anomalies syndrome (ICF) results from a mutated DNA methyltransferase gene, *DNMT3B*. The phenotype, physical and cytogenetic changes, can be regarded as secondary to a failure of methylation. The defective methylation results in chromatin decondensation, which becomes apparent at the juxtacentromeric heterochromatin of chromosomes 1, 9, and 16. As a consequence, these chromosomes can form "windmill" multiradials by interchange within heterochromatic regions [69].

3.7.5 Roberts Syndrome/SC Phocomelia

Roberts syndrome/SC phocomelia (RBS) is an autosomal recessive disorder of symmetric limb defects, craniofacial abnormalities, and pre- and postnatal growth retardation. Intellect is normal. Most affected individuals (about 80%) show a chromosomal phenomenon known as premature centromere separation, sometimes also referred to as "heterochromatin repulsion." This is due to lack of cohesion at the heterochromatic regions around centromeres and the long arm of the Y chromosome, which results in reduced growth capacity, and hypersensitivity to DNA-damaging agents. RBS is caused by mutations in *ESCO2*, which encodes a protein involved in regulating sister chromatid cohesion [92] (see also Sect. 3.3.3).

3.7.6 Mosaic Variegated Aneuploidy

Mosaic variegated aneuploidy (MVA) is a recessive condition characterized by mosaic aneuploidies, predominantly trisomies and monosomies, involving multiple different chromosomes and tissues. The proportion of aneuploid cells varies, but is usually >25% and is substantially greater than in normal individuals. Affected individuals typically present with severe intrauterine growth retardation and microcephaly. Eye anomalies, mild dysmorphism, variable developmental delay, and a broad spectrum of additional congenital abnormalities and medical conditions may also occur. The risk of malignancy is high, with rhabdomyosarcoma, Wilms tumor, and leukemia reported in several cases.

In five families with this disorder, truncating and missense mutations of *BUB1B*, which encodes BUBR1, a key protein in the mitotic spindle checkpoint, were found. These data suggest that germline mutations in a spindle checkpoint gene may increase the rate of aneuploidy and strongly support a causal link between aneuploidy and cancer development [99].

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From Genes to Genomics to Proteomics

4

Michael R. Speicher

Abstract In human genetics many initial research initiatives focused on single genes or were performed on a gene-by-gene basis. However, recent findings, especially those about the extensive transcriptional activity of the genome, changed the concept of what a gene is supposed to be. In addition, novel high-throughput approaches and numerous innovative technologies, such as gene and expression microarrays, mass spectrometry, new sequencing methods, and many more, now enable us to address complex diseases and to unravel underlying involved regulatory patterns. These high-throughput assays resulted in a shift from studying Mendelian disorders towards multifactorial diseases, although monogenic diseases still provide a unique opportunity for elucidating gene function. This chapter describes current concepts about the definition of a gene, possible consequences of mutations and the latest developments in the areas of genomics, transcriptomics, and proteomics and their potential to add to a better understanding of factors contributing to phenotypic features.

Contents

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4.1 Single-Gene Approaches

Prior to the era of high-throughput analyses, typical research initiatives focused on single genes or were performed on a gene-by-gene basis. However, even research focusing on a single gene may already represent a very complex challenge. Some principles of working with single genes are described below. A particular focus will be on the limitations which have propelled the development of numerous innovative technologies, such as gene and expression microarrays, mass spectrometry, and proteomics, and many more, which now allow investigators to reveal underlying complex regulatory patterns.

4.1.1 What Is a Gene?

Before discussing the steps from genes to proteomics we should reflect on what a "gene" is actually supposed to be. In 1909 the term "gene" was used for the first time by Wilhelm Johannsen. Ever since, the concept of a gene has been under constant development, and numerous gene definitions have been proposed and adjusted as our knowledge of genes has evolved over the past decades. A somewhat surprising result is that although the term "gene" is one of the most commonly used expressions in genetics and although genes are constantly being characterized and more and more mutations in genes are being linked to diseases, the term itself in fact remains poorly defined. An excellent history of operational definitions of a gene over the past decades together with an attempt at an updated definition was recently provided by Gerstein et al. [25]. The authors rightfully argue that the provocative findings of the ENCODE Project [17], which elucidated the complexity of the RNA transcripts produced by the genome, have to change previous definitions of a gene. The preceding views of a gene were centered on protein coding (Fig. 4.1) and did not take the extensive transcriptional activity of the genome into account, most likely because the full extent of transcriptional activity was unknown prior to the ENCODE Project.

Based on the knowledge derived from the ENCODE Project, Gerstein et al. [25] proposed the following, updated definition for a gene: "The gene is a union of genomic sequences encoding a coherent set of potentially overlapping functional products." An illustration of how to apply this definition is provided in Fig. 4.2.

Another implication of this definition is that 5′ and 3′ untranslated regions (UTRs), despite their importance for translation, regulation, stability, and/or localization of mRNAs, would not be part of a gene because they do not participate in encoding the final product of a protein-coding gene. In order to compensate for this, Gerstein et al. [25] suggested a new "category" for regulatory and untranslated regions playing an important part in gene expression, by naming these regions "gene-associated." This terminology may help to acknowledge that additional DNA sequences outside

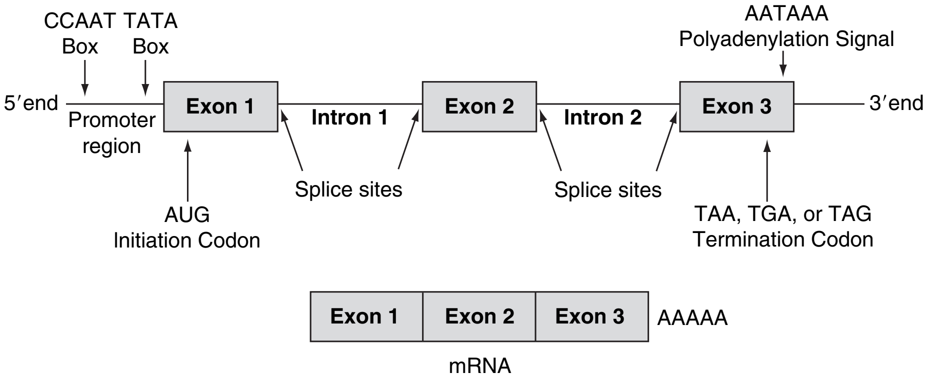


Fig. 4.1 Representative classic view of a gene. Transcription may be initiated from the promoter region located at the 5′ side of a gene. The promoter region often contains a TATA or a CCAAT box and is enriched for the paired nucleotides cytosine and guanine (CG islands). Genes consist of translated (exons) and noncoding (introns) portions. The open reading frame (*ORF*) is situated between the initiation codon (*AUG*) and the termination codon (*TAA*, *TGA*, or *TAG*). Sequences encoding the polyA tail of the protein are located at the end of a gene. The precursor RNA is spliced so that intronic sequences are removed and messenger RNA (mRNA) is formed

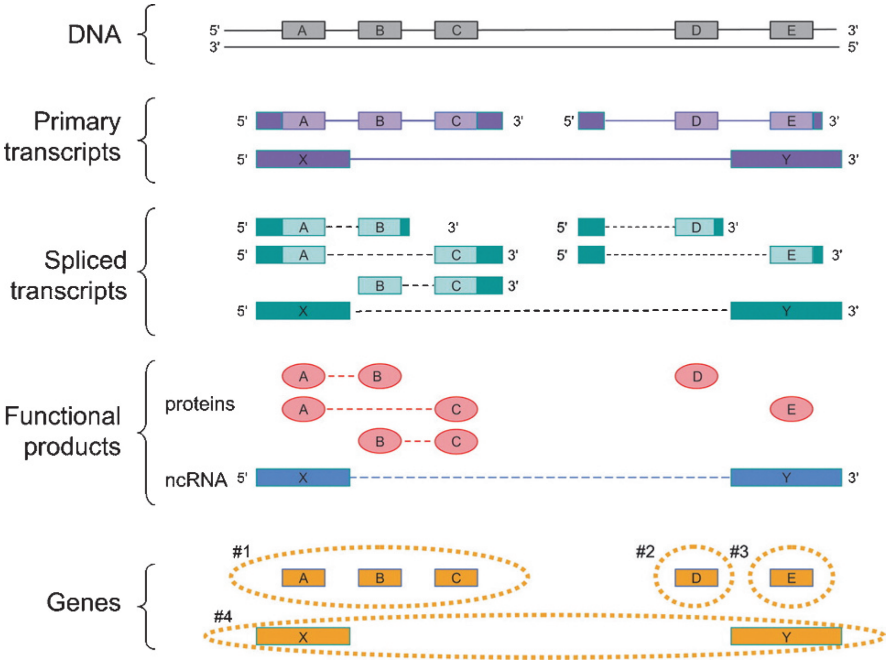


Fig. 4.2 Gerstein et al. [25] proposed a new definition for genes, and this figure illustrates how this definition should be applied. In this region the *gray rectangles* correspond to exonic/protein-coding sequences. Three primary transcripts originate from this genomic region. Two of these transcripts consist, in addition to the 5′ and 3′ un-translated regions, of some of the exons (*A*, *B*, *C* or *D*, and *E*); intronic sequences are represented by *solid lines*. The third transcript (*X* and *Y*) does not encode a protein but is a noncoding RNA (ncRNA) product. Therefore, such a transcript may share its genomic sequences with protein-coding segments; however, it usually does not exactly correspond to exons. The result of alternative splicing in this example is that the first two transcripts encode five protein products (*A-B*, *A-C*, *B-C*, *D*, and *E*; the *dashed lines* illustrate connectivity between the RNA sequences). Thus, exons A, B, and C generate transcripts, each derived from two of these DNA segments. In contrast, the products originating from D and E share a 5′ untranslated region, but their translated regions do not overlap after alternative splicing. The noncoding RNA product is not a coproduct of the protein-coding genes. The functional products are 5 different proteins shown in *ovals* (connected by *dashed lines*) and one RNA product (*rectangles*, also connected by a *dashed line*). As a consequence this region harbors four genes indicated at the bottom within the *orange dashed lines*. Sequence segments A, B, and C comprise gene 1, whereas gene 2 contains D, gene 3 E and gene 4 X and Y. From [25]

of the respective gene themselves have important roles in contributing to gene function.

From this new definition it follows that only continuous DNA sequences coding for a protein or RNA product without overlapping products correspond to the classic and most commonly used view of gene. In fact, the vast majority of our knowledge about "genes" and their functions centers on this subclass of genes. Thus, with these new evolving concepts it is obvious that even "monogenic" disorders are at present incompletely explored and a lot remains to be discovered.

As the updated definition emphasizes the final products of a gene, it disregards intermediate products originating from a genomic region that may happen to overlap. This implies that the number of genes in the human genome is going to increase significantly when the survey of the human transcriptome has been completed.

4.1.2 Mutations

The aforementioned summary of the complexity of a gene and its possible transcripts also suggests that the distinction between pathogenic and nonpathogenic mutations is often very difficult. In general, there are three different types of mutations. *Deletions* involve the loss of at least one nucleotide, whereas *insertions* represent the addition of at least one nucleotide. Both deletions and insertions cause a shift of the reading frame and are therefore also referred to as frameshift mutations. Usually the resulting sequences no longer code for a functional gene product and are thus dubbed "nonsense mutations." Since insertions and deletions usually disturb the gene function significantly, they are often associated with diseases and are therefore frequently pathogenic.

In contrast, a contribution to specific phenotypic features of the *substitution* or *exchange* of a single nucleotide is often very difficult to establish. An exchange of one purine for another purine or of one pyrimidine for another is called transition, whereas an exchange of a purine for a pyrimidine or vice versa is a transversion. A nucleotide substitution does not result in a shift of the reading frame, and possible consequences depend on how a codon has been altered. For example, a substitution may alter a codon so that a wrong amino acid will be present at this site, which is referred to as a "missense mutation." Such missense mutations may have consequences ranging from no changes to severe functional changes, and it is often very difficult to establish the outcome of such mutations. A nucleotide substitution is called a "silent mutation" if the resulting codon still corresponds to the same amino acid. This is possible because of the redundancy of the genetic code, as different nucleotide sequences may code for the same amino acid sequence. For example, the four nucleotide base pairs GCC, GCG, GCT and GCA all code for the amino acid alanine. If GCC represented a codon within an open reading frame a substitution at the third position from C to G or from C to A would still represent a codon with the nucleotide sequence for alanine. Much has been learnt about the phenotypic consequences of mutations, but there are many examples of missense mutations, variants in DNA elements of unknown function, and silent changes in coding regions for which pathogenicity is questionable. Thus, another difficult challenge is to prove that an altered allele is causal to the disease in question.

For example, silent mutations frequently have no consequences for the phenotype. However, in order to illustrate the often enormous difficulties in determining the significance of mutations, two striking examples demonstrating that even "silent" mutations may have severe consequences for a phenotype will be discussed below.

4.1.3 Silent Mutations and Phenotypic Consequences

Two particularly fascinating "silent" mutations with significant consequences for the phenotype are described here.

Hutchinson–Gilford progeria syndrome (HGPS) is a rare genetic disorder. Affected individuals show very early signs of aging, such as loss of hair, lipodystrophy, scleroderma, decreased joint mobility, osteolysis, and facial features resembling those of aged persons, and they die at an average age of 13. In the vast majority (90%), progressive atherosclerosis of the coronary and cerebrovascular arteries is the cause of death [30]. HGPS belongs to a group of conditions called laminopathies, which affect nuclear lamins. The lamins belong to the multiprotein family of intermediate filaments and can be regarded as the main determinants of the nuclear architecture. HGPS is caused by mutations in *LMNA*, resulting in an abnormally formed lamin A. In the majority of progeria patients a classic p.G608G (c.1824C>T) mutation in exon 11 can be found. It is predicted that this mutation is a silent mutation, as it does not cause any change at the amino acid level. However, this change improves the match to a consensus splice donor, activating a cryptic splice site [14, 18]. Owing to this activation of a cryptic splice site, 150 nucleotides, up to the start codon of exon 12, are removed [14, 18]. The last step in the posttranslational processing of prelamin A cannot occur without these nucleotides, so that the mutant prelamin A persists. The mutant prelamin A is called progerin, and it is the presence of progerin, and not the lack of normal lamin A, that causes the phenotype [55].

The second example is the identification of a synonymous *single-nucleotide polymorphism* (SNP), which did not produce altered coding sequences in the *Multidrug Resistance* 1 (*MDR*1) gene [35]. The *MDR*1 gene product is a P-glycoprotein multiple-transmembrane protein pump contributing to the pharmacokinetics of drugs, which is associated with the multidrug resistance of cancer cells. Although *MDR1* harbors many SNPs, some SNPs have been associated with reduced functionality of the pump. This was observed for two SNPs (e.g., C1236T and C3435T) even though neither changes the amino acid sequence of P-glycoprotein. For example, the C1236T polymorphism changes at amino acid position 412a GGC codon to GGT and both encode glycine, whereas the C3435T polymorphism changes at position 1145 ATC to ATT, which in each case encodes isoleucine. However, Kimchi-Sarfaty et al. [35] were able to demonstrate that both polymorphisms resulted in changes from frequent to infrequent codons. As a consequence, ribosome trafficking is slowed down at the corresponding mRNA regions. These alterations likely affect the cotranslational folding pathway of P-glycoprotein, resulting in a different final conformation and eventually in altered substrate specificity. Thus, silent mutations of synonymous codons (changing from frequent to infrequent) in certain genes may alter translation kinetics of mRNA, which might in turn affect final protein conformation.

4.1.4 Mutation Detection by Sanger Sequencing

In 1977 Fred Sanger published three seminal method papers on the rapid determination of DNA sequences [53, 54, 60], for which he received his second Nobel prize in Chemistry in 1980. This technology, which besides Sanger sequencing is also referred to as dideoxynucleotide sequencing, provided a tool for deciphering complete genes and later entire genomes. In fact, Sanger sequencing evolved into the only DNA sequencing method used for three decades after it was first described. DNA can be prepared for sequencing by two approaches: for targeted resequencing, which is done in most diagnostic routine applications: primers flanking the target regions are used to amplify the respective region. In contrast, for shotgun de novo sequencing, DNA is randomly fragmented and cloned into a plasmid, which is subsequently used to transform *Escherichia coli* (Fig. 4.3a). The latter approach played a pivotal role in deciphering the human genome. The

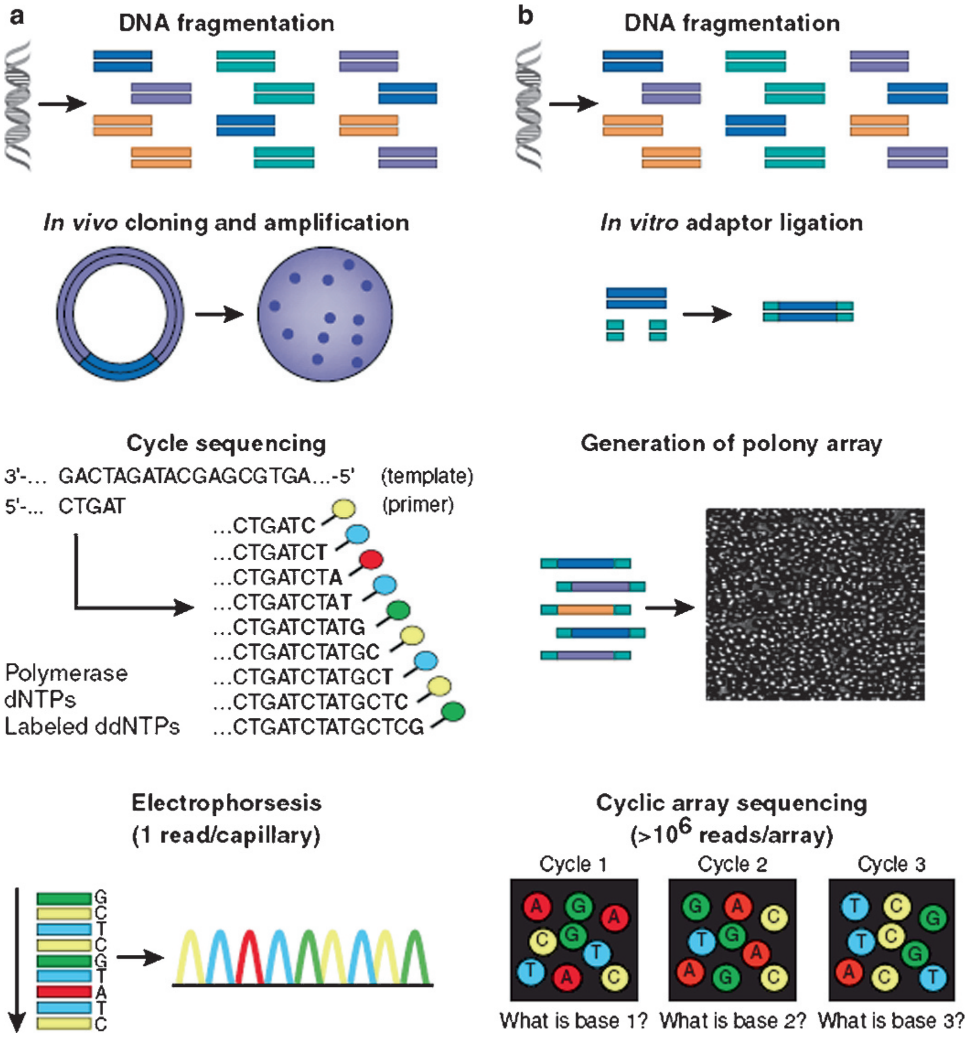


Fig. 4.3 Comparison between (**a**) Sanger and (**b**) next-generation sequencing. (Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology [58], copyright 2008)

results of both approaches are multiple templates, which are then subjected to the sequencing reactions, consisting of repeated rounds of template denaturation, primer annealing, and primer extension. In each cycle of the sequencing reaction the primer extension is stochastically terminated by the integration of dideoxynucleotides (ddNTPs), which are labeled with a fluorochrome. This results in a mixture of extension products of different lengths, and the label of the respective terminating ddNTPs reflects the nucleotide identity of its terminal position. Subsequently the sequence can be determined by high-resolution electrophoretic separation of the single-stranded, end-labeled extension products in a capillary-based polymer gel. The DNA sequence is deciphered by analysis of the fluorescent labels at the end of the fragments. The discrete lengths of the fragments determine the nucleotide position, and the nucleotide itself is encoded by laser excitation of the fluorescent labels and a four-color detection of the emission spectra, which are then translated into DNA sequence by appropriate software (Fig. 4.3a).

The application of Sanger sequencing for deciphering the entire human genome represented particularly large-scale sequencing efforts, which were conducted in factory-like environments called sequencing centers. These centers had a specialized and dedicated infrastructure consisting of hundreds of DNA-sequencing instruments, robotics, bioinformatics, computer databases, instrumentation, and a large number of personnel. The aim of deciphering the entire human genome dramatically changed the throughput requirements of DNA sequencing and propelled developments such as automated capillary electrophoresis. Many capillary-based sequencing systems have 96 or more capillaries, meaning that 96 sequence reads can be processed in parallel. However, a simple increase in the number of capillaries was not sufficient for the new enduring tasks in genomics, which required the development of entirely new technologies, as summarized in the next paragraph.

4.1.5 Next-Generation Sequencing

The next-generation sequencing revolution started in 2005 with two seminal papers describing a sequence-by-synthesis technology [47] and a multiplex polony-sequencing protocol [59]. The parallel sequencing throughput capacity is perhaps the most important feature setting next-generation sequencers apart from conventional capillary-based sequencing. In fact, instead of running 96 capillaries or samples at a time, next-generation sequencing allows the processing of millions of sequence reads simultaneously (Fig. 4.3b). This massive parallel sequencing requires only one or two instruments instead of several hundred Sanger-type DNA capillary sequencers and naturally involves significantly fewer personnel operating the machines. Another important difference is that next-generation sequence reads do not depend on vector-based cloning, but are instead derived from fragment libraries. This alone allows a significant speeding up of sequencing (Fig. 4.4). Another difference is that read lengths are shorter (35–250 bp for next-generation sequencing, as against 650–800 bp for capillary sequencers). Next-generation sequencing, often also referred to as second-generation sequencing, and the evolving third-generation sequencing will be discussed in greater detail in Sect. 4.4.

4.1.6 The Importance of Monogenic Mendelian Disorders

The quest for high-throughput assays is also accomanied by a shift away from the Mendelian disorders towards multifactorial diseases. This neglects the fact that linking naturally occurring pathogenic mutations with monogenic disorders provides a unique opportunity for elucidating gene function [1]. Studies on Mendelian traits reveal irreplaceable insights into mutation processes and their associated molecular pathophysiology. Furthermore, it was the investigations of Mendelian disorders that disclosed the existence of genetic phenomena, such as uniparental disomy or parental imprinting.

Only in-depth analysis of monogenic disorders can unravel the consequences of different mutations within the same gene that can give rise to distinct phenotypes. For example, among the most striking examples are mutations in the aforementioned *LMNA* gene, which can cause not only the Hutchinson–Gilford progeria syndrome but also several other, different phenotypes, which are often summarized as primary laminopathies. Phenotypic consequences of mutations in *LMNA* can be further subdivided into laminopathies with striated muscular atrophy [including

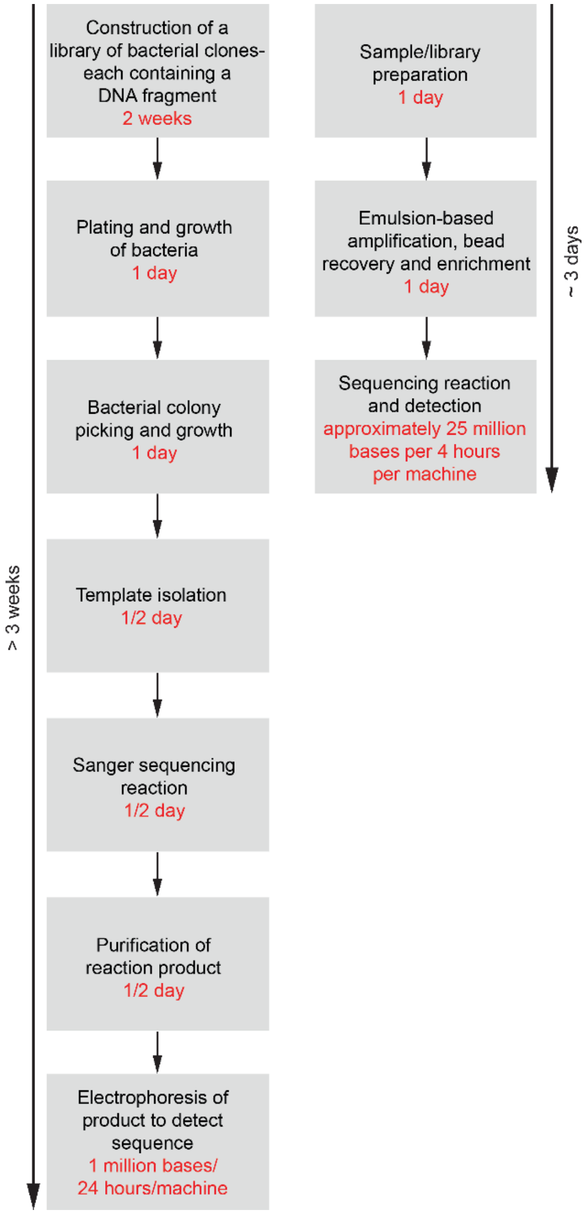

Construction of a library of bacterial clones-each containing a DNA fragment
2 weeks
Plating and growth of bacteria
1 day
Bacterial colony picking and growth
1 day
Template isolation
1/2 day
Sanger sequencing reaction
1/2 day
Purification of reaction product
1/2 day
Electrophoresis of product to detect sequence
1 million bases/ 24 hours/machine
> 3 weeks
Sample/library preparation
1 day
Emulsion-based amplification, bead recovery and enrichment
1 day
Sequencing reaction and detection
approximately 25 million bases per 4 hours per machine
~ 3 days

Fig. 4.4 Flow diagrams comparing the time-lapse needed for traditional Sanger sequencing (*left*) and massively parallel sequencing as used for the 454 system (*right*). (Reprinted by permission from Macmillan Publishers Ltd: Nature [52], copyright 2005)

Emery-Dreifuss muscular dystrophy (EDMD2; OMIM 181350), autosomal dominant limb girdle muscular dystrophy 1B (LGMD1B; OMIM 159001) and dilated cardiomyopathy, 1A (DCM1A; OMIM 115200)], laminopathies affecting peripheral nerves [(Charcot-Marie-Tooth disease type 2B1 (CMT2B1; OMIM 605588)] and laminopathies with loss of or reduced adipose tissues [familial partial lipodystrophy, Dunnigan type (FPLD2; OMIM 151660) and congenital generalized lipodystrophy, type 2 (CGL2, OMIM 269700)] [41]. A probably new *LMNA*-associated disease entity that may be classified as a congenital muscular dystrophy (LMNA-related congenital muscular dystrophy, or L-CMD) has recently been described [51] and suggests that even more phenotypes may be caused by mutations in this gene.

Even monogenic diseases have considerable phenotypic complexity, often depending on the genetic background and the status of modifier genes, which may modulate the consequences of specific mutations. In addition, such epigenetic changes as the genomic distribution of 5-methylcytosine DNA and histone acetylation may change the outcome of a mutation, and to make these issues even more complicated, such epigenetic modifications may change as we age [22]. Thus, monogenic disorders are in fact examples of oligogenic inheritance and vary along a continuum from simple to complex disorders [1]. Allelic variation in genes or other functional DNA sequences that modify the phenotypic severity of a monogenic disorder or control variation in gene expression provide links to additional genomic causes related to phenotypic variability.

4.2 Gene Regulation

Genes can be regulated by various means (Fig. 4.5). Obviously there is a “many-to-many” relationship between regulatory regions, epigenetic mechanisms, small RNAs, and genes. In fact, gene expression is a multilevel process, which is controlled by regulatory proteins and DNA sequences (genetic regulation) and by chromatin remodeling and the position of chromosomes in the nucleus (epigenetic regulation). In addition, gene regulation may be affected by complex sets of RNAs that do not produce proteins.

4.2.1 Genetic Regulation

At the beginning of transcription the base sequences of genes are transcribed into RNA by RNA polymerase II. Multiple accessory factors determine the transcriptional start and end points for RNA polymerase II. An important component is the promoter, typically located close to the gene it regulates, which facilitates the transcription of a gene.

Promoters comprise two interacting parts, i.e., the basal promoter elements and the enhancer elements. Basal promoter elements bind accessory transcription initiation factors that position RNA polymerase II in the right place and direction. These basal elements are composed of short, low-complexity sequences (such as the TATA element). Enhancer elements bind regulatory factors that specify the physiological conditions or cell types where the gene will be expressed. The enhancer and basal promoter complexes interact at both functional and physical levels to determine how often an RNA transcript is produced. Enhancers can work over large distances of DNA in both directions.

4.2.2 Epigenetic Regulation

In addition, there are several epigenetic components influencing gene expression, such as histone modifications, DNA methylation, and position effects (Fig. 4.5a).

The major mechanism for suppressing widespread transcription is probably sequestration of potential transcription start sites by wrapping most of the genome in nucleosomes (see Sect. 3.2.2). Typical transcription start sites are found in nucleosome-free regions generated by DNA sequences that are intrinsically resistant to nucleosome wrapping. Another mechanism is the targeted modification and removal of nucleosomes in order to expose the underlying promoter sequences (see Sect. 3.2.2). Thus, functional eukaryotic promoters must not only attract RNA

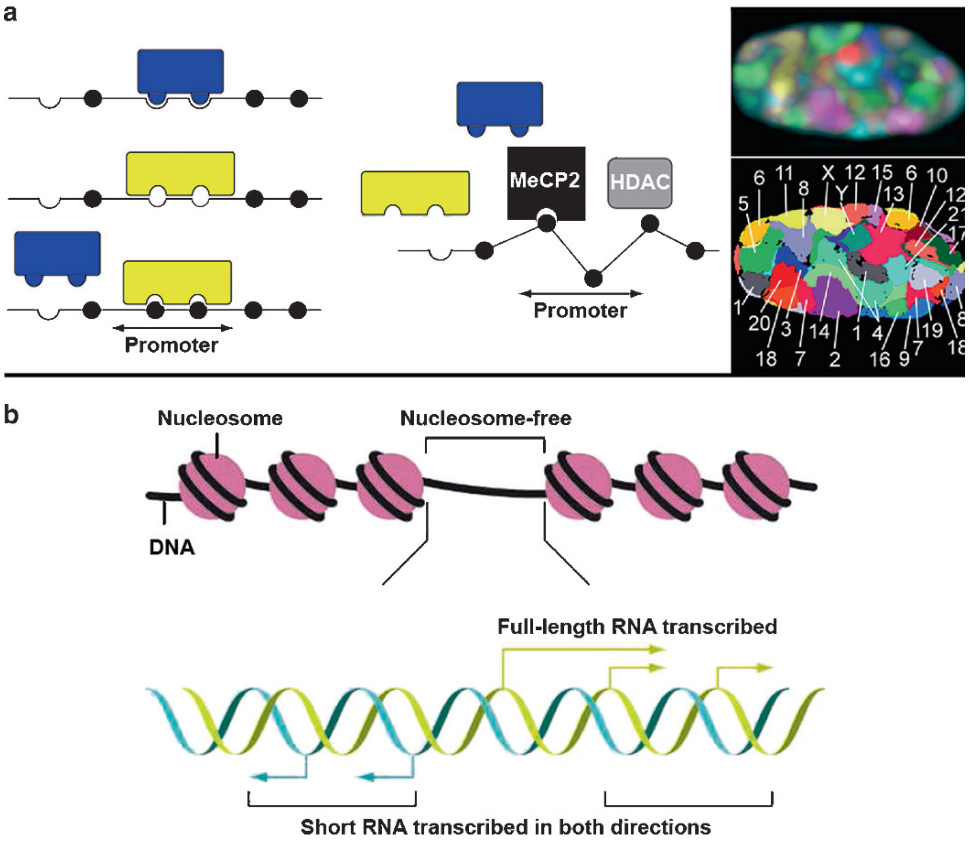


Fig. 4.5 (**a**, **b**) Different means of gene regulation. (**a**) *Left panel*: Consequences of epigenetic regulation by histone methylation: the configuration of the promoter region changes so that transcription factors cannot bind and expression of the respective gene is suppressed; *right panel*: Chromosomes occupy nonrandom positions in cell nuclei, these position effects influence genes expression. From [4]. (**b**) Example for possible gene regulation by small RNAs: promotor-associated transcripts (transcription start sites and transcripts are represented as *bent arrows*) within nucleosome-free DNA close to the promoters may influence gene expression. (From [6]. Reprinted with permission from AAAS)

polymerase II, but also evade nucleosomal repression. The epigenetic modes of gene regulation by histone modifications, DNA methylation, and position effects are discussed in detail in Sects. 3.2.2, 3.4.4.2, and 3.6.2.1.

4.2.3 Regulatory Transcripts of Small RNAs

More recently it has become clear that gene regulation may be affected by complex sets of small (20–30 nucleotides) RNAs that do not produce proteins, i.e., noncoding RNAs (ncRNAs; Fig. 4.5b). In general, effects of small RNAs on gene expression are inhibitory, as small RNAs may bind effector proteins to target nucleic acid molecules through base-pairing interactions. Therefore, activities of small RNAs are frequently summed up as "RNA silencing."

In humans the two main categories of small RNAs – among several classes – are short, interfering RNAs (siRNAs) and microRNAs (miRNAs) [2, 8]. These small RNAs are important regulators of gene expression that control both physiological and pathologic processes (e.g., development and cancer) miRNAs are regulators of endogenous genes, whereas siRNAs are defenders of genome integrity in response to foreign or invasive nucleic acids such as transposons and transgenes. An important distinction between miRNAs and siRNAs is whether or not they silence their own expression. Almost all siRNAs silence the same locus as they were derived from, and they only sometimes have the ability to silence other loci as well. In contrast, most miRNAs do not silence their own loci but do silence other genes. Both RNAs have double-stranded precursors and depend upon the same two families of proteins: Dicer enzymes to excise them from their precursors and Ago proteins to support their silencing effector functions [2, 8]. Single-stranded forms of both miRNAs and siRNAs associate with effector assemblies, which have been dubbed RNA-induced silencing complexes (RISCs).

The genes to be silenced are determined by the small RNA component, which identifies the respective complementary nucleotide sequence. The silencing can be monitored by increased expression of small RNAs or, conversely, by dilution or removal of old ones.

Furthermore, a new class of short RNA transcripts begins near the expected transcription start sites upstream of protein-encoding sequences (Fig. 4.5b). These RNAs often occur in the direction opposite to that of the protein-coding region [12, 29, 50, 57]. Although the function of these RNAs is presently not well defined, they may have an impact on how promoters delineate transcription start sites. These new RNAs are largely derived from DNA in nucleosome-free regions and may therefore arise from random, weak basal promoter elements that escape suppression [12, 29, 50, 57]. Hence, these short promoter-associated RNAs may simply result from incomplete suppression of cryptic initiation which, however, does not exclude an associated function by affecting the expression of the nearby gene.

4.3 "-omics" Sciences

Single-biomarker analysis is increasingly being replaced by multiparametric analysis of genes, transcripts, or proteins, now subsumed under the term "omics" sciences. The current nomenclature of omics sciences includes genomics for DNA variants, transcriptomics for mRNA, proteomics for proteins, and metabolomics for intermediate products of metabolism. The omics sciences use high-throughput techniques often allowing simultaneous examination of changes in the genome (DNA), transcriptome (messenger RNA [mRNA]), proteome (proteins), or metabolome (metabolites) in a biological sample, with the goal of understanding the physiology or mechanisms of disease. Insights derived from the complementary fields of omics sciences are expected to assist the development of new diagnostic, prognostic, and therapeutic tools. The omics sciences have in common that they require the development of novel informatic applications and sophisticated dimensionality reduction strategies. They have an enormous potential to unravel disease and physiological mechanisms and can identify clinically exploitable biomarkers from huge experimental datasets and offer insights into the molecular mechanisms of diseases.

The characteristics of the individual omics sciences and their integration to systems biology can be summarized as follows:

Genomics: Genomics seeks to define our genetic substrate and describes the study of the genomes of organisms.

Transcriptomics: Transcriptomics refers to the detailed analysis of the entire transcriptome, i.e., of all expressed sequences.

Proteomics: Proteomics explores the structure and function of proteins, which are the end-effectors of our genes. Proteomics has been revolutionized in the past decade by the application of techniques such as protein arrays, two-dimensional gel electrophoresis, and mass spectrometry. These techniques have tremendous potential for biomarker development, target validation, diagnosis, prognosis, and an optimization of treatment in medical care, especially in the field of clinical oncology.

Systems Biology: The integration of omic techniques is called "systems biology." This discipline aims at defining the interrelationships of several, or ideally all, of the elements in a system, rather than studying each element independently. Thus, systems biology will capture information from genomics, transcriptomics, proteomics, metabolomics, etc. and combine it with theoretical models in order to predict the behavior of a cell or organism.

4.4 Genomics

Genomics is the systematic study of the genomes of organisms. The field includes intensive efforts to determine the entire DNA sequence of organisms and fine-scale genetic mapping efforts. The investigation of single genes does not usually fit the definition of genomics. However, as the function of a single gene may affect many other genes, the border between single-gene analysis and genomics is often blurred.

4.4.1 Genomes of Organisms

A major branch of genomics is still concerned with the sequencing of the genomes of various organisms. The genome of the first free-living organism that was completely sequenced (*Haemophilus influenzae* in 1995) had a size of 1.8 Mb [21]. This was followed by complete sequences for *Mycoplasma genitalium* [23] and *Mycobacterium tuberculosis* [11], and subsequently by many other archeal, bacterial, and eukaryotic genomes. A rough draft of the human genome was presented in 2001 [39, 67], followed by an auspiciously completed version in 2004 [32]. Today sequencing efforts for other genomes continue. However, especially the resequencing of genomes, e.g., of human genomes to establish the variability between the genomes of different individuals, was propelled by the new possibilities of next-generation sequencing, which have added the sequences of other human individuals or of the first entire tumor genomes.

4.4.2 Array and Other Technologies

Genomics has certainly benefited from various array technologies that allowed the systematic analysis of entire genomes with various resolutions. These array technologies and other currently frequently employed important diagnostic tools, such as ChIP on chip and MLPA, are described and discussed in detail in Chap. 3 (Sect. 3.4.4.4). However, perhaps the most important recent development in genomics stems from next-generation (also referred to as second-generation) sequencing and the evolving third-generation sequencing (also referred to as single-molecule DNA sequencing).

4.4.3 Next-Generation Sequencing

Next-generation sequencing has already been introduced briefly in Sect. 4.1. An important issue of the new sequencing technologies is a significant reduction in costs: the public Human Genome Project spent US $3 × 10^9 to sequence the human genome, and the National Human Genome Research Institute at the National Institutes of Health aimed at a reduction of these costs to US $ 10^3 by 2014 (www.genome.gov/12513210). The new DNA-sequencing platforms now available do indeed have the potential to achieve the same sequencing results of the Human Genome Project at perhaps 1% of the cost. However, the data obtained with next-generation sequencing depends heavily on the high-quality reference sequence produced by the Human Genome Project. The key to the increased efficiency of the new methods lies in massive parallelization of the biochemical and measurement steps. The second important issue is a significant increase in DNA sequencing speed.

So far there are several commercial next-generation DNA sequencing systems, such as Roche's (454) Genome Sequencer 20/FLX Genome Analyzer, Illumina's Solexa 1G sequencer, Applied Biosystem's SOLiD system, and the Polonator G.007 (Dover Systems/Harvard).

4.4.3.1 Roche's (454) GS FLX Genome Analyzer

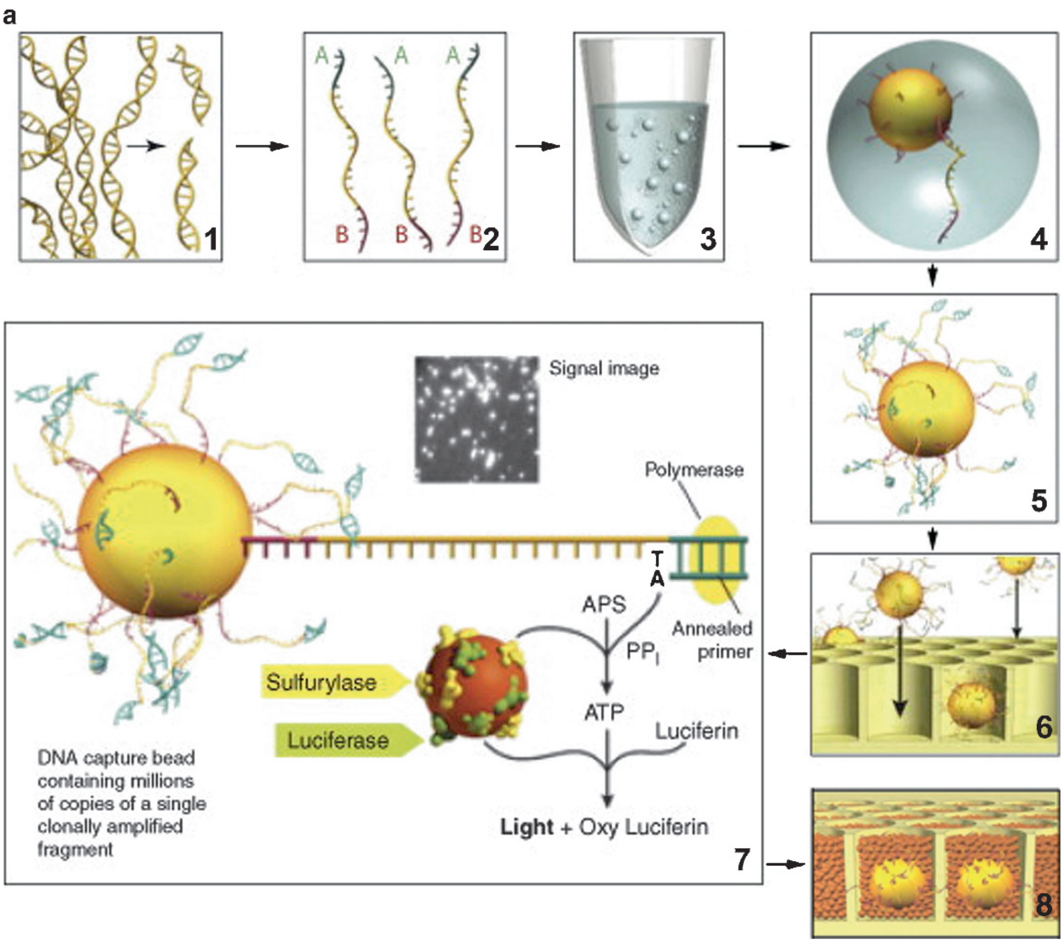
This system was commercially introduced in 2004 [47] and is based on pyrosequencing [49]. The sample preparation starts with fragmentation of the genomic DNA (Fig. 4.6a, b). In a next step, adapter sequences are attached to the ends of the DNA pieces to allow the DNA fragments to bind to beads, which have millions of oligomers attached to their surfaces, each with a complementary sequence to the adapter sequences. This is done under conditions allowing only one DNA fragment to bind to each bead. Subsequently, the DNA strands of the library are amplified by emulsion PCR: the beads, each with a single unique DNA fragment, are encased in droplets of oil, which isolate individual agarose beads and keep them apart from their neighbors to ensure that the amplification is uncontaminated. Each droplet contains all reactants needed to amplify the DNA, so that after some hours each agarose bead surface contains more than 1,000,000 copies of the original annealed DNA fragment. This number of DNA strands is needed to produce a detectable signal in the subsequent sequencing reaction. For this sequencing reaction the DNA template-carrying beads are loaded into picoliter reactor wells, each of which just has space for one bead. In these wells pyrosequencing [49], a sequencing-by-synthesis method, takes place, because DNA complementary to each template strand is synthesized. The pyrosequencing reactions flow through each well, and nucleotide and reagent solutions are delivered into it in a sequential fashion. The nucleotide bases used for sequencing release a chemical group as the base forms a bond with the growing DNA chain. This group drives a light-emitting reaction in the presence of specific enzymes and luciferin. The light from the luciferase activity reflects which templates are adding that particular nucleotide, and the emitted light is directly proportional to the amount of the particular nucleotide incorporated. Average read length per sample (or per bead) is about 250 bp.

4.4.3.2 Illumina's Solexa IG Sequencer

Illumina's Genome Analyzer, also commonly referred to as the "Solexa," was the second system commercially launched, in 2006. It is based on "sequencing by synthesis" [3] and is the only next-generation sequencing system that employs bridge-PCR [19] rather than emulsion-PCR (Fig. 4.7). The system applies high-density clonal single-molecule arrays consisting of genomic DNA fragments immobilized to the surface of a reaction chamber. In a first step, DNA fragments are generated by random shearing, and these are then ligated to a pair of oligonucleotides in a forked adapter configuration (Fig. 4.7a). These products can be amplified with two different oligonucleotide primers, which result in double-stranded DNA fragments with different adapter sequences at either end (Fig. 4.7a). In a next step these DNA fragments are denatured and a microfluid cluster station is used to anneal the single strands to the respective complementary oligonucleotides, which are covalently attached to the surface of a glass flow cell (Fig. 4.7b). A new strand is generated using the original strand as template in an extension reaction with an isothermal polymerase. Accordingly, the original strand is removed by denaturation. The adapter sequence of each newly generated strand is annealed to another surface-bound complementary oligonucleotide. This leads to formation of a bridge, and a new site for synthesis of a second strand is generated (Fig. 4.7b). Repeated cycles of annealing, extension, and denaturation result in growth of clusters, each apparently about 1 μm in diameter (Fig. 4.7c). Approximately 50×10^6 separate clusters can be generated per flow cell. For sequencing, each cluster is supplied with polymerase and four differently labeled fluorescent nucleotides (Fig. 4.7d). Based on the concept of "sequencing-by-synthesis," each base incorporation is followed by an imaging step to identify the incorporated nucleotide at each cluster. This iterative process needs about 2.5 days to generate read lengths of 36 bases. As each flow cell has 50×10^6 clusters, each analytical run generates more than 1 billion base pairs (Gb).

4.4.3.3 Applied Biosystem's SOLiD System

The SOLiD (sequencing by *o*ligo *l*igation and *d*etection) system was commercially released in 2007 and represents a development of work published in 2005 [59]. It



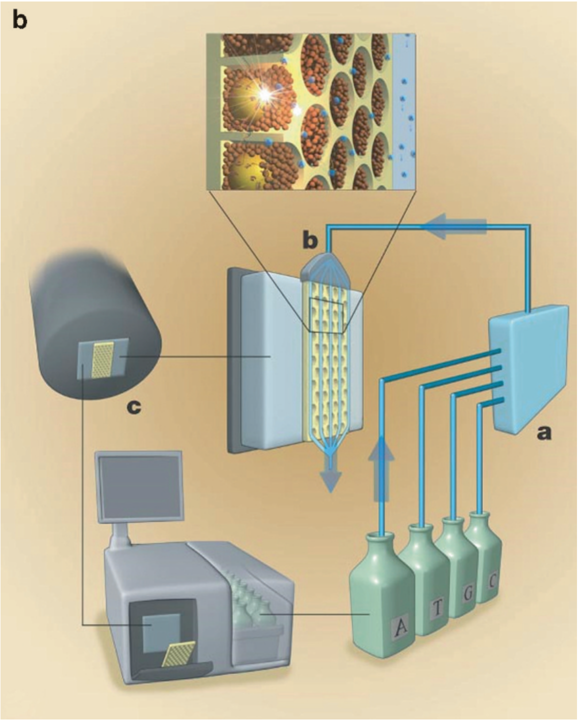


Fig. 4.6 (**a**, **b**) Steps involved in sequencing with the 454 system. (**a**) After isolation genomic DNA is fragmented (*1*) and ligated to adapters (*2*). The DNA is denatured to prepare them for emulsion PCR (*3*). Fragments are bound to beads under conditions that usually allow only one fragment per bead (*4*). The beads are captured in the droplets of a PCR reaction mixture-in-oil emulsion so that a PCR-amplification can be performed within each droplet (*5*). As a result, each bead carries 10 million copies of a unique DNA template. After breaking the emulsion the DNA strands are denatured, and beads carrying single-stranded DNA clones are placed into picotiter plates, i.e., wells of a fiberoptic slide (*6*). In these wells the pyrosequencing reaction takes place (*7* and *8*). (A composite from figures in [46] and [47]) (**b**) Major subsystems of the 454 sequencing instrument: (**b***a*) fluidic assembly; (**b***b*) flow chamber including the well-containing fibre-optic slide; (**b***c*) CCD camera, which captures the light emitted during the pyrosequencing reaction and a computer for instrument control. From [47], reprinted by permission from Macmillan Publishers Ltd: *Nature*, copyright 2005

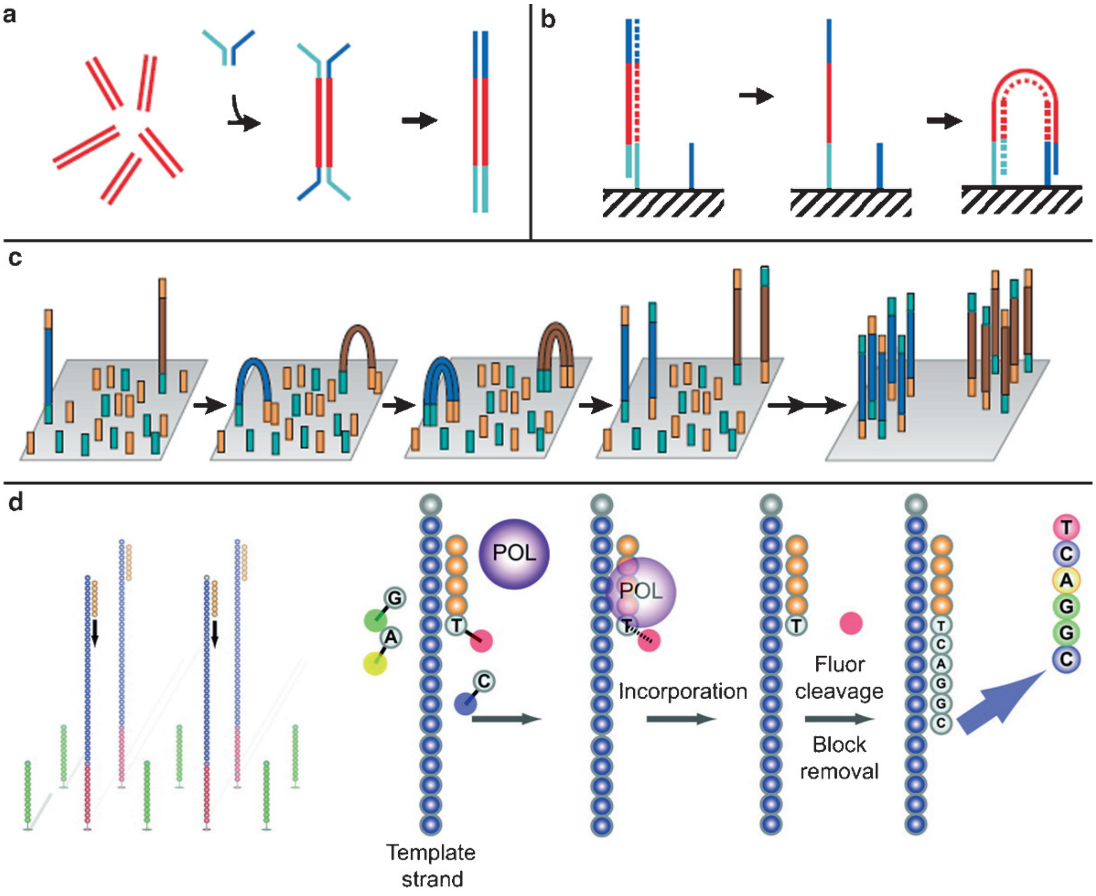


Fig. 4.7 (**a–d**) Steps involved in sequencing with the Illumina system. (**a**) DNA is fragmented by random shearing, and the fragments are then ligated to a pair of oligonucleotides. (**b**) The DNA fragments are denatured and annealed to the respective complementary oligonucleotides, which are covalently attached to the surface of a glass flow cell. A new strand is generated using the original strand as template. The "bridge" amplification relies on captured DNA strands arching over to that they can hybridize to an adjacent anchor oligonucleotide. By this means a bridge is formed and a new site for synthesis of a second strand is generated. (**c**) The adapter sequence of each newly generated strand is annealed to another surface-bound complementary oligonucleotide. Repeated cycles of annealing, extension, and denaturation result in growth of clusters, each appearing about 1 μm in diameter (**c**). (**d**) For sequencing the clusters are denatured, and after a chemical cleavage reaction and wash only forward strands remain for single-end sequencing. Each cluster is supplied with polymerase and four differently labeled fluorescent nucleotides, and each base incorporation is followed by an imaging step to identify the incorporated nucleotide at each cluster. (Reprinted by permission from Macmillan Publishers Ltd: (a,b) Nature [3], (c) Nature Biotechnology [58], copyright 2008)

also employs emulsion-PCR. After amplification the emulsion is broken and beads are covalently attached to the surface of a solid planar substrate, resulting in a dense, disordered array. The ligation-based sequencing process starts with the annealing of a universal primer complementary to the specific adapters on the library fragments. In each sequencing cycle a partially degenerate population of 8mer fluorescently labeled octamers is added (Fig. 4.8). These semi-degenerate oligos are structured in such a way that the label correlates with the identity of the central 2 bp in the octamer ("XX" in Fig. 4.8; the correlation with 2 bp, rather than 1 bp, is the basis of two-base encoding). When an 8mer oligo matches, it can hybridize adjacent to the universal primer 3′ end. The DNA ligase can then seal the phosphate backbone. After oligo-ligation a fluorescent readout consisting of imaging in four channels identifies the fixed base with the fluorescence label (the fifth position in Fig. 4.8). Subsequently, a chemical cleavage step removes the sixth through eighth bases ("zzz" in Fig. 4.8) via a modified linkage between bases 5 and 6, which deletes the fluorescent

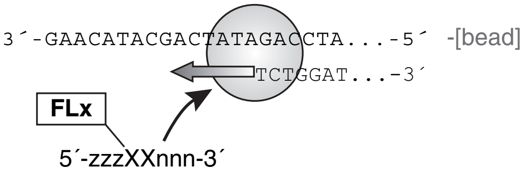


Fig. 4.8 Steps involved in sequencing with the Abi SOLiD system. Sample preparation is similar to that used in 454 technology, because DNA fragments are also ligated to oligonucleotide adapters linked to beads and clonally amplified by emulsion PCR. The ligation-based sequencing process starts with the annealing of a universal primer (5′-zzzXXnnn-3′) complementary to the specific adapters on the library fragments. These semidegenerate oligos are structured in such a way that the label correlates with the identity of the central 2 bp in the octamer ("XX"). Matching 8mer oligos can hybridize adjacent to the universal primer 3′ end and DNA ligase can then seal the phosphate backbone. After oligoligation a fluorescent readout consisting of imaging in four channels identifies the fixed base with the fluorescence label (here the fifth position). Subsequently, a chemical cleavage step removes the sixth through eighth bases (*zzz*), which leaves a free end for another cycle of ligation. For more details see text. (Reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology [58], copyright 2008)

group and leaves a free end for another cycle of ligation. Several cycles of that kind will iteratively interrogate an evenly spaced, discontinuous set of bases, in this example the sequence of each fragment at five nucleotide intervals. The system is then reset (by denaturation of the extended primer), and the process is repeated with a different offset (e.g., a primer set back from the original position by one or several bases) so that a different set of discontinuous bases is interrogated on the next round of serial ligations. A 6-day instrument run generates sequence read lengths of 35 bases. Placing two flow-cell slides in the instrument per analytical run can produce a combined output of more than 4 Gb of sequence.

A system related to the SOLiD system is the Polonator, which was also developed by the group of George M. Church at Havard [59].

4.4.4 Third-Generation Sequencing

Unlike many of the aforementioned high-speed sequencing technologies currently in use, third-generation sequencing is still under development. This technology is also often referred to as "single-molecule" sequencing, and it reads from individual DNA fragments without the need for amplification, or the risk of introducing errors, or the use of expensive reagents, such as fluorescent tags. As a consequence, third-generation sequencing has the potential to be even faster and cheaper than next-generation sequencing.

There are several different third-generation sequencing approaches, such as exonuclease sequencing, sequencing by synthesis, nanopore sequencing, and transmission electron microscopy [27]. For example, the principle of nanopore sequencing is that DNA can be detected as it passes through a pore by the interruption in the flow of ions through the aperture. The pores, made from a ring of seven α-hemolysin membrane proteins, are the same as those pushed into the membranes of other cells by the infectious bacterium *Staphylococcus aureus* in order to create damaging holes. The identity of each of the four bases traversing the hole might be revealed by distinctive changes in ion flow, which can be read as an electrical signal.

Companies which will likely offer commercial products within the near future include Helicos Bioscience, Complete Genomics, Pacific Biosciences, and Oxford Nanopore.

4.4.5 Personalized Genomics

In April 2008, 454 Life Science sequenced the entire genome of James Watson within 2 months for less than US$1 million [70]. In November 2008, Illumina reported the sequence of the human genome of a person of West African descent [3] and of a person of Han Chinese descent [69], each obtained for about US$250,000 within 8 weeks. At the same time, using the same technology the first complete DNA sequencing of a cytogenetically normal acute myeloid leukemia genome was reported [40]. Thus, the cheap sequencing enabled by next-generation sequencing heralds an era of "personal genomics." In fact, the routine use of whole-genome sequencing as a research tool in human genetics is now possible. At present it actually seems impossible to imagine the potential of third-generation sequencing. For example, Pacific Biosciences, which uses a single-molecule technology with DNA polymerase, aims at producing entire human genomes in less than 3 min by 2013. If these ambitious goals can be realized, the sequencing of an entire genome for about US$1,000 becomes reality and may introduce personalized genomics to the routine work-up in human genetics.

The rapid progress in genetic screening assays and DNA sequencing techniques promises to increase our understanding of the complex relationship between the human genetic make-up (the genotype) and its associated traits (the phenotype). However, what can we expect to learn from the sequences of individual genomes? The first complete genomes demonstrated that it will be extremely difficult to extract medically, or even biologically, reliable inferences from individual sequences. Without any doubt, whole-genome sequencing allows the identification of SNPs, as well as insertion/deletion polymorphisms and structural variations. However, at present they do not accurately define copy-number variants (CNVs, Sect. 3.4.4.4) at the nucleotide level. Thus, next-generation sequencing will improve the catalogue of variants existing in human genomes – SNPs by the million, insertion/deletion polymorphisms by the hundred thousand and structural variants by the thousand. The numbers of these variants will not directly provide information about how such polymorphisms contribute to the wide spectrum of human traits, yet they do provide a necessary step toward accurately defining genomic loci that are likely to be implicated in those traits. Therefore, association studies using complete individual genomes may become the approach of choice for understanding the complexity of human biology and disease.

4.4.6 Gene Function

Regardless of what definition of gene is being used, there is no question that genotype determines phenotype, often together with some environmental factors. At the molecular level, DNA sequences determine the sequences of functional molecules. Thus, an important consequence of the new gene definition as discussed in Sect. 4.1 is that the protein or RNA products must be functional for the purpose of assigning them to a particular gene [25]. This of course results in the important question of, “What is a function?”. Many genes remain functionally uncharacterized in the physiological context of disease development. Importantly, the same pathologic mutation may – depending on the genetic background in which it occurs – have different consequences on the phenotype, which is often referred to as expressivity or penetrance. Therefore, high-throughput biochemical and mutational assays, molecular profiling, and interaction studies are needed to define function on a large scale. This is one of the purposes of the -omics sciences.

Gene functions must be clearly defined. This is a tremendous task, considering that biological function has many facets owing to the diversity of cellular activities. Defining the function of a gene is difficult, and it may be influenced by a membership in a specific pathway or a complex network in which the gene product interacts. Depending on this, the function of a gene may have effects across a wide range of spatial and temporal scales.

The gene ontology (GO) database uses a clearly defined and computationally friendly vocabulary for representing the cellular, biochemical, and physiological roles of gene products in a systematic fashion [28]. GO provides a standardized way to assess whether a given number of genes have similar functions. GO terms are organized in a tree-like structure, starting from more general at the root to the most specific at the leaves distributed across three main semantic domains – molecular function, biological process, and cellular location. However, GO describes many, but not all specific biological properties of known genes. In addition to GO, there are many other publicly available data sources, which can be used to get some information about possible gene-product functions (e.g., [31]; Chaps. 29.1–29.3).

Furthermore, there are multiple computational and statistical methods which can be used to deduce the functions of poorly characterized genes from genomic and proteomic datasets via association networks [31].

Many efforts have been made to assign functions to genes computationally. These gene-function predictions are based on parameters such as sequence similarity, the co-occurrence of the protein products in the same macromolecular complex, similarity in mRNA, and protein-expression patterns [71].

A particular challenge in the postgenome era is the deciphering of the biological function of individual genes and gene networks that drive disease. Therefore, at present, alternatives to traditional forward genetics approaches are sought. Such alternatives could consist in the construction of molecular networks defining the molecular states of a system underlying disease. Unlike classic genetics approaches aiming at the indentification of genes underlying genetic loci associated with disease, such approaches seek to identify whole gene networks responding *in trans* to genetic loci driving disease, and in turn leading to variations in the disease traits. The promise of these studies is that investigating how a network of gene interactions affects disease will come to complement more strongly the classic focus of how a single protein or RNA affects disease. Thus, a more detailed picture of the particular network states driving disease may be derived. This in turn may pave the way for more progressive treatments of disease, which may ultimately involve targeting whole networks, as opposed to current therapeutic strategies focused on targeting one or two genes [9].

4.5 Transcriptomics

4.5.1 Capturing the Cellular Transcriptome, Expression Arrays, and SAGE

A detailed analysis of the entire transcriptome requires sophisticated high-throughput approaches. Quantitative real-time PCR (qRT-PCR) represents a very effective technology for gene expression analysis, as it is indeed very quantitative and has a high sensitivity, enabling very accurate measurements of low-abundance transcripts. However, qRT-PCR provides less throughput than the technologies listed in the following paragraphs. Still, many see qRT-PCR as the "gold standard" against which other methods are validated.

Microarray chips have evolved to the most successful and most commonly-used technology for gene expression profiling [13, 56]. Numerous commercially available high-density microarray platforms are accessible, allowing the analysis of more or less entire transcriptomes of complex organisms with relative technical simplicity at low cost.

In parallel with the development of microarrays, computational methods for the analysis of the resulting large data sets were improved and standardized reporting and interpretation guidelines were developed [5]. In principle, two approaches are used for microarray analysis: First, as with CGH (Sect. 3.4.3.5.3), the two differently labeled RNAs are hybridized to the same array and the different fluorescence intensities are compared with one another. In the second approach only one RNA is hybridized to an oligonucleotide platform and stored reference data are being used to derive a comparison.

Microarray-based experiments are performed with RNA isolated from a specific tissue source, which is labeled with a detectable marker. This labeled RNA is then hybridized to arrays comprised of gene-specific probes representing thousands of individual genes.

Each experiment creates a massive amount of data requiring analysis by elaborate computational tools. There are two principle forms of data analysis, i.e., unsupervised and supervised hierarchical clustering analysis. The latter approach detects gene-expression patterns that discriminate tumors on the basis of predefined clinical information [16, 26].

Microarray-based gene expression has propelled our knowledge about transcriptome changes in disease and in physiological conditions. For example, the transcriptome of a normal cell type can be compared with the transcriptome of the same cell type with a specific disease, e.g., after malignant transformation, to elucidate disease-specific alterations. Another frequent application is the analysis of physiological changes, e.g., the comparison of the transcriptome of young versus old cell donors to decipher aging-related changes in the transcriptome [24, 42].

Serial analysis of gene expression (SAGE) [65] represents another approach for gene expression analysis (Fig. 4.9). SAGE is an RNA library-based technology which requires the sequencing of millions of cDNA tags from each library. These tags are then assigned to their genomic location by bioinformatics tools. The main advantage of SAGE is that the transcriptome analysis does not depend on the sequences represented on an array platform. However, SAGE involves significant sequencing efforts, making cost an important issue, so that this technology has not been affordable for many laboratories. Still, the aforementioned new next-generation or third-generation sequencing technologies should significantly decrease costs and may make SAGE even more attractive.

Other, more recent transcriptome analysis approaches are cap analysis of gene expression (CAGE) [7, 36] and polony multiplex analysis of gene expression (PMAGE) [34].

4.5.2 Regulatory Networks

The particular challenges in transcriptomics are to identify every transcript of each cell type and the analysis

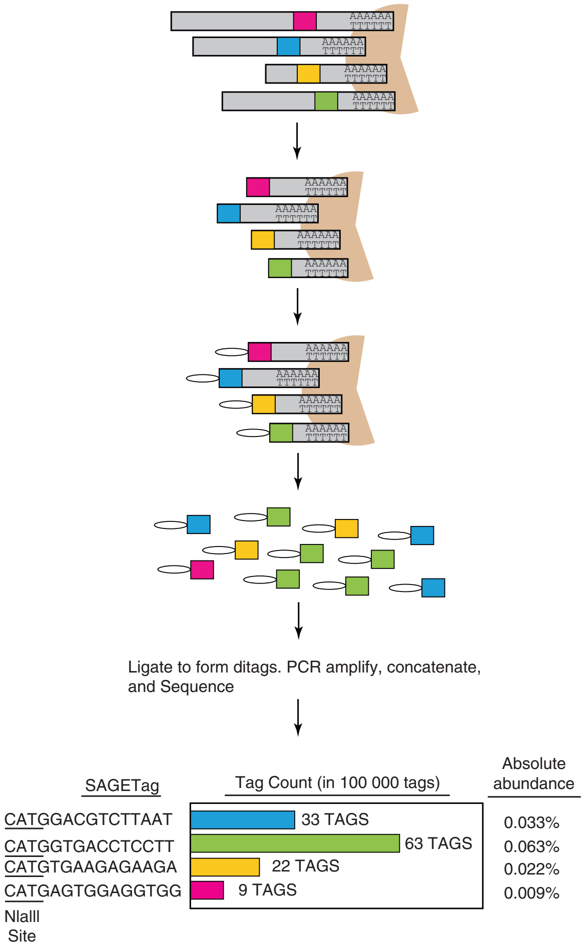


Fig. 4.9 Outline of serial analysis of gene expression (SAGE). In a first step poly-A RNA is captured on oligo-dT-coated beads and subjected to double-stranded cDNA synthesis. The poly-A RNA is cut at defined positions within each transcript by cleavage with an anchoring enzyme (usually NlaIII). Subsequently, linkers are ligated to the immobilized cDNA fragments. These linkers harbor a restriction enzyme type IIs site so that a "tagging enzyme" (usually BsmFI) cuts a short (15-bp) tag from the cDNA. These tags are ligated to form ditags, which can be amplified by PCR. The amplification products are then concatemerized and cloned. Individual tags are then identified by sequencing of concatemere clones. Absolute abundances of tags are calculated by dividing the observed abundance of any tag by the total number of tags analyzed. (Reprinted from [66], with permission from Elsevier)

of how transcription changes during development, with time and space, and especially according to environmental alterations. An integral part of these research efforts is to unravel the control mechanisms which regulate the transcriptome. One aim is the identification of regulatory networks for each cell type under different conditions, which may be an important prerequisite for the development of new therapeutic options. As a consequence, system approaches have been developed over the past years to elucidate transcriptional regulatory networks from high-throughput data [61].

The definition of networks includes the identification of all expressed transcripts under any developmental and growth condition. Furthermore, all possible physical interactions between transcriptional regulators and regulatory elements have to be delineated.

As complete transcriptomes of cells are cataloged at increasingly finer levels of detail, we may be able to discern the rules that determine where RNAs are made and how they are processed. However, such rules may change under certain conditions. For example, a cryptic transcription start site upstream of the "correct" initiation site might produce an RNA with additional protein-coding sequence or altered translation efficiency. A minor transcription start site within a gene could produce a truncated protein variant targeted at a different subcellular location. If any of these events provide some selective advantage the cryptic transcription start site could, over the course of time, become an alternative one and eventually the real transcription start site. Such evolutions can only be addressed if entire networks are being analyzed.

4.5.3 Outlier Profile Analysis

A particular challenge in transcriptome analysis could be the inability to extract the essence of recurring specific characteristics that may only be present on a subset of cases within a group. This may be especially true in RNA that has been extracted from cancer samples and which may show heterogeneous patterns of gene amplification, fusion, mutation, or deletion. To overcome these problems, a novel bioinformatics approach dubbed "cancer outlier profile analysis" has been developed as a means of identifying recurring patterns of gene overexpression that may characterize distinct subsets of known cancer types, but may not be detectable with traditional analysis methods (such as *t*-tests or signal-to-noise ratios) [62]. By using cancer outlier profile analysis, two members of the ETS family of transcription factors, ETV1 and ERG, were identified as outliers in prostate cancer. Additional analysis of cDNA transcripts of ERG and ETV1 in prostate cancer cell lines indicated fusion of the 5′ untranslated region of *TMPRSS2* (a prostate-specific, strongly androgen-regulated gene) to either ERG or ETV1. Indeed, cytogenetic analyses performed subsequently confirmed the presence of translocations involving the *TMPRSS2* locus on chromosome 21q22.3 and the corresponding chromosomes harboring one of the ETS family genes. Thus, purely computational manipulation and meta-analysis of existing high-throughput gene expression datasets has eventually led to discovery of a novel group of recurring chromosomal translocations in prostate cancer, which had been neglected by all previously performed cytogenetic or molecular cytogenetic technologies [62].

4.5.4 High-Throughput Long- and Short-Read Transcriptome Sequencing

The same group as initiated outlier profile analysis developed an integrative analysis of high-throughput long- and short-read transcriptome sequencing of cancer cells to discover novel gene fusions [45]. This strategy may represent a powerful tool for the discovery of novel gene chimeras using high-throughput sequencing, opening up an important class of cancer-related mutations for comprehensive characterization [45]. At the same time it becomes obvious that the new sequencing technologies can also be applied to the transcriptome and that they will have a tremendous impact on transcriptomics.

4.5.5 Disease Classification

Interestingly, it has been shown that cancer types can be subclassified based on their *gene* expression patterns. Therefore, gene expression data are often referred to as “signatures” or “molecular portraits,” because most tumors show unique expression patterns [10]. Together with appropriate statistical analysis, new or improved classifications have been developed based on expression microarrays for a variety of tumors, such as breast, ovary, prostate, colon, gastric, lung, kidney, brain, leukemia, and lymphoma (reviewed in [10]). These analyses demonstrated that some gene pathways, especially those involved in cell-cycle control, adhesion and motility, apoptosis, and angiogenesis, are frequently affected. Furthermore, these analyses point to pathways, which may represent especially promising targets for therapeutic interventions.

4.5.6 Tools for Prognosis Estimation

Gene expression data have also been used to establish prognostic categories, e.g., in leukemias, breast cancers, and other tumor types [38]. For example, several studies suggest that a panel of 70 genes is sufficient to classify breast cancer into prognostic categories [63, 64]. These analyses resulted in the first multigene panel test approved by the FDA for predicting breast cancer relapse [63].

However, a meta-analysis of seven of the most prominent studies on cancer prognosis based on microarray-expression profiling failed to reproduce the original data in five of these studies [48]. The other two studies yielded much weaker prognostic information than the original data. This suggests that larger sample sizes and careful validation are needed before definite statements about the clinical usefulness of such prognosis predictors can be made. Thus, at present the use of these gene arrays as diagnostic markers cannot yet be recommended [38].

4.6 Proteomics

The proteome is the entire set of proteins encoded by the genome, whereas proteomics is the discipline which studies the global set of proteins and their expression, function, and structure. Proteomics is – after genomics – often considered as a next step in the study of biological systems. Whereas an organism’s genome is relatively stable, and therefore more or less constant, the proteome differs from cell to cell and from time to time, making the analysis of the proteome more complicated. Even within a particular cell type, cells may make different sets of proteins at different times or under different conditions. Furthermore, any protein can undergo a wide range of posttranslational modifications, such as phosphorylation, ubiquitination, methylation, acetylation, and so on. As a particular gene can generate multiple distinct proteins, the number of proteins exceeds the number of genes in the corresponding genome by far.

As neither DNA nor mRNA reflects the function of proteins, a number of sophisticated technologies are needed to study individual proteins or the proteome.

4.6.1 From Low-Throughput to High-Throughput Techniques

There are a number of low-throughput techniques which allow testing for the presence of proteins and which can quantify them accurately. These analyses are often performed under certain conditions, e.g., to measure any protein changes during a particular physiological setting or during defined disease stages. Such techniques include Western blot, immunohistochemical staining, and enzyme-linked immunosorbent assay (ELISA). However, in a similar way to DNA or RNA analyses, the study of a protein can quickly become very complex. A frequent aim of proteomics is the identification of biomarkers. This usually requires a detailed understanding of multiple proteins and the complexities of protein-protein interactions. With such an amount of complexity, high-throughput approaches are needed.

At the beginning of proteomics, protein composition studies were performed on two-dimensional gel electrophoresis, which separates proteins in one dimension by molecular weight and in the second dimension by isoelectric point. Spots in the polyacrylamide gel can be cut, and proteins are identified using trypsin digestion and mass spectrometry (MS; Fig. 4.10). The MS tracing provides information on the mass/charge ratio (*m/z* ratio) of ions. These ratio values can be used to search protein databases. Such a two-dimensional polyacrylamide gel electrophoresis is suitable for high-throughput protein profiling. Basically, procedures to identify biomarkers from clinical specimens can be classified into two principle methodologies: mass spectrometer-based methods and antibody array-based methods, which are similar to DNA microarrays. Mass spectrometry-based approaches are more suitable in cases where the nature of the biomarkers or biosignatures is unknown. In contrast, targeted antibody arrays, which appear to be more cost effective, are more popular for testing proteins for known key pathways.

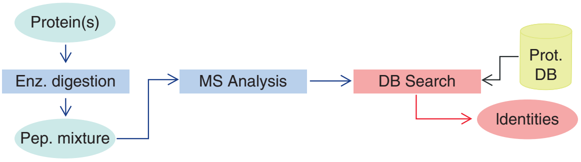


Fig. 4.10 Outline of an experiment in which proteins from 2D gel electrophoresis are identified after enzymatic digestion to create a protein mixture and mass spectrometry of the resulting peptides. The MS tracing provides information on the mass/charge ratio (*m/z* ratio) of ions, which can be used to search protein databases. (Adapted from [15]. Reprinted with permission from AAAS)

4.6.2 Mass Spectrometer-Based Methods

The central analytical technique for protein research and for the study of biomolecules is mass spectrometry (MS) [15]. MS is the method most commonly used for the investigation and identification of proteins. MS operates to create ions from neutral proteins, peptides, or metabolites. Therefore, MS depends on effective technologies to softly ionize and to transfer the ionized molecules from the condensed phase into the gas phase without excessive fragmentation. Thus, an MS consists of two main components – an ionization source and a mass analyzer. There are two commonly used techniques to transfer molecules into the gas phase and ionize them prior to mass separation, i.e., electrospray ionization (ESI) [20] and matrix-assisted laser desorption/ionization (MALDI) [33]. After ionization the mass analyzer utilizes the electric charge of the particulates for their separation by speed and/or direction, dependent on the intrinsic m/z of the ion. The types of ion mass separation may include, for example, time-of-flight (ToF), quadrupole electric fields (Q), ion trap (IT), Fourier transform ion cyclotron resonance (FT-ICR) and the Orbitrap [15]. The mass spectrum is characteristic of the molecular mass and/or structure of the metabolite.

Single-stage mass spectrometers are used to evaluate the molecular mass of a polypeptide. However, MS can also provide information about additional structural features, such as amino acid sequence or types of posttranslational modifications. Such analyses are performed after the initial mass determination. Specific ions are selected and fragmented, and structural features of the respective peptides can be deduced from the analysis of these fragments' masses. As two MS analyses are sequentially performed these analyses are usually referred to as tandem MS (MS/MS) [15].

However, like all other approaches, the promising proteomic profiling technologies via MS also have some shortcomings. These include potential artifacts attributable to sample collection and storage, the inherent qualitative nature of mass spectrometers defined by instrument sensitivity, resolution, mass accuracy, dynamic range and throughput, and finally potential artifacts introduced by high-abundance proteins in the serum [38].

4.6.3 Antibody Array-Based Methods

Alternative proteomic strategies include protein microarrays, which depend on immobilization of proteins on a solid support in a way that preserves their folded conformations [44]. For example, antibodies are spotted on the solid surface onto which unmodified proteins are applied. After binding of the proteins to their respective antibodies, a second antibody, which recognizes the same protein and which is labeled for detection by fluorescence, is applied. Such an approach has been referred to as a "sandwich ELISA assay" [37].

Rather like DNA arrays, the direct chemical modification of proteins provides a direct assay mode. Proteins can be labeled with different fluorescent dyes, e.g., as Cy3 and Cy5, and can then be applied to the antibody-spotted slide. This allows the simultaneous analyses of hundreds of target proteins on the same slide. Such an assay is semiquantitative and makes the comparison of two samples applied on the same array, e.g., control versus treated, or normal versus cancer, as in CGH experiments, possible. As in DNA arrays, false-positive or false-negative results have to be excluded, making further validation with other methodologies necessary.

The use of antibody arrays is mainly intended for initial screening of large numbers of proteins to identify candidates for further research. Additional applications include the analysis of posttranslational modifications (such as phosphorylation, acetylation, glycosylation, among others) in complex mixtures of proteins and the analysis of protein/protein interactions [43].

4.6.4 Proteomic Strategies

Several strategies for the analysis of proteins or the proteome have evolved. *MS analysis of substantially purified proteins* corresponds to the aforementioned, classic approach: two-dimensional (2D) gel electrophoresis followed by the mass-spectrometric identification of the protein(s) in a single gel spot. The targeted proteins are digested and identified by mass spectrometry.

In contrast, for *MS analysis of complex peptide mixtures,* also referred to as shotgun proteomics, complex protein samples are digested. The resulting peptide samples are extensively fractionated and analyzed by automated MS/MS. Such an approach allows the analysis of protein samples derived from complete cell lysates or tissue extracts, subcellular fractions, isolated organelles, or other subproteomes.

Furthermore, the establishment of comparative peptide patterns is an important issue. Beside the aforementioned antibody arrays to which two differently labeled protein samples are applied, such a comparison can also be made by 2D gel electrophoresis. For each sample to be analyzed, 2D patterns are generated and the patterns are compared to identify quantitative or qualitative changes. Observed differences can then be further characterized, for example, by sequencing or by determining their posttranslationally modified state.

Future strategies aim at more efficient approaches than those available at present. Such strategies may avoid the situation where the proteome is rediscovered in every experiment. Instead, it would be desirable to use the information from prior proteomic experiments as a guideline for new experiments. This requires the generation of extensive (complete) databases with information to both known and theoretical peptides and their respective proteins to facilitate the targeted, nonredundant analysis of information-rich peptides [15].

4.6.5 Proteomics for Screening and Diagnosis of Disease (Diagnostic and Prognostic Biomarkers)

MS and antibody arrays have evolved into popular platforms for protein screening. It is of special importance that they offer the advantage of multiplexing, can be performed with low sample requirement, and they have the potential for up-scaling using automation.

The availability of methods for measuring the abundance of proteins simultaneously in multiplexed assay formats has opened up opportunities in basic and disease-related research. These technologies can be applied to studies requiring large surveys of changes in protein abundance, to biomarker identification and validation, and to clinical diagnostics using selected targets.

The technologies have matured and can now be used not only for broad protein expression analysis, but also for defining signal transduction pathways, for molecular classification of diseases, for compound profiling and toxicology studies, and for the analysis of patients' individual sensitivities to drugs.

4.7 Conclusions

In human biology the elucidation of gene-product function and regulation is a fundamental objective. In most scenarios a focused single-gene approach is insufficient, making omics sciences indispensible. Owing to its relative stability, the in-depth analysis of the human genome now represents, especially because of new sequencing technologies, an amenable task, although the real extent of genomic variability is so far unknown. The recent completion of the genomic sequences of human and other mammalian species provides researchers with access to a wealth of relevant sequence information necessary for the functional characterization of gene products in a systematic and comprehensive manner. However, analyses of both transcriptome and proteome appear to be significantly more complex than the analysis of the genome. At transcriptome level, the functional characterization of noncoding RNAs represents what will presumably be the greatest challenge. Furthermore, proper biological activity and cellular homeostasis depend on spatially and temporally restricted partitioning of functionally related sets of gene products. A basic and conserved mode of biological control is the organ- and organelle-selective protein accumulation. Therefore, the fundamental biological information encrypted in the human genome can only be understood by the study of the global patterns of protein synthesis and subcellular localization across the major mammalian organ systems. However, at present, much of the human proteome remains poorly annotated in terms of tissue- and organelle-selective expression.

One of the outstanding questions in expression profiling is how well mRNA levels indeed reflect protein abundance and may represent the biological basis for any measurable differences. Although protein synthesis is dependent on mRNA, in many studies often only a modest relationship between mRNA and protein levels was reported. There may be numerous causes for incomplete proteome/transcriptome coverage, such as sample complexity, unknown protein modifications, poor recovery and detection of lower abundance and membrane-associated proteins, and the fact that certain proteins may also be transported between tissues, particularly those associated with circulatory or endocrine functions. This hampers a rigorous definition of the expressed proteome. Hence, the biological significance of differences in mRNA abundance detected among tissues remains to be elaborated at the protein level.

Furthermore, another limitation of transcriptional profiling is that little information is gleaned with respect to the subcellular localization of the translated gene products. Therefore, proteomic methods of examining protein expression and subcellular localization on a genome-wide scale should provide additional insight into the biological context of uncharacterized gene products that can naturally lead to testable hypotheses regarding function.

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Formal Genetics of Humans: Modes of Inheritance*

5

Arno G. Motulsky

Revised by S.E. Antonarakis and M.R. Speicher

Contents

The law of combination of the differing traits, according to which the hybrids develop, finds its foundation and explanation in the proven statement that the hybrids produce germ and pollen cells … which originate from the combination of the traits by fertilization.
G. Mendel, Versuche über Pflanzenhybriden, 1865

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5.1 Mendel's Modes of Inheritance and Their Application to Humans

Mendel's fundamental discoveries are usually summarized in three laws:

1. Crosses between organisms homozygous for two different alleles at one gene locus lead to genetically identical offspring (F_1 generation), heterozygous for this allele. It is unimportant which of the two homozygotes is male and which is female (law of uniformity and reciprocity). Such reciprocity applies only for genes not located on sex chromosomes.
2. When these F_1 heterozygotes are crossed with each other (intercross), various genotypes segregate: one-half are heterozygous again, and one-quarter are homozygous for each of the parental types. This segregation 1:2:1 is repeated after crossing of heterozygotes in the following generations, whereas the two types of homozygotes breed pure. As noted previously (Chap. 1), Mendel interpreted this result correctly, assuming formation of two types of germ cells with a 1:1 ratio in heterozygotes (law of segregation and law of purity of gametes).
3. When organisms differing in more than one gene pair are crossed, every single gene pair segregates independently, and the resulting segregation ratios follow the statistical law of independent segregation (law of free combination of genes).

This third law applies only when there is no linkage (Chap. 6). Human diploid cells have 46 chromosomes: the two sex chromosomes and 44 autosomes forming 22 pairs of two homologues each. The pairs of homologues are separated during meiosis, forming haploid germ cells or gametes. After impregnation, paternal and maternal germ cells unite to form the zygote, which is diploid again. Sex is determined genotypically; women normally have two X chromosomes, men have one X and one Y chromosome (Chap. 3).

For an understanding of the statistical character of segregation ratios in humans it is important to realize that the number of germ cells formed is very large, particularly among males. Only a very small sample comes to fertilization. Regarding single gene loci this sampling process can generally be regarded as random.

Two alleles may be termed A and A′. The set of combinations described in Fig. 5.1 are possible. As noted above, these theoretical segregation ratios are probabilities; segregation ratios found empirically should be tested by statistical methods to determine whether they are compatible with the theoretical ratios implied by the genetic hypothesis.

The mating type of identical homozygotes (AA × AA or A′A′ × A′A′) is uninteresting except where it permits conclusions regarding genetic heterogeneity of a recessive condition (Sect. 5.3.5). Mating between the two different homozygous types (AA × A′A′) is usually rare and is therefore of little practical importance. Matings between homozygotes and heterozygotes (AA′ × AA) and between two heterozygotes (A′A × A′A) are most important practically, as explained below.

Mendel found that a genotype does not always determine one distinct phenotype. Frequently heterozygotes resemble (more or less) one of the homozygotes. Mendel called the allele that determines the phenotype of the heterozygote dominant, the other recessive. With more penetrating analysis, some human geneticists have concluded that these terms may be misleading and should be abandoned. In fact, at the level of gene action, genes are not dominant or recessive. At the phenotypic level, however, the distinction is important and useful. Biochemical mechanisms of dominant hereditary diseases usually differ from those of recessive conditions. Hence the mode of inheritance gives a hint regarding the biochemical mechanism likely to be involved.

There are a number of instances in which each of two alleles in a heterozygous state has a distinct phenotypic expression. If both are inherited and phenotypically expressed, this mode of inheritance is sometimes called codominant.

5.1.1 Codominant Mode of Inheritance

The first examples of codominance in man were found in the genetics of blood groups; the MN blood types (111300; numbers refer to identifying numbers of diseases listed in [52]) may serve as an example (Table 5.1). When methods for genetic analysis at the protein level became available, many more examples were soon discovered. The example in Table 5.1 clearly points to a genetic model with two alleles, M and N, the phenotypes M and N being the two homozygotes and MN the heterozygote. This example is used below for a statistical comparison between expected and observed

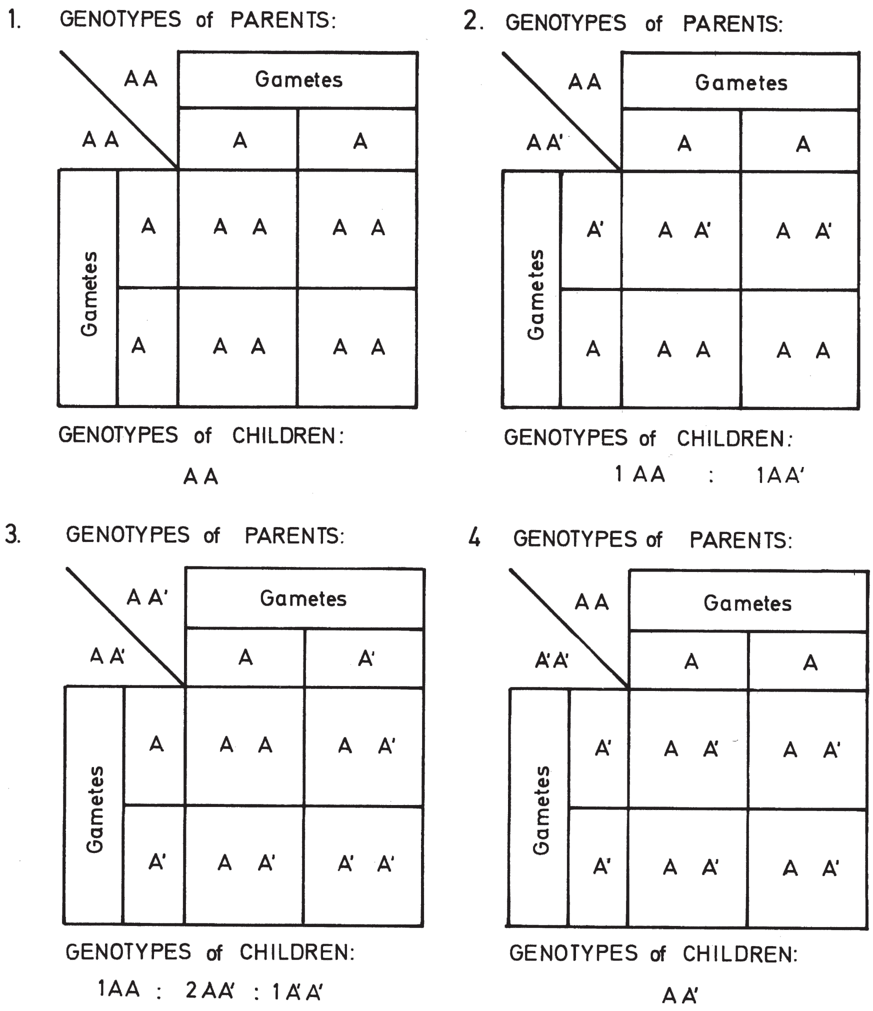


Fig. 5.1 Mating types with two alleles

Table 5.1 Family studies of the genetics of MN blood types from Wiener et al. [92]

	Number of	Types of children			Total
Mating type	families	M	N	MN	children
M × M	153	326	0	(1)	327
M × N	179	(1)	0	376	377
N × N	57	0	106	0	106
MN × M	463	499	(1)	473	973
MN × N	351	(3)	382	411	796
MN × MN	377	199	196	405	800
	1,580	1,028	685	1,666	3,379

Parentheses, false paternity.

segregation ratios. The "aberrant" cases in parentheses, which at first glance seem to contradict the genetic hypothesis, were the result of false paternity – a frequent finding in most such investigations.

5.1.2 Autosomal Dominant Mode of Inheritance

The first description of a pedigree showing autosomal dominant inheritance of a human anomaly was Farabee's [22] paper in 1905 on "Inheritance of Digital

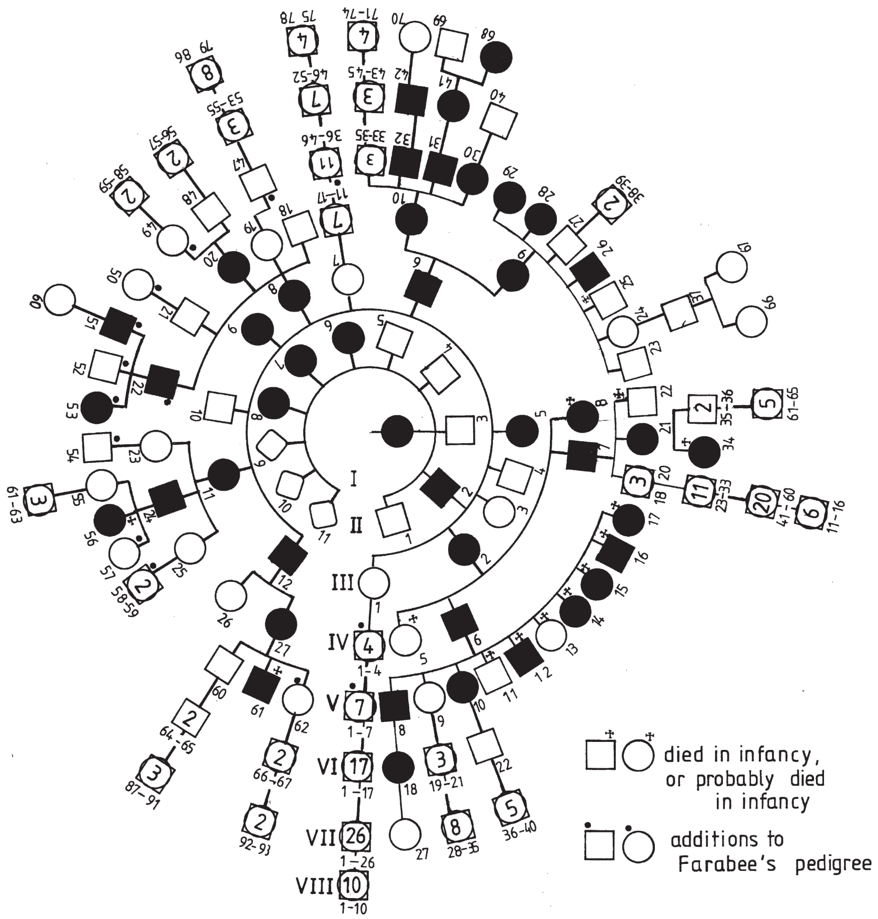

I
II
III
IV
V
VI
VII
VIII
died in infancy, or probably died in infancy
additions to Farabee's pedigree

Fig. 5.2 The brachyphalangy pedigree of Farabee [22]. *Black symbols*, affected females (●) and males (■); *numbers,* point to their position in the pedigree

Malformations in Man" (Fig. 5.2). Textbooks usually refer to the condition as brachydactyly (short digits), but from the original paper it is clear that not only were the phalanges of hands and feet shortened, but the number of phalanges was also reduced (Fig. 5.3). In addition, stature was low (average of 159 cm in three males), apparently due to shortness of legs and inferentially also of arms. In every other aspect, Farabee wrote,

> The people appear perfectly normal …and seem to suffer very little inconvenience on account of their malformation. The ladies complain of but one disadvantage in short fingers, and that is in playing the piano; they cannot reach the full octave and hence are not good players.
>
> Figure 5.3 shows the pedigree. There are 36 affected in generations II–V, 13 of which are male and 23 female. Among the unaffected 18 are male and 15 female. The trait is transmitted from one of the parents to about half the children; transmission is independent of sex. Unfortunately, Farabee did not consider the children of the unaffected. Had he done so, he would have found them free from the anomaly. Many other pedigrees have shown absence of the trait among offspring of parents who do not carry the dominant gene. More recently the family has been reexamined [38]. The children of the unaffected family members and some affected family members were added, and X-ray examination confirmed that not only hands and feet were affected but the distal limb bones as well. The basic defect is thought to affect the epiphyseal cartilage.

The condition described by Farabee is now referred to as brachydactyly A-1 (BDA1; OMIM 112500). As pointed out by Farabee, characteristics include shortness of all middle phalanges of the hands and toes,

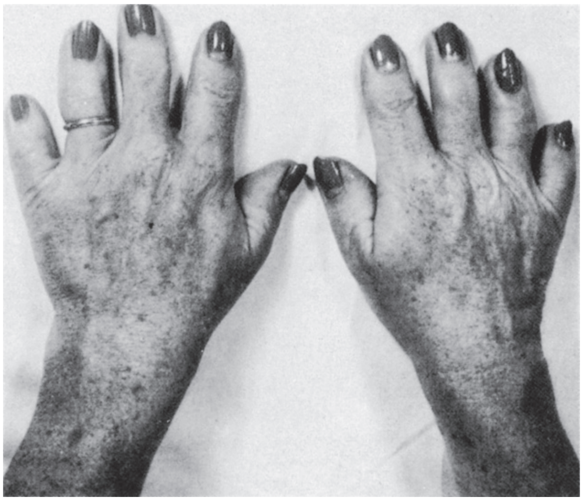


Fig. 5.3 Brachyphalangy in one member of a younger generation of Farabee's pedigree. From Haws and McKusick [38]

occasional terminal symphalangism, shortness of the proximal phalanges of the first digit, and short stature. In 2002 mutations in the Indian Hedgehog gene (*IHH*) were found in descendants of Farabee's family resolving an almost 100-year-old mystery [50, 51]. BDA1 is a heterogeneous condition as an additional locus in another BDA1 family was mapped to 5p13.3-p13.2 [1] (OMIM 607004), and in another BDA1-affected family both the *IHH* locus and the 5p13.3-p13.2 region were excluded [44] suggesting that other, yet unidentified mutations may cause the BDA1 phenotype.

Affected patients are heterozygous for an autosomal allele leading to a clearcut and regular abnormality in the heterozygote. Therefore the trait is, by definition, dominant. The family shows two other characteristics that have since been found to be widespread:

1. The anomalies were described as being almost identical in all family members, and in each person appearing in all four extremities. This is a frequent finding in malformations with a regular mode of inheritance. The reason for the symmetry is evident considering that the same genes act on all four extremities.
2. The anomaly affected the well-being of its bearers only very little. This lack of health impairment is typical for such extended pedigrees. Reproduction is normal. Otherwise the trait would not be transmitted and would soon disappear. This is why, especially in the more serious dominant conditions, extended pedigrees are the exception rather than the rule. Most diseases caused by mutations observed in the present generation have originated rather recently, often even in the germ cell of one of the parents.

5.1.2.1 Late Manifestation, Incomplete Penetrance, and Variable Expressivity

Sometimes a severe dominant condition manifests only during or after the age of reproduction. Here extended pedigrees are usually observed in spite of the severity of the condition. The classic example is Huntington disease (HD) (143100), a degenerative disease of the nerve cells in the basal ganglia (caudate nucleus and putamen) leading to involuntary extrapyramidal movements, personality changes, and a slow deterioration of mental abilities.

Wendt and Drohm [88] carried out a comprehensive study of all cases of HD in the former West Germany. The distribution of ages at onset is presented in Fig. 5.4. The great majority of their patients were married when they developed clinical symptoms. Even among thousands of patients the authors were not able to locate a single case that could be ascribed with confidence to a new mutation. For these reasons and based on results from other early studies, the existence of de novo mutations in HD had long been debated. HD is caused by an increased (CAG) trinucleotide repeat number within the huntingtin gene (*HD*) on 4p16. The unaffected range is $(CAG)_{6–35}$ repeats. Alleles with a length of $(CAG)_{40}$ and above are fully penetrant, i.e., they will cause HD within a normal lifespan. In contrast, alleles of $(CAG)_{36–39}$ confer an increasing risk of developing HD [3]. Analysis of apparently sporadic HD cases revealed that nonpathogenic alleles in the high normal range ($(CAG)_{27–35}$) have the potential to expand into the pathogenic range [57]. In fact $(CAG)_{27–35}$ alleles can be unstable during transmission and have a relatively high mutation rate for *HD* of ≥ 10% in each generation [23]. Analysis of the gene is described in Chapter 9.

Another phenomenon occasionally encountered in dominant traits is incomplete penetrance [72]). Penetrance is a statistical concept and refers to the fraction of cases carrying a given gene that manifests a

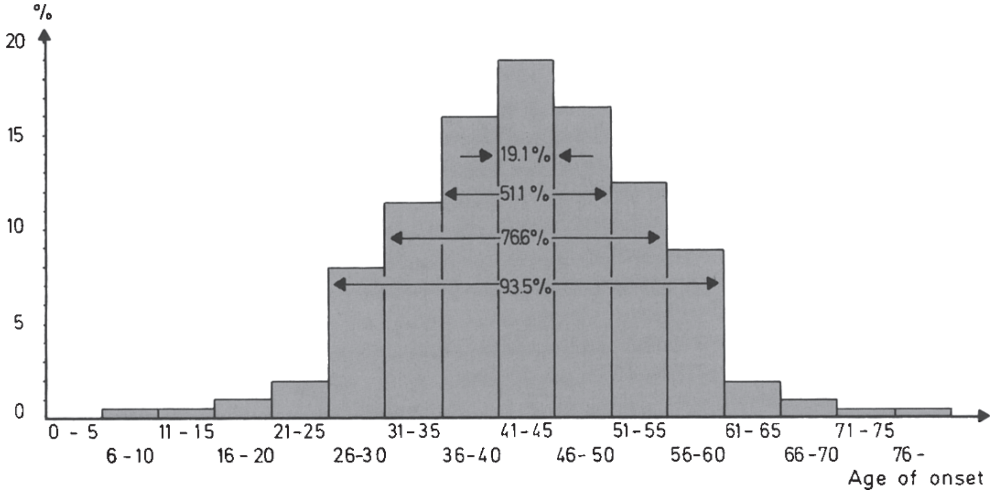


Fig. 5.4 Distribution of ages at onset in 802 cases of Huntington's disease. From Wendt and Drohm [88]

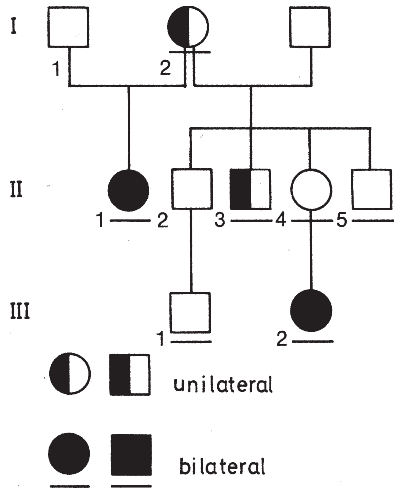


Fig. 5.5 Incomplete penetrance in retinoblastoma. The unaffected woman II,4 must be heterozygous, her mother I,2 and her daughter III,2 being affected; □ personally observed. (Personal observation, F. V.)

specified phenotype. The transmission seems occasionally to skip one generation, leaving out a person who judging from the pedigree must be heterozygous, or the fraction of those affected among sibs (after appropriate corrections, Sect. 5.3.4) turns out to be lower than the expected segregation ratio. An example is retinoblastoma (180200), a malignant eye tumor of children. Bilateral cases (and cases with more than one primary tumor) are always dominantly inherited, whereas most unilateral, single tumors are nonhereditary, probably being caused by somatic mutation (Chap. 10). Even in pedigrees otherwise showing regular dominant inheritance, however, apparent skipping of a generation is observed occasionally (Fig. 5.5). Calculation of the segregation ratio in a large sample showed that about 45% of sibs were affected instead of the 50% expected in regular dominant inheritance. The penetrance of all cases (unilateral and bilateral) is therefore about 90%. Penetrance in families with bilateral cases is higher than in those with unilateral cases.

In many cases, penetrance is a function of the methods used for examination; higher penetrance is observed with detection methods (clinical or laboratory) that are closer to gene action.

In many dominant conditions the gene may manifest in all heterozygotes, but the *degree of manifestation* may be different. An example is neurofibromatosis (162200). Some cases may show the full-blown picture with many tumors of the skin, café-au-lait spots, and systemic involvement, whereas other cases – even in the same families – may show only a few café-au-lait spots. The term used to describe this phenomenon is "variable expressivity" [72]. While such terms as "incomplete penetrance" and "variable expressivity" are often needed to convey quick understanding about certain phenomena, they may become dangerous if we forget that they do not explain a biological mechanism but rather are labels for our ignorance.

It is indeed somewhat surprising that so many dominant conditions show such a large interindividual variability in age at onset and severity of manifestation. It would be more understandable if such variability were observed only between different families. Our knowledge of molecular biology (Chap. 10) suggests that the mutational events leading to these conditions are almost always slightly different between families. Indeed, there is usually an intrafamilial correlation between age at onset and severity of manifestation. For HD, for example, Wendt and Drohm [88] calculated a correlation coefficient of +0.57 for age at onset for affected family members. But there usually remains appreciable variability within families, in which the abnormal genes are identical by descent. It is again no more than a label for our ignorance when we invoke the "genetic background" or the action of all other genes for help. In HD, molecular analysis of the gene has provided at least a partial explanation: the number of repeats in the DNA triplet CAG is higher in patients with onset at a very young age. Alleles of $(CAG)_{70}$ repeats or more invariably cause a juvenile onset [3]. Unfortunately, there is no correlation between the number of repeats and age at onset in most patients who develop their clinical disease in the fourth to sixth decades of life.

5.1.2.2 Effect of Homozygosity on Manifestation of Abnormal Dominant Genes

An abnormal gene is called dominant when the heterozygote clearly deviates from the normal. Indeed, almost all bearers of dominant conditions in the human population are heterozygotes. From time to time, however, two bearers of the same anomaly do marry and have children. One quarter of these are then homozygous. This has been observed in several instances, especially when the spouses were relatives. The first example was probably that described by Mohr and Wriedt in [54]. In a consanguineous marriage between two bearers of a moderate brachydactyly (112600) a child was born who not only lacked fingers and toes but also showed multiple malformations of the skeleton and died at the age of 1 year. A sister, however, had only the moderate anomaly, as did her parents [54].

Further examples of homozygosity of dominant anomalies are known. In one family, two parents with hereditary hemorrhagic teleangiectasia had a child showing multiple, severe internal and external telangiectasias who died at the age of 2.5 months [70]. Similarly, a very severe form of epidermolysis bullosa was observed in two of eight children of a couple, both of whom were afflicted with a mild type of this disease.

Another couple, both having a myopathy affecting the distal limb muscles, had 16 children, three of whom showed atypical and especially severe symptoms: the long flexors and the proximal hip muscles were also afflicted, and onset was earlier in life [87].

Epithelioma adenoides cysticum (132700) is a dominant skin disease characterized by multiple nodular tumors. One female patient, whose parents were both affected, had especially severe symptoms, and her eight children all showed this anomaly (Fig. 5.6) [28]. Further examples include achondroplasia (100800), Ehlers-Danlos syndrome (130000), and others. All these cases indicate that homozygotes of dominant anomalies are more severely affected than heterozygotes. It is therefore of interest that there appears to be no clinical difference between heterozygotes and homozygotes for HD, which is therefore a truly dominant disease as defined by Mendel. Clearly a different mechanism must apply to the pathogenesis of such a condition as compared with most other autosomal-dominant diseases, where dose effects are observed [89].

Given what we know about gene action, this is not surprising. In familial hypercholesterolemia (143890) for example, the mechanism of action of a dominant gene is known. A decreased number of receptors for a regulatory substance (low-density lipoprotein) showed the expected differences between heterozygotes and affected homozygotes: 50% decrease and complete absence or very much reduced activity of receptors,

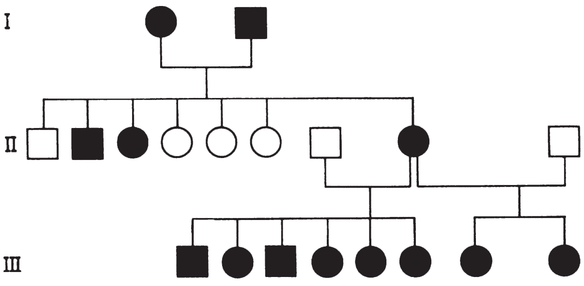


Fig. 5.6 Woman homozygous for epithelioma adenoides cysticum and her progeny in two marriages. From Gaul [28]. The pedigree was complemented in 1958 by Ollendorff-Curth [59]

respectively. Affected homozygotes show massive hypercholesteremia and usually die of myocardial infarction before the age of 30 years.

As noted above, Mendel called a gene dominant when the phenotype of the heterozygote resembled that of one homozygote. The examples of more severe manifestation of dominant genes in the homozygous than in the heterozygous state show that this strict definition is not maintained in human genetics. Here, all conditions are called dominant in which the heterozygote deviates consistently and perceptibly from the normal homozygote – irrespective of the phenotype of the anomalous homozygote. In Mendel's strict definition, most or even all dominant conditions in humans would be "intermediate." However, the more lenient connotation of "dominance" is now in general use.

5.1.3 Autosomal-Recessive Mode of Inheritance

The mode of inheritance is called recessive when the heterozygote does not differ phenotypically from the normal homozygote. In many cases special methods uncover slight detectable differences. Contrary to dominant inheritance, in which almost all crosses are between heterozygotes and homozygous normals (Sect. 5.1.2), the great majority of matings observed in recessive anomalies involve heterozygous and phenotypically normal individuals. Since the three genotypes AA, Aa, and aa occur in the ratio 1:2:1 among the offspring, the probability of a child's being affected is 25%. At the turn of the century when Garrod wrote his paper on alkaptonuria (Chap. 1) the "familial" character of recessive diseases was evident, as family size was large. Today, however, two-children families are generally predominant in industrialized societies. This means that the patient with a recessive disease is very often the only one affected in an otherwise healthy family. However, once an affected child has been born, the genetic risk for any further child of the same parents is 25%. This is important for genetic counseling.

Xeroderma pigmentosum is an autosomal recessive disease (278700). After exposure to ultraviolet light erythema develops, especially in the face, followed by atrophy and telangiectases (Fig. 5.7a). Finally, skin cancers develop that, if untreated, lead to death. Figure 5.7b shows a typical pedigree; here the parents are first cousins. The rate of consanguinity among parents of patients with rare recessive diseases is well above the population average. Usually these parents have inherited this gene from a common ancestor. In Garrod's days this was a powerful tool for recognizing rare recessive diseases; among ten families of alkaptonurics for which this information was available, the parents were first cousins in six cases. Today, however, the consanguinity rate has decreased in most industrialized societies. Hence, even if the rate of consanguinity in families with affected children is substantially increased above the population average, this does not

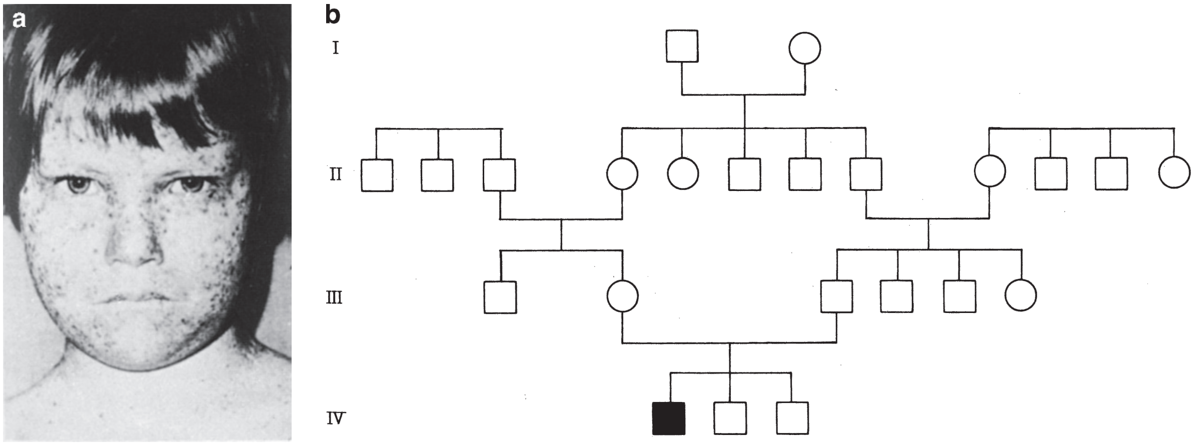


Fig. 5.7 (**a, b**) Xeroderma pigmentosum. (**a**) Girl with this condition (Courtesy of Dr. U. W. Schnyder) (**b**) Pedigree of single case with first-cousin marriage. From Dorn [19]

necessarily lead to the appearance of consanguineous mating when a limited number of families are studied particularly if the abnormal gene is not too rare. This phenomenon together with the small average family size makes it increasingly difficult to recognize an autosomal-recessive mode of inheritance with certainty. Fortunately, however, we no longer need to depend solely on formal genetics. When a rare disease, especially in a child, shows signs of being an inborn error of metabolism, and especially when an enzyme defect can be demonstrated, a recessive mode of inheritance can be inferred in the absence of evidence to the contrary. For purposes of genetic counseling, it must be assumed.

As a rule the vast majority of patients with autosomal-recessive diseases are children of two heterozygotes. Especially decisive for recessive inheritance are the rare matings of two homozygotes with the same anomaly. If both parents are homozygous for the same recessive gene, their mating should exclusively produce affected children. A number of such examples are reported in oculocutaneous albinism (OCA). Some marriages between albinos, however, have produced normally pigmented children [78]. Unless these children are all illegitimate, this proves that the parents must be homozygous for different albino mutations, i.e., more than one albino locus must exist in man. This is the kind of proof that formal genetics can provide to indicate genetic heterogeneity of diseases demonstrating an autosomal recessive mode of inheritance and the same (or a very similar) phenotype. Today, OCA is known as a group of inherited disorders of melanin biosynthesis characterized by a generalized reduction in pigmentation of hair, skin, and eyes. Several types of OCA can be distinguished, with OCA1A (OMIM: 203100) being the most severe type, while OCA1B (OMIM: 606952), OCA2 (OMIM: 203200), OCA3 (OMIM: 203290), and OCA4 (OMIM: 606574) represent milder forms. Each of these four types of OCA is inherited as an autosomal-recessive disorder and at least four genes are responsible for the different types of the disease (i.e., *TYR*, *OCA2*, *TYRP1* and *MATP*) [31].

Another condition for which genetic heterogeneity has been proven in this way is deaf-mutism (Fig. 5.8). Since environmental causes can also cause deafness, it is remarkable that in the pedigree shown here both spouses have an affected sibling, and both parents are consanguineous. Up to date at least 46 genes have been implicated in nonsyndromic hearing loss. The most frequent gene associated with autosomal-recessive nonsyndromic hearing loss is *GJB2*, which is responsible for more than half of cases. Other, relatively frequently implicated genes are *SLC26A4*, *MYO15A*, *OTOF*, *CDH23,* and *TMC1* [39]. Thus, it is likely that in the family shown in Fig. 5.8 the hearing loss was caused by mutations in different genes, e.g., by *GJB2* mutations in one family and *SLC26A4* mutations in the other family. In this scenario the two sons in generation IV would be heterozygous mutation carriers for these two genes; however, this does not result in hearing loss.

5.1.3.1 Pseudodominance in Autosomal Recessive Inheritance

Occasionally matings between an unaffected heterozygote and an affected homozygote are observed. One parent is affected, and the expected segregation ratio among children is 1:1. Since this segregation pattern mimics that found with dominant inheritance,

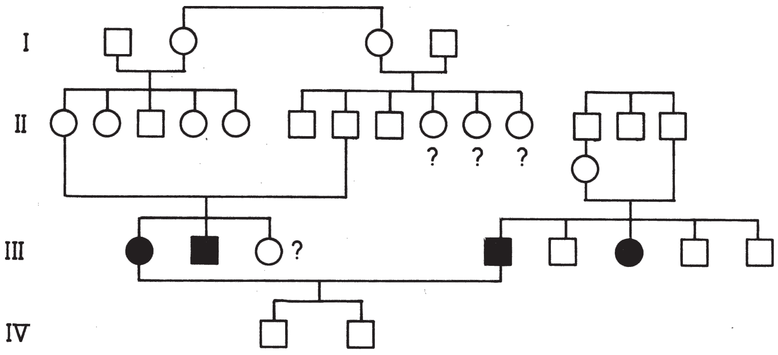


Fig. 5.8 Pedigree of deaf-mutism showing genetic heterogeneity. Both parents are affected with a hereditary type of deaf-mutism; they have affected sibs and come from consanguineous marriages; however, the two sons are not deaf. They are compound heterozygotes for different deaf mutism genes. From Mühlmann [56]

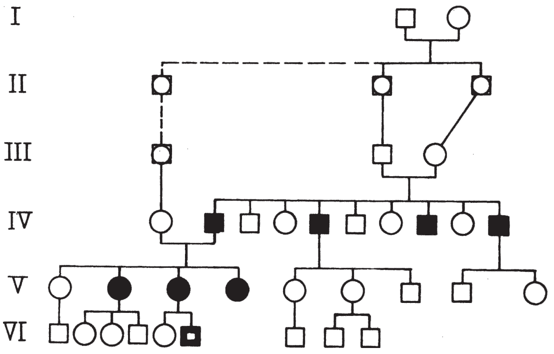


Fig. 5.9 Pedigree of pseudodominance of alkaptonuria, an autosomal-recessive condition. ▣, Suspected alcaptonuric; ⦿, sex unknown. (From Milch [53])

this situation is aptly named "pseudodominance." Fortunately for genetic analysis, such matings are very rare.

Garrod's alkaptonuria (203500) provides an example. In all families described since Garrod the autosomal-recessive mode of inheritance had been confirmed until 1956 when a family with a phenotypically similar but apparently dominant form was reported (Fig. 5.9) – a surprising finding. Some years later the authors had to disavow their conclusions: further family investigations had shown typical, recessive alkaptonuria. A number of marriages between relatives (homozygotes × heterozygotes) had led to pseudodominance. If an individual suffering from a recessive disease mates with a normal homozygote, all children are heterozygotes and hence phenotypically normal. As soon as we learn to treat recessive diseases successfully, marriages of affected but treated homozygotes will increase.

Expressivity is generally more uniform within the same family in recessive than in dominant disorders. Incomplete penetrance seems to be rare. Variability between families, however, may be appreciable.

5.1.3.2 Compound Heterozygotes

When a more penetrating biochemical analysis becomes possible, alleles of different origin frequently have slightly different properties. In an increasing number of instances when the gene is analyzed, and the mutations can be identified, such differences can be explained by the properties of the gene-determined proteins and the impairment of their specific functions. The genes of hemoglobin α and β chains offer an extreme example. Homozygosity of a mutation within the Hbβ gene, for example, may lead to sickle cell anemia or thalassemia major, depending on the precise place of the base substitution. If there are different substitutions within the two alleles, the resulting phenotype might differ from any one of the two true homozygotes. The phenotype of the compound heterozygote who has the sickle cell mutation in one allele and the HbC mutation in the other is different from that of either homozygote (SS or CC). It depends on the population structure how often homozygous patients with a recessive disease are true homozygotes carrying precisely the same mutation twice, and how often they are compound heterozygotes who carry in their two chromosomes different mutations of homologous genes (Fig. 5.10).

We can be reasonably sure that an affected homozygote carries two copies of the same mutation if both copies have a common origin; for example, if his parents are first cousins and if the condition is very rare. Another source of identity by descent are cases from

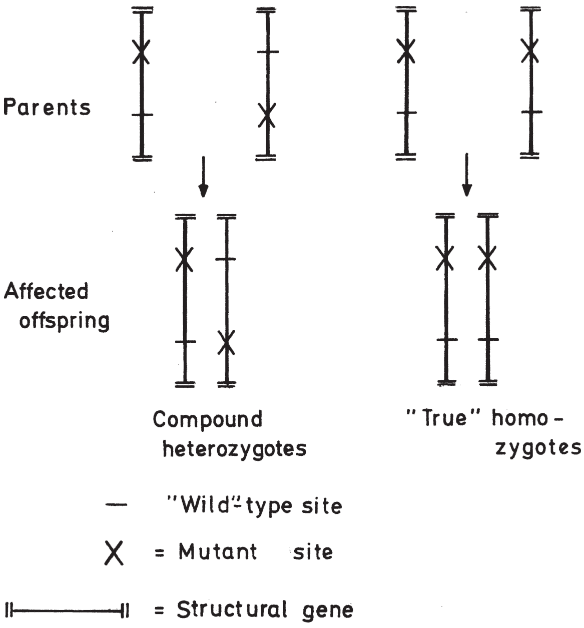


Fig. 5.10 Formation of a compound heterozygote. Each *line* represents the mutant locus on one chromosome in a parent. Among the many possibilities for mutation, two are shown. If parents are heterozygous for mutations that are at identical sites, the affected child is a "true" homozygote; otherwise, he or she is a compound heterozygote

an isolate in which a single mutation – which has been introduced by one individual – became frequent, such as the skin disease called Mal de Meleda (OMIM 248300) on the Croatian island of Mljet. Even in a larger and genetically heterogeneous population group, however, the majority of homozygotes may carry the same gene twice. This happens especially when the gene had a selective advantage some time in the past. The *CFTR* (cystic fibrosis) gene is one example: about 60–70% of all abnormal alleles in northwestern European populations are of the type delta 508, meaning that about 40–50% of patients are indeed homozygous for this mutation ($0.7 \times 0.7 = 0.49$). In other diseases the great majority of "homozygous" individuals are in fact compound heterozygotes. With the progress of DNA studies of human genes this question will be answered directly in an increasing number of instances.

5.1.4 X-Linked Modes of Inheritance

In humans, every mating is a Mendelian backcross with respect to the X and Y chromosomes:

		Paternal gametes	
		X	Y
Maternal gametes	X	$^{1}/_{4}$ XX	$^{1}/_{4}$ XY
	X	$^{1}/_{4}$ XX	$^{1}/_{4}$ XY
Total		$^{1}/_{2}$ XX♀ +	$^{1}/_{2}$ XY♂

This implies that on average female and male zygotes are formed at a 1:1 ratio. This, however, is not quite true. The sex ratio at birth (known as the secondary sex ratio in contrast to the primary sex ratio at conception) is slightly shifted in favor of boys (102–106 boys/100 girls). The primary sex ratio is not known exactly, but there are hints that it is also somewhat variable. The formal characteristics of X-linked modes of inheritance can easily be derived from the mode of sex determination. Many studies on the (primary and secondary) sex ratio have been published. Chromosome studies on abortions should reflect the primary sex ratio and point to a value not too far from 100 (boys and girls in a ratio of 1:1). However, the primary and secondary sex ratio also depend on the interval between sexual intercourse and ovulation, frequency of intercourse, general cultural conditions, and even war and peace. After artificial insemination, the fraction of male offspring appears to be appreciably increased.

5.1.4.1 X-Linked Recessive Mode of Inheritance

If we use A for the dominant, normal wild-type and a for the recessive alleles, the following matings are possible:

(a) AA♀ × A♂. All children have the phenotype A. Neither this nor the analogous mating aa × a is useful for genetic analysis.

(b) AA♀ × a♂. All sons have one of the mother's normal alleles. They are healthy. All daughters are heterozygous Aa. They are phenotypically healthy, but carriers of the abnormal allele. In the analogous, very rare mating aa♀ + A♂ all sons are affected (a), and all daughters are heterozygous (Aa).

(c) Aa♀ + A♂. This type is most important. All daughters are phenotypically normal; half are heterozygous carriers. Half of their sons are hemizygous a and affected. The analogous mating Aa♀ × a♂ is extremely rare. There is a 1:1 ratio of affected and heterozygotes among female children and an 1:1 ratio of affected and normals among males.

The principal formal characteristics of X-linked recessive inheritance can be summarized as follows: Males are predominantly – and in rare X-linked conditions almost exclusively – affected. All their phenotypically healthy but heterozygous daughters are carriers. If no new mutation has occurred, and the mother of the affected male is heterozygous, half of his sisters are heterozygous carriers. Among sons of heterozygous women, there is a 1:1 ratio between affected and unaffected.

Strictly speaking, transmission from affected grandfathers via healthy mothers to affected grandsons is helpful, but not altogether decisive for locating the gene on the X chromosome. An autosomal gene with manifestation limited to the male sex could show the same pattern. The fact that all sons of affected men are unaffected, however, is decisive unless the wife is a heterozygous carrier which may not be unusual for common X-linked traits. This criterion can create difficulties in interpretation when a disease is so severe that the patients do not reproduce.

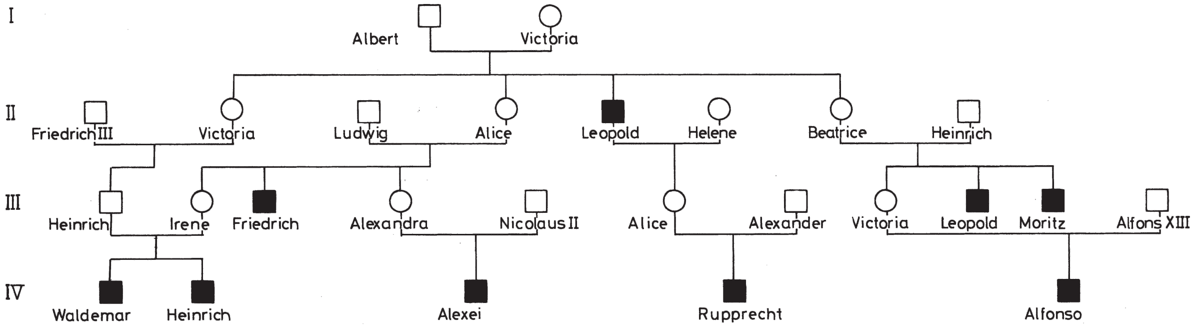


Fig. 5.11 Pedigree of X-linked recessive hemophilia A in the European royal houses. Queen Victoria (I,2) was heterozygous; she transmitted the mutant gene to one hemophilic son and to three daughters

The two most famous and, from a practical standpoint, very important examples are hemophilia A and B (306700, 306900). Due to its alarming manifestations, hemophilia has been known to doctors for a long time and has given rise to the formulation of Nasse's rule (Chap. 1). Figure 5.11 shows the famous pedigree of Queen Victoria's descendants in the European royal houses. One of the hemophilics was the Czarevich Alexei of Russia, and in this case genetic disease influenced politics. Rasputin's power over the imperial couple was based at least partially on his ability to comfort the Czarevich when he was frightened by bleedings. Much larger pedigrees have been described, probably the most extensive being that of hemophilia B in Tenna, Switzerland. As a rule, however, the pedigrees observed in practice are much smaller. Frequently there is only one sibship with affected brothers, or the patient is even the only one affected in an otherwise healthy family. Again, as in dominant conditions (Sect. 5.1.2), this is caused by the reduced reproductive capacity of the patients, which leads to the elimination of most severe hemophilia genes within one or a few generations after they have been produced by new mutation. As expected, almost all hemophilia patients are males. However, there are a few exceptions. Figure 5.12 shows a pedigree from former Czechoslovakia in which a hemophilic had married a heterozygote (who was his double first cousin because in their parents' generation two brothers had married two sisters). The homozygous sisters both had moderately severe hemophilia similar to their affected male relatives.

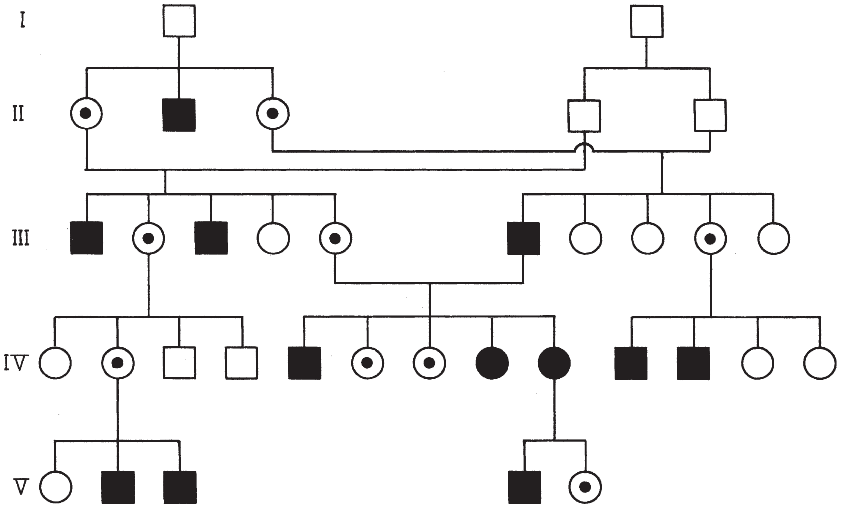


Fig. 5.12 Pedigree of two female homozygotes for X-linked hemophilia. The parents are double first cousins. ⊙, Obligatory heterozygotes. From Pola and Svojitka [64]

Some X-linked conditions have reached considerable frequencies. The most widespread are red-green color vision defects and variants of the enzyme glucose-6-phosphate dehydrogenase, but various types of X-linked mental retardation are also common.

5.1.4.2 X-Linked Dominant Mode of Inheritance

An X-linked dominant condition manifests itself in hemizygous men and heterozygous women. However, all sons of affected males are free of the trait unless their mothers are also affected, and the sons' children are also unaffected. On the other hand, all daughters of affected males are affected. Among children of affected women the segregation ratio is 1:1 regardless of the child's sex, just as in autosomal-dominant inheritance. If affected individuals have a normal rate of reproduction, about twice as many affected females as males are found in the population.

Since only children of affected males provide information in discriminating X-linked dominant from autosomal-dominant inheritance, it is difficult or even impossible to distinguish between these modes of inheritance when the available data are scarce.

The first clearcut example was described by Siemens in [67] in a skin disease that he named "keratosis follicularis spinulosa decalvans (KFSD) cum ophiasi" (308800). The disease manifests follicular hyperkeratosis leading to partial or total loss of eyelashes, eyebrows, and head hair. Severe manifestations were, however, confined to the male members of this family. KFSD is an extremely rare condition as in the last 50 years only 43 additional KFSD cases were identified. A disease-causing gene has not yet been identified [14].

Since then it has been confirmed for all traits with an X-linked dominant mode of inheritance that males are on average more severely affected than females. This finding is no surprise since heterozygous women have a normal allele for compensation, but a satisfactory explanation became possible only when random inactivation of one of the X chromosomes in females was discovered.

Another example of X-linked dominant inheritance is vitamin D-resistant rickets with hypophosphatemia (307800) [93]. In the pedigree shown in Fig. 5.13, all 11 daughters of the affected men suffered from rickets or had hypophosphatemia; all 10 of their sons, however, were healthy. The affected women have both affected and healthy sons and daughters. The probability for the mode of inheritance to be autosomal-dominant and for the affected males to have only affected daughters and only healthy sons is less than 1:10,000. Moreover, in this family male members also tended to be more severely affected than females. Meanwhile, it is established that X-linked hypophosphatemia is caused by mutations in the phosphate-regulating endopeptidase gene (PHEX) [41].

5.1.4.3 X-Linked Dominant Inheritance with Lethality of the Male Hemizygotes [90]

Females with X-chromosomal diseases tend to have milder symptoms than males, as noted above. In some cases the male zygotes may be so severely affected that

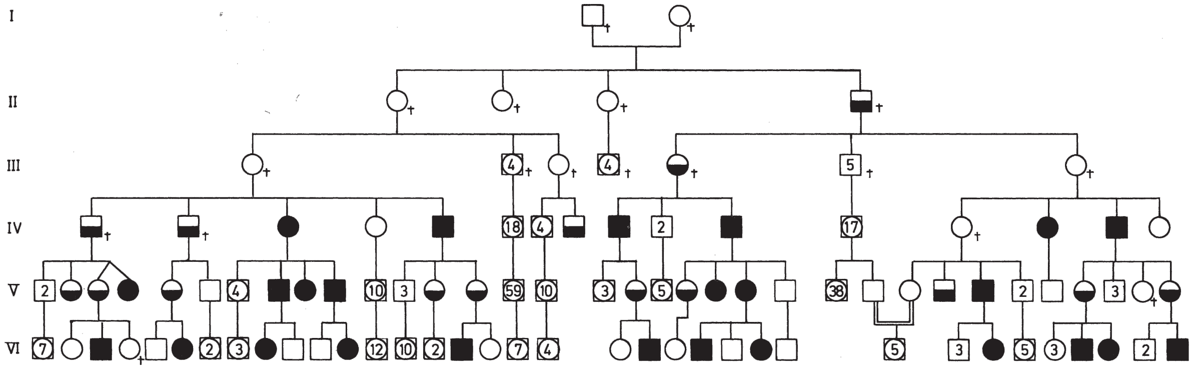


Fig. 5.13 Pedigree of X-linked dominant vitamin D resistant rickets and hypophosphatemia. ■, Hypophosphatemia and rickets; ⬓ , hypophosphatemia without rickets. From Winters et al. [93]

they die before birth, and only the females survive. This would result in pedigrees containing only affected females, and among their children affected daughters, normal daughters, and normal sons would be found in the ratio of 1:1:1. Among the male hemizygotes who did not die in very early pregnancy, spontaneous abortions (or male stillbirths) would be expected. W. Lenz in [47] was the first to show that this mode of inheritance exists in humans in the condition known as incontinentia pigmenti (Bloch-Sulzberger; 308300).

Around the time of birth the girls affected with this disease develop inflammatory erythematous and vesicular skin disorders. Later, marblecakelike pigmentations appear (Fig. 5.14a). The syndrome additionally comprises tooth anomalies. Figure 5.14b shows a typical pedigree. The alternative hypothesis would be that of an autosomal-dominant mode of inheritance with manifestation limited to the female sex. The two hypotheses would have the following consequences:

a) With autosomal-dominant sex-limited inheritance, and after proper correction (Sect. 5.3.4), there would be a 1:1 ratio of affected to unaffected among sisters of propositae. All brothers would be healthy. If the population sex ratio is assumed to be 1:1, a sex ratio of 2♂:1♀ would be expected among healthy sibs. With X-linked inheritance, on the other hand, the expected number of healthy brothers is much lower, because one-half of the male zygotes are expected to die before birth (possibly leading to an increased rate of spontaneous miscarriages). Among healthy sibs a 1♂:1♀ ratio would be expected.

b) With autosomal-dominant inheritance the abnormal gene may come from the father or from the mother. Therefore more remotely related affected relatives are to be expected among paternal as well as among maternal relatives. With X-linked inheritance, on the other hand, the gene must come from the mother. Considering the rarity of the condition, additional cases would not occur in the father's family.

c) With autosomal-dominant inheritance the loss of mutant genes per generation would be relatively small compared to the total number of these mutations in the population, since the male carriers, being free of symptoms, would reproduce normally. Therefore, assuming genetic equilibrium, the number of new mutations would be small compared to the overall number of cases in the population. With X-linked inheritance, on the other hand, the loss of zygotes is high due to death

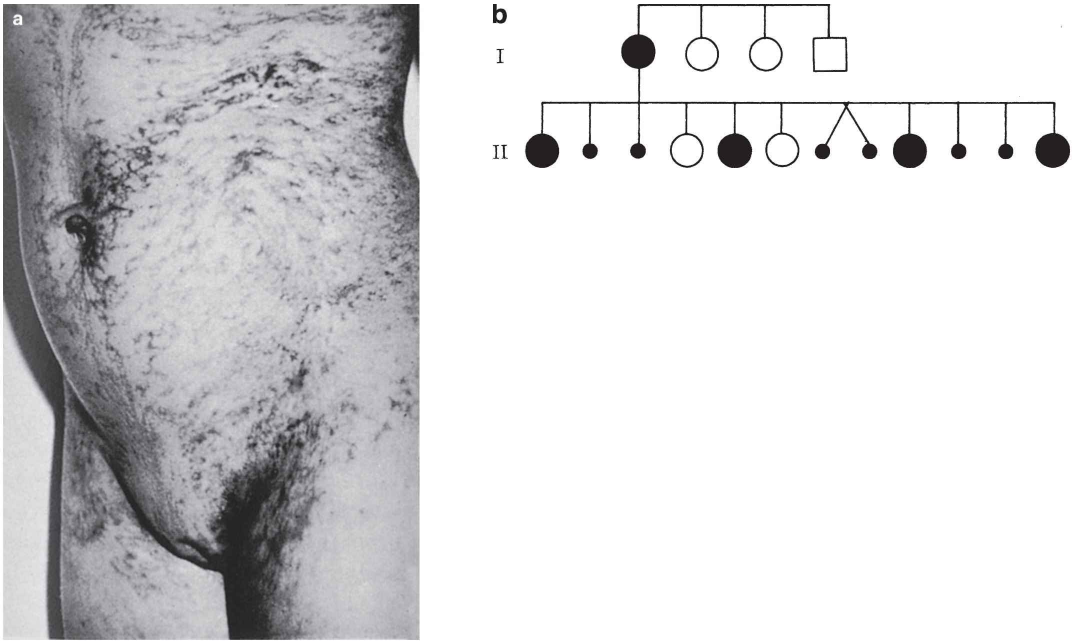


Fig. 5.14 (**a**) Incontinentia pigmenti (Bloch-Sulzberger; courtesy of Dr. W. Fuhrmann). Note the marble cake appearance of skin. (**b**) Pedigree of incontinentia pigmenti. •, Spontaneous abortion; ●, incontinentia pigmenti. From Lenz [47]

of the hemizygote. Hence many of the cases in the population are caused by recent mutation, and extensive pedigrees are rare [7].

The available statistical evidence has consistently supported the hypothesis of an X-linked dominant mode of inheritance with lethality of the male hemizygote. According to Carney et al. [13], 593 female and 16 male cases have been reported. Among the female patients 55% had a positive family history. How can the sporadic males be explained? Of course, the phenomenon of *Durchbrenners* (Hadorn [32] used the term "escapers" – the occasional survival of individuals affected with a lethal genotype) is well known, but Lenz [48] suggested a more specific explanation, assuming, on the basis of a suggestion by Gartler and Francke [27], that a mutation occurs in only one half-strand of the DNA double helix of either the sperm or the oocyte.

Several X-linked syndromes which occur predominantly among females have now been identified. The rareness of affected males in these syndromes is usually attributed to male lethality, which may often occur in the form of early pregnancy loss. About half of the X-linked conditions with predominant expression in females are associated with impairment of cognitive function. Examples include, in addition to the aforementioned incontinentia pigmenti: Aicardi syndrome (OMIM: 304050), focal dermal hypoplasia (Goltz syndrome; OMIM: 305600), Microphthalmia with linear skin defects syndrome (MLS; MIDAS; OMIM: 309801), oral-facial-digital syndrome I (OMIM: 311200), and Rett syndrome (OMIM: 312750). An example for an X-linked syndrome occurring predominantly among females without mental retardation is CHILD syndrome (Congenital hemidysplasia with ichthyosiform erythroderma and limb defects; OMIM: 308050) [74].

5.1.4.4 Genes on the Y Chromosome

Until the 1950s most geneticists were convinced that the human Y chromosome contained genes that occasionally mutate, giving rise to a Y-linked (or holandric) mode of inheritance with male-to-male transmission and males solely being affected. Stern in [72] reviewed the evidence with the result that the time-honored textbook example of Y-linked inheritance of the porcupine man (severe ichthyosis) could no longer be maintained as valid. The only characteristics for which Y-linked inheritance can still be discussed are hairy pinnae, i.e., hair on the outer rim of the ear. A number of extensive pedigrees have been published that show male-to-male transmission. However, the late onset, usually in the third decade of life, and the extremely variable expressivity and high prevalence in some populations (up to 30), makes distinction from a multifactorial mode of inheritance with sex limitation very difficult. Y-linkage can therefore not be fully accepted for this trait.

The Y chromosome contains genes for male differentiation as well as for spermatogenesis.

In experimental animals, segregation ratios deviating from those expected from Mendelian expectations were occasionally reported, one example being the T locus of the mouse [9].

Other cases for which abnormal segregation has been asserted are less well-documented. Since families with many children have become the exception in most industrial societies, the prospect for tracking down and verifying abnormal segregation of pathological genes is becoming more difficult.

5.1.5 "Lethal" Factors [32]

5.1.5.1 Animal Models

Mutations showing a simple mode of inheritance often lead to more or less severe impairment of their bearer's health. There is even evidence (Sect. 5.1.4) that some X-linked conditions prevent the male hemizygote from surviving to birth. It can be assumed that mutations exist which interfere with embryonic development of their carriers so severely as to cause prenatal death.

The first reported case of a lethal mutation in mammalian genetics was the so-called yellow mouse. L. Cuénot [17] reported an apparent deviation from Mendel's law in 1905. A mutant mouse with yellow fur color did not breed true. When yellow animals were crossed with each other, normal gray mice always segregated out. All yellow mice were heterozygous. They all had the same genetic constitution A^Y/A^+; A^Y is a dominant allele of the agouti series, the wild allele of which is termed A^+. When A^Y/A^+ heterozygotes were mated with A^+/A^+ homozygotes, the expected 1:1

ratio between yellow and gray mice was observed. In 1910 it was found that A^Y/A^Y homozygotes are formed but die in utero. Abnormal embryos were later discovered in the expected frequency of 25%.

In this case the allele that is lethal in the homozygous state can be recognized in the heterozygotes by the yellow fur color.

Cases of this sort are exceptional. Generally heterozygotes of lethals are not readily recognizable; therefore lethals occurring spontaneously are difficult to ascertain even in experimental animals and much more so in man.

Usually a lethal mutation kills the embryo in a characteristic phase of its development ("effective lethal phase" [32]). This can easily be explained by the assumption that the action of the mutant gene would be required for further development in this phase.

5.1.5.2 Lethals in Humans

In humans many different types of lethals must occur since many metabolic pathways and their enzymes are essential for survival. It is likely that many still undetected enzyme defects do indeed occur but are not compatible with zygote survival. Moreover, many types of defects of inducer substances needed during embryonic development, and enzymes involved in nucleic acid and protein synthesis, may occur and add to the high incidence of zygote death, which has so far been unexplainable genetically. This problem is discussed from a different standpoint in the context of population genetics (Chap. 16).

According to current estimates, about 15–20% of all recognized human pregnancies end in spontaneous miscarriage. Studies on other mammals suggest that an appreciable number of additional zygote losses go unnoticed, as death occurs during migration through the fallopian tubes. How much of this zygote wastage is due to genetic factors is unknown. A high proportion is caused by numerical or structural chromosome aberrations (Chap. 3). However, there are certainly other maternal causes for abortion as well. While it seemed hopeless to try to relate any proportion of antenatal (or even postnatal) zygote loss to autosomal-dominant or recessive lethals, it appeared more reasonable to speculate about X-linked lethals, as these could influence the sex ratio.

5.1.6 Modifying Genes

So far we have considered phenotypic traits depending on one gene only. However, the phenotypic expression of one gene is usually influenced by other genes. Experiments with animals, especially mammals, show the importance of this "genetic background." One way to overcome analytic difficulties caused by such variation is the use of inbred strains where all animals are genetically alike.

The genetic background is a fairly diffuse concept, but in a number of cases it has been possible to show that penetrance or expressivity of a certain gene can be influenced by another, which is called a "modifier gene" when expressivity is influenced. When penetrance is suppressed altogether, the term "epistasis" (and "hypostasis" of the suppressed gene) is used. In experimental animals cases have been analyzed in which the interaction of two mutations at different loci leads to a completely new phenotype. The classic example is the cross of chickens with "rose" combs and "pea" combs, which leads to the "walnut" comb in homozygotes for both of these mutations. To the best of our knowledge, a similar situation has not been described in man. Modifier genes and epistasis, however, have been demonstrated.

5.1.6.1 Modifying Genes in the AB0 Blood Group System

The best analyzed examples of modifying genes are offered by the AB0 blood group systems. Occurrence of the ABH antigens in saliva (and other secretions) depends on the secretor gene Se. Homozygotes se/se are nonsecretors; heterozygotes Se/se and homozygotes Se/Se are secretors. Hence, se is a recessive suppressor gene. Other rare suppressor genes even prevent the expression of ABH antigens on the surface of erythrocytes.

Bhende et al. [11] discovered a phenotype in 1952 which they called "Bombay" (211100). The erythrocytes were not agglutinated either by anti-A, anti-B or anti-H. The serum contained all three of these agglutinins. Later another family was discovered showing that the bearers of this unusual phenotype did have normal AB0 alleles, but that their manifestation was suppressed (Fig. 5.15; a woman, II, 6, has a Bombay phenotype but

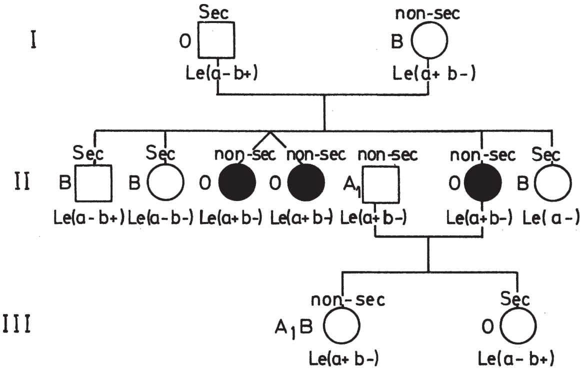


Fig. 5.15 The Bombay blood type. Manifestation of the B antigen is suppressed by a recessive gene x. Note that an O mother (II,6) has an A_1B child. From Bhende et al. [11]

transmitted the B allele to one of her daughters). It was further shown that A can also be suppressed, and the available family data suggested an autosomal-recessive mode of inheritance. In the family shown in Fig. 5.15, the parents of the proposita are first cousins.

The locus is not linked to the AB0 locus. The gene pair was named H, h, the Bombay phenotype representing the homozygote, h/h. The gene has been cloned (see [52]). Depending on the nature of the suppressed allele, the phenotype is designated O_hA_1, O_hA_2, or O_hB. The phenotype has a frequency of about 1 in 13,000 among Maharati-speaking Indians in and around Bombay. A variant with reduced activity is common in the population isolate on Reunion Island [29]. It is caused by the defect of an enzyme that converts a precursor substance into the H antigen, which in turn is a precursor of the A and B antigens [37, 60, 65]. A second gene pair Yy, the rare homozygous conditions of which partially suppresses the A antigen, has been postulated, and subsequently a number of additional families with this condition have been reported.

5.1.6.2 Modifying Genes in Cystic Fibrosis

Cystic fibrosis (CF) is characterized by progressive bronchiectasis, exocrine pancreatic dysfunction, and recurrent sinopulmonary infections. It is a common autosomal recessive disorder with significant morbidity and mortality. The gene, which causes CF, *CFTR*, was already identified 1989; however, the significant phenotypic variation observed in CF suggests that in addition to different mutations in the disease-causing gene and environmental factors, genetic modifiers may contribute to this variability. The identification of such modifiers would have a great potential to improve care for individuals with CF. However, such a modifying effect could to date only be established for a small number of genes. The majority of studies examined the phenotype of lung function and using this parameter, certain alleles of two genes, i.e., transforming growth factor β1 (*TGFβ1*) and mannose binding lectin 1 (*MBL2*), were shown to have an effect on lung function [16]. The efforts of identifying modifier genes show some general problems: First, measurable parameters such as lung function are needed to establish a modifying effect. Second, in a disease affecting multiple organs, such as CF, a modifying effect may have an impact only on one organ but not on others. Third, effects of modifying genes are usually moderate and therefore difficult to identify. Newer tools, such as genome-wide association studies, may further contribute to the elucidation of such modifying genes.

5.1.6.3 Sex-Limiting Modifying Genes

In other, less directly accessible traits the action of modifying genes has been analyzed with statistical methods.

Haldane [33] tried in 1941 to identify such genes in HD, using the family data assembled by Bell in [8]. Harris in [36] examined the problem in a condition called diaphyseal aclasis (133700), which is characterized by multiple exostoses near the cartilaginous epiphyses.

The mode of inheritance is dominant; however, the condition is about twice as common in males as in females. It may be transmitted in some families through unaffected females but not through unaffected males. Statistical analysis of the comprehensive pedigree data collected by Stocks and Barrington [75] suggests in part of the families independent segregation of a factor leading to incomplete penetrance only in females: a sex-limiting modifying gene.

5.1.6.4 Modification by the Other Allele

Phenotypic expression of a gene may be modified not only by genes at other loci but also by the "normal" allele. One example comes from the genetics of the Rh

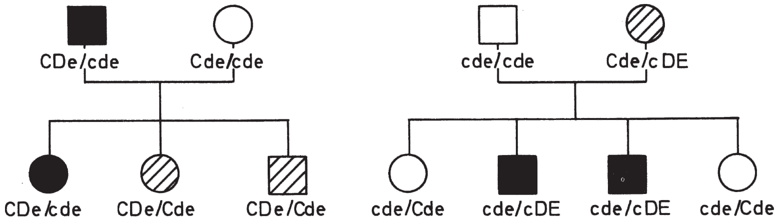


Fig. 5.16 Modification by the homologous allele in the Rh system. ●, D$^+$ blood with normal reaction; ▨ , weak reaction (D^u variant); ○, D$^-$ blood. The haplotype Cde reduces expression of the D factor to D^u. From Ceppellini et al. [15]

factor (Sect. 6.2.4). Occasional blood specimens, when tested with an anti-Rh D serum, give neither a strong positive nor a negative reaction but an attenuated positive reaction. These are called D^u. In most cases a special allele is responsible for this effect, but there are exceptions. In several families the D^u reaction was observed only in family members having Cde as the homologous allele (Fig. 5.16).

5.1.6.5 Modification by Variation in Related Genes

Sickle cell anemia caused by homozygosity for HbS (see Chap. 11) becomes clinically less severe in the presence of several genetic conditions that increase the amount of fetal hemoglobin in the affected red cells. Similarly, the presence of the common alpha thalassemia gene (see Chap. 11) makes for a milder disease manifestation.

5.1.6.6 Modification by a DNA Polymorphism Within the Same Gene

Analysis at the molecular level is revealing new and unsuspected phenomena, including those regarding modification of gene action. Prions are especially interesting proteins. Mutations within the prion gene (176 640) may cause hereditary diseases such as Creutzfeldt-Jakob disease (CJD), Gerstmann-Straussler disease (GSD), or familial fatal insomnia (FFI). The same mutation (Asp → Asn-178) may lead either to CJD or to FFI, depending on a normal polymorphism within the same gene but at a different site: the allele Val 129 segregated in CJD and the allele Met 129 segregated in FFI [30].

Study of various modifying genes and their mechanism is promising to be an important feature for our understanding of the variability of genetic diseases.

The causes of clinical variability in monogenic diseases are:

- Genetic heterogeneity
  - Intra-allelic: different mutations at same locus
  - Inter-allelic: different mutations at other loci
- Modifying genes
  - Additional polymorphisms altering protein conformation
  - Other, as yet unknown mechanisms
- Exposure to various environmental factors required for clinical end result
- Random additional somatic mutations of allele at same locus (e.g., tumors)
- Imprinting (parental origin of mutation)

5.1.7 *Anticipation*

A time-honored concept popular among physicians in the nineteenth and early twentieth centuries was anticipation. They observed that some hereditary diseases begin earlier in life and follow a more severe course as they progress through generations: the grandfather appeared to be mildly affected; the father was definitely ill, and in the son the disease manifests itself with full force. Anticipation was closely associated with another concept called "degeneration": in some families general, mental, and physical qualities were thought to deteriorate through the generations. These ideas became popular not only among physicians but also among the general public, and were expressed in literary works such as Thomas Mann's novel *Die Buddenbrocks*. In two diseases that tend to manifest during adult life, anticipation seemed to be obvious: HD and myotonic dystrophy (160900) [25]. In the latter, myotonia is associated with relatively mild muscular dystrophy, cataracts, and sometimes mental retardation, or dementia. This disease shows an unusual degree of variability in age at onset, and earlier onset

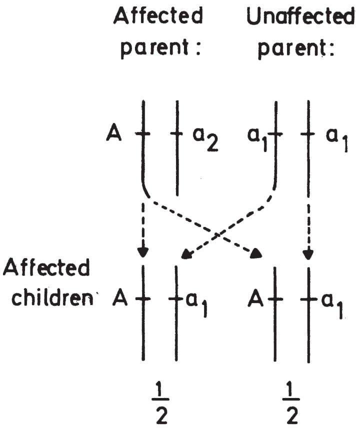


Fig. 5.17 Allelic modification. If manifestation of a dominant, abnormal gene A is modified by the normal allele, and if the allele a_1 causes severe and a_2 milder manifestation of A, there is a correlation in the degree of manifestation between affected sibs but not between affected parent and child. An affected child cannot receive the modifying a_2 allele

as well as a more severe course in some patients of the most recent generation.

When Mendel's laws were rediscovered, anticipation did not fit the new, and otherwise so successful, theory. Therefore scientists interested in genetic problems tried to explain these phenomena away with sophisticated arguments (which were also used in the first two editions of this book). Weinberg [86] pointed out, for example, that anticipation can easily be simulated if families were ascertained directly by patients of the youngest generation who were affected early in life. Their parents and grandparents, on the other hand, who were ascertained through these young probands, could be recognized only if the onset of the disease was so late that they had a chance to have children.

Penrose [63], one of the best human geneticists of his time, explained in great detail that anticipation could be mimicked if ascertainment through the youngest generation combined with dissimilarity of age at onset between parents and children, but similarity between sibs. This would be expected if, in a dominant condition, the normal allele influenced the degree of manifestation of the mutant allele (allelic modification; Fig. 5.17). There can be little doubt that explanations given by Weinberg and Penrose are correct in some instances. However, in HD and myotonic dystrophy molecular analysis revealed specific types of mutations whose effects increase with passage through succeeding generations.

In HD, patients with early onset are more likely to have inherited their mutant genes from the father, whereas late onset is more common when the gene comes from the mother. In myotonic dystrophy, on the other hand, cases of very early onset are less rare; the babies have signs of the disease even at birth. This occurs almost exclusively when the mothers are affected.

Such differences have also been observed in some other monogenic diseases (Table 5.2). On page 169 the huntingtin gene (initially designated "IT15" for "important transcript 15") and the mutations leading to HD were described: amplification of a $(CAG)_n$ repeat beyond 40 copies causes the disease. Moreover, these amplification products are unstable; the predominant tendency appears to be toward an increase in copy numbers by further rounds of amplification; a reduction in copy numbers may occur but is apparently rarer. Higher copy number, on the other side, correlates with earlier onset: a convincing explanation for anticipation.

In myotonic dystrophy an analogous explanation has been found [12, 26, 35]. Here an unstable, amplified sequence was found in the 3′ untranslated region of a gene whose product was predicted to be a member of a protein kinase gene family [12]. It is a $(CTG)_n$ repeat. In normal individuals between 4 and 37 CTG

Table 5.2 Dominant diseases in which parental origin influences the disease (modified from Reik [66])

Disorder	Chromosome	Observations
Huntington disease	4	Early onset frequently associated with paternal transmission
Spinocerebellar ataxia	6	Early onset with paternal transmission
Myotonic dystrophy	19	Congenital form almost exclusively with maternal transmission
Neurofibromatosis I	17	Increased severity with maternal transmission
Neurofibromatosis II	22	Earlier onset with maternal transmission
Wilms tumor	11	Loss of maternal alleles in sporadic tumor
Osteo-sarcoma	13	Loss of maternal alleles in sporadic tumors

repeats are found, 38 to 49 CTG repeats are a premutation. Affected patients may have between 50 and some 2,000 repeats or even more. The repeat number tends to increase over the generations [35]; it is correlated with age at onset and severity of the disease, explaining anticipation.

Thus, the sex of the transmitting parent is an important factor that determines the trinucleotide repeat allele size in the offspring. In the case of myotonic dystrophy it has been speculated that because expansion of the CTG repeat is more rapid with male transmission, negative selection during spermatogenesis may be required to explain the almost exclusive maternal inheritance of severe congenital onset myotonic dystrophy.

5.1.8 Total Number of Conditions with Simple Modes of Inheritance Known So Far in Humans

For many years McKusick has undertaken the task of collecting and documenting known conditions with simple modes of inheritance in man. This extremely valuable resource is now known as OMIM (Online Mendelian Inheritance in Man; www.ncbi.nlm.nih.gov/omim). This web-based full-text, referenced compendium of human genes and genetic phenotypes has the advantage that it can be updated daily, and the entries contain links to other genetics resources. OMIM contains information on all known Mendelian disorders. Table 5.3 provides the number of OMIM entries for autosomal, X-linked, Y-linked, and mitochondrial genes as of 25 May 2009 with information regarding known sequences and phenotypes. Enumeration of dominant and recessive entries was discontinued by OMIM 10 years ago. Note a total number of 19,462 entries, which should be compared with the estimation of about 25,000 human genes based on molecular data. While genetic polymorphisms are included, most conditions listed in this register are rare. Many are rare hereditary diseases. At first glance the list is impressive. However, more detailed scrutiny of the conditions shows that our knowledge of these rare diseases is not nearly as good as it should and could be. There are several reasons:

(a) Most hereditary diseases have become known by occasional observation of affected patients and their families. With rare diseases it is difficult to assess whether they do or do not have a genetic basis. Here, next-generation sequencing or third-generation sequencing may pave the way to finding possible genetic bases in rare diseases.

(b) Some recessive diseases have become known because they happened to be frequent in special populations, primarily in isolates. Isolate studies permit examination of the manifestation of recessive diseases caused by a single mutation. One problem with this approach is that chance determines which genes are studied.

(c) Most human and medical geneticists are working in relatively few industrialized countries. However, genes for rare diseases show a very unequal distribution in different populations. This is particularly true for recessives but has also been shown for dominants with normal or only slightly lowered biological fitness, i.e., when the incidence is not determined by the mutation rate. Hence the developing countries can be expected to abound with hereditary anomalies and diseases that are unclassified to date. Any medical geneticist who has ever walked through, say, an Indian village

Table 5.3 Number of OMIM Entries, 25 May 2009

	Autosomal	X-Linked	Y-Linked	Mitochondrial	Total
* Gene with known sequence	12,111	581	48	37	12,777
+ Gene with known sequence phenotype	347	25	0	0	372
# Phenotype description, molecular basis known	2,293	207	2	26	2,528
% Mendelian phenotype or locus, molecular basis unknown	1,598	141	5	0	1,744
Other, mainly phenotypes with suspected Mendelian basis	1,900	139	2	0	2,041
Total	18,249	1,093	57	63	19,462

knows that this suggestion is not merely a theoretical speculation.

(d) Genetic defects with simple modes of inheritance have a good chance of being detected when they show a clearcut phenotype that is readily recognizable. This is why the inherited conditions of the skin and eye are relatively well known. Other defects, however, may cause anomalies or diseases in some families that are precipitated by environmental factors. Most of such hidden defects are unknown at present.

(e) The real significance of hereditary disease and its total impact can be established only by studies in large populations, using epidemiological methods. Such studies offer the opportunity to detect heterogeneity in etiology and to aid in distinguishing genetic and nongenetic causes. They afford the only basis on which genetic parameters such as mutation rates, biological fitness, and the relative incidence of mild and severe mutations of the same gene can be established. They also help in predicting the long-term and public health effects of medical therapy and of genetic counseling for future generations.

5.1.8.1 Difference in the Relative Frequencies of Dominant and Recessive Conditions in Humans and Animals?

At first glance, there appears to be a difference between humans and experimental animals in the relative frequencies of dominant and recessive conditions. Of the better known mutants of *Drosophila melanogaster* 200 are recessive and only 13 (6.1%) dominant. In the chicken, 40 recessive and 28 dominant mutations have been reported. In the mouse only 17 of 74 mutants are dominant (23%) and the rest recessive. In the rabbit 32 recessive and 6 dominant mutations have been found. (Instances of multiple allelism are counted as one gene locus.) In humans, on the other hand, more dominant than recessive conditions are known. This discrepancy, however, is likely to be caused by diagnostic bias. Our species observes itself most carefully; therefore, defects are detectable that would probably escape observation when present in experimental animals. It would be difficult, for example, to detect brachydactyly in the mouse. This condition, however, leads to a much more severe defect when homozygous. Hence such a defect, dominant in man, would be counted as recessive in the mouse. Another reason might be that the population of industrialized countries is not in equilibrium for recessive genes. The frequency of consanguineous matings has dropped sharply, and therefore the chance of a recessive gene meeting another mutation in the same gene and becoming homozygous is reduced. A new equilibrium will be reached only in the very distant future when recessive genes could become sufficiently frequent again. In our opinion, there is no significant reason to assume that humans are unique in regard to the ratio of dominant and recessive mutations.

5.1.9 Uniparental Disomy and Genomic Imprinting

In 1980 Eric Engel of the University of Geneva published a paper in which he discussed the possibility of having a chromosomal pair derived from only one parent [21]. He termed this possibility “uniparental disomy” (UPD). The original article included calculations on the potential frequency of UPD; he predicted that as many as 3 individuals out of 10,000 might have UPD for one of the chromosomes involved in common aneuploidies such as 15, 16, 21, 22, and sex chromosomes. Eight years later, the team of Art Beaudet published in the *American Journal of Human Genetics* a case of UPD for chromosome 7 in a female with short stature, cystic fibrosis, and growth hormone deficiency [4]. The authors published a list of possibilities for the mechanism of UPD7 and favored a monosomy 7 conception followed by mitotic nondisjunction or replication of the solitary chromosome 7. The UPD7 in this case was maternal in origin, i.e., there were two chromosomes 7 from the mother and no chromosome 7 contribution from the father. Nonpaternity was obviously convincingly excluded. Isodisomy refers to the case in which the two homologues are identical in sequence (one parental chromosome duplicated); heterodisomy refers to the case in which the two homologues differ (both parental chromosomes inherited). Isodisomy and heterodisomy could be complete, i.e., for the entire chromosome, or partial (segmental) due to recombination events in the parental chromosomes.

The detection of UPD could be done with DNA analysis of the proband and the parents. Single nucleotide polymorphisms, or short sequence repeat polymorphisms, could be used to mark the parental chromosomes, to follow the inheritance, and determine the UPD. In addition the genotyping of the DNA variants could determine the iso- or heterodisomy, either complete of segmental.

UPD has been observed for almost all human chromosomes [20]. The mechanisms resulting in UPD are multiple and include:

(a) "Trisomy rescue" refers to the loss of a chromosome from an initial trisomy (Fig. 5.18). Such reduction from a trisomy to a disomy results from two errors, one meiotic leading to a trisomy state after fertilization by a normal gamete, the other mitotic, removing the supernumerary chromosome by nondisjunction or anaphase lag. Trisomy rescue as a cause of UPD contributes primarily to cases of maternal UPD since most segregation errors occur in oogenesis.

(b) "Gamete complementation" is a mechanism by which a nullisomic gamete meets a disomy gamete. This mechanism implies two errors, one in each sex (Fig. 5.19).

(c) "Rescue of a monosomy" refers to the duplication of a singly inherited chromosome (Fig. 5.20). Such "correction" from a monosomy to a disomy results also from two errors, one meiotic leading to monosomy, the other mitotic duplicating the solitary chromosome.

(d) Somatic recombination (i.e., somatic crossing-over, the symmetrical "trading" of a paternal and maternal homologous chromatid segment) may also be the source of segregants producing cells with segmental UPD (Fig. 5.21).

(e) Chromosomal translocations particularly of acrocentric chromosomes have been found in numerous cases of UPD (Fig. 5.22). Heterologous Robertsonian translocations (of different acrocentrics), or homologous Robertsonian translocations (of the same acrocentric), as well as other translocations provide increased risk for UPD.

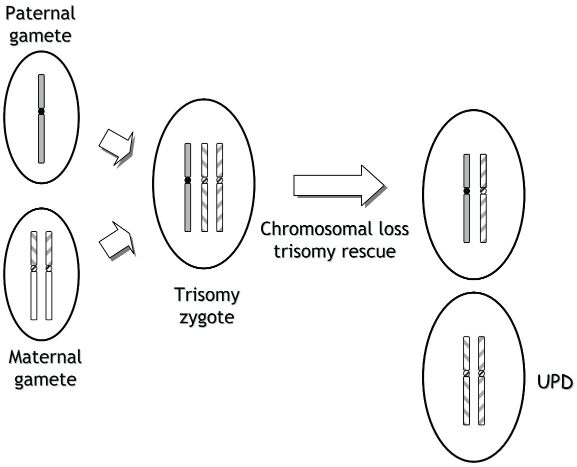


Fig. 5.18 Schematic representation of the mechanism of UPD due to trisomy rescue

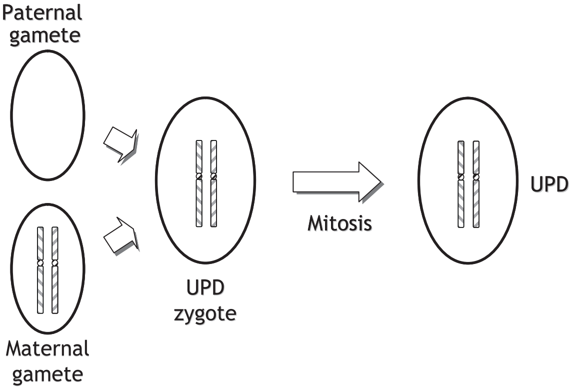


Fig. 5.19 Schematic representation of the mechanism of UPD due to gamete complementation

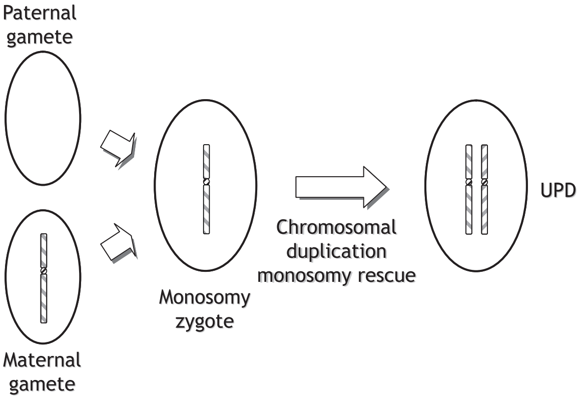
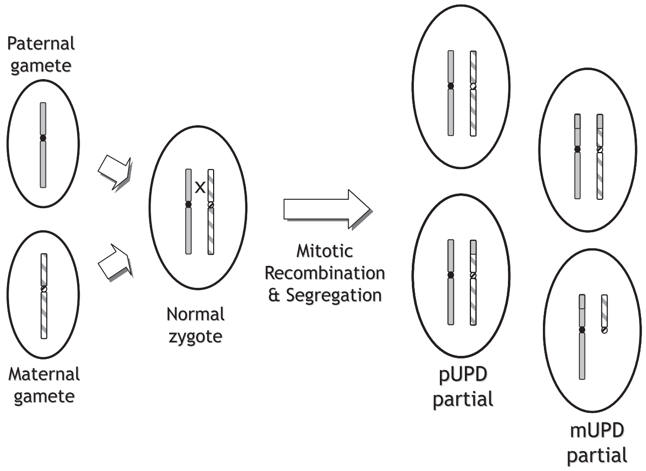


Fig. 5.20 Schematic representation of the mechanism of UPD due to monosomy rescue

5.1.9.1 Phenotypic Consequences of UPD

There are two main reasons for the phenotypic consequences of UPD:

Fig. 5.21 Schematic representation of the mechanism of partial UPD due to somatic–mitotic recombination



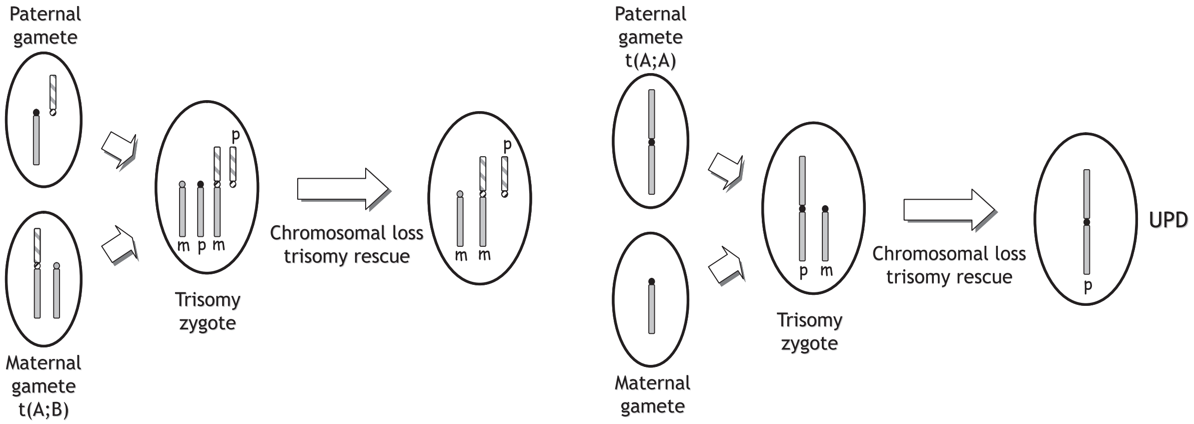


Fig. 5.22 Schematic representation of the mechanism of partial UPD due to translocations of acrocentric chromosomes. The *left panel* depicts a case of a translocation involving two different acrocentrics; the *right panel* shows a case of a translocation involving homologous acrocentrics

1. Duplication of autosomal recessive alleles. In isodisomy, two copies of a mutant allele would result in the disease phenotype. In the originally described case of maternal UPD7, cystic fibrosis was due to two maternally derived copies of the Gly542Ter mutation of the CFTR genes [4] (the mother in that case was a heterozygous carrier of this mutation).

2. Parental imprinting effects. Genomic imprinting refers to parent-of-origin dependent gene expression. Some genes are monoallelically expressed either from the paternally or the maternally derived chromosome. Thus for a paternally-only expressed gene, maternal UPD would result in a null phenotype for this gene. On the other hand, for a maternally-only expressed gene, paternal UPD would result in a similar null phenotype. An example of the former is Prader-Willi syndrome caused by matUPD15; and of the latter is Angelmann syndrome caused by patUPD15 [57].

A considerable number of imprinted genes have been identified in human and mouse [55].

5.1.9.2 Human Disorders Involving UPD

Rare cases of UPD for almost all chromosomes have been identified; the phenotypes are variable. Among them there are some recognizable syndromes which include: (a) Prader-Willi syndrome (matUPD15); (b) Angelmann syndrome (patUPD15); (c) Beckwith-Wiedemann syndrome (patUPD11p15); (d) neonatal transient diabetes mellitus in patUPD6; (e) maternal and paternal UPD14 syndromes; (f) some cases of Russell-Silver syndrome (matUPD7).

5.1.10 Diseases Due to Mutations in the Mitochondrial Genome

As shown in Chapter 2, the mitochondrial genome, mtDNA, consists of a ring-shaped chromosome with 16 596 bp. It encodes a small (12 S) and a large (16 S) rRNA for mitochondrial RNA translation, 22 tRNAs, and 13 genes encoding subunits of the respiratory chain. All these polypeptides are subunits of the mitochondrial energy-generating pathway, oxidative phosphorylation (OXPHOS). OXPHOS encompasses five multiunit enzyme complexes, arrayed within the mitochondrial inner membrane; most of the peptides necessary for building these enzyme complexes are encoded in nuclear genes.

At fertilization the oocyte contains about 200,000 mtDNAs. Once fertilized, the nuclear DNA replicates and the oocyte cleaves, but the mtDNA does not replicate until after the blastocyst is formed. Since the blastocyst cells that are destined to become the embryo proper constitute only a small fraction of all blastocyst cells, and only a fraction of these cells enter the female germ line, few of the oocyte's mtDNA molecules are found in the primordial germ cells. However, it is questionable whether this mechanism is sufficient for creating an mtDNA "population" in human cells that is as homogeneous, as is normally found, especially if we consider the fact that a single mitochondrium contains 5–10 mtDNA molecules.

Most proteins necessary for development of the mitochondria themselves are produced by nuclear genes. Therefore some of the diseases due to malfunction of mitochondria are caused by defects of such genes; they follow classical Mendelian modes of inheritance [82, 84]. On the other hand, diseases due to defects of genes in the mitochondrial genome are transmitted as the mitochondria themselves, i.e., from the mother to all children, irrespective of sex. However, considering the great number of mitochondria that a oocyte contains, and the number of genomes per mitochondrium, it is not surprising that a child may inherit from its mother more than one type of mitochondrial genome; cells containing variable proportions of affected mitochondria are "heteroplasmic." During further development, one genome may become more abundant; different cell lineages may even become "homoplasmic" for different mitochondrial genomes. This may explain in part the enormous phenotypic variation between individuals with the same mitochondrial disease. A heteroplasmic mtDNA mutation may reduce the function of the gene-determined peptide. In most instances this is unimportant, but in a few cells the fraction of mitochondria containing the mutant increases to the extent that OXPHOS enzyme activity decreases until it falls below the cellular or tissue energetic threshold, i.e., the minimum activity necessary to sustain oxidative phosphorylation. Because OXPHOS is necessary for nearly all cells, any organ can be affected in mitochondrial diseases. Thus, respiratory chain deficiencies caused by mitochondrial disorders may generate almost any symptom, in any organ system, and at any stage of life. The heteroplasmy produces marked variability in the severity and symptom patterns of these conditions. The most severe inherited mitochondrial disorders become clinically apparent during infancy, whereas other disorders of mitochondrial function may have an adult onset.

Four categories of diseases due to mutations in the mitochondrial genome may be distinguished (Fig. 5.23) [84]. In the first we find missense mutations with relatively mild phenotypic effects. These are transmitted maternally and appear to be homoplasmic. The second category comprises deleterious point mutations. Of course they can be transmitted maternally only if they are heteroplasmic. The third category, deletion mutants, occur by new mutations during early development, and these are therefore heteroplasmic. In the fourth category of diseases, certain mutations may be present that diminish OXPHOS activity somewhat at onset but not sufficiently to cause functional damage. During life time, however, additional random mutations accumulate in somatic cells, reducing their OXPHOS capacity until the threshold is reached. Then a degenerative disease of advanced age such as Alzheimer or Parkinson disease might ensue.

5.1.10.1 Leber Optical Atrophy

An example of the first category is Leber's hereditary optical neuropathy, (LHON; 308900) [82, 83]. In this disease, rapid vision loss occurs during young adult age; cardiac dysrhythmia is common. Variation in severity of the disease is strong; males are more often and on average more severely affected than females; the proportion of transmitting females in the family is much larger than expected if the mutation were

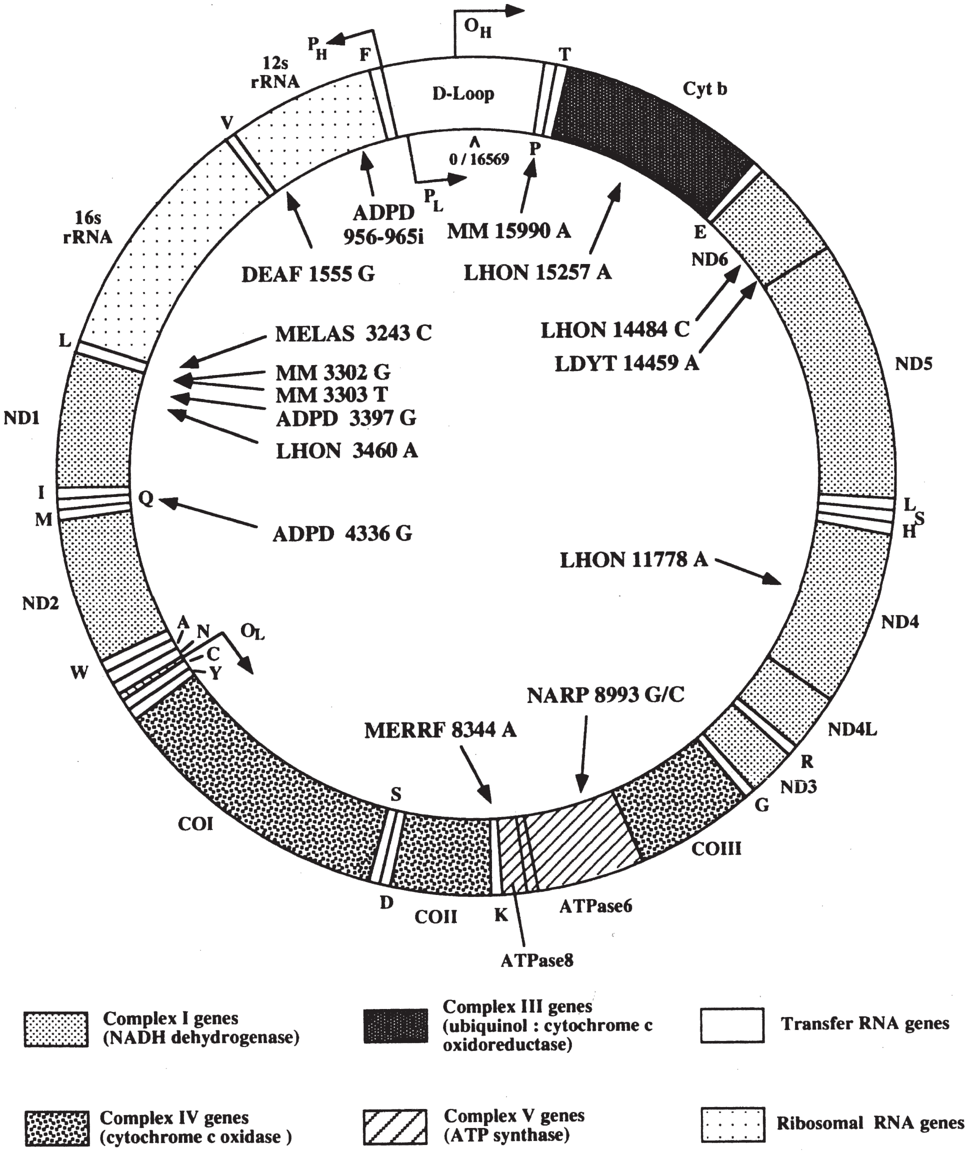


Fig. 5.23 Human tDNA map showing locations of genes and mutations Definitions of gene symbols and mutations, example: MTTK*MERRF8344A.MTTK is the altered mtDNA (MT) gene for tRNA (Lys) (TK); Myoclonic epilepsy and Ragged Red Fiber disease (MERRF) is the most characteristic clinical presentation, 8344 is the altered nucleotide, and A is the pathogenic base. From Wallace [84]

X-linked. Transmission, however, occurs exclusively through females [80]. Molecular analysis revealed a G → A transition (G3460A) leading to an Arg → His replacement in the gene for the NADH subunit 4. The Arg residue must be important for function since it has been conserved in evolution from flagellates and fungi to humans. The mutation is homoplasmic; hence the clinical variability as well as the sex difference must have other causes that are still unknown. Other mitochondrial mutations in closely related genes have occasionally been described [40].

Two diseases apparently belong to the second category – deleterious but heteroplasmic point mutations. In a large kindred, Leber disease was found to be associated with infantile bilateral striatal necrosis. In this family four phenotypes were found: normal, Leber disease, striatal necrosis, and the combination of the two diseases. All members were related through the female line. Since careful analysis has shown no deletion, the disease appears to be due to a deleterious but heteroplasmic point mutation. Depending on the preponderance of the aberrant mtDNA, the clinical signs vary [82]. The second disease of this class is one combining myoclonic epilepsy and mitochondrial myopathy – both conditions with huge interindividual variation [18].

5.1.10.2 Deletions

The third category is that of sporadic and heteroplasmic deletions. These occur as somatic mutations; since all deletions in one individual are identical, they must have arisen by clonal expansion of a single molecular event. Therefore a selective advantage of mutant cells has been suggested [82]. Figure 5.24 shows such deletions. Clinical manifestations again depend on the distribution of mutant mitochondria. A family has been described [94] in which multiple deletions of mtDNA behaved as one autosomal-dominant trait. The affected individuals suffered from progressive external ophthalmoplegia, progressive proximal weakness, bilateral cateract, and precocious death.

5.1.10.3 Diseases of Advanced Age

The fourth category comprises diseases of advanced age that have not found satisfactory explanations so far. In both Alzheimer and Parkinson diseases, for example, pedigrees have been observed in which relatively early onset in middle age is combined with an autosomal-dominant mode of inheritance. In the majority of these cases, however, an accumulation of affected individuals within families is found but no combination of clearcut Mendelian mode of inheritance with onset at more advanced age. Here, mildly to moderately deleterious germ line mutations established in the distant past, and present in a certain proportion of the population in combination with somatic mutations occurring during lifetime of the individual, may lead to such degenerative diseases. For example, a homoplasmic mutation among whites at nucleotide base pair 4,336 leading to a tRNA mutant has been observed in 5% of Alzheimer and Parkinson disease mutations, but appears to be much rarer in the general population. It may contribute to the multifactorial origin of these diseases [76].

In general, mutations within the mitochondrial genome affect mainly organ systems that depend on intact oxidation – central nervous system and muscles. Probably the number of known diseases due to mutations in the mitochondrial genome will increase in future (Table 5.4).

5.1.10.4 Interaction Between Nuclear and Mitochondrial Genomes

Several subunits of the electron transport chain are not encoded within the mitochondrial DNA but by the nuclear DNA. As a consequence, mutations in the nuclear genome can cause secondary mitochondrial DNA information loss. Hence, there are some clinical syndromes in which defects in OXPHOS follow classic Mendelian patterns of dominant-recessive transmission and not the maternal pattern, which is usually associated with this group of disorders. An example is the mitochondrial neurogastrointestinal encephalopathy syndrome (MNGIE; OMIM: 603041) which can be caused by mutations in the gene encoding thymidine phosphorylase (ECGF1).

5.1.11 Unusual, "Near Mendelian" Modes of Inheritance

As a "bridge" between monogenic and polygenic phenotypes it is worth mentioning two concepts that provide an understanding of the increased complexity between genotype and phenotype [2].

5.1.11.1 Digenic Inheritance

In this case, the phenotype is due to one mutant allele in each of two different genes. The first example was that of one form of retinitis pigmentosa published in 1994 from the laboratory of T. Dryja [42]. Individuals with a mutation in the ROM1 gene (OMIM 180721 on chromosome 11q13) *AND* a mutation in the RDS gene (OMIM 179605 on chromosome 6p21) manifest the disease (Fig. 5.25). However, individuals with only heterozygosity of the ROM1 gene mutation, or with only heterozygosity of the RDS gene mutation, were not affected

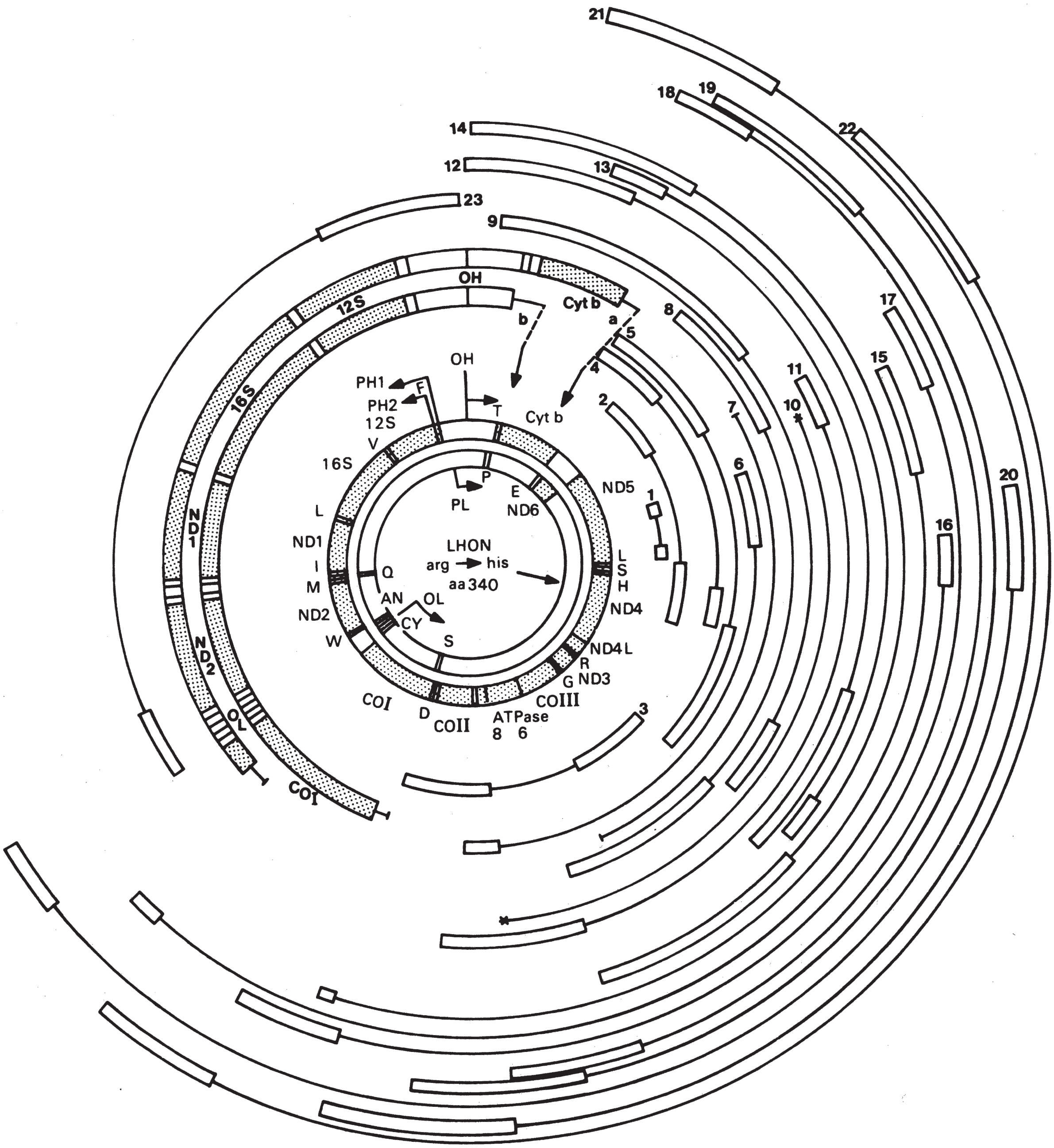


Fig. 5.24 Deletion map of human mtDNA. The *inner circles* show localization of genes and mutations. (See also Fig. 5.23). The arcs no. 1–23 show the mtDNA regions that were lost in various deletions. The *open bars* at the end of the arcs show regions of uncertainty. Deletion 1 was found in a patient with Myoclonic Epilepsy and Ragged Red Fibre Disease (MERRF) together with stroke-like symptoms. Deletions 2–23 were found in ocular myopathy patients with symptoms of varying severity. Deletion 10 was found in about one third of all ocular myopathy patients. The * at the ends of deletion 10 represents the associated 13 base pairs direct repeat. The two partial mtDNA maps labeled "a" and "b" to the left of the function map indicate the regions that were tandemly duplicated in patients with ocular myopathy associated with diabetes mellitus. The insertion sites around MTCYB (cytb) are indicated by *arrows*. From Wallace [83]

Table 5.4 The Mitochondrial Chromosome from McKusick [52]

Location (nt)	Symbol	Title	MIM	Disorder[a]
577–647	MTTF	tRNA phenylalanine	590070	
648–1,601	MTRNR1	12 S rRNA	561000	Deafness, aminoglycoside-induced, 580,000
1,602–1,670	MTTV	tRNA valine	590105	
1,671–3,229	MTRNR2	16 S rRNA	561010	Cloramphenicol resistance, 515,000
3,230–3,304	MTTL1	tRNA leucine 1 (UUA/G)	590050	MELAS syndrome, 540,000; MERRF syndrome, 545,000; Cardiomyopathy; Diabetes-deafness syndrome, 520,000
3,307–4,262	MTND1	NADH dehydrogenase 1	516000	Leber optic atrophy, 535,000
4,263–4,331	MTTI	tRNA isoleucine	590045	Cardiomyopathy
4,400–4,329[b]	MTTQ	tRNA glutamine	590030	Cardiomyopathy
4,402–4,469	MTTM	tRNA methionine	590065	
4,470–5,511	MTND2	NADH dehydrogenase 2	516001	Leber optic atrophy, 535,000
5,512–5,576	MTTW	tRNA tryptophan	590095	
5,655–5,587[b]	MTTA	tRNA alanine	590000	
5,729–5,657[b]	MTTN	tRNA asparagine	590010	Ophthalmoplegia, isolated
5,826–5,761[b]	MTTC	tRNA cysteine	590020	Ophthalmoplegia, isolated
5,891–5,826[b]	MTTY	tRNA tyrosine	590100	
5,904–7,444	MTCO1	cytochrome c oxidase I	516030	
7,516–7,445[b]	MTTS1	tRNA serine 1 (UCN)	590080	
7,518–7,585	MTTD	tRNA aspartic acid	590015	
7,586–8,262	MTCO2	cytochrome c oxidase II	516040	
8,295–8,364	MTTK	tRNA lysine	590060	MERRF syndrome, 545,000
8,366–8,572	MTATP8	ATP synthase 8	516070	
8,527–9,207	MTATP6	ATP synthase 6	516060	Leigh syndrome; NARP syndrome, 551,500
9,207–9,990	MTCO3	cytochrome c oxidase III	516050	Leber optic atrophy 535,000
9,991–10,058	MTTG	tRNA glycine	590035	
10,059–10,404	MTND3	NADH dehydrogenase 3	516002	Leber optic atrophy, 535,000
10,405–10,469	MTTR	tRNA arginine	590005	Leber optic atrophy, 535,000
10,470–10,766	MTND4L	NADH dehydrogenase 4 L	516004	
10,760–12,137	MTND4	NADH dehydrogenase 4	516003	Leber optic atrophy, 535,000
12,138–12,206	MTTH	tRNA histidine	590040	
12,207–12,265	MTTS2	tRNA serine 2 (AGU/C)	590085	
12,266–12,336	MTTL2	tRNA leucine 2 (CUN)	590055	
12,337–14,148	MTND5	NADH dehydrogenase 5	516005	
14,673–14,149[b]	MTND6	NADH dehydrogenase 6	516006	Leber optic atrophy, 535,000

(continued)

Table 5.4 (continued)

Location (nt)	Symbol	Title	MIM	Disorder[a]
14,742–14,674[b]	MTTE	tRNA glutamic acid	590025	
14,747–15,887	MTCYB	cytochrome b	516020	
15,888–15,953	MTTT	tRNA threonine	590090	
16,023–15,955[b]	MTTP	tRNA proline	590075	

[a] In addition to the disorders caused by point mutations in individual genes, deletions involving more than one mitochondrial gene have been identified in Pearson syndrome (557,000), early-onset chronic diarrhea with villus atrophy (520,100), and Kearns-Sayre syndrome (530,000), among others[b]Transcribed from light chain (L) in opposite direction from all the other genes which are transcribed from the heavy chain (H)

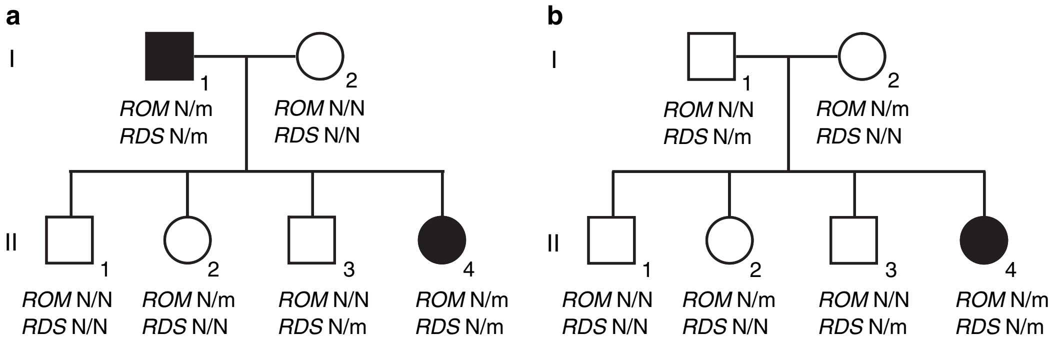


Fig. 5.25 Schematic representation of pedigrees with digenic inheritance; affected individuals are shown in *black*. (**a**) Pedigree with an apparently dominant inheritance. *N* normal allele, *m* mutant allele at the two different genes. (**b**) Pedigree with an apparently recessive mode of inheritance. From [43]

with retinitis pigmentosa. The mode of transmission of this condition resembles an autosomal dominant trait in some pedigrees (vertical transmission, males and females affected) but with 25% affected offspring from an affected parent. In some pedigrees, the transmission resembles a recessive trait when the two parents are heterozygotes for a pathogenic mutation in two different genes. In this case the affected offspring are also 25%.

5.1.11.2 Triallelic Inheritance

A more complicated case is that observed in several forms of Bardet-Biedl syndrome (BBS). The laboratories of N. Katsanis and J.R. Lupski described in 2001 BBS families in which *three* different mutations were necessary to cause the phenotypes of this syndrome [43]: for example, homozygous mutant alleles in the BBS2 gene (OMIM 606151 on chromosome 16q21) *AND* a heterozygous mutation in the BBS6 gene (OMIM 604896 on chromosome 20p12) needed to be present for the affected status (Fig. 5.26). Homozygous mutations only in the BBS2 gene and normal BBS6 gene were not sufficient for the phenotypic manifestations.

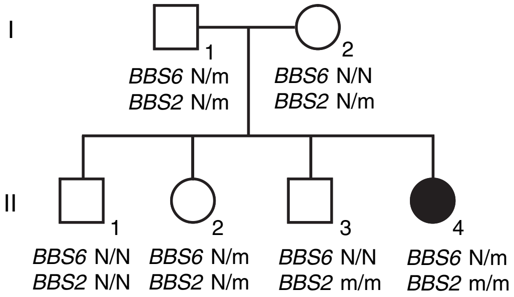


Fig. 5.26 Schematic representation of a pedigree with digenic inheritance; the affected individual II4 is shown in *black*. *N* normal allele, *m* mutant allele Only individuals with three mutant alleles are affected. Note that individual II2 who is heterozygous for mutant alleles in the *BBS6* and *BBS2* genes is not affected; furthermore, individual II3 who is homozygous for mutant alleles in the *BBS2* gene is also not affected. From [43]

Digenic and triallelic inheritance may not be rare in more complex phenotypes, and accumulation of mutations in a few or several genes (also referred to as oligogenic inheritance) may be necessary for complex polygenic disorders.

5.1.12 *Multifactorial Inheritance*

The majority of human phenotypic features, such as size, weight, intelligence, and others follow multifactorial inheritance. This mode of inheritance is described in Chaps. 4 and 8.

5.2 Hardy–Weinberg Law and Its Applications

5.2.1 *Formal Basis*

So far the application of Mendel's laws in man has been considered from the standpoint of the single family. What, however, are the consequences for the genetic composition of the population? The field of research that considers this problem is called population genetics. Some basic concepts are introduced here.

These concepts revolve around the so-called Hardy–Weinberg law, discovered by these two authors independently in 1908 [34, 85]. In 1904 Pearson [62] – in the process of reconciling the consequences of Mendel's laws for the population with biometric results – had already derived this law for the special case of equal gene frequencies of two alleles.

The law in its more general form may be formulated as follows: Let the gene frequencies of two alleles in a certain population be p for the allele A and q for the allele B; $(p+q=1)$. Let mating and reproduction be random with respect to this gene locus. The gene frequencies then remain the same, and the genotypes AA, AB, and BB in the F_1 generation occur in the relative frequencies p^2, $2pq$, and q^2, the terms of the binomial expression $(p+q)^2$. In autosomal genes, and in the absence of disturbing influences, this proportion is maintained through all subsequent generations.

5.2.1.1 Derivations from the Hardy–Weinberg Law

We assume that at the beginning the proportions of genotypes AA, AB, and BB in the population of both males and females are D, $2H$, and R, respectively. Symbolically, the distribution of genotypes in both sexes may be written as:

$$D \times \mathrm{AA} + 2H \times \mathrm{AB} + R \times \mathrm{BB} \qquad (5.1)$$

From this the distribution of mating types for random mating is obtained by formal squaring:

$$\begin{aligned}&(D \times \mathrm{AA} + 2H \times \mathrm{AB} + R \times \mathrm{BB})^2\\ &= D^2 \times \mathrm{AA} \times \mathrm{AA} + 4DH\\ &\quad \times \mathrm{AA} \times \mathrm{AB} + 2DR \times \mathrm{AA} \times \mathrm{BB} + 4H^2\\ &\quad \times \mathrm{AB} \times \mathrm{AB} + 4HR \times \mathrm{AB} \times \mathrm{BB} + R^2\\ &\quad \times \mathrm{BB} \times \mathrm{BB}\end{aligned}$$

The distribution of genotypes in the offspring of the different mating types is:

AA × AA	AA
AA × AB	$^1/_2$AA + $^1/_2$AB
AA × BB	AB
AB × AB	$^1/_4$AA + $^1/_2$AB + $^1/_4$BB
AB × BB	$^1/_2$AB + $^1/_2$BB
BB × BB	BB

Inserting these distributions for the mating types into (5.1) yields the distribution of genotypes in the F_1 generation:

$$\begin{aligned}&(D^2 + 2\,DH + H^2)\mathrm{AA} + (2\,DH + 2\,DR\\ &+2H^2 + 2HR)\mathrm{AB} + (H^2 + 2HR + R^2)\mathrm{BB}\\ &= p^2\mathrm{AA} + 2pq\mathrm{AB} + q^2\mathrm{BB}\end{aligned}$$

where $p=D+H$, $q=H+R$ are the frequencies of the alleles A and B, respectively, in the parental generation. Thus, the distribution of genotypes in the offspring generation is uniquely determined by the gene frequencies in the parental population:

$$D' = p^2, 2H' = 2pq, R' = q^2.$$

As:

$$p' = D' + H' = p^2 + pq = p,$$
$$q' = H' + R' = pq + q^2 = q,$$

the gene frequencies in the F_1 generation are equal to those in the parental generation. Thus, the genotype distribution in the next generation (F_2) is also the same as in the F_1 generation, and this holds true for all following generations.

This means that in autosomal inheritance these proportions are expected in the first generation and are maintained in the following generations. For X-linked genes the situation is slightly more complicated. At the same time, the concept of gene frequencies $p+q=1$ was created.

The Hardy–Weinberg law can also be rephrased, indicating that random mating is equivalent to drawing random samples of size 2 from a pool of genes containing the two alleles A and a with relative frequencies p and q. One of the advantages of this law is that frequencies of genetic traits in different populations can be expressed and compared in terms of gene frequencies.

Apart from making it possible to simplify population descriptions, the Hardy–Weinberg law can also help to elucidate modes of inheritance in cases where the straightforward approach through family studies would be too difficult. The classic examples are the AB0 blood types.

5.2.2 Hardy–Weinberg Expectations Establish the Genetic Basis of AB0 Blood Group Alleles

5.2.2.1 Multiple Allelisms

So far, only two different alleles for each locus have been considered. Frequently, however, more than two different states for one gene locus, i.e., more than two alleles, are possible. Examples of such "multiple allelism" in humans and experimental animals abound. Two of the classics are the white series in *Drosophila melanogaster* and the albino series in rabbits.

The formal characteristics can easily be derived:

(a) In any one individual a maximum of only two alleles can be present (unless there are more than two homologous chromosomes, as in trisomics).

(b) Between these alleles, crossing over can be disregarded as they are located at homologous loci. Here the simplest formal model is described, using the AB0 blood groups as an example.

5.2.2.2 Genetics of the AB0 Blood Groups

The AB0 blood groups were discovered by Landsteiner in [46]. Compared to other blood group systems their most important property is the presence of isoantibodies that have led to frequent transfusion accidents. These accidents helped in the discovery of blood groups. The first relevant genetic theory was developed by von Dungern and Hirszfeld in [79]. To explain the four phenotypes A, B, 0, and AB they assumed two independent pairs of alleles (A, 0; B, 0), with dominance of A and B. In 1925 Bernstein [10] tested this hypothesis using the Hardy–Weinberg expectations for the first time. He found their concept to be wrong and replaced it by the correct explanation – three alleles with six genotypes, leading to the four phenotypes due to the dominance of A and B over 0.

The most obvious method to discriminate between these two hypotheses is by family investigation. However, differences between them are to be expected only in matings in which at least one parent carries group AB (Table 5.5). The two-locus hypothesis allows for 0 children while the three-allele hypothesis does not. Although AB is the rarest group, the early literature contained some reports of supposedly 0 children with AB parents; these children were either misclassified or illegitimate. Bernstein, however, was not misled by these observations. His argument goes as follows. It may be assumed that the two-gene pair theory is correct; p may be the gene frequency of A, $1-p=p'$ of a; q the frequency of B, $1-q=q'$ of b. The frequencies to be expected in the population are presented in Table 5.6.

Table 5.5 Comparison of the two theories for inheritance of AB0 blood groups (adapted from Wiener [91])

	Children expected from the hypothesis of	
Parents	Two gene pairs	Multiple alleles
0 × 0	0	0
0 × A	0, A	0, A
0 × B	0, B	0, B
A × A	0, A	0, A
A × B	0, A, B, AB	0, A, B, AB
B × B	0, B	0, B
0 × AB	0, A, B, AB	A, B
A × AB	0, A, B, AB	A, B, AB
B × AB	0, A, B, AB	A, B, AB
AB × AB	0, A, B, AB	A, B, AB

Table 5.6 Expectations from multiple allele hypothesis for the AB0 system from Bernstein [10]

Phenotype	Genotype	Frequency	
0	aabb	$(1-p)^2(1-q)^2=p'^2q'^2$	
B	aaBB	$(1-p)^2q^2$	$=p'^2(1-q'^2)$
	aaBb	$2(1-p)^2q(1-q)$	
A	AAbb	$p^2(1-q)^2$	$=(1-p'^2)q'^2$
	Aabb	$2\,p(1-p)(1-q)^2$	
AB	AABB	p^2q^2	$=(1-p'^2)(1-q'^2)$
	AaBB	$2\,p(1-p)q^2$	
	AABb	$2\,p^2q(1-q)$	
	AaBb	$2\,p(1-p)2\,q(1-q)$	

This leads to the following relationships ($\overline{\mathrm{A}}, \overline{\mathrm{B}}$: frequencies of phenotypes):

$$\overline{0}\times\overline{\mathrm{A}}\overline{\mathrm{B}} = \overline{\mathrm{A}}+\overline{\mathrm{B}}$$

and

$$+\mathrm{A}++\mathrm{AB}=1-p'^2;+\mathrm{B}++\mathrm{A}B=1-q'^2$$

Thus, it follows:

$$(+\mathrm{A}++\mathrm{AB})\times(+\mathrm{B}++\mathrm{AB})=+\mathrm{AB}$$

These identities can be tested. It turned out – and has turned out ever since – that $(\overline{\mathrm{A}}+\overline{\mathrm{A}}\overline{\mathrm{B}})$ × $(\overline{\mathrm{A}}\overline{\mathrm{B}}+\overline{\mathrm{A}}\overline{\mathrm{B}})>\overline{\mathrm{A}}\overline{\mathrm{B}}$, and $\overline{0}$ × $\overline{\mathrm{A}}\overline{\mathrm{B}}<\overline{\mathrm{A}}+\overline{\mathrm{B}}$. The differences are so large – and so consistent – that an explanation by chance deviations is inadequate. The first alternative possibility considered by Bernstein was heterogeneity within the examined population. This explanation, however, proved insufficient. On the other hand, it could be shown that the distributions in all populations for which data were available are in perfect agreement with expectations derived from the multiple-allele hypothesis.

To understand Bernstein's argument a fresh look at the Hardy–Weinberg law is necessary. Up to now it has been derived here for the special case of two alleles only. However, it can also be shown to apply for more than two alleles. Assuming n alleles p_1, p_2, …, p_n, the relative frequencies of genotypes are given by the terms of the expansion of $(p_1+p_2+\ldots p_n)^2$. It follows for the special case of A, B, and 0 with the frequencies p, q, and r that the distribution of genotypes is:

$$p^2(\mathrm{AA})+2pq(\mathrm{AB})+2pr(\mathrm{A0})$$
$$+q^2(\mathrm{BB})+2pr(\mathrm{B0})+r^2(\mathrm{B0}).$$

Now, we follow Bernstein again (our translation): "for the classes" (phenotypes):

$$\overline{0}=00 \quad \overline{\mathrm{B}}=\mathrm{B0}+\mathrm{BB}$$
$$\overline{\mathrm{A}}=\mathrm{A0}+\mathrm{AA} \quad \overline{\mathrm{AB}}=\mathrm{AB}$$

the following probabilities can be derived:

$$r^2 \quad 2qr+q^2 \quad 2pr+p^2 \quad 2pq$$

It follows:

$$\overline{0}+\overline{\mathrm{A}}=(r+p)^2$$
$$\overline{0}+\overline{\mathrm{B}}=(r+q)^2$$

and therefore:

$$\mathrm{q}=1-\sqrt{\overline{0}+\overline{\mathrm{A}}}$$
$$\mathrm{q}=1-\sqrt{\overline{0}+\overline{\mathrm{B}}}$$
$$\mathrm{q}=1-\sqrt{\overline{0}}$$

and the relation:

$$1=\mathrm{p}+\mathrm{q}+\mathrm{r}=1-\sqrt{\overline{0}+\overline{\mathrm{B}}}+1-\sqrt{\overline{0}+\overline{\mathrm{A}}}+\sqrt{\overline{0}}$$

This can be tested using the AB0 phenotype distributions in various populations of the world. The criterion is that the gene frequencies calculated with this formula must add to 1. In addition, expected genotype frequencies can be calculated from these gene frequencies and can be compared with observed frequencies. Apart from the correctness of the genetic hypothesis, however, this result requires still another condition. There must be random mating with regard to this characteristic.

In the data analyzed by Bernstein the agreement already was excellent, and this has proven to hold true for the huge amount of data collected ever since. One example may help in understanding the principle of calculation. The following phenotype frequencies were reported from the city of Berlin ($n = 21{,}104$): 43.23% A ($n = 9{,}123$), 14.15% B ($n = 2{,}987$), 36.60% 0 ($n = 7{,}725$), and 6.01% AB ($n = 1{,}269$).

Using Bernstein's formula, the gene frequencies are:

$$p = 1 - \sqrt{(0.3660 + 0.1415)} = 0.2876$$
$$q = 1 - \sqrt{(0.3600 + 0.4323)} = 0.1065$$
$$r = \sqrt{0.3660} = \frac{0.6050}{0.9991}$$

Thus:

$$p + q + r = 0.9991$$

At first glance, this result agrees well with the expectation, i.e., 1. As a statistical test for examining whether the deviation is significant, the χ^2 method can be applied [73]:

$$\chi_1^2 = 2n\left(1 + \frac{r}{pq}\right)D^2$$

$$D = 1 - (p + q + r)$$

In our example, the result is:

$$\chi_1^2 = 0.88$$

This confirms that the values found are in good agreement with the genetic hypothesis and with the assumptions of random mating for the AB0 system.

In a later paper Bernstein showed how the difference D may be utilized to correct the calculated gene frequencies. The uncorrected gene frequencies may be named p', q', and r', and the following formulas may be used:

$$p = p'(1 + D/2)$$
$$q = q'(1 + D/2)$$
$$r = (r' + D/2)(1 + D/2)$$

and for the example:

$$p = 0.2876(1 + 0.00045) = 0.2877$$
$$q = 0.1065(1 + 0.00045) = 0.1065$$
$$r = (0.6050 - 0.00045)(1 + 0.00045) = 0.6057$$

In the process of testing the two genetic hypotheses for the AB0 system Bernstein developed a method for calculating gene frequencies.

5.2.2.3 Meaning of a Hardy–Weinberg Equilibrium

Populations showing agreement of the observed genotype proportions with the expectations of the Hardy–Weinberg Law are said to be "in Hardy–Weinberg equilibrium." This equilibrium must be distinguished from that between alleles, which is discussed in the contexts of selection and of mutation. The Hardy–Weinberg equilibrium is an equilibrium of the distribution of genes in the population ("gene pool") among the various genotypes. Under random mating this equilibrium is reestablished after one generation, possibly with changed gene frequencies if it is disturbed by opposing forces.

It follows from our discussion, however, that the Hardy–Weinberg law can be expected to be valid only when the following prerequisites are not violated:

(a) The matings must be random with respect to the genotype in question. This can safely be assumed for such traits as blood groups or enzyme polymorphisms. It cannot be assumed for visible characteristics such as stature, and still less for behavioral characteristics such as intelligence. This should be kept in mind when measures used in quantitative genetics, (for example, correlations between relatives), are interpreted in genetic terms.

(b) A deviation from random mating is caused by consanguineous matings. If the consanguinity rate in a population is high, an increase in the number of homozygotes must be expected (Chap. 17). It is even possible to estimate the frequency of consanguinity in a population by means of the deviations from the Hardy–Weinberg proportions.

(c) Recent migrations might disturb the Hardy–Weinberg proportions.

(d) Occasionally selection is mentioned as a factor leading to deviations. This may be true but need not necessarily apply. As a rule, selection tends to cause changes in gene frequencies; selection before reproductive age, for example, in the prenatal period, or during childhood and youth, does not influence the Hardy–Weinberg proportions in the next generation at all. If genotypes are tested among adults in a situation in which a certain genotype had been selected against in children, this genotype is found to decrease in frequency. Even assuming appreciable selection in a suitable age group, ascertainment of statistically significant deviations from Hardy–Weinberg proportions requires large sample sizes – larger than are usually available. Sometimes the absence of significant selection is inferred from the observation that Hardy–Weinberg proportions are preserved in a population. This conclusion, however, unless carefully qualified may easily be wrong. Considering all the theoretical possibilities for disturbance, it is indeed amazing how frequently the Hardy–Weinberg proportions are found to be preserved in the human population.

(e) Formally, a deviation from the Hardy–Weinberg law may be observed if the population is a mixture of subpopulations that do not completely interbreed (random mating only within subpopulations), and consequently the gene frequencies in these subpopulations differ. This was first described by Wahlund in [81], who gave a formula for calculating the coefficient F of the apparent inbreeding from the variance of the gene frequencies between the subpopulations.

(f) Another cause of deviation may be the existence of a hitherto undetected ("silent") allele, a heterozygous carrier of which cannot be distinguished from a homozygous carrier of the usual allele. C.A.B. Smith [69], however, has pointed out that a silent allele causes a significant deviation from the Hardy–Weinberg law only when it occurs at a sufficiently high frequency for the homozygote to be detected.

5.2.3 Gene Frequencies

5.2.3.1 One Gene Pair: Only Two Phenotypes Known

In rare autosomal-recessive diseases only one gene pair is present, and only two phenotypes are usually known when the heterozygotes cannot be identified, or, as is usually the case, when direct data on population frequencies of heterozygotes are not available. This also applies for blood group systems for which only one type of antiserum is available. Here the frequency of homozygotes aa being q^2, the gene frequency is simply. There is no way to test the assumption of random mating.

Table 5.7 [49] is slightly oversimplified; some of the frequencies given vary in different populations. However, the data point out how much more frequent the heterozygotes are, especially for rare conditions. This is important for genetic counseling, and for the much-discussed problem of the number of lethal or detrimental genes for which the average human being might be heterozygous.

5.3 Statistical Methods in Formal Genetics: Analysis of Segregation Ratios

5.3.1 Segregation Ratios as Probabilities

During meiosis – and in the absence of disturbances – germ cells are formed in exactly the relative frequencies expected from Mendel's laws. A diploid spermatocyte heterozygous for alleles A and a produces two haploid sperms with A, and two with a. If all the sperms of a given male come to fertilization, and none of the zygotes die before birth, the segregation ratio among his offspring would be exactly 1:1. There would be no place for any statistics.

Organisms in which such an analysis is indeed possible are yeast and the bread mould *Neurospora crassa,* which has become important in biochemical genetics. In the development of such an organism,

Table 5.7 Differing homozygote and heterozygote frequencies for different gene frequencies (with examples of recessive conditions; adapted from Lenz [49])

Homozygote frequency q^2	Gene frequency q	Heterozygote frequency $2pq$	Approximate homozygote frequencies in European populations
0.64	0.8	0.32	Lp(a-) lipoprotein variant
0.49	0.7	0.42	Acetyl transferase, “slow” variant (Sect. X56)
0.36	0.6	0.48	Blood group 0
0.25	0.5	0.50	Nonsecretor (se/se)
0.16	0.4	0.48	Rh negative (dd)
0.09	0.3	0.42	Lactose restriction (northwestern Germany)
0.04	0.2	0.32	Le(a–b–) negative
0.01	0.1	0.18	β-Thalessemia (Cyprus)
1:2500	1:50	1:25	Pseudocholinesterase (dibucaine-resistant variant), cystic fibrosis; α_1-antitrypsin deficiency
1:4,900	1:70	1:35	Adrenogenital syndrome (Canton Zurich)
1:10,000	1:100	1:50	Phenylketonuria (Switzerland; USA)
1:22,500	1:150	1:75	Albinism; adrenogenital syndrome with loss of NaCl
1:40,000	1:200	1:100	Cystinosis
1:90,000	1:300	1:150	Mucopolysaccharidosis type 1
1:1,000,000	1:1,000	1:500	Afibrinogenemia

there is a phase in which the diploid state has just been reduced to the haploid, and all four meiotic products lie in a regular sequence. They can be removed separately, grown, and examined (“tetrad analysis”). Expected segregation ratios are found with precision.

In higher plants and animals, including humans, only a minute sample of all germ cells comes to fertilization. In the human female about 6.8×10^6 oogonia are formed; the number of spermatogonial stem cells in the male is estimated at about 1.2×10^9; the actual number of sperm is a multiple of this figure. Hence any given germ cell has a very small probability of coming to fertilization. In addition, the sampling process is usually random with respect to a given gene pair A,a. This means that for the distribution of genotypes among germ cells coming to fertilization the rules of probability theory apply, and empirically found segregation ratios may show deviations from their statistical expectations.

Modern humans are fairly accustomed to thinking in statistical terms when solving daily problems. These experiences help us to understand simple applications of probability theory. Everyone, for example, readily recognizes that the following rationale is wrong.

A young mother had always wished to have four children. After the third, however, there was a long pause. The grandmother asked her daughter whether she had now decided differently. Answered the daughter: “Yes, in principle, I would still like four children. But I read in the newspaper that every fourth child born is Chinese. And a Chinese child …there I am reluctant.”

In another example, the mistake is less obvious. The parents of two albino children visit a physician for genetic counseling. They wish to know the risk of a third child also being albino. The physician knows that albinism is an autosomal-recessive condition, with an expected segregation ratio of 1:3 among children of heterozygous parents. He also knows that sibships in which all sibs are affected are very rare. Hence, he informs the parents: “As you already have two affected children, the chance that the third child will also be affected is very small. The next child should be healthy.” The actual risk, of course, remains 25% (Sect. 5.3.2).

A textbook on human genetics cannot teach probability theory and basic statistics. Therefore, it is assumed that the reader has some knowledge of the basic concepts of probability theory, that he knows the most important distributions (binomial, normal, and Poisson distribution), and has some idea of standard statistical methods. The following presents some applications to problems in human genetics. We are aware of the danger that this section may be used as a “cookbook,” without understanding of the basic principles and recommend that the reader become familiar with these principles, for example, in the opening chapters of Feller's *Probability Theory and Its Applications* [24].

5.3.2 Simple Probability Problems in Human Genetics

5.3.2.1 Independent Sampling and Prediction in Genetic Counseling

The physician who gave the wrong genetic counsel to the couple with two albino children did not take into account that the fertilization events leading to the three children are independent of each other, and that each child has the probability of $^1/_4$ to be affected, regardless of the genotypes of any other children. The probabilities for each child must be multiplied. He was right when he said that illness of all three children is rare in a recessive condition: The probability is $(^1/_4)^3 = {^1/_{64}}$ for all three children to be affected; the family to be counseled however, already had two such children and the probability of this occurring was only $(^1/_4)^2 = {^1/_{16}}$. It takes only one event with the probability $^1/_4$ to complete the three-child family with albinism, $^1/_{16} \times {^1/_4} = {^1/_{64}}$. It is also intuitively obvious that there is no way for a given zygote to influence the sampling of gametes of the same parents many years later. Chance has no memory!

All possible combinations of affected and unaffected siblings in three-child families can be enumerated as follows (A = affected; U = unaffected):

UUU,AUU,UAU,AAU,\quad UUA,AUA,UAA, AAA

In recessive inheritance, the event U has the probability $^3/_4$. Thus, the first of the eight combinations *(UUU)* has the probability $(^3/_4)^3 = {^{27}/_{64}}$. This means that of all heterozygous couples having three children $^{27}/_{64}$, or fewer than 50% have only healthy children. On the other hand, all three children are affected in $(^1/_4)^3 = {^1/_{64}}$ of all such families. There remain the intermediate groups. Three-child families with one affected child and two healthy ones in that order obviously have the probability $^1/_4 \times {^3/_4} \times {^3/_4} = {^9/_{64}}$. However, we are not particularly interested in the sequence of healthy and affected children. Therefore the three cases of such families, *UUA, UAU,* and *AUU*, can be treated as equivalent, giving $3 \times {^9/_{64}} = {^{27}/_{64}}$. The group with two affected can be treated accordingly, giving $3 \times {^1/_4} \times {^1/_4} \times {^3/_4} = {^9/_{64}}$. As a control, let us consider whether the various probabilities add up to 1:

$$\frac{27+27+9+1}{64}$$

This is a special case of the binomial distribution. There are two consequences for Mendelian segregation ratios one theoretical, the other extremely practical. First, it follows that among all families for which a certain segregation ratio must be expected, an appreciable percentage – 27 of 64 in a three-child family with recessive inheritance – cannot be observed because chance has favored them by not producing any affected homozygotes. Hence, the segregation ratio in the remainder is systematically distorted. Special methods have been devised to correct for this "ascertainment bias" (Sect. 5.3.4). Secondly, and this is a most practical conclusion, with limitation of the number of children to two or three, most parents both of whom are heterozygous for a recessive disease will not have more than one affected child. Since the probability of affected children occurring in another branch of the family is very low – and the rate of consanguinity in current populations of industrialized countries has likewise decreased – almost all affected children represent sporadic cases in an otherwise healthy family; there is no distinct sign of recessive inheritance. Any subsequent child, however, again runs the risk of $^1/_4$. The layman usually does not know that the condition is inherited. Therefore, genetic counseling must be actively offered to these families.

5.3.2.2 Differentiation Between Different Modes of Inheritance

In Sect. 5.1.4, an X-linked dominant pedigree is shown (Fig. 5.13) for vitamin D-resistant rickets and hypophosphatemia. What is the probability of such pedigree structure if the gene is in fact located on one autosome? Only the children of affected males are informative because among children of affected women a 1:1 segregation irrespective of sex must be expected. The seven affected fathers have 11 daughters, all of whom are affected. The probability of this outcome with autosomal inheritance is $(^1/_2)^{11}$. The same fathers have 10 sons who are all healthy, giving a probability of $(^1/_2)^{10}$. Hence, the combined probability of 11 affected daughters and 10 healthy sons is:

$$(^1/_2)^{21} = \frac{1}{2\ 097\ 152}$$

This probability is so tiny that the alternative hypothesis of an autosomal-dominant mode of inheritance is convincingly rejected. The only reasonable alternative is the X-linked dominant mode. This hypothesis is corroborated independently by the observation (Sect. 5.1.4) that on average male patients are more severely affected than female.

This is different for a rare skin disease (Brauer keratoma dissipatum). For this condition a Y-chromosomal mode of inheritance has been considered – and indeed all nine sons of affected fathers in a published pedigree show the trait, whereas five daughters in both generations are unaffected. This gives:

$$({}^{1}/_{2})^{9} \times ({}^{1}/_{2})^{5} = ({}^{1}/_{2})^{14} = \frac{1}{16\ 384}$$

Hence, the probability of this pedigree having occurred by chance as an autosomal-dominant trait is very low indeed. There is an important difference, however, from the example of vitamin D-resistant rickets. Other pedigrees showing autosomal-dominant inheritance are unknown for this type of rickets, and all observations confirm the location of this gene on the X chromosome. For Brauer keratoma dissipatum, on the other hand, some families have been observed exhibiting very similar phenotypes that show clearcut autosomal-dominant inheritance. It is therefore likely that the described pedigree has been selected from an unknown number of observations because of its peculiar transmission. The calculation is misleading as the "universe" from which this sample of observations was drawn (all pedigrees with the same phenotype) is much larger (and ill-defined), and the sample (the pedigree) is biased. The trait seems to be autosomal-dominant.

Another, more obvious example of an error in the definition of the sample space is the mother, above, who did not want a Chinese baby.

5.3.3 Testing for Segregation Ratios Without Ascertainment Bias: Codominant Inheritance

Apart from these limiting cases, calculation of exact probabilities for certain families or groups of families is usually impracticable. Therefore statistical methods are used that are either based on the parameters of the "normal" distribution, which is a good approximation of the binomial distribution (parametric tests), or derive directly from probabilistic reasoning (nonparametric tests). One method that is especially well suited for genetic comparisons is the χ^2 test. This enables us to compare frequencies of observations in two or more discrete classes with their expectations. The most usual form is:

$$\chi^2 = \Sigma \frac{(E-O)^2}{E}$$

(E=expected number; O=observed number). In Farabee's pedigree with dominant inheritance (Sect. 5.1.2), there are 36 affected and 33 unaffected children of affected parents. With dominant inheritance, E is ${}^{1}/_{2}$ of all children, i.e., 34.5:

$$\chi_1^2 = \frac{(36-34.5)^2}{34.5} = \frac{(33-34.5)^2}{34.5} = 0.13$$

The probability p for an equal or greater deviation from expectation can be taken from a χ^2 table for 1 degree of freedom. The number of degrees of freedom indicates in how many different ways the frequencies in the different classes can be changed without altering the total number of observations. In this case the content of class 2, unaffected, is unequivocally fixed by the content of class 1. Therefore, the number of degrees of freedom is 1. In general the number of degrees of freedom is equal to the number of classes less 1.

A second example is taken from the codominant mode of inheritance (Sect. 5.1.2). Table 5.1 summarizes Wiener's family data for the MN blood types. Are the resultant segregation ratios compatible with the genetic hypothesis? For this problem, matings MM × MM, MM × NN, and NN × NN give no information. Expectations in the matings MM × MN and NN × MN are 1:1, in the mating MN × MN 1:2:1. This leads to Table 5.8 for the χ^2 test: For 4 degrees of freedom we find in the χ^2 table: p=0.75. This is very good agreement with expectation.

5.3.3.1 Dominance

The situation becomes slightly more complicated when one allele is dominant and the other recessive. This is the case, for example, in the AB0 blood group system. Here, the phenotype A consists of the genotypes AA and A0. The expected segregation ratios among their offsprings differ. Some of the heterozygous parents A0 can be

Table 5.8 Comparison between expected and observed segregation figures in the MN data of Wiener et al. (Table 4.1 [92])

Mating type	MM	MN	NN	χ^2	Degrees of freedom
MM × MN	$\frac{(499-486)^2}{486}$	$\frac{(473-486)^2}{486}$	–	0.6955	1
MN × MN	$\frac{(199-200)^2}{200}$	$\frac{(405-400)^2}{400}$	$\frac{(196-200)^2}{200}$	0.1475	2
MN × NN	–	$\frac{(411-396.5)^2}{396.5}$	$\frac{(382-396.5)^2}{396.5}$	1.0605	1

recognized, for example, in matings with 0 partners by the finding of 0 children. Others have only A children just by chance. Special statistical methods are necessary to calculate correct expectations and to compare empirical observations with these expectations [68].

5.3.4 *Testing for Segregation Ratios: Rare Traits*

5.3.4.1 Principal Biases

If the condition under examination is rare, families are usually not ascertained at random; one starts with a "proband" or "propositus," i.e., a person showing the condition. This leads to an *ascertainment bias,* which must be corrected. The bias can be of different kinds, depending on the way in which the patients have been ascertained.

(a) Family or truncate selection. All individuals suffering from a specific disease in a certain population at a certain time (or within certain time limits) are ascertained. The individual patients are ascertained independently of each other, i.e., the second case in a sibship would always have been found. Such truncate ascertainment is possible, for example, if the condition always leads to medical treatment, and all physicians report every case to a certain registry – as when an institute carries out an epidemiological study. As a rule, case collections approaching completeness are possible only in ad hoc studies of research workers specializing in a condition or group of conditions.
Here, the ascertainment bias is caused exclusively by the fact that only those sibships are ascertained that contain at least one patient. As noted above, however (Sect. 5.3.3), this leaves out all sibships in which no affected individual has occurred just by chance. Their expected number is:

$$\sum_s q^s n_s \qquad (5.2)$$

(s = number of siblings/sibship; p = segregation ratio; $q = 1 - p$; n_s = number of sibships of size s). In recessive disorders, $p = 0.25$. The smaller the average sibship size, the stronger is the deviation from the 3:1 ratio in the ascertained families.

(b) Incomplete multiple (proband) selection; single selection as limiting case. It is rare that all individuals in a population are ascertained; frequently a study starts, for example, with all patients in a hospital population who have a certain condition. Here an additional bias must be considered: the more affected members a sibship has, the higher is its chance to be represented in the sample. This bias causes a systematic excess of affected persons, which is added to the excess caused by truncate selection as explained above.

Koller [45] gave a simple example that demonstrates the nature of this excess. Let us assume that the probands are ascertained during examination of only a single year's group of conscripts. The population comprises a number of families with three children, at least one of whom has the disease, and one of whom is a member of the current year's group. Ascertainment of the family depends on the presence of an affected child in the 1 year group examined. Thus, all families with three affected siblings but only two-thirds of the families with two affected and one-third of those with only one affected are ascertained.
The methods of correction described below are reliable only if the probability for ascertainment

of consecutive siblings is independent of the ascertainment of the first one. In an examination of conscripts, as described above, this may be the case. Most studies, however, begin with a hospital population or some other group of medically treated persons. Here, according to general experience, subsequently affected children are much more frequently brought to a hospital when another child has been treated successfully. The opposite trend, however, is also possible. Becker [3], for example, collected all cases of X-linked recessive Duchenne's muscular dystrophy in a restricted area of southwestern Germany. He had good reason to think that ascertainment was complete for this area. Nevertheless, brothers developing muscular dystrophy as the second or later cases in their sibships were generally not ascertained as probands (i.e., through hospitals and physicians) but through the first proband in the family. In his interviews with the parents Becker found the reason. In the case of the first patient in the sibship the parents usually consulted a physician. Then, however, they discovered that in spite of examinations and therapeutic attempts, the course of the disease could not be influenced. Hence they refrained from presenting a second child to the hospital or the physician.

(c) Apart from these biases, which can be statistically corrected to a certain degree, there are other biases that cannot be corrected. Frequently, for example, a genetic hypothesis is discussed on the basis of families sampled from the literature. Experience shows that such sampling usually leads to reasonable results in autosomal-dominant and X-linked recessive disorders. Autosomal-recessive diseases, however, are more difficult to handle. Families with an impressive accumulation of affected sibs have a higher chance of being reported than those with only one or two affected members. This selection for "interesting" cases was more important early in the twentieth century because families generally had more children. Furthermore, recessive conditions discovered today are usually interesting from a clinical and biochemical point of view as well.

These biases can be avoided only by publishing all cases and by critical interpretation of data from the literature. A statistically sound correction is impossible, as such bias has no simple and reproducible direction.

To summarize, the method of segregation analysis depends on the way in which families are ascertained. It follows that the method of ascertainment should always be described carefully. Above all, the probands should always be fully indicated. It is also of interest whether the author during his case collection has become aware of any ascertainment biases.

These considerations show that complete (truncate) ascertainment of cases in a population, and within defined time limits, is the optimal method of data collection.

5.3.4.2 Methods for Correcting Bias

Two different types of correction are possible: test methods and estimation methods.

In a test method the observed values are compared with the expected values, which have been corrected for ascertainment bias. The first such test method was published by Bernstein in [45]; it corrected for truncate selection. The expected number of affected E_r is:

$$\mathrm{E_r} = \mathrm{sn_s} \frac{\mathrm{p}}{1-q^s} \qquad (5.3)$$

in all sibships of size *n* (definition of symbols as in (5.2)). A similar test method can also be used for proband selection.

Test methods answer a specific question: do the observed proportions fit the expected values according to a certain genetic hypothesis?

In many if not in most actual cases, the question is more general: What is the unbiased segregation ratio in the observed sibships? This is an estimation problem. The earliest method was published in 1912 by Weinberg [86] and was called the sib method. Starting from every affected sib in the sibship, the number of affected and unaffected among the sibs is determined. This method is adequate for "truncate selection," i.e., when each affected person is, at the same time, a proband. The sib method is the limiting case of the "proband method" used when the families are ascertained by incomplete multiple proband selection. The number of affected and unaffected siblings is counted, starting from every proband. A limiting case is single selection. Here each sibship has only one proband, and the counting is done once among the sibs.

These estimates converge with increasing sample size to the parameter *p,* the true segregation ratio; they are *consistent.* It was realized early, however, that they are not fully *efficient,* except for the limiting case of single selection, i.e., they do not make optimal use of all available information. Therefore improvements have been devised by a number of authors. Today such simple methods are no longer used. Moreover, the problems to be solved by segregation analysis are usually more complex. For example, the families to be analyzed may be a mixture of genetic types with various modes of inheritance; there may be admixture of "sporadic" cases, due either to new mutation or to environmental factors; penetrance may be incomplete, or the simple model of a monogenic mode of inheritance may be inadequate for explaining familial aggregation, and a multifactorial genetic model must be used (for the conceptual basis of such multifactorial models, see Chap. 8). Computer programs are now available for carrying out such analyses; they are available either from their authors' institutions or through an international network of program packages. Some of these also offer programs for comparing predictions from various genetic models.

5.3.5 Discrimination of Genetic Entities: Genetic Heterogeneity

It is a common experience in clinical genetics that similar or identical phenotypes are caused by a variety of genotypes. The splitting of a group of patients with a given disease into smaller but genetically more uniform subgroups has been a major topic of research in medical genetics over recent decades. Frequently such heterogeneity analysis is another aspect of the application of Mendel's paradigm and its consequences: carrying genetic analysis through different levels ever closer to gene action.

It appears at first glance that with modern biological methods discrimination of genetic entities on descriptive grounds, i.e., on the level of the clinical phenotype, would no longer hold interest. In our opinion, however, knowledge of the phenotypic variability of genetic disease in humans is needed for many reasons:

(a) Such knowledge provides heuristic hypotheses for systematic application of the more penetrating methods from biochemistry, molecular biology, immunology, micromorphology, and other fields.
(b) Treatment will often depend upon manipulation of gene disordered biochemistry and pathophysiology of a given disease.
(c) We require insight into the genetic burden of the human population.
(d) Better data are needed for many of our attempts to understand the problems of spontaneous and induced mutation.

5.3.5.1 Genetic Analysis of Muscular Dystrophy as an Example

One group of diseases in which analysis using the clinical phenotype together with the mode of inheritance proved to be successful are the muscular dystrophies. These conditions have in common a tendency to slow muscular degeneration, incapacitating affected patients who often ultimately die from respiratory failure. There are major differences in age at onset, location of the first signs of muscular weakness, progression of clinical symptoms, and mode of inheritance. These criteria were used by medical geneticists to arrive at the following classification of muscular dystrophies:

1. X-linked muscular dystrophies
   a. Severe type (Duchenne) (310200)
   b. Juvenile or benign type (Becker; 310100)
   c. Benign type with early contracture (Cestan-Lejonne and Emery-Dreifuss; 310300)
   d. Hemizygous lethal type (Henson-Muller-de Myer; 309950)
2. Autosomal-dominant dystrophy Facio-scapulo-humeral type (Erb-Landouzy-Déjérine; 158900)
3. Autosomal-recessive muscular dystrophies
   a. Infantile type
   b. Juvenile type
   c. Adult type
   d. Shoulder girdle type

This classification is based on many reports from various populations and, for the rarer variants, on reports of pedigrees. It does not include pedigrees in which affected members showed involvement only of restricted parts of the muscular system, such as distal and ocular types. Congenital myopathies were also excluded. The main criteria for discrimination are obvious from the descriptive terms used in the tabulation; for details, see Becker [6]. At present, various mutations of the X-linked dystrophin gene are known at the molecular level which lead to the Duchenne and Becker types. The gene for Emery-Dreyfus disease has been localized to distal Xq28.

5.3.5.2 Multivariate Statistics

The critical human mind is an excellent discriminator. However, statistical methods for identifying subgroups within a population on the basis of multiple characteristics are now available (multivariate statistics). Such methods can also be applied to the problem of making discrimination of genetic entities more objective.

5.3.6 Conditions Without Simple Modes of Inheritance

The methods discussed so far are used mainly for genetic analysis of conditions thought to follow a simple mode of inheritance. In many diseases, however, especially in some that are both serious and frequent, there are problems:

(a) Diagnosis of the condition may be difficult. There are borderline cases. Expressed more formally: the distribution of affected and unaffected in the population is not an outright alternative (examples: schizophrenia; hypertension; diabetes).
(b) It is known from various investigations, including twin studies, that the condition is not entirely genetic but that certain environmental factors influence manifestation (example: decline of diabetes in European countries during and after World War II).
(c) The condition is so frequent that clustering of affected patients in some families must be expected simply by chance (examples: some types of cancer).
(d) It can be concluded from our knowledge of pathogenic mechanisms that the condition is not a single disease but a complex of symptoms common to a number of different causes (example: epilepsy). In fact, it is becoming apparent that diagnoses such as hypertension and diabetes subsume groups of heterogeneous disease entities.

In no such case can a genetic analysis that starts from the phenotype be expected to lead to simple modes of inheritance. However, for many such conditions, two questions of practical importance arise:

1. What is the risk of relatives of various degrees being affected? Is it higher than the population average?
2. What is the contribution of genetic factors to the disease? Under what conditions does the disease manifest itself?

Familial aggregation can be assessed by calculation of empirical risk figures. Twin studies and comparisons of incidence among relatives of probands with those in the general population are required to answer the questions. Here, we discuss risk figures.

5.3.6.1 Empirical Risk Figures

The expression “empirical risk” is used in contrast to “theoretic risks” as expected by Mendelian rules in conditions with simple modes of inheritance. The early methods were developed largely by the Munich school of psychiatric genetics in the 1920s with the goal of obtaining risk figures for psychiatric diseases.

The basic concept is to examine a sufficiently large sample of affected patients and their relatives. From this material, unbiased risk figures for defined classes of relatives are calculated. These figures are used to predict the risk for relatives in future cases. This approach makes the implicit assumption that risk figures are generally constant “in space and time”, i.e., among various populations and under changing conditions within the same population. Considering the environmental changes influencing the occurrence of many diseases such as diabetes, this assumption is not necessarily true but is useful as a first approximation.

The approach can be extended to include the question of whether two conditions A and B have a common genetic component, leading to increased occurrence of patients with disease A among close relatives of patients with disease B.

5.3.6.2 Selecting and Examining Probands and Their Families

In conditions that have simple modes of inheritance, the selection of probands is usually straightforward. The modes of ascertainment are discussed in Sect. 5.3.4. For empirical risk studies the same rules apply. In fairly frequent conditions, complete ascertainment of cases in a population is rarely if ever feasible and is also unnecessary in these investigations. In most situations, a defined sample of probands, such as all cases coming to a certain hospital for the first time during a predefined time period can be used. The mode of ascertainment is single selection, or very close to it. This approach simplifies correction of the ascertainment bias among sibs of probands. The empirical risk figures can be calculated by counting affected and unaffected among the sibs, excluding the proband. Risk figures among children ascertained through the parental generation are unbiased and need no correction.

Frequently, the diagnostic categories are not clearcut. In these cases, criteria for accepting a person as a proband must be defined unambiguously beforehand, and all possible biases of selection should be considered. Are more severe cases normally admitted to the hospital selected for study? Are patients selected from a particular social or ethnic group? Are there any other biases that might influence the comparability of the results? Genuinely unbiased samples are hardly if ever available, but the biases should be known. Most importantly, such biases should be independent of the problem to be analyzed. For example, it would be a mistake to consider only patients who have similarly affected relatives.

The goal of the examinations is to obtain maximal and precise information about the probands and their families as far as possible. Methods for achieving this goal, however, vary. Clinical experience and the study of publications on similar surveys are helpful.

Once the proband and his family are ascertained, the relatives should be noted as completely as possible, and information on their health status must be collected. Here, personal examination by the investigator and historical information provided by the patients and their relatives are indispensible. Such data should be backed by hospital records and various laboratory and radiological studies. Even results of clinical examinations should be regarded with scepticism since not all physicians are equally knowledgeable and careful, and official documents, such as death certificates, are often unreliable regarding diagnostic criteria.

In most cases, the determination of genetic risk figures answers the question of whether the risk is higher than in the average population. Sometimes adequate incidence and/or prevalence data from a complete population in which the study is carried out or a very similar one are available. More often than not, however, a control series must be examined with the same criteria as used for the "test" populations. If possible, examination on normal controls and their relatives should be performed in a "blind" way; i.e., the examiners should be unaware of whether the persons studied come from the patient or the control series. It is a good idea to use matched controls, i.e., to examine for every patient a control person matched in all criteria but not related to the condition to be investigated (such as age, sex, ethnic origin, etc.).

5.3.6.3 Statistical Evaluation, Age Correction

In conditions that manifest at birth, such as congenital malformations affecting the visible parts of the body, further calculations are straightforward: the empirical risk for children is given by the proportion of affected in the sample. In many cases, however, onset occurs during later life, and the period at risk may be extended. Here the question asked is: What is the risk of a person's becoming affected with the condition, provided he or she lives beyond the manifestation period? The appropriate methods of age correction have been discussed extensively in the earlier literature [45]; one much-used is Weinberg's "shortened method." First, the period of manifestation is defined on the basis of a sufficiently large sample (usually larger than the sample of the study itself). Then all relatives who dropped out of the study before the age of manifestation are discarded. The dropping out may be for any of a variety of reasons: death, loss of contact due to change of residence, or termination of the study. All persons dropping out during the age of manifestation are counted as one-half, and all who have survived the upper limit of manifestation age are counted full.

5.3.6.4 Example

Among children of schizophrenics, 50 were affected and 200 unaffected. Of these, 100 have reached the age of 45 and 100 are between the age of 15 and 45 (i.e., the age of manifestation for the great majority of schizophrenic cases).

Thus, the corrected number of unaffected is: 200 $-{}^{1}/_{2} \times 100 = 150$; the empirical risk is:

$$\frac{50}{150+50} = 25\%$$

Chapter 23.7 deals in detail with practical problems, taking schizophrenia and affective disorders as examples.

5.3.6.5 Selection of Probands for Genome-Wide Association Studies

Genome-wide association studies have expanded our possibilities to identify new traits in conditions without simple modes of inheritance. The selection of probands or of big cohorts of individuals with a certain phenotypic feature follows different strategies to the aforementioned examples. This will be explained in detail in Chapters 8.1 and 8.

5.3.6.6 Theoretical Risk Figures Derived from Heritability Estimates?

There are suggestions that empirical risk figures should be replaced by theoretical risk figures computed from heritability estimates for the multifactorial model (Chap. 8), after data are found to agree with expectations from such a model. This could be done when the data compared with a simple diallelic model. Such heritability estimates can be achieved by comparing the incidence of the condition in the general population with that in certain categories of relatives, for example, sibs or, with caution, from twin data. In theory the method permits inclusion of environmental, for example, maternal, effects. Its disadvantage, however, is that it depends critically on the assumption that the genetic model fits the actual situation sufficiently well. Since the genetic model chosen may not apply to the data at hand, there is danger that the sophisticated statistical approach suggests a spuriously high degree of precision of the results.

5.4 Conclusions

The transmission of traits determined by single genes, including hereditary diseases, follows Mendel's laws. Autosomal-dominant, autosomal-recessive, and X-linked modes of inheritance can be identified on the basis of the location of mutant genes on autosomes or on the X chromosome, and noting the phenotypic distinction between homozygotes and heterozygotes. Mutations in mitochondrial DNA are transmitted from the mother to all children. Deviations from the classical Mendelian transmission scheme may occur as a consequence of "genomic imprinting," where the parental origin of the mutation determines the phenotype. "Anticipation," with earlier age of onset in succeeding generations, may owe its origin to unstable mutations. Genotype frequencies in populations follow the Hardy–Weinberg Law, which can be used to estimate gene frequencies. In rare traits, such as those in most hereditary diseases, pedigrees are often ascertained via affected individuals and their sibships; when such pedigrees are used to calculate Mendelian segregation ratios, the resulting "ascertainment bias" in favor of affected persons must be corrected by appropriate statistical methods. New sequencing approaches will now enable researches to find disease-causing genes even in relatively small families. Furthermore, genome-wide association studies have paved the way for identifying genomic loci associated with multifactorial inheritance.

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Linkage Analysis for Monogenic Traits

6

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Revised by M.R. Speicher

Abstract Linkage analysis, that is the observation of cosegregation of adjacent genetic markers or traits, is the principal means of constructing genetic maps, and locating genes that cause disease, or genetic traits. Although these methods have been partially supplanted by newer methods such as whole genome sequencing, linkage analysis still has considerable utility. The history of the method and the mathematical basis of linkage analysis are presented as well as specific applications to human genetics. Gene clusters consist of groups of adjacent genes that exist largely though mechanisms of gene duplication. In addition there exist clusters of genes that have related function, the best studied of which is the Major Histocompatibility Complex. The structure and evolutionary history of these clusters provides insight into the history of mammalian genomes.

Contents

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If "to take a possible example, an equally close linkage" (as between the genes for hemophilia and color blindness) "were found between the genes for blood group" and that "determining Huntington's chorea, we should be able, in many cases, to predict which children of an affected person would develop this disease and to advise on the desirability or otherwise of their marriage."

J.B.S. Haldane and J. Bell (1937) The linkage between the genes for colour-blindness and haemophilia in man. Proc. Roy. Soc. B 123, 119.

6.1 Linkage: Localization of Genes on Chromosomes

Genes are located in a linear fashion on the chromosomes. This has the logical consequence that genes located on the same chromosome are transmitted

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together, i.e., that their segregation is not independent. On the other hand, it is known from cytogenetics that chiasmata are formed during the first meiotic division, and that certain chromosome segments are exchanged between homologous chromosomes (crossing over; see Sect. 3.5). Hence, even genes located on the same chromosome are not always transmitted together; the probability of transmission of two linked genes depends on the distance between them and on the frequency with which they are separated by crossing over. If located on a fairly long chromosome, and if the distance is large enough that numerous crossing over events occur between them, genes located on the same chromosome may even seem to segregate independently. Such genes are syntenic but not linked. It was the great achievement of Morgan and his school in the first two decades of the twentieth century to exploit linkage for localizing genes relative to each other on chromosomes and developing gene maps in the fruit fly *Drosophila melanogaster*.

Studies on linkage and gene mapping in humans lagged behind this development for decades. Sophisticated statistical techniques were developed to get around the difficulty that directed breeding experiments are impossible in humans, and information from naturally occurring families must be used. The application of such techniques, however, was only sparsely rewarded by detection of linkage. A breakthrough occurred only when the new techniques of somatic cell genetics and especially cell fusion were introduced. These techniques permitted the assignment of genes to specific chromosomes and even chromosome segments. Later, methods taken from molecular biology, especially the development of restriction fragment length polymorphisms (RFLPs) revolutionized this progress [9]. Radiation hybrid methods and YAC and BAC clones provided the first high-resolution physical maps and paved the way to the complete sequence of the human genome [50, 104]. Annotation of the human genome sequence has allowed the location of all the known genes to be determined.

In the following we describe first the principle of the classic approach to gene localization as introduced by Morgan and his followers. This provides an opportunity to introduce some general concepts. We then discuss statistical methods for detecting and measuring linkage in humans. The various groups of DNA markers are described next, followed by the principle of cell fusion and its use in localizing genes on chromosomes, as well as the application of radioactive and nonisotopic in situ hybridization for this purpose. Genetic maps are compared to physical maps, and the use of linkage studies as analytical tools in genetic analysis of common diseases with complex etiology and pathogenesis is assessed.

6.1.1 Classic Approaches in Experimental Genetics: Breeding Experiments and Giant Chromosomes

According to Mendel's third law, segregation of two different pairs of alleles is independent; all possible zygotes of two pairs of alleles are formed by free recombination. Mating between the double heterozygote AaBb and the double homozygote aabb leads to:

Paternal gametes	AB	Ab	aB	ab
Maternal gametes ab	$^1/_4$ AaBb	$^1/_4$ Aabb	$^1/_4$ aaBb	$^1/_4$ aabb

The four genotypes are formed in equal proportions.

Soon after Mendel's laws were rediscovered Bateson et al. [4] found an exception from this rule in the vetch, *Lathyrus odoratus*. Certain combinations were observed more frequently and others less frequently than expected. In some cases, the two parental combinations – in our example AaBb (father) and aabb (mother) – were increased among the progeny; in other cases the two other types Aabb or aaBb were more frequent.

Paternal gametes	AB	Ab	aB	ab
Maternal gametes ab	AaBb	Aabb	aaBb	aabb
First case (coupling)	$\frac{1}{2} - \Theta$	Θ	Θ	$\frac{1}{2} - \Theta$
Second case (repulsion)	Θ	$\frac{1}{2} - \Theta$	$\frac{1}{2} - \Theta$	Θ

Θ = Recombination fraction Θ.

The alleles of the parental combination seemed either to attract one another or to repel one another. Bateson et al. [4] coined the terms "coupling" for the former phase and "repulsion" for the latter. Morgan in [65] recognized that coupling and repulsion are two aspects of the same phenomenon (i.e., location of two genes on the same or homologous chromosomes). He coined the term "linkage." Coupling occurs when the genes A and B are localized in the doubly heterozygous parent on the same chromosome $\frac{AB}{ab}$, and repulsion occurs when they are localized on homologous chromosomes $\frac{Ab}{aB}$. The terms *cis* and *trans* are more frequently used to refer to genes in coupling or repulsion, respectively. If linkage is complete, only two types of progeny can occur. More frequently, however, all four types are found, albeit two types in smaller numbers. Morgan explained this finding by exchange of chromosome pieces between homologous chromosomes during meiotic crossing over. He also recognized that the frequency of crossing over depends on the distance between two gene loci in one chromosome. Using recombination analysis as an analytic tool, he and his coworkers succeeded in locating a great number of gene loci in *Drosophila* and in establishing chromosome maps. Their results were confirmed in the early 1930s when Heitz, Bauer, and Painter discovered the giant chromosomes of some *Dipterae.* With this experimental tool many gene localizations known from indirect evidence could be confirmed by direct inspection when they were accompanied by small structural chromosomal variation. In the meantime linkage analyses have been carried out in a great number of species.

6.1.1.1 Linkage and Association

It is sometimes assumed that genes which are linked should always show a certain association in the population, i.e., that the chromosomal combinations AB or ab (coupling) occur more frequently than the combinations Ab or aB (repulsion). However, this is not the case in a randomly mating population. Even if the linkage is fairly close, repeated crossing over in many generations causes all four combinations, AB, ab, Ab, and aB, to be randomly distributed in the long run. As a rule, association of genetic traits does not point to linkage. This rule, however, has exceptions. Some combinations of closely linked genes do indeed occur more often than expected with random distribution. Such "linkage disequilibrium" was first postulated in humans for the rhesus blood types (Sect. 6.2.4) and has also been proven for the major histocompatibility complex (MHC), especially the HLA system (Sect. 6.2.5) and for many DNA polymorphisms. It has now been shown that there are blocks of linkage disequilibrium throughout the genome and these blocks vary by location and by population [25] (Sect. 16.3). Linkage disequilibrium may occur for three reasons:

1. The population under examination originated from a mixture of two populations with different frequencies of the alleles A,a and B,b, and the time elapsed since the mixing of the populations was not sufficient for complete randomization (admixture linkage disequilibrium).
2. Two mutants, for example, DNA markers, are located so closely together that an insufficient number of generations has elapsed to separate them by recombination since the two mutations occurred in one chromosome.
3. Certain combinations of alleles at linked gene loci are maintained in high frequency by natural selection.

These problems are discussed in greater detail in connection with the MHC system (Sect. 6.2.5) and in the discussion on association between HLA and disease (Sect. 6.2.5.4).

6.1.2 Linkage Analysis in Humans

6.1.2.1 Direct Observation of Pedigrees

Linkage analysis by classic methods in humans is difficult since no directed breeding occurs. However, in some cases pedigree inspection can provide information. Linkage is excluded, for example, if one of the genes under scrutiny can be localized to the X chromosome while the other is on an autosome. By the same token, there is a high probability of demonstrating formal linkage if both genes are X-linked. Even in this case, however, formal linkage may not be demonstrable since the loci may be so far from each other that crossing over separates them. Similar considerations hold for genes located on a given autosomal chromosome. The term synteny refers to two or more genes being situated on the same chromosome, regardless of whether formal linkage can be demonstrated. Either a large pedigree or a number of smaller pedigrees must be screened to assess the extent of crossing over. Figure 6.1a shows a pedigree with red–green color blindness (303800, 303900) and hemophilia (306700). The males in the sibships at risk either have both conditions or are normal. The genes are in the coupling (or *cis*) state. The pedigree in Fig. 6.1b shows the opposite; here these genes are in the repulsion (or *trans*) phase.

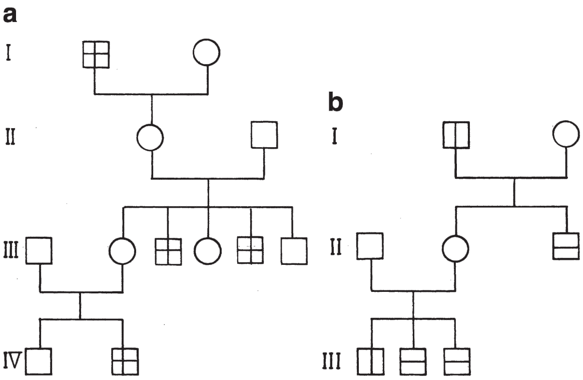

Fig. 6.1 (**a**, **b**) Pedigrees with red-green color blindness (⊟), hemophilia (◫), or both conditions (⊞). (**a**) Both abnormal genes in coupling. (From Madlener 1928) (**b**) In repulsion. From Stern [98]

In some exceptional cases linkage between gene loci localized on an autosome can also be established by simple inspection of an extensive pedigree. Figure 6.2 shows a large pedigree in which Huntington disease segregates together with a *Hin*dIII DNA polymorphism detected by a DNA marker, which was named “G 8” [29]. Four allelic variants of this probe are observed in this pedigree, A, B, C, and D. The Huntington gene invariably segregates together with allele C. One individual, VI, 5 (arrow), so far has been unaffected by Huntington disease, but she will be affected later, provided that her father, (who has not been tested), does not happen to have transmitted another chromosome that carries a C allele not linked to the Huntington gene. The pedigree points to close linkage between the locus for this DNA polymorphism and the Huntington gene. Some cross-overs in other such pedigrees have been detected, and the recombination fraction is 4% or less.

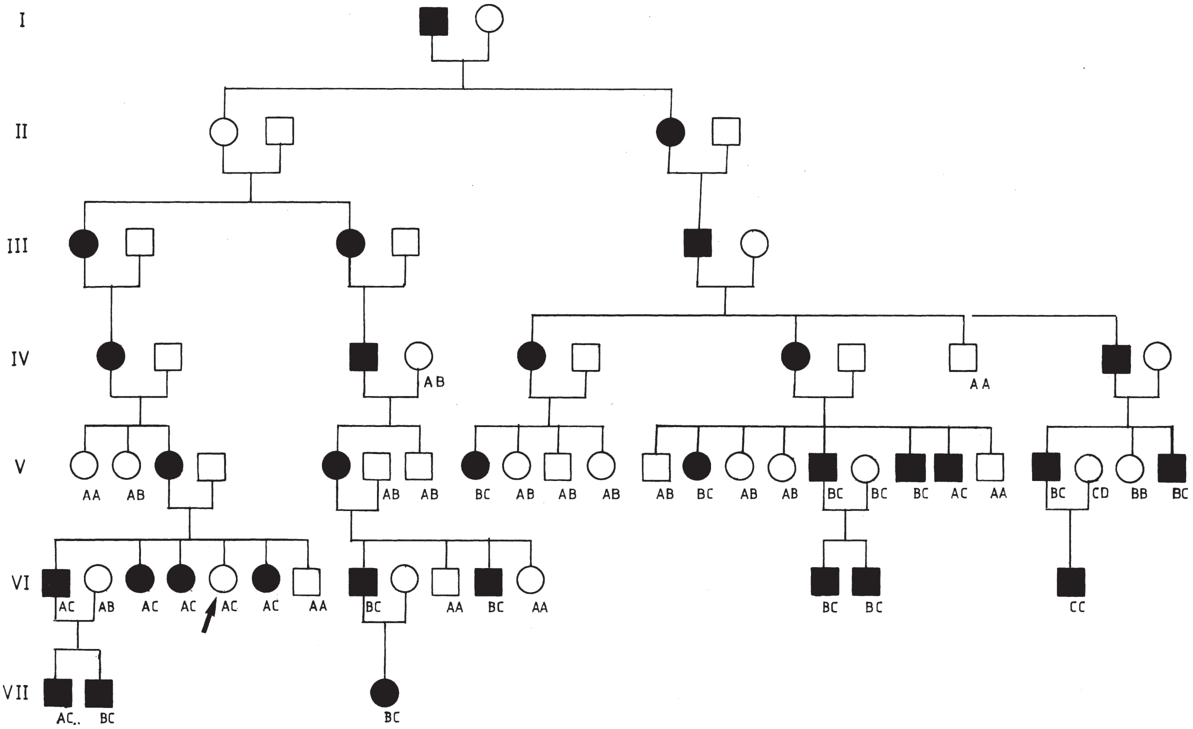

Fig. 6.2 Large pedigree from Venezuela with Huntington disease. *A, B, C, D*, four different “alleles” of a DNA polymorphism. The Huntington gene is transmitted together with “allele” C. One individual, VI,5 (*arrow*) has so far been unaffected. She will most likely be affected later (See text). From Gusella et al. [28]

The example of this pedigree shows that the chromosomal phase of alleles at two loci (*cis* or *trans*) can often be ascertained with great precision even in one large pedigree, and that recombinants can be identified if (at least) three generations are available for analysis, and if there are many sibs.

6.1.2.2 Statistical Analysis

In most cases linkage analysis is more difficult. Extensive pedigrees such as that in Fig. 6.2 are exceptional; most available families consist of two parents and their children. Here the problem is that the chromosomal phase is usually unknown: a double heterozygote may be AB/ab *(cis)* or Ab/aB *(trans)*. When the alleles are randomly distributed in the population (linkage equilibrium), the two types are expected in about equal frequencies: an AB/ab person forms germ cells in the ratio:

AB	Ab	aB	ab
$\frac{1-\Theta}{2}$	$\frac{\Theta}{2}$	$\frac{\Theta}{2}$	$\frac{1-\Theta}{2}$

whereas a heterozygote Ab/aB forms germ cells in the ratio:

AB	Ab	aB	ab
$\frac{\Theta}{2}$	$\frac{1-\Theta}{2}$	$\frac{1-\Theta}{2}$	$\frac{\Theta}{2}$

Expectations for germ cells are then in any case:

AB	Ab	aB	ab
$\frac{1-\Theta}{2}$	$\frac{\Theta}{2}$	$\frac{\Theta}{2}$	$\frac{1-\Theta}{2}$

or

$\frac{\Theta}{2}$	$\frac{1-\Theta}{2}$	$\frac{1-\Theta}{2}$	$\frac{\Theta}{2}$

which adds up to:

$\frac{1}{4}$	$\frac{1}{4}$	$\frac{1}{4}$	$\frac{1}{4}$

irrespective of Θ. It even remains true if $\Theta=0$ (very close linkage).

All four types of germ cells occur with the same frequencies, regardless of the probability of recombination Θ. Linkage does not lead to any association of alleles A,B or a,b in the population (Exception: linkage disequilibrium; Sect. 6.2). Another criterion for linkage must be found, one that is independent of the phase of the double heterozygote.

Such a criterion would be the *distribution* of children within sibships. In mating of AB/ab persons (*cis* phase) most children show the allele combinations of their parents; in matings of Ab/aB (*trans* phase) most children show these alleles in a new combination. How can these deviations from random distribution within sibships be measured and used for establishing linkage and determining the probability of recombination? Bernstein in [6] was the first to develop such a method. It has now been replaced by the method of "logarithm of differences" (LOD) scores as developed by Haldane and Smith [31] and Morton [65–66] and is generally used to assess linkage. Its principle can be described as follows:

The probability P_2 that the observed family data conform to the behavior of two loci under full recombination without any linkage is calculated and similarly, the probability P_1 that the identical family data are the result of two linked loci under a specified recombination fraction (Θ) is estimated for various families. The ratio of these two probabilities is the likelihood ratio and expresses the odds for and against linkage. This ratio $\frac{P_1(F/\Theta)}{P_2(F/(^1/_2))}$ must be calculated for each family *F*.

A man may be doubly heterozygous for the gene pairs A,a and B,b. His wife may be homozygous for the two recessive alleles aa, bb. Assume that his two sons, as the father, are doubly heterozygous, i.e., they inherited the dominant alleles A and B from the father. This probability is $^1/_2 \times {}^1/_2 = {}^1/_4$ for each son if the genes segregate independently. If the gene loci are closely linked without crossing over, the probability for occurrence of this pedigree may be calculated as follows. Either the genes occur in coupling state: AB/ab, then the possibility for common transmission to each of the two sons is $^1/_2$ (transmission of the combination ab would also have a probability of $^1/_2$), or the genes occur in repulsion state Ab/aB, where transmission of both dominant alleles to the same son requires crossing over. With close linkage and in absence of crossing over the probability here of common transmission = 0. Hence, the total probability for transmission of the combination aB to either son is $^1/_2$ and the likelihood ratio is $P_1/P_2 = (^1/_2)/(^1/_4) = 2$ in favor of close linkage. Likelihood ratios for the various degree of loose linkage can be calculated in the same way.

For convenience the logarithm of the ratio is used, and a LOD score *z* (meaning "log odds" or "log probability ratio") is used:

$$z = \log_{10} \frac{P(F|\Theta)}{P(F|(^1/_2))} \qquad (6.1)$$

Here, $P\ (F\ |\Theta)$ denotes the probability of occurrence for a family F when the recombination fraction is Θ. Using the logarithms instead of the probabilities themselves has the advantage that the score of any newly found family can be added, giving a combined score $z = S\ z_{\mathrm{i}}$ for all families examined.

Equation (6.1) implies an identical recombination fraction for both sexes. Since sex differences in recombination rates have been described [82] (see below), the z score in actual data should be computed separately for the sexes:

$$z = \log_{10} \frac{P(F/\Theta,\Theta')}{P(F/(^1/_2,{}^1/_2))} \qquad (6.2)$$

where Θ is the recombination fraction in females and Θ' in males.

It follows from the definition of the likelihood ratio that the higher its numerator, the stronger is the deviation in the direction of linkage. In terms of logarithms the higher the z score, the better is the evidence for linkage. A LOD score of 3 or higher is generally considered as proof of linkage. Minor corrections for dominance and for ascertainment of pedigrees with rare traits but are not dealt with here [93].

The score $z(\Theta,\Theta')$ for the entire set of data is the sum of the scores of the separate families. For a first approach $\Theta = \Theta'$ is assumed to simplify the calculations. After linkage has been established, a possible sex difference can be looked for.

Numerous computer programs for detection and estimation of linkage are available (for example, see http://linkage.rockefeller.edu/soft, which lists multiple software tools for genetic linkage analysis of human pedigrees). They also allow for testing whether a part only of the observed families show linkage (=linkage heterogeneity). These programs permit to make optimal use of linkage information even in large and sometimes complicated pedigrees. For a detailed account of reasoning on linkage as well as methods of analysis, see Ott [74].

6.1.2.3 The Use of LOD Scores

The ideal mating for linkage studies involves a double heterozygote, i.e., a person heterozygous for two different traits, with a person homozygous for the two genes. The following types of families do not contribute information regarding linkage:

(a) Families in which neither parent is doubly heterozygous
(b) Families in which there cannot be any observable segregation
(c) Families in which the phases of the parents are unknown and there is only one examined child

Most linkage studies involve analysis of two common markers or of a common gene with a gene for a rare genetic disease. Opportunities to study linkage between two rare genes hardly ever exist. The ideal family for linkage studies is a kindred with at least three generations, many matings, and a large number of offspring. Such families are becoming rare in Western societies. An alternative approach involves testing of many small families. This may even have an advantage if more than one gene locus causes a special phenotype. In these instances the study of a single, large pedigree with linkage may create the impression that this gene locus is the only one whose mutations cause the phenotype in question, whereas analysis of many, smaller pedigrees may point to other loci as well, and hence to genetic heterogeneity.

When linkage has been established and a maximum likelihood estimate of Θ achieved, the question of heterogeneity should be examined. If, for example, linkage between the locus for a genetic polymorphism and a rare dominant condition has been established, linkage analysis can help to prove genetic heterogeneity if only part of the family data shows linkage. This occurs very often [61]; the statistical problem posed by such a situation is tricky. Ott [72] has proposed using the χ^2 statistic to compare hypotheses: linkage without heterogeneity vs. nonlinkage, linkage with heterogeneity vs. linkage without heterogeneity, and linkage with heterogeneity vs. nonlinkage. It is also possible to estimate the proportion of families showing linkage in the data set studied.

The human genome is so saturated with genetic markers that one can estimate linkage not only for two loci but for several markers at once (multipoint linkage). Appropriate computer programs for linkage analysis, for example, the LINKAGE package (http://linkage.rockefeller.edu/), have proven to be very useful. In fact, linkage analysis is now often performed using SNP arrays covering several hundred thousand markers which can easily be analyzed with other software tools such as dChip (http://www.biostat.harvard.edu/complab/dchip/).

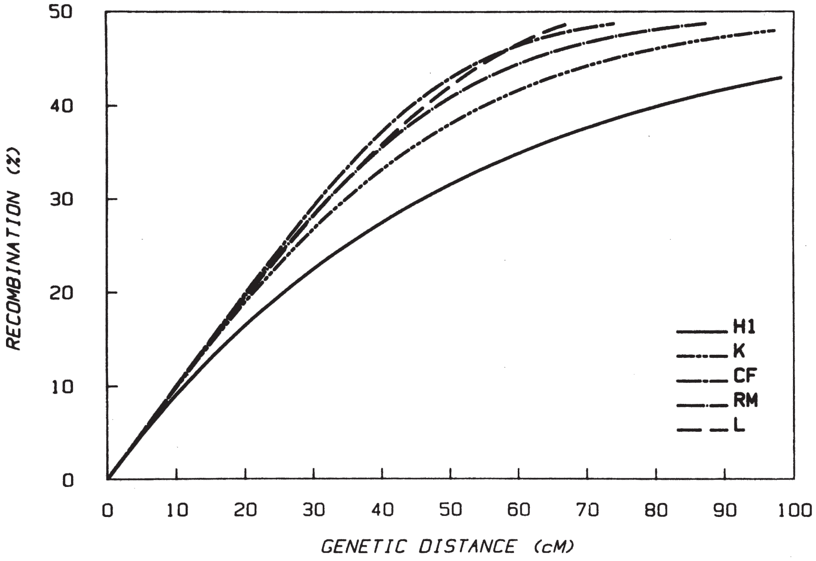

Fig. 6.3 Genetic distance (in centimorgans) in relation to the recombination fraction (in percentage), according to estimates from various authors. *H1* Haldane function with no interference; *K* Kosambi; *CF* Carter and Falconer; *RM* Rao and Morton; *L* Ludwig. From White and Lalouel [110]

6.1.2.4 Recombination Probabilities and Map Distances

Once a number of linkages have been established, the next step is to estimate map distances between these loci. These distances are expressed in morgans and centimorgans, 1 cM (map unit) meaning 1% recombination ($\Theta=0.01$) for small map distances. For larger distances this value must be corrected for double crossing over. Various methods have been proposed. Given a recombination frequency Θ, the map distance (cm) can be read directly from Fig. 6.3.

6.1.2.5 The Sib Pair Method

The use of LOD scores is the ideal method if the mode of inheritance of the two traits to be tested for linkage has been established. Examples include testing of linkage for two genetic markers or for a marker and a clearcut monogenic disease. At least two generations should be available. The analysis becomes more difficult if penetrance of the mutant gene is incomplete, and a definitive phenotype cannot be assigned. While inclusion of a penetrance term in the analysis may be possible, introduction of this and other adjustments can be hazardous since it may lead to false claims, particularly if the data are manipulated in various ways until a "positive" linkage result is obtained.

In general, if the mode of inheritance cannot be established, or when data from only one generation are available, it is preferable to use the sib method first suggested by Penrose in the 1930s [76] (see also [7, 82–84, pp. 90–92]; Fig. 6.4). Its rediscovery has been called by Ott [73] "the cutting of the Gordian knot." (Alexander the Great was challenged to disentangle this knot, which no one had been able to do previously; he cut through it with his sword.) This is because the detection of linkage with this method does not depend on correct assignment of the mode of inheritance but only on the influence of a gene that contributes nonnegligibly to the trait and on linkage of this gene with a marker. Such approaches are termed "nonparametric." Penrose explains, "The method is based on the principle that, when pairs of sibs are taken at random, certain types of sibling pairs will be more frequent if there is linkage than if there is free assortment of the characters studied." The method is used as follows: Codominant genetic markers with several alleles are studied in a series of sib pairs (or other pairs of relatives) both of whom are affected with a disease whose linkage relationship to the marker is to be investigated. If there is *no* linkage to the marker, 25% of affected sib pairs share both maternal and paternal alleles of the

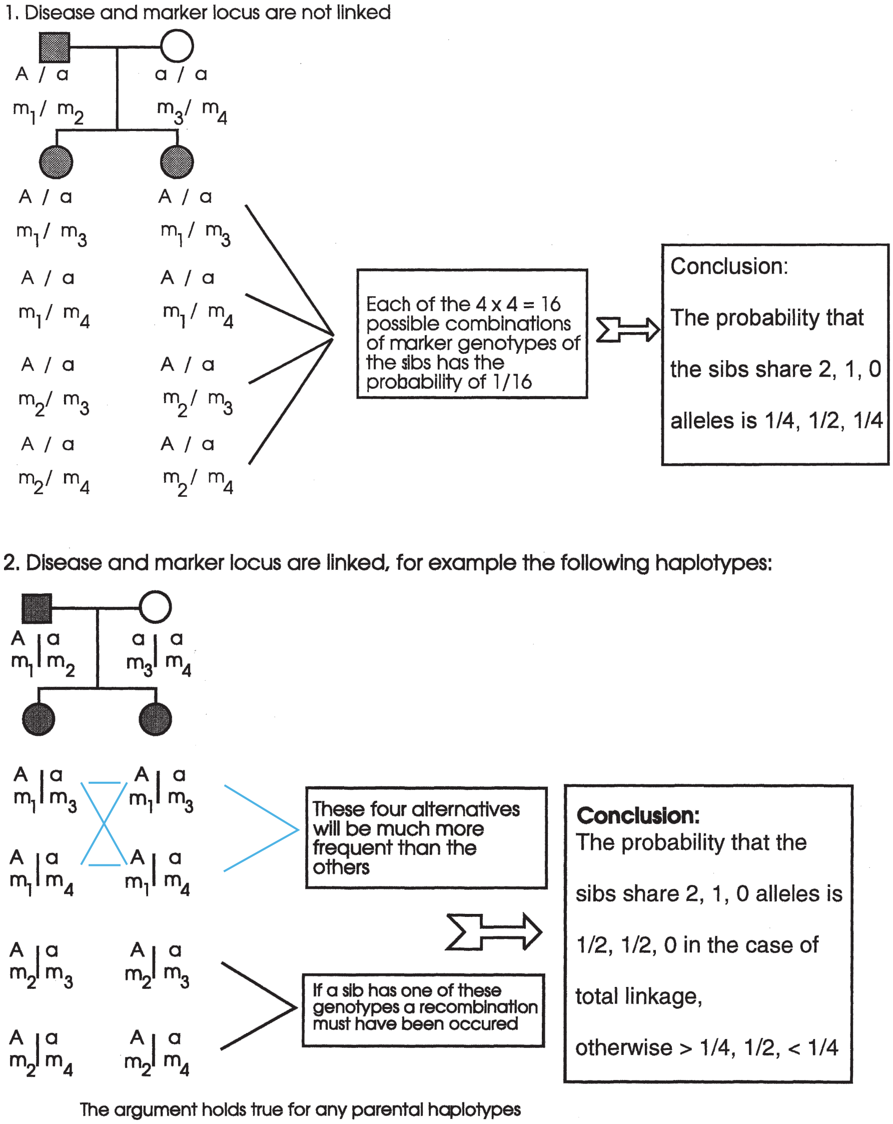
1. Disease and marker locus are not linked
A / a
m1 / m2
a / a
m3 / m4
A / a m1 / m3
A / a m1 / m3
A / a m1 / m4
A / a m1 / m4
A / a m2 / m3
A / a m2 / m3
A / a m2 / m4
A / a m2 / m4
Each of the 4 x 4 = 16 possible combinations of marker genotypes of the sibs has the probability of 1/16
Conclusion:
The probability that the sibs share 2, 1, 0 alleles is 1/4, 1/2, 1/4
2. Disease and marker locus are linked, for example the following haplotypes:
A | a m1 | m2
a | a m3 | m4
A | a m1 | m3
A | a m1 | m3
A | a m1 | m4
A | a m1 | m4
A | a m2 | m3
A | a m2 | m3
A | a m2 | m4
A | a m2 | m4
These four alternatives will be much more frequent than the others
If a sib has one of these genotypes a recombination must have been occured
Conclusion:
The probability that the sibs share 2, 1, 0 alleles is 1/2, 1/2, 0 in the case of total linkage,
otherwise > 1/4, 1/2, < 1/4
The argument holds true for any parental haplotypes

Fig. 6.4 Principle of the sib pair method for finding linkage. Assume a mating between a parent heterozygous for a dominant disease allele A and a parent homozygous for a normal allele a; each of the four parental chromosomes have different marker alleles at a marker locus. The possible alternatives are presented. Blue bars connect the more common sib pairs

marker, 50% share one of the marker alleles, and 25% differ for both marker alleles. If the marker is linked to a gene that contributes to causing the disease, the proportion of sib pairs with the disease who share one or two marker alleles is increased over the expected 25% or 50%, respectively.

The problem is straightforward when the marker status of both parents is known, and the identity by descent of the marker can be established. However, the method can also be used, albeit with more difficulty, if only marker information on the affected sib pairs is available, and the parents are not investigated (see also [13]). Problems arise here if the affected parent is homozygous for the marker, or if the unaffected parent contributes a marker allele to the child that is identical to the marker that cosegregates with the disease gene [67]. This problem can be avoided in either of two ways: (a) *Unaffected* sib pairs in the same families can be studied. Such pairs are expected to be more similar in the alternative marker alleles. However, the disease must have 100% penetrance so that there is certainty that an unaffected sib really does not carry the disease gene. (b) Another approach is to study haplotypes of several closely linked markers or of multiallelic variable-number tandem repeats (VNTRs) or microsatellites rather than of only a single restriction fragment length polymorphism (RFLP) marker. Under these conditions each parent often has a unique haplotype at the site under study with four haplotypes between the two parents. A child inherits only two haplotypes, one from each parent. With no linkage 25% of sib pairs share two haplotypes, 50% are identical for one, and 25% for none. With linkage, statistically significant increases over the 25% and 50% proportions of shared haplotypes are obtained.

This method has been adapted for pairs of relatives other than sibs as well; computer programs for testing linkage and estimating map distances are available (http://linkage.rockefeller.edu/soft). Risch and Merikangas have shown that the sib pair methods have relatively low power to detect loci with moderate odds ratios [85].

Haplotype analysis is especially useful if general circumstances favor the view that all patients suffer from a genetic disease which can be traced back to one single mutation. (See the discussion of such "founder effects" in Sect. 17.1.2) Here, the time since this mutation occurred may not have been sufficient to randomize marker loci around this mutation by repeated crossing over; it may still exist within the same haplotype in most instances. If the mode of inheritance is autosomal-recessive, patients may be homozygous for this haplotype. In this way, the autosomal-recessive gene for benign recurrent intrahepatic cholestasis (BRIC), an autosomal-recessive condition occurring in the Tyrolian Alps, was mapped to chromosome 18. If its main precondition – evidence for a founder effect – is met, the method is very efficient statistically [35], and the gene can be identified from a single pedigree with a modest number of siblings.

6.1.2.6 Results for Autosomal Linkage, Sex Difference, and Parental Age

The first autosomal linkage in man was found by Mohr [64] between the Lutheran blood groups and the AB0 secretor locus. Some years later linkage between the Rh loci and elliptocytosis (166900) was established and used to detect genetic heterogeneity of elliptocytosis, since not all families with elliptocytosis showed this linkage. A short time later, linkage between the AB0 blood group locus and the dominant nail-patella syndrome (161200) locus was found. This linkage established for the first time a sex difference of recombination probabilities in humans: map distance between these loci was 8 cM for males and 14 cM for females. A great many linkages have since been examined for sex differences; in the majority of instances a higher recombination fraction has been observed in females than in males. The same sex difference had been known for a while in the mouse [80]. It conforms to Haldane's rule [30] that crossing over is generally more frequent in the homogametic than in the heterogametic sex. In humans, however, this rule has exceptions. In the distal portion of 11p, for example, the recombination frequency appears to be higher in males [111]. It appears that such a higher male recombination rate may be characteristic for chromosome parts close to telomeres. There is also good evidence that the absolute exchange rate is higher in chromosome parts close to the telomere [52, 110]. However, the overall recombination rate is definitely higher in females [22, 45]. In *Drosophila,* there is no crossing over at all in the male. Typing of individual sperm shows that the recombination rate in a specific region can be different between individuals [14, 45]. The mechanism controlling this variation in humans has remained elusive. However, recently sequence variants in the 4p16.3 region were identified, which correlated with recombination rates. Interestingly these variants were mapped to the *RNF212* gene. This gene is a putative ortholog of the *ZHP-3* gene that is essential for recombinations and chiasma formation in *Caenorhabditis elegans*. An intriguing finding was that the haplotype formed by two single-nucleotide polymorphisms (SNPs) was associated with the highest recombination rate in males whereas the same haplotype was associated with a low recombination rate in females [46].

There has been considerable discussion as to whether recombination frequency is also influenced by parental age. In the mouse, the data are consistent with decreasing recombination rates with aging in females and increasing rates in males. Weitkamp [109] found a significantly increased incidence of recombinants with increasing birth order in humans for eight closely linked pairs of loci, indicating a parental age effect. There was no difference between males and females for this effect. A similar parental age effect was found for the Lutheran/secretor and Lutheran/myotonic dystrophy (160900) pairs but not for AB0/nail-patella or Rh/PGD pairs.

In a survey of cytogenetically determined chiasma frequencies from 204 males reported in the literature, little or no linear trend with age was found [53]. No cytogenetic data are available for females. The discrepancy between formal recombination data and chiasma frequencies is unexplained [110].

6.1.2.7 Information from Chromosome Morphology

Pairs or clusters of autosomal loci found to be linked (linkage groups) could not be assigned to specific chromosomes by a formal methodology of family study. The first chromosomal localization was accomplished as follows [21, 81].

The long arms of chromosome 1 frequently show a secondary constriction close to the centromere. In about 0.5 % of the population, this constriction appears much thinner and longer than normal. The variant is dominantly inherited. An uncoiler locus (Un-l) appears to be localized on chromosome 1. Linkage studies show close linkage between the blood group Duffy locus and the Un-l trait; $\Theta=0.05$. Linkage between Duffy and congenital zonular cataract (116200) had been discovered earlier. Hence, a linkage group with three loci, cataract, Duffy, Un-l could be assigned [21].

Another possibility to localize genes on specific chromosomes was afforded by deletions. If a gene locus whose mutation has a dominant effect is lost by deletion, the absence of that gene may occasionally have a phenotype similar to the dominant mutation. More extensive symptoms may also be present, since more genetic material than a single gene would be expected to be lost. In 1963 a retarded child with bilateral retinoblastoma was found to have a deletion of the long arm of one D chromosome [56]. This chromosome was later identified as no. 13, and this 13q14 deletion has been found in a number of other cases with retinoblastoma and additional anomalies. Patients with retinoblastoma without additional symptoms usually have no deletion. The localization of this gene (RB1) has since been confirmed by DNA marker studies and the gene has been cloned [24, 55] (see Sect. 14.1.2).

Another approach, thought to be more generally useful, is the quantitative examination of enzyme activities in cases with chromosome anomalies. Most enzymes show a clearcut gene dose effect in heterozygotes, i.e., heterozygotes for an enzyme deficiency have approx. 50% of enzyme activity. Therefore a similar gene dose effect might be expected when a gene locus is localized on a chromosome segment that has been lost by deletion.

The results of many early studies of this sort proved disappointing. Later, however, an increasing number of such gene dosage effects have been described in vitro, on trisomic and monosomic cells [48] (Sect. 3.6). To mention only one example, the activity of the enzyme phosphoribosylglycinamide synthetase was studied in several cases of partial monosomy and full and partial trisomy 21, as earlier studies had suggested a gene dosage effect for this enzyme. In regular trisomy 21 an excess was found with a ratio of trisomy 21 to normal of 1.55. A ratio of 0.99 was found in 21q21 → 21pter monosomy; 0.54 in 21q22 → 21qter monosomy; 0.88 in 21q21 → 21pter trisomy; and 1.46 in 21q22.1 trisomy. Therefore the phosphoribosylglycinamide synthetase gene locus could be localized in subband 21q22.1 [15]. Utilization of variants in chromosome morphology (heteromorphisms), such as the secondary constriction on chromosome 1 mentioned above, along with gene dosage studies, slowly opened the way to linkage and gene localization. Another method, using cell fusion, has led to much more rapid progress.

6.1.3 Linkage Analysis in Humans: Cell Hybridization and DNA Techniques

6.1.3.1 First Observations on Cell Fusion

The history of cell fusion is related by Harris [33]. Binucleate cells were observed in 1838 by J. Mueller in tumors, and afterwards by Robin in bone marrow, by Rokitansky in tuberculous tissue, and by Virchow both in normal tissues and in inflammatory and neoplastic lesions. The view that some of these cells were produced by fusion of mononucleate cells derived from the work of de Bary in 1859, who observed that the life cycle of certain myxomycetes involves the fusion of single cells to form multinucleated plasmodia. The earliest reports of multinucleated cells in lesions that can be identified with certainty as being of viral origin appear to be those of Luginbuehl (1873) and Weigert (1874), who described such cells at the periphery of smallpox pustules.

Following the introduction of tissue culture methods, numerous observations were made on cell fusion in cultures of animal tissue (see [32]). Enders and Peebles (1954) found that the measles virus induces cells in tissue culture to fuse to form multinucleated syncytia. Okada (1958) showed that animal tumor cells in suspension can be fused rapidly to form multinucleated giant cells using high concentrations of hemagglutinating parainfluenza virus (Sendai virus).

In 1960 Barski, identified cells generated by spontaneous fusion in a mixed culture of two different but related mouse tumor cell lines. These cells contained the chromosome complements of both parent cells within a single nucleus. This phenomenon was then examined by Ephrussi et al., who concluded that not only closely related mouse cells could be hybridized; even larger genetic differences did not exclude spontaneous cell fusion. However, it soon became obvious that the frequency of spontaneous cell fusion is very low, and that many cell types never fuse spontaneously. Fusion frequency must be increased in some manner. Furthermore, isolation of hybrid cells was possible only when culture conditions gave these cells a selective advantage.

Both problems were soon solved. Littlefield (1964) isolated the rare products of spontaneous fusion in mixed cultures by a technique adopted from microbial genetics. Fusion of two cells deficient in two different enzymes resulted in hybrids that recovered the complete enzyme set by complementation. Only these cells survived selection against the deficient cells.

Harris and Watkins [33] enhanced the fusion rate of various cells by treatment with UV-inactivated Sendai virus. Along with introduction of this method, they showed that fusion can be induced between cells from widely different species, and that the fused cells are viable. With this work, widespread use of the cell fusion method in various branches of cell biology began.

6.1.3.2 First Observation of Chromosome Loss in Human–Mouse Cell Hybrids and First Assignment of a Gene Locus

Weiss and Green [108] fused a stable, aneuploid mouse cell line, a subline of mouse L cells, with a diploid strain of human embryonic fibroblasts. The mouse cell line was deficient in the thymidine kinase (TK) locus and did not grow in hypoxanthine-aminopterin-thymidine (HAT) medium, a culture medium selective for cells containing the human TK locus (188300).

Cultures were initiated by mixing the two types of cells and growing them on standard medium. After 4 days cultures were placed in the selective HAT medium. This led to degeneration of the mouse cells, leaving a single layer of human cells. After 14–21 days hybrid colonies could be detected growing on the human cell monolayer. A number of these colonies were then isolated, grown for a longer time period, and examined. They turned out to maintain the mouse chromosome complement, but 75–95% of the human chromosomes were lost. One human chromosome, however, was present in almost all cells growing in the HAT medium. This suggested that the locus for thymidine kinase is localized on this chromosome. Therefore control experiments were carried out with a bromodeoxyuridine-containing medium. Bromodeoxyuridine, a base analogue for thymine, is accepted by TK in place of thymine and selects against cells containing this enzyme. A special chromosome described as "having a distinctive appearance" was present in almost all HAT cultures but in none of the bromodeoxyuridine cultures. It was concluded that the TK locus is indeed localized to this chromosome. Shortly thereafter the chromosome bearing the TK locus was found to be no. 17 [63] (Fig. 6.5).

This work led to two principles which were later decisive for the use of cell hybridization in linkage work:

1. Hybrids between mouse and human cells tend to lose many human chromosomes. It was later shown that this loss is random, and therefore examining a great number of hybrids one can expect to find a cell that has kept any one specified chromosome.
2. By using an appropriate selective system it is possible to select cells with a certain enzyme activity and to localize the gene loci specifying this enzyme to a specific chromosome.

Whereas genetics has historically been the science of genetic variability within a species, the hybridization method permits the localization of genes that do not show genetic variability in humans, provided only that the gene products of the human and nonhuman cells can be identified. One means of identification is the use of a selective system.

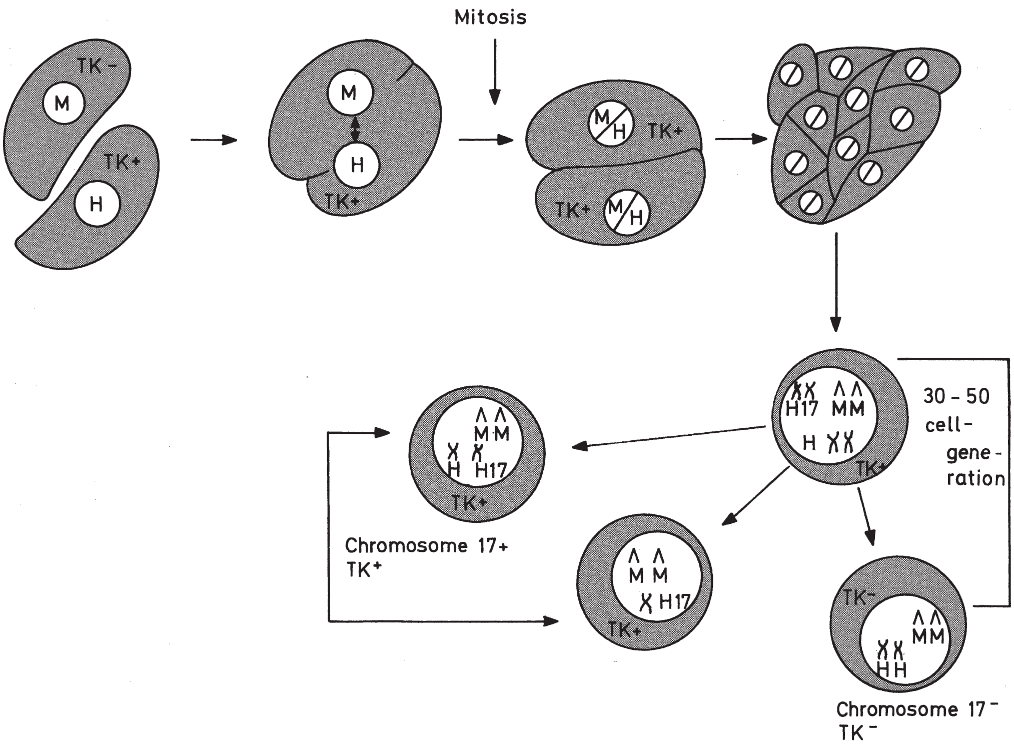

Fig. 6.5 The principle of gene localization on an autosome. Thymidine kinase deficient mouse cells (*M, TK*⁻) are grown in mixed cell culture with normal human cells (*H, TK*⁺). The cells are fused spontaneously, chemically, or by Sendai virus. After 30–50 cell generations the cells have lost part of their human chromosomes. Only cells having kept chromosome 17 show thymidine kinase activity (two cells at *left*). Cells without chromosome 17 show no *TK* activity (cell at *bottom right*)

Since 1967 selective systems have been developed for several enzymes. One example uses the hypoxanthine phosphoribosyltransferase locus on the X chromosome. This system can be used for selection not only of other X-linked loci but also of autosomal loci if a part of the autosome has undergone translocation with the X chromosome. It is also possible to assign loci for which no selective system exists, provided that enzymes produced by the two species have recognizable differences such as electrophoretic variation.

In hybrid cultures chromosome breakage and rearrangement are relatively frequent events. This chromosomal behavior made possible the suitable selection of hybrid clones containing identifiable parts of chromosomes, thereby combining the advantages of deletion mapping and hybridization.

Later the use of irradiation of the donor chromosome led to the development of radiation hybrids. These panels contain many chromosome fragments and were used to map genes and RFLP- and PCR-based markers more precisely [18, 54].

6.1.3.3 Other Sources of Information for Gene Localization

In situ hybridization technologies have also been instrumental for the localization of genes on chromosomes (see Sect. 3.4.4).

6.1.3.4 DNA Polymorphisms and Gene Assignment

The detection of a large number of DNA restriction site polymorphisms and other DNA markers opened up an additional approach to mapping of the human genome. In addition to RFLPs, other markers have been detected, among them the minisatellites [40], short DNA sequences distributed in great number over the human genome and occurring with variable numbers of repeats; this number is different in almost every individual. Therefore the information content for linkage studies is very high. Another such system, that of so-called microsatellites, consists of $(CA)_n$ repeats that occur in great numbers in the genome; the number of repeats per unit is extremely variable, as well. Localization of individual $(CA)_n$ probes in the genome has been achieved by identifying and using specific DNA sequences on both sides of these markers that allows amplification by the polymerase chain reaction (PCR). Genes for many important hereditary diseases could be localized on specific chromosomal sites using such markers.

Immortalized lymphoblastoid cell lines from large three-generation families with known genotypes for many marker loci are available for the study of new markers [111]. These pedigrees, the CEPH families, consist of samples collected in Salt Lake City, Utah, France, and Venezuela [19, 22]. The CEPH families (both parents and a great number of children) have been typed with many genetic markers. DNA from such families is available to the scientific community for further mapping. With increasing numbers of available markers, analysis of linkage relationships not only between two gene loci (e.g., one disease gene and one marker) but also between a disease gene and a set of markers – a haplotype – is routinely carried out. Such haplotypes are used in research studies as well as in genetic counseling and prenatal diagnosis. The proportion of kindreds in which the combination of genotypes is informative for linkage – and hence for prenatal diagnosis – can be enhanced appreciably by using such sets of markers.

6.1.3.5 Gene Symbols to Be Used

In cooperative scientific activities such as mapping of the human genome, certain terminological conventions are necessary. Human gene names are now assigned by the Human Genome Organization (HUGO) gene nomenclature committee. The rules for gene symbols include: only uppercase letters, no hyphens, no more than four or five letters or numbers, etc. For details see http://www.gene.ucl.ac.uk/nomenclature. Standardized nomenclature to describe mutations and genetic variants has also been established [3].

6.1.3.6 Linkage of X-Linked Gene Loci

Assignment of loci to the X chromosome is straightforward when the pedigrees show the typical pattern of X-linked inheritance. The X chromosome is the chromosome most completely saturated with disease loci. Well-known genes for human diseases have been localized. But even with these localizations many X-linked genes need to be mapped; for example, it is estimated that up to 260 genes causing syndromic and nonsydromic X-linked mental retardation (XLMR) may exist. The most common known cause of XLMR is an expansion of a trinucleotide repeat in *FMR1*. Large-scale systematic resequencing of the coding exons of the X chromosome in males with XLMR now allow the identification even of rare, disease-causing sequence variants [101].

6.1.3.7 Genetic and Physical Map of the Homologous Segment of X and Y Chromosomes

When genetic maps of a chromosome were determined, these were complemented by a physical map. The final goal was to identify the DNA sequence of all genes within this area. The following presents an introduction into these methods using the pseudoautosomal region of Xp and Yp as examples.

This region is located in the Giemsa light band Xp22.3 and in Yp11.32 (see Sect. 3.6.3.2). Various authors constructed partial physical maps; and the region comprises about 2,560 kb in its entire length [79]. There is a certain interindividual length variation. A number of families from the CEPH family pool [19] were studied with 11 exactly localized DNA markers; this established very high recombination frequencies in males and lower but clearly elevated frequencies in females. In males, the genetic map is 55 cM long and in females 8–9 cM. Hence the sex difference in this case is opposite to Haldane's rule. Moreover, it is about 20–25 times higher than the average of all chromosomes in the male and 6 times higher in the female. Differences in recombination rates between chromosome regions have also been demonstrated for other chromosomes.

The first step of physical mapping often involved restriction of this area with "rare cutter enzymes" which cut DNA into regions with many CpG islands [49]. As a rule, CpG islands indicate the presence of genes [87]. Closer scrutiny of this terminal area by chromosome jumping revealed at least five regions in which the CpG islands are concentrated. Further analysis, by, constructing of a contig of YAC clones, allowed the first-generation maps to be generated.

6.1.3.8 The Y Chromosome

It is the Y chromosome [105, 113] which determines male sex. Genetic analyses succeeded in localizing specific factors involved in sex determination to certain segments of this chromosome. As in many other instances, analysis of pathological conditions has contributed to understanding of the normal state, such as the study of men with two X but apparently no Y chromosome. As early as 1966, Ferguson-Smith [23] postulated XY translocations which were expected to transfer to the X a small – but for male development decisive – part of the Y chromosome. This expectation has been confirmed by many observations [113]. Since meiotic pairing of X and Y chromosomes occurs in the pseudoautosomal and in adjacent nonhomologous regions, and since pairing errors provide a plausible mechanism for such translocations, the search for the testis-determining factor (TDF) soon concentrated on the short arm. Here the *SRY* (sex reversal gene on Y) gene was finally identified [91]. The mechanism of *SRY* action has been elusive. Recent discoveries shed some light on the role of *SRY*, which is thought to act synergistically with SF1, a nuclear receptor, through an enhancer of *SOX9* to promote Sertoli cell differentiation. *SOX9* is probably the pivotal factor in regulating the gene activity that defines Sertoli cells. Both *SOX9* and SF1 synergize to activate transcription of several downstream genes [88].

The Y chromosome is now known to contain 89 protein-coding genes and at least 27 distinct proteins [92]; www.ensembl.org]. A region within the euchromatic segment of the long arm appears to be important for normal spermatogenesis, since deletions within this region lead to arrested sperm formation either in an early stage, i.e., not even functional spermatogonia are formed, or in postmeiotic stages [105]. The first discovered deletions were so large that they could be recognized by cytogenetic methods [102]. Small deletions were later identified by molecular techniques [105], and their recognition has become important for differential diagnosis of male infertility.

In addition to genes involved in testis development and spermatogenesis, the Y chromosome harbors genes encoding transcription factors, initiation factors, ribosomal proteins, and kinases. Comparisons of the Y chromosomes of human and chimp have provided unique insight into the evolution of Y-chromosome genes [36].

6.1.3.9 DNA Variants in Linkage

The HapMap project has set out to characterize the majority of the SNPs in the human genome and has led to the identification of over 3 million variants [37]. The large number of DNA polymorphisms provide many new markers, and most linkage work is now being carried out with DNA variants often on arrays with 100,000 to 1 million SNPs [59] (Sect. 4.4.2).

Linkage disequilibrium (i.e., failure to demonstrate free assortment; see Sect. 6.2) has frequently been found between the sites of various markers at a given locus. Since these sites are physically very close, crossovers between them are rare, and many generations must pass before linkage equilibrium is reached. Furthermore, current data suggest that recombination rates at closely linked markers may vary considerably between different chromosomal locations. Thus, both "hot" and "cold" spots of recombination appear to exist [26, 68, 69].

6.1.3.10 Practical Application of Results from Linkage Studies

In the past the main interest of linkage studies was theoretical. Practical applications, however, are frequently employed. If, for example, gene A causes a rare hereditary disease manifesting itself later in life, and B is a genetic marker closely linked to A and segregating in the same family, the disease was predicted in a prenatal sample or young individual, and this prediction used in genetic counseling (Sect. 25). Today genetic diagnosis is routinely performed by direct study of the mutant gene itself.

6.1.4 Biology and Statistics of Positional Cloning

For disease loci localized by linkage analysis identifying the gene and mutations involved is termed "positional cloning" [17]. This process involves identifying genes in the interval defined by linkage, and analyzing affected individuals to identify mutations. This process can proceed very rapidly if a gene of obvious biological interest is identified in the interval, and the mutations readily identified. However, the process can be very time consuming if the interval is large, or the disease gene small or poorly expressed, or if there are few affected individuals for mutation screening. The cystic fibrosis gene was localized to an interval now know to be 1.4 Mb in size [106, 112]. It took over 4 years of intense effort to positionally clone the cystic fibrosis gene [86]. With the completion of the sequence of the human genome, this process is greatly aided as most of the genes in an interval are known. Therefore an investigator can sequence all of the coding exons of the positional candidate genes to identify mutations.

To date, most of the genes responsible for the common Mendelian disorders have been identified. However, identification of genes in less common Mendelian disorders with the aforementioned tools will continue to be important [2]. Nowadays, positional cloning is more often applied to complex diseases. This process is similar – a genetic interval is defined via sib pair linkage or whole-genome association and the relevant gene and "disease-causing variant" need to be identified [20]. This process has resulted in identifying genes for such complex diseases as macular degeneration, diabetes, Parkinson disease, obesity, and others. However, many gene haplotypes associated with common disease do not have coding sequence variants, and regulatory effects are proposed.

6.2 Gene Loci Located Close to Each Other and Having Related Functions

6.2.1 Some Phenomena Observedin Experimental Genetics

6.2.1.1 Closely Linked Loci May Show a Cis-Trans Effect

When series of multiple alleles were analyzed in *Drosophila,* crossing over within these series was observed occasionally, indicating that what had been considered as one "gene" can be subdivided by genetic recombination. Such alleles were termed "pseudoalleles" by McClintock in [60]. In some a so-called *cis-trans* effect was shown. When two mutations were located side by side on the same chromosome (*cis* position), the animal was phenotypically normal, but when they were localized on homologous chromosomes (*trans* position), a phenotypic anomaly was seen [58].

6.2.1.2 Explanation in Terms of Molecular Biology

In fungi, bacteria, and phages, genetic recombination is normally observed within functional genes, i.e., DNA regions carrying information for one polypeptide chain. A *cis-trans* effect is now considered to be typical for two mutations that are not able to complement each other functionally, i.e., that are located within the same structural gene. Complementation between two mutations, by the same token, is regarded as an indication that these mutations are located in different functional genes. A gene has many mutational sites and may be subdivided by recombination. Complementation tests are often used to test genetic, biochemically characterized conditions for heterogeneity.

6.2.1.3 A Number of Genes May Be Closely Linked

Close linkage has frequently been described between mutations affecting closely related functions, which are perfectly able to complement each other functionally and show no *cis-trans* effect. In bacteria such as *E. coli,* gene loci for enzymes acting in one sequence have been found to be closely linked and arranged in the sequence of their metabolic pathway. Their activity is subject to a regulating mechanism by a common operator and promoter [44].

6.2.2 Some Observations in the Human Linkage Map

6.2.2.1 Types of Gene Clusters That Have Been Observed

The first impression when examining the human linkage map and DNA sequence is that while most loci are distributed fairly at random, there are a large number of clusters of closely related genes. Here are a few examples:

(a) The loci for human hemoglobins γ, δ, and β are closely linked.

(b) The immune globulin region comprises a number of loci responsible for synthesis of immunoglobulin chains. The same is true for genes of the T cell receptor (chromosome 14q11). The major histocompatibility complex (MHC) cluster including various components of complement on chromosome 6.

(c) No less than four gene loci involved in the glycolytic pathway are located on chromosome 1.

(d) A number of genes determining closely related enzymes are closely linked, for example, pancreatic and salivary amylase on chromosome 1, and guanylate kinase 1 and 2 on the same chromosome.

(e) The protan and deutan loci for red–green color blindness are located in the same cluster on the X chromosome.

6.2.2.2 Clusters Not Observed So Far

As mentioned above, functionally related genes in bacteria are frequently closely linked; they are subject to common control within an operon. One might predict that, in humans, such operons would also occur, but functionally related genes are rarely clustered. Two genes linked in the same operon in bacteria are those for galactose-1-phosphate uridyltransferase and galactokinase. In humans these genes are located on chromosomes 3 and 17, respectively. Similarly, the gene for G6PD is located on the X chromosome, and that for 6-PGD, the following enzyme in the shunt pathway, is situated on chromosome 1. Genes belonging to one gene family are sometimes but by no means always located close together. For genes involved in the immune system, including those for immunoglobulin synthesis, T cell receptors, and genes in the MHC system, this location has functional significance.

6.2.3 Why Do Gene Clusters Exist?

6.2.3.1 They Are Traces of Evolutionary History

In some cases clustering is simply left over from the evolutionary history of these genes. Early in evolution there was one locus for a given gene. Then gene duplication occurred and offered the opportunity of functional diversification [70]. The first duplication paved the way for further duplications due to unequal crossing over (Sect. 6.2.8) and hence for further functional specialization.

With no further chromosomal rearrangements the gene clusters remain closely linked. It is unknown whether in these cases close linkage is necessary for orderly function. While it may be so in some cases, this explanation is not needed to explain clustering. Evolutionary explanations are sufficient. For example, the red and green color vision genes appear to have arisen by gene duplication.

6.2.3.2 Duplication and Clustering May Be Used for Improvement of Function

The clustering of genes is without obvious functional significance. It would be surprising, however, if evolution were never to take advantage of this situation, combining products of such gene clusters to form higher functional units. This may be the case for the hemoglobin molecule since in the β cluster the ε, γ, β, and δ genes are arranged in the sequence of their successive activation during individual development (Sect. 11.3). In the immunoglobulins and T cell receptors close linkage of a number of genes, possibly a great many, has become important functionally, as their gene products combine to form various classes of functional molecules. In fact, segmental duplications in the human genome are selectively enriched for genes involved in immunity. In this respect, one of the most fascinating, recent discoveries was the identification of interindividual and interpopulation differences in the copy number of a segmental duplication encompassing the gene encoding CCL3L1 (MIP-1alphaP). This gene is a potent human immunodeficiency virus-1 (HIV-1)-suppressive chemokine and ligand for the HIV coreceptor CCR5. Individuals with a CCL3L1 copy number lower than the population average have a markedly enhanced HIV/acquired immunodeficiency syndrome (AIDS) susceptibility [27].

6.2.4 Blood Groups: Rh Complex (111700), Linkage Disequilibrium

The history of the rhesus blood types provides a fascinating illustration of how science develops. First, a new phenomenon was discovered. Scientists soon realized that it eludes explanation by conventional concepts. Then a long-lasting scientific controversy arose as to the most appropriate extension of these concepts. In this controversy, a new explanatory principle was created that survived the controversy in this special case, and that could be applied to an increasing number of other observations. Finally, the problem was solved, and the controversy ended – by new methods.

6.2.4.1 History

In 1939 Levine and Stetson [57] discovered a novel antibody in the serum of a woman who had just delivered a macerated stillborn child and had received blood transfusions from her AB0-compatible husband. Of 101 type 0 bloods only 21 showed a negative reaction with this antibody. There was no association with AB0, MN, or P systems.

The following year Landsteiner and Wiener [51], immunizing rabbits with the blood of rhesus monkeys, obtained an immune serum that gave positive reactions with the erythrocytes of 39 of 45 individuals. Later the antibodies were compared with those of Levine and Stetson and thought to give reactions with the same antigens. This was subsequently found to be not quite true, and now the antigen uncovered by the true anti-rhesus antibody is called LW –, in honor of Landsteiner and Wiener. Rh typing in humans is always carried out with sera of human origin, i.e., according to Levine and Stetson's observation. The following discussion relates only to reactions with these human sera.

The great practical importance of the rhesus system became apparent when transfusion accidents were traced to this antibody, and especially when erythroblastosis fetalis, a common hemolytic disease of the newborn, was explained by Rh-induced incompatibilities between mother and fetus. The red blood cells of about 85% of all whites give positive reactions; family examinations showed that Rh-positive individuals are homozygous Rh/Rh or heterozygous Rh/rh, whereas the rh-negative individuals are homozygous rh/rh.

In 1941 Wiener discovered a different antibody that reacted with the cells of 70% of all individuals and was independent of the basic Rh factor (Rh', according to Wiener). A third related factor was discovered in 1943. These three factors were found in all possible combinations with one another, and the combinations were inherited together. Wiener proposed the hypothesis that these serological "factors" are properties of "agglutinogens," and that these agglutinogens are determined by one allele each of a series of multiple alleles. The agglutinogens were thus thought to determine the factors in different combinations. This descriptive hypothesis is so general that it indeed explains all the complexities discovered later.

6.2.4.2 Fisher's Hypothesis of Two Closely Linked Loci

R.A. Fisher developed a more specific hypothesis. At that time another antibody had been detected, anti-Hr. In 1943 Fisher (see [78]) examined a tabulation prepared by Race, containing the data accumulated so far. He recognized that Rh' and Hr were complementary. All humans have either Rh', Hr, or both. Individuals with both antigens never transmit them together to the same child, and a child always receives one of the two. Fisher explained these findings by proposing one pair of alleles for the two antigens. The pair was named C/c. In analogy, an additional pair of alleles D/d was postulated for the original antigens Rh^{+} and rh^{-}, and a third pair of alleles for the third factor that had been discovered. To explain the genetic data close linkage between these three loci was assumed.

Fisher's hypothesis predicted discovery of the two missing complementary antigens d and e. This prediction was later fulfilled for e but not for d. In developing this hypothesis Fisher went one step further. In the British population, there are three classes of frequency of the Rh gene complexes (Figs. 6.6, 6.7). Fisher explained this finding by suggesting that the rare combinations could have originated from the more frequent ones by occasional crossing over. All four combinations of the less common class may have originated from occasional crossing over between the

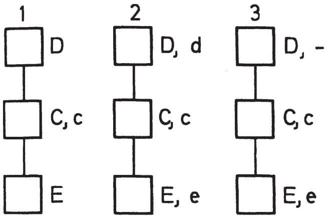

Fig. 6.6 A hypothetical structure of the Rh complex. *1,* On the basis of the evidence known in 1941; *2,* antigens predicted by Fisher and Race; *3,* antigens discovered; antigen *d* was not found

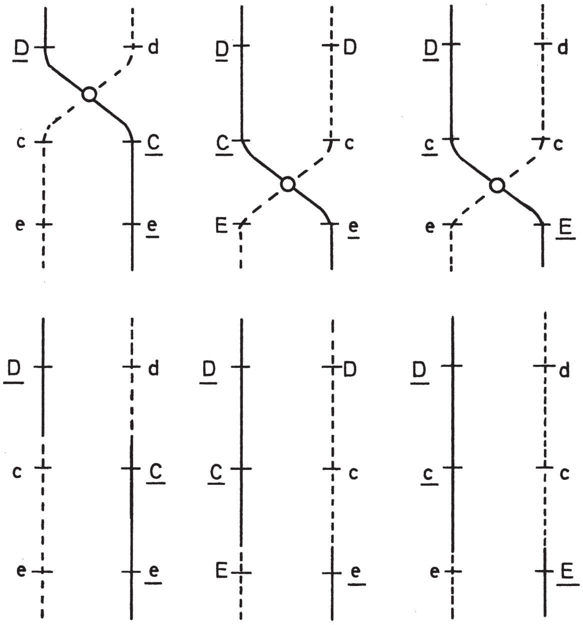

Fig. 6.7 Postulated production of three rare Rh haplotypes from the more common ones by crossing over. Each diagram refers to a different crossing over event. From Race and Sanger [78]

most frequent combinations; not, however, CdE. This complex needs inclusion of a second-order chromosome. Therefore the hypothesis explains why CdE is so rare. Still another prediction is possible. In every crossing over leading, on the one hand, to Cde, CDE, or cdE the complex cDe must also be produced. It follows that the frequency of the three former combinations together should equal the frequency of cDe. Frequencies actually found were: cDe, 0.0257; Cde+cdE+CDE, 0.0241 (in blacks, however, cDe has a high frequency).

Furthermore, Fisher believed the sequence of the three loci to be D-C-E, since cdE – which must have originated by crossing over between D and E from the genotype cDE/cde – is much more frequent in comparison with this genotype than Cde in relation to genotype CDe/cde (crossing over between C and D) and CDE in relation to CDe/cDE (crossing over between C and E).

6.2.4.3 Confirmation and Tentative Interpretation of the Sequential Order

Since Fisher's hypothesis many new observations have been made, the most important for the question of sequence being the combined antigens, for example, ce. These compound antigens were all compatible with the sequence D-C-E, whereas no such antigen suggesting close linkage between D/d and E/e has emerged. Fisher's hypothesis leads to two questions:

1. If the rare types have been formed by occasional crossing over from the more frequent ones, cases of crossing over should occasionally turn up in family studies. One such family has indeed been reported [97]: the father was CDe/cde, the mother cde/cde, four children were cde/cde, and three others CDe/cde, all in concordance with genetic theory. The sixth-born child, however, was Cde/cde. As the discrepancy involved father and child, it could be argued that the child was illegitimate. This, however, was made unlikely by blood and serum groups and by the fact that the family belonged to a religious sect with especially strict rules against adultery.
2. How should we envision the structure of the Rh gene(s) in the light of evidence from molecular genetics? There are two possibilities in principle:

   (a) The Rh complex is one gene with many mutational sites. Mutational changes are expressed as antigenic differences.

   (b) The Rh complex is composed of a number of closely linked genes, possibly three, and the main antigens reflect genetic variability at these genes. One important criterion is the *cis-trans* effect found in mutations affecting the same functional gene. As the ce compound antigen can be found in *cis*-phase CE/ce but not in *trans*-phase Ce/cE, Race and Sanger [77] tentatively concluded that C/c and E/e may be located within the same functional gene.

6.2.4.4 Molecular Basis

The Rh specificities have now been identified as membrane polypeptides. Molecular studies of the gene(s) have shown that in all D^+ individuals, two closely related Rh genes in each haploid genome appear to be present. One of these genes is missing in D^- individuals [16]. The authors concluded that one of the two genes controls the D polypeptide whereas the C/c and E/e specificities are coded by the second gene, a result confirmed by the molecular cloning [16, 107]. These observations explain at the molecular level why no anti-d serum has been found. They also confirm the sequence D-C-E postulated by Fisher, as well as the above-mentioned conclusion of Race and Sanger [77] that C/c and E/e appear to be located in the same gene product. Thus, both original hypotheses were partially correct: the specificities C,c,E,e are located within the same gene-determined protein, as postulated by Wiener (which does not exclude occasional intragenic crossing over); the D specificity, on the other hand, is located in a second, closely linked gene, as postulated by Fisher. Moreover, attempts at understanding genetics of the Rh system led to the development of a new concept by Fisher that has found widespread application in many fields of human genetics: linkage disequilibrium.

6.2.4.5 Blood Groups: Linkage Disequilibrium

Linkage normally does not lead to association between certain traits in the population (Sect. 6.1.1). Even if initially there is a nonrandom distribution of linkage phases, repeated crossing over randomizes the linkage groups, and in the end the coupling and repulsion phases for two linked loci are equally frequent. There is linkage equilibrium. However, when the population begins with a deviation from this equilibrium, for example, because two populations with different gene frequencies have merged, or because a new mutation has occurred on one chromosome, the time required to reach equilibrium depends on the closeness of linkage: the closer the linkage, the longer the time until equilibrium is reached [12]. It is never reached if certain types have a selective disadvantage.

A selective disadvantage for certain Rh complexes that could lead to their becoming less frequent has not been demonstrated so far; selection works against heterozygotes (Sect. 18.3.3), but this does not mean that a general disadvantage has never existed; neither has a conclusive explanation in terms of population history been postulated. Fisher's hypothesis, by answering some questions, has posed a number of others. However, the concept of linkage disequilibrium proved to be still more important in population genetics and in the genetic analysis of DNA polymorphisms (Sect. 2) and the major histocompatibility (MHC) complex:

6.2.5 Major Histocompatibility Complex [105, 111]

6.2.5.1 History

It had long been known that skin grafts from one individual to another (allotransplants) are usually rejected after a short time. In 1927 K. H. Bauer [5] observed that rejection does not occur when skin is transplanted from one monozygotic twin to the other (isotransplant). Such a transplant is accepted just as a transplant in the same individual (autotransplant). This showed the rejection reaction to be genetically determined. In the following years skin, and later kidney, transplantations between monozygotic twins were occasionally reported. Research on histocompatibility antigens in humans began only when leukocytes were shown to be useful as test cells.

Dausset observed in 1954 that some sera of polytransfused patients contain agglutinins against leukocytes. He later showed that sera from seven such patients agglutinated leukocytes from about 60 % of the French population, but not the leukocytes of the patients themselves. Twin and family investigations soon established that these isoantigens are genetically determined. Other isoantigens (now part of the HLA-B) were discovered by van Rood. Another important achievement was the microlymphocyte toxicity test introduced by Terasaki and McClelland in 1964, which is now the most frequently used method (Figs. 6.8 and 6.9). Subsequently the number of detected leukocyte antigens increased rapidly, and in 1965 it was suggested that most of these antigens were components of the same genetic system. At the histocompatibility workshop in 1967, 16 different teams typed identical samples from Italian families. Here the basic relationships among the different antigens were established. Finally, Kissmeyer-Nielsen [42] proposed the hypothesis of two closely linked loci (now A and B) controlling two series of alleles.

More recently, especially since the PCR technique became available, scientists study MHC genes directly at the DNA level. This has led to a splitting up of serologically defined gene loci, at both the class I and class II antigens (HLA D-DR; see Fig. 6.10) There are over 2,100 alleles described at the MHC locus with the HLA-B gene alone having 728 alleles (http://www.ebi.ac.uk/imgt/hla/stats.html), and several haplotypes have been completely sequenced [34].

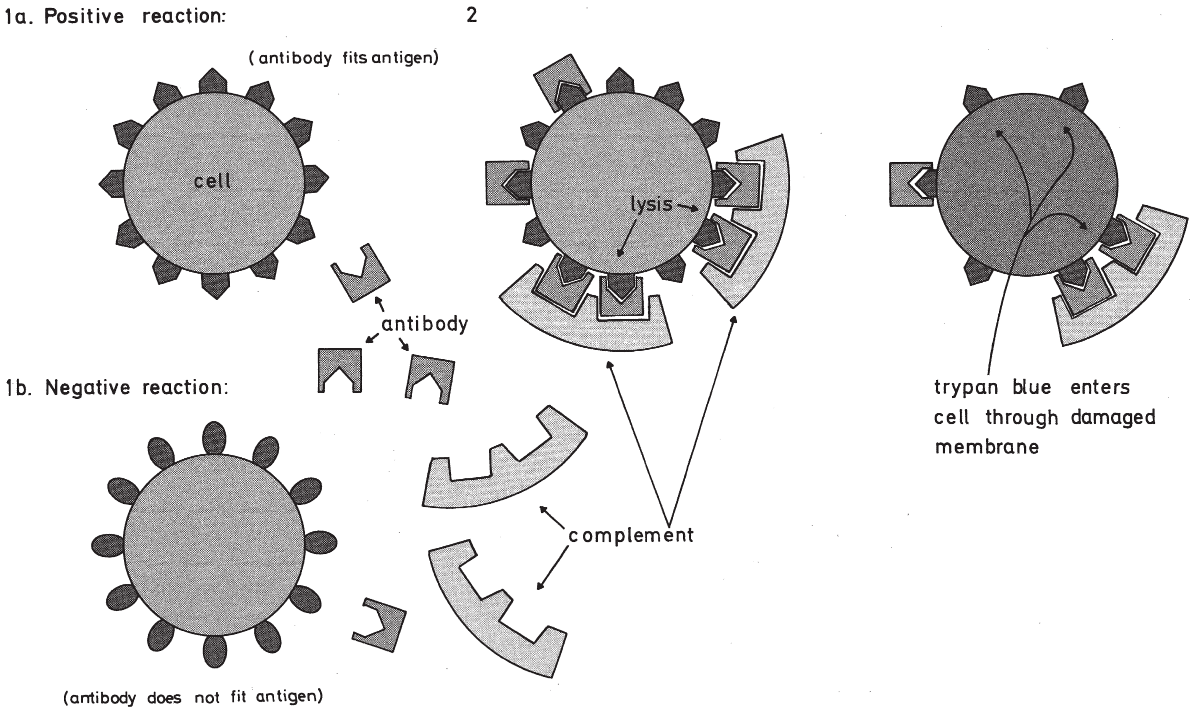

Fig. 6.8 Principle of the lymphocytotoxicity test: A cell having an appropriate antigen reacts with a specific antibody and complement. As a result, trypan blue enters the cell through the damaged membrane and indicates that the cell surface antigen has been recognized by a specific antibody

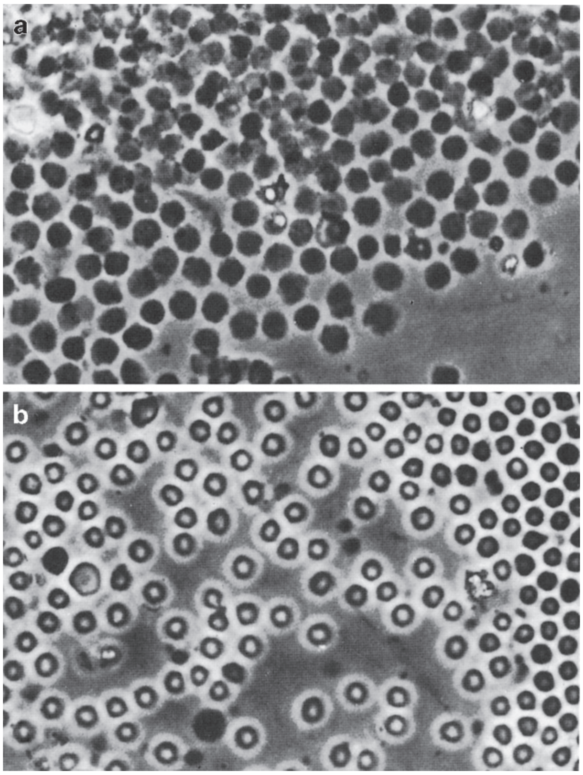

Fig. 6.9 (**a**, **b**) Lymphocytotoxicity test. (**a**) Positive reaction. (**b**) Negative reaction. Positive reaction is indicated by staining of the cells. (Courtesy of Dr. J. Greiner)

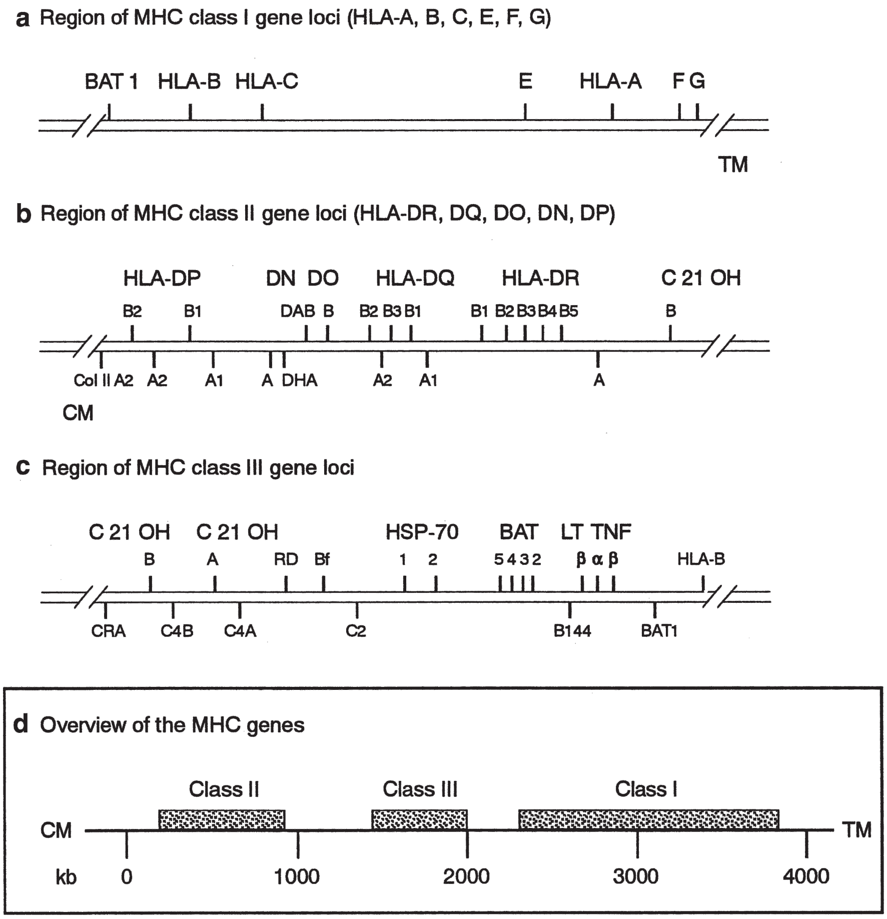
a Region of MHC class I gene loci (HLA-A, B, C, E, F, G)
BAT 1
HLA-B
HLA-C
E
HLA-A
F G
TM
b Region of MHC class II gene loci (HLA-DR, DQ, DO, DN, DP)
HLA-DP
DN DO
HLA-DQ
HLA-DR
C 21 OH
B2 B1
DAB B
B2 B3 B1
B1 B2 B3 B4 B5
B
Col II A2 A2 A1 A DHA A2 A1 A
CM
c Region of MHC class III gene loci
C 21 OH
C 21 OH
HSP-70
BAT
LT TNF
B A RD Bf 1 2 5 4 3 2 β α β HLA-B
CRA C4B C4A C2 B144 BAT1
d Overview of the MHC genes
Class II
Class III
Class I
CM
TM
kb 0 1000 2000 3000 4000

Fig. 6.10 (**a**–**d**) Sequence MHC class I, II, and III genes on the short arm of chromosome 6. (**a**–**c**) Detailed view of the three regions, together with their subregions. (**d**) Overview of approx. 4,000 kb. *TM,* Telomere; CM, centromere. *Letters, numbers, and their combinations,* the genes and their subregions. Genes of class III (HSP-70, DAT, C2/OH, CT) have no direct functional relationship with the immune response. From Albert [1]

6.2.5.2 Main Components of the MHC on Chromosome 6

The linkage group of the MHC is presented in Fig. 6.10. There are now three classes of MHC antigens. As revealed by studies using mainly molecular methods, each class can be subdivided into a great number of subclasses which are not described here (for details see [103]). Class I comprises the HLA-B, HLA-C, and HLA-A loci (in this order). In class II the HLA-D loci are found together with some other related, transcribed areas. Between these two classes a heterogeneous group of genes is located which have been named MHC class III despite the fact that at least some of these genes, such as those for 21-hydroxylase appear to have no functional relationship to the MHC system, and many non-HLA genes are located between the class I genes.

The function of this system has been elucidated; it plays an important role in the immune response. Here only some genetic aspects are considered.

The concept of four series of alleles is based on the following lines of evidence:

(a) No individual possesses more than two antigens from any of the series.
(b) Recombination between these series has been observed, for example, between the loci for A and B series, 40 crossovers among 4,614 meiotic divisions were described up to 1975, giving a combined (♀+♂) recombination frequency of 40/4614 = 0.0087 = 0.87 cM. Ten A-B recombinants

informative for the C series have been reported. In eight of these the C antigen followed B, and in two it followed A. Therefore C is located between A and B, closer to B, a fact confirmed by molecular studies.

(c) When two antigens from the same series are present together in a parent, he or she always transmits one of them – never both or none – to the child. The segregation ratio is 0.5, corresponding to a simple codominant mode of inheritance.

(d) Hardy–Weinberg proportions have been demonstrated for each of the allele series separately in large population samples.

(e) Serological cross reactions occur almost exclusively within the series, not between them. This points to a close biochemical relationship of the antigens within a given series.

(f) Complete sequencing of several MHC haplotypes confirms the presence of four class I genes.

6.2.5.3 Complement Components

Complement consists of a series of at least ten different factors present in fresh serum. The factors are called C1, C2, C3, etc., and C1 is activated by antibodies that react to their corresponding antigens. Then C1 activates C4, this activates C2, and so on. The end result of this "complement cascade" is damage to the cell membrane carrying the antigen and often lysis of the cell. Moreover, activated complement components have a number of other biological properties, such as chemotaxis or histamine release. They are important immune mediators in the body's defense against microbial infection.

The complement system can be activated not only via C1 (the classic pathway) but also via C3 through an alternative pathway involving the "properdin factors." The factor B(BF) acts as "proactivator" for C3.

For some of the complement factors hereditary deficiencies have been described, and polymorphisms are known. BF, C2, C3, and C4, are polymorphic. The loci of C2 and C4 A and B are in class III, together with the properdin factor B with the main alleles BF^F and BF^S. The locus for C3, on the other hand, is located on chromosome 19. Several regulatory factors such as complement factors H and I (CFH, CFI) are also located on autosomes [12, 90].

6.2.5.4 Significance of HLA in Transplantation

One of the main motives for rapid development of our knowledge of HLA antigens has been the hope of improving the survival rate of transplanted organs, primarily kidneys. Indeed, kidneys from HLA-identical and AB0-compatible siblings have a survival rate in the recipient almost equaling that of monozygotic twins. The survival rate is worse in unrelated recipients even if HLA matching is as perfect as possible, and AB0 compatibility is secured. This shows that, apart from the major histocompatibility system – the HLA system – there must be other systems of importance for graft survival. This is not surprising. A great number of such systems are known in the mouse. These systems lead to host-versus-graft reactions in almost all transplantations (Fig. 6.11). These reactions can be managed by immunosuppressive therapy. The chances for survival, and the survival times, of transplanted kidneys have increased substantially. The same is true for transplantation of other organs, such as heart, liver, bone marrow, and pancreas.

Considering the high degree of polymorphism and the low gene frequencies of HLA alleles, successful matching of potential recipients with donor kidneys from others than sibs requires large-scale international organizations. Once kidneys – or other transplantable organs – become available due to the accidental death of an individual, a center is notified in which persons in need of such an organ are registered, together with their HLA status. The donor is typed, and the recipient whose HLA status best fits receives the organ.

6.2.5.5 HLA: Linkage Disequilibrium

One of the most conspicuous properties of the HLA system is that some HLA alleles tend to occur more frequently together than expected by chance. Table 6.1 shows some examples. The A1,B8 haplotype, for example, occurs about five times as often as expected.

Consider two alleles at two linked loci, with frequencies p_1 and p_2. With free recombination between them their combined frequency, i.e., the haplotype frequency h, should be $p_1 \times p_2$. If such a result is obtained, the two loci are said to be in linkage equilibrium. If the haplotype frequency h is higher than expected with free recombination, there is linkage disequilibrium (Δ, deviation from linkage equilibrium), which is often

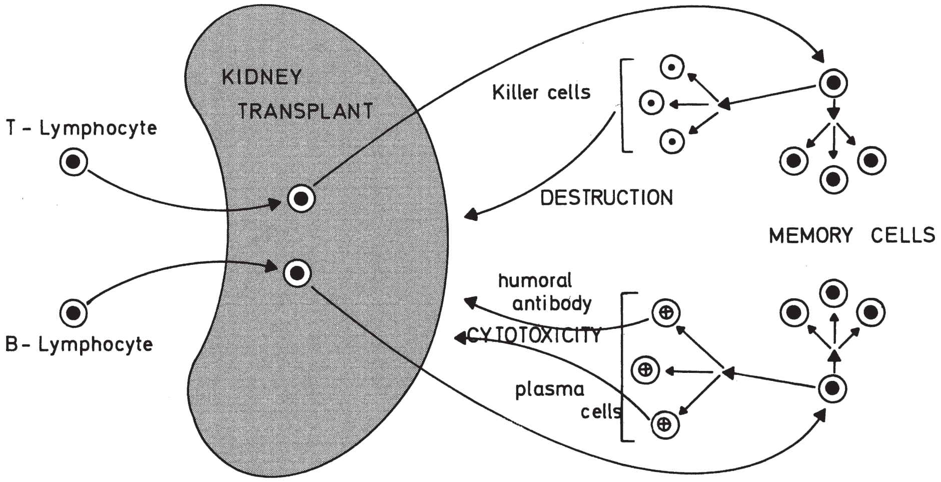

Fig. 6.11 Simplified diagram of the activation of the immune system by a kidney allotransplant. The transplant is recognized as foreign to the host organism by its T and B lymphocytes. This leads to activation of cellular and tumor immune response. From Svejgaard et al. [100]

Table 6.1 Linkage disequilibrium (gametic association; from Svejgaard et al. [100])

Haplotype			Frequency (%)	
A	B	D	Observed	Expected
A1	B8		9.8	2.1
A3	B7		5.4	2.1
	B8	Dw3	8.6	1.4
	B7	Dw2	3.9	1.8

The expected haplotype frequencies were calculated under the assumption of no association

Table 6.2 Association of HLA-A1 and B8 in unrelated Danes (2×2 table; from Svejgaard et al. [100])

	Number of individuals		
	B8$^+$	B8$^-$	Total
A1$^+$	376	235	611
A1$^-$	91	1,265	1,356
Total	467	1,500	1,967

First antigen	Second antigen	+/+ a	+/– b	–/+ c	–/– d	Total *n*
A1	B8	376	235	91	1,265	1,967

where, for example, +/– means number of individuals possessing the first character (A1) and lacking the second (B8). The χ^2 is:

$$\chi_1^2 = \frac{(\mathrm{ad}-\mathrm{bc})^2\,N}{(\mathrm{a}+\mathrm{b})(\mathrm{c}+\mathrm{d})(\mathrm{a}+\mathrm{c})(\mathrm{b}+\mathrm{d})} = 699.4$$

corresponding to the correlation coefficient:

$$r = \sqrt{\chi^2/n} = \sqrt{699.4/1967} = 0.60$$

Gene frequencies for A1 and B8 can be calculated by Bernstein's formula:

$$p = 1 - \sqrt{1-\alpha}$$

(where *a* is the antigen frequency) as 0.170 and 0.127, respectively. The Δ value can be calculated by the formula

$$\Delta = \sqrt{\frac{d}{n}} - \sqrt{\frac{(\mathrm{b}+\mathrm{d})(\mathrm{c}+\mathrm{d})}{n^2}} = 0.077$$

Thus, the frequency of the HLA-A1, B8 haplotype is
$\mathrm{h}_{\mathrm{A1,B8}} = \mathrm{p}_{\mathrm{A1}}\mathrm{p}_{\mathrm{B8}} + \Delta_{\mathrm{A1,B8}} = 0.170 \times 0.127 + 0.077 = 0.099$.

symbolized as $D=h-p_1p_2$. Haplotype and gene frequencies can be estimated from family and population data. In families the haplotypes of parents can in most cases be derived from those of their children (Table 6.2).

In the HLA system the deviations from linkage equilibrium are indeed striking. The situation is similar to that encountered with the Rh system (Sect. 6.2.4), but there is one important difference: in the Rh system only one case of recombination has been discovered, whereas many cases are known for the HLA system. Hence, genetic data point to much closer linkage in the Rh system than among the MHC genes. This conclusion has been corroborated by molecular studies in both systems (see above).

The observation of linkage disequilibrium – together with identification of immune response (Ir) genes in the mouse – initiated the investigations of HLA associations with diseases.

Linkage disequilibrium may have either of two main causes:

1. Two populations homozygous for different haplotypes mixed a relatively short time ago, and repeated

crossing over at a low rate has so far not been sufficient to lead to random distribution of alleles.

2. Certain combinations of alleles on closely linked gene loci caused a selective advantage for their bearers and have therefore been preserved.

To be able to decide between these two possibilities Bodmer [8] calculated how long a linkage disequilibrium would need to disappear in a random mating population.

For these calculations he used the work of Jennings [39], according to which *Δ* decreases to zero at a rate of 1–*Θ* per generation, where *Θ* is the recombination fraction between the two loci. Between the HLA-A and HLA-B loci *Θ* was found to be of the order of magnitude of 0.008. Taking linkage disequilibrium between HLA-A1 and B8 as an example, *Δ* values of about 0.06–0.1 have been found in European populations. On the other hand, *Δ* values between 0.01–0.02 are not statistically significant with reasonable sample sizes. Therefore it is meaningful to examine how many generations are needed to reduce *Δ* from 0.1 by a factor of 5 to 0.02.

Using the above principle of Jennings, we obtain:

$$(1-\theta)^n(1-0.008)^n = 1/5; n \approx 200$$

This means that *Δ* would be reduced to an insignificant value within about 200 generations of random mating, i.e., 5,000 years, taking a generation as around 25 years.

This period is approximately the length of time since agriculture first came to parts of northern Europe and is certainly a very short time considering the evolutionary life span of the human species. The fact that such a significant *Δ* could be eroded in so short a time in the absence of selection suggests at least that this particular combination of HLA-A1, B8 is being maintained at its comparatively high frequency by some sort of interactive selection [50]. We consider it likely that selection will also be found to explain some of the other common cases of linkage disequilibrium and that the effect of recent population mixture will be shown to be of minor importance. Certain haplotypes seem to have a selective advantage that keeps them more frequent than others. This selective advantage, on the other hand, cannot be directly related to the diseases for which associations have been shown so far, as they are too rare. Besides, the onset of most of them is usually delayed until after the age of reproduction. Infectious diseases have probably been the most important selective forces for maintaining the MHC polymorphism as well as linkage disequilibrium. This topic is discussed in Sect. 16).

6.2.5.6 The Normal Function of the System

The HLA determinants are localized at the surface of the cell and are strong antigens. They exhibit the most pronounced polymorphism of expressed genes known so far in humans, with abundant linkage disequilibrium. Disease associations have been shown between HLA antigens and diseases for which an autoimmune mechanism had previously been suspected. Furthermore, similar systems are known in all other mammals examined so far (see [1, 43, 114]). Finally, there is close linkage with other loci concerned with the immune response. All this evidence together is very suggestive of a system that regulates the contact of cells with their environment. In recent years, this function has been elucidated in detail. These genes are important mediators of the immune reaction. Such cell recognition mechanisms may be important in embryonic development and differentiation, especially when they are present on only certain cell types. However, such hypothesis would not explain the selective advantage of the high degree of polymorphism in this system.

Another possible function is protection against viral or bacterial infection. Antigenic material of human origin may be incorporated in the outer membrane of the virus, which is thereby made less recognizable to another human host. However, if the virus contains MHC material from a genetically different individual, it is more readily inactivated by the immune system. Such a mechanism would also explain why the extreme polymorphism of the MHC system has a selective advantage. Further elucidation of the MHC will teach us a great deal about how the organism handles its interaction with the environment. This knowledge is important to our understanding of how natural selection has shaped our genetic constitution in the past, and how recent changes in our environment may influence it in the future.

To broaden the empirical basis for such understanding, however, it may be useful to ask whether there are other examples in nature of such gene clusters with related functions? Can their analysis provide us with hints for a better understanding of the MHC cluster? There is indeed one such example that has been analyzed very carefully – mimicry in butterflies. It cannot be described here for lack of space, since it has no direct relationship with human genetics. But for the reader interested in more general, philosophical aspects of science, it is highly interesting showing how certain general principles may be used by nature in quite different contexts (see also earlier editions of this book).

6.2.6 *Unequal Crossing Over*

6.2.6.1 Discovery of Unequal Crossing Over

In the early years of work with *Drosophila* some authors observed that the bar mutation, an X-linked dominant character, occasionally reverts to normal, whereas in other cases homozygotes for the allele produce offspring with a new and more extreme allele, later called "double bar." Sturtevant [99] showed that this peculiar behavior is not due to mutations but to unequal crossing over, producing, on the one hand, a chromosome with two bar loci (double bar) and, on the other, a chromosome with no bar locus at all. When the giant salivary chromosomes of *Drosophila* permitted visual testing of genetic hypotheses, Bridges [11] showed that the simple, dominant bar mutation is caused by a duplication of some chromosomal bands. The reversion corresponds to the unduplicated state, whereas double bar is caused by a triplication of that band. Both reversion and triplication can be produced by a single event of unequal crossing over. Bridges did not yet formulate clearly the obvious reason for this event: the mispairing of "structure-homologous" but not "position homologous" chromosome sites (Fig. 6.12).

6.2.6.2 Unequal Crossing Over in Human Genetics

Haptoglobin [10], a transport protein for hemoglobin, is found in the blood serum and shows a polymorphism, the most common alleles being HP^{1F}, HP^{1S} and HP^2. Smithies et al. [94] discovered that the allele HP^2 is almost twice the length of each of the two alleles HP^{1F} and HP^{1S}, as evidenced by the composition of its polypeptide chain. In the HP^2 chain the amino acid sequence of the HP^1 chain is repeated almost completely. They concluded that the HP^2 allele might have been produced by gene duplication. Moreover, they predicted that unequal crossing over might again occur with a relatively high probability between HP^2 alleles, producing, on the one hand, an allele similar to HP^1

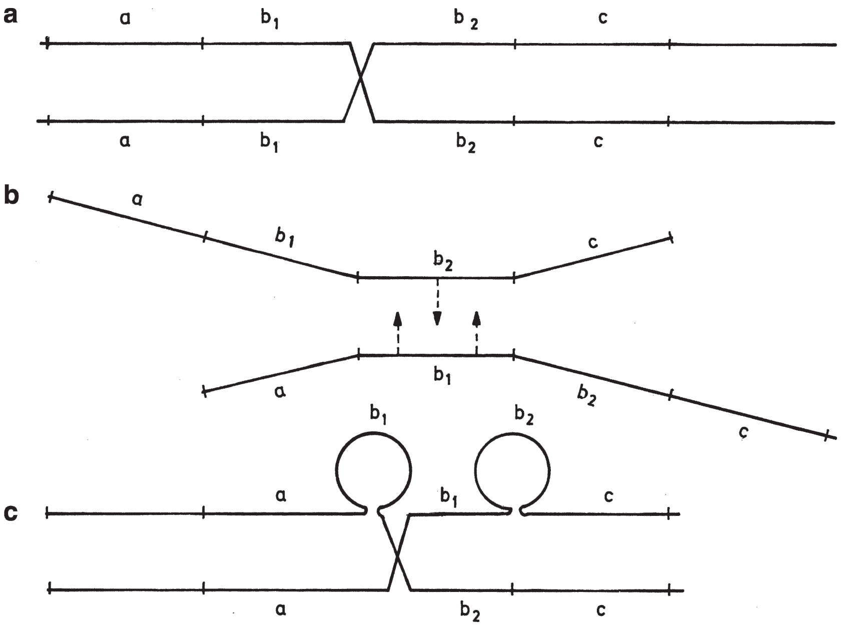

Fig. 6.12 (**a–c**) The principle of unequal crossing over. (**a**) Normal pairing and crossing over. The two genes b_1 and b_2 are assumed to have very similar DNA sequences. (**b**) Genes b_1 and b_2 are pairing. This leads to a shift of the two homologous chromosomes relative to each other. (**c**) Such pairing requires formation of two loops in the upper chromosome

and, on the other, an allele comprising the genetic information almost in triplicate. Repeated occurrence of this event might lead to still longer alleles and hence to a polymorphism of allele lengths in the population. Indeed, such alleles have occasionally been observed and are known as Johnson-type alleles [96].

There is an essential difference between the first unique event that produces the almost double-sized gene (for example, HP^2) from a single gene HP^1, and the unequal but homologous crossing over that becomes possible as soon as the first duplicated allele is present in the population [47].

6.2.6.3 First Event

Given a pair of homologous chromosomes, both partner chromosomes consist of largely identical sequences of nucleotides. Normally these partner chromosomes pair at meiosis, and there can be no unequal crossing over. To allow mispairing and thus unequal crossing over, an initial duplication is necessary. Mechanisms for such a duplication are known in cytogenetics, the simplest being two breaks at slightly different sites in adjacent homologous chromatids during meiosis and subsequent crosswise reunion. Another mechanism would be mispairing due to homology of short base sequences in nonhomologous positions. Our present knowledge of the structure of DNA sequences suggests ample opportunities for such a mispairing (slippage).

If the sites of breakage are separated only by the length of one structural gene, this event results in two gametes that do not contain this gene at all, together with two others containing it in duplicate (Fig. 6.13). The gametes containing a relatively large deletion have a high risk of not being transmitted because of lethality of the ensuing embryo. On the other hand, a gamete with the duplication is likely to develop into a diploid

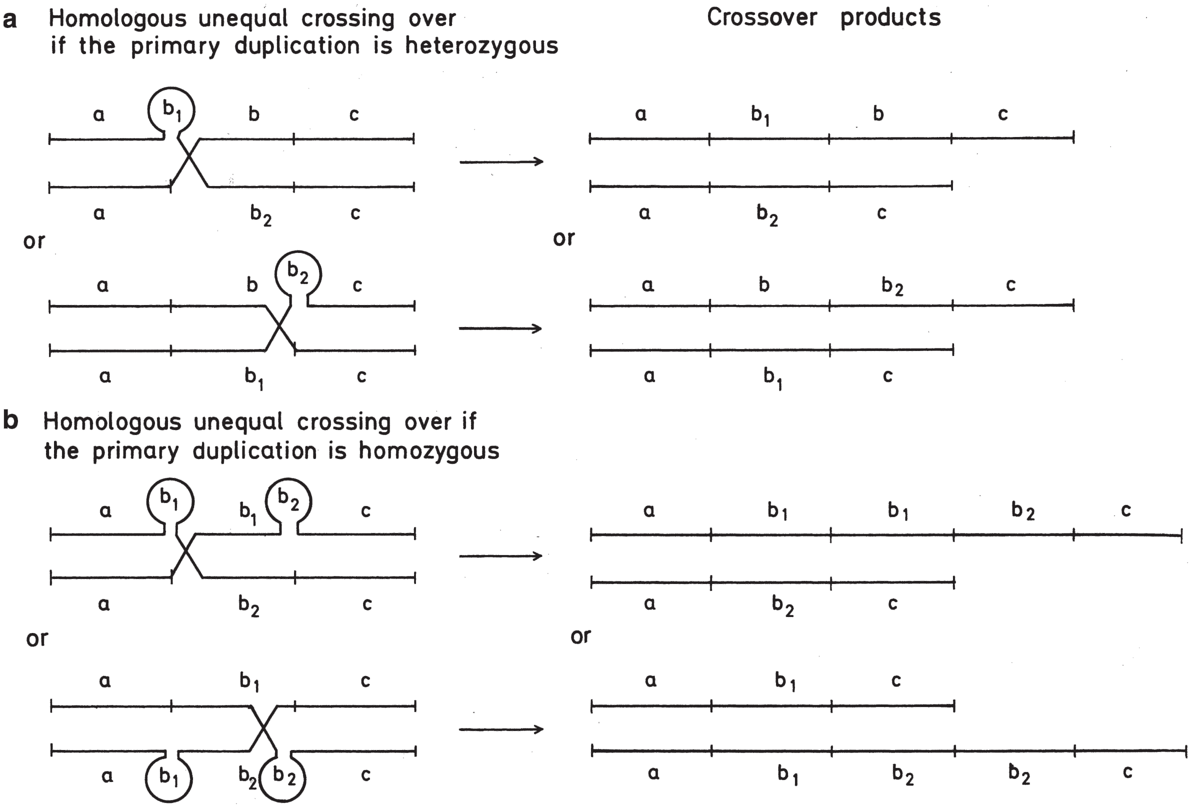
a Homologous unequal crossing over if the primary duplication is heterozygous
Crossover products
a
b_1
b
c
a
b_2
c
or
or
a
b
b_2
c
a
b_1
c
b Homologous unequal crossing over if the primary duplication is homozygous
a
b_1
b_1
b_2
c
a
b_2
c
a
b_1
c
a
b_1
b_2
b_2
c

Fig. 6.13 (**a**, **b**) Unequal crossing over between structure-homologous but not position-homologous genes. (**a**) Unequal crossing over always leads to one crossover product with two genes b (b_1b or bb_2) and to another with only one gene (**b**). Formation of larger allele sequences becomes possible if the primary duplication is homozygous. In this case a chromosome with three alleles b (b_1 b_1 b_2 or b_1 b_2 b_2) may be formed. From Krüger and Vogel [47] individual, providing for the first time a chance for mispairing of homologous sequences and therefore for unequal crossing over.

6.2.6.4 Consequences of Unequal Crossing Over

The consequences are seen in Fig. 6.13. As long as the duplication remains heterozygous, all gametes contain either one or two copies of the duplicated gene. When the duplication becomes homozygous, however, larger allele sequences may be formed. Unequal crossing over may lead, on the one hand, to gametes with only one copy and, on the other, to gametes containing three, and in subsequent generations, more than three copies (Figs. 6.13 and 6.14).

If the probability of unequal crossing over is not too low, high variability is soon found in the number of homologous chromosomal segments that resemble each other in structure but not in position. If selection favors a certain number of such chromosomal segments, which may be as small as a single gene, this number soon becomes the most common one. Selection relaxation leads to an increase in variability in both directions: the proportion of individuals with a very high number of such genes as well as those with a low gene number gradually increases [47]. Another genetic mechanism resembling unequal crossing over in some aspects is gene conversion where nonreciprocal products result.

Other examples besides the haptoglobin genes are the closely linked hemoglobin *β*- and *δ*-genes, the color vision pigment locus, and the natural killer cell receptor (KIR) genes [40]. Here the Lepore-type mutants, the X-linked color vision genes, and the diversity of KIR haplotypes are caused by unequal crossing over. Moreover, there are many examples for moderately or highly repetitive DNA sequences within which unequal crossing over should be possible. The presence of short repetitive DNA sequences such as minisatellites (Sect. 2.1.2) provides ample opportunities for pairing "slippage," leading to unequal crossing over. The high mutation rate within such areas (sometimes even a few percent per meiosis (Sect. 3.5) a well as the resulting huge interindividual variability show that this is not merely a theoretical speculation. Other repeated DNA sequences are those coding for the immunoglobulins. Increasing knowledge of the functional significance of repeated DNA sequences will bring a better understanding of the significance of unequal crossing over.

Recently there have been described large DNA segments that are duplicated in tandem or on other chromosomes. These "segmental duplications" can be up to 1 Mb is size or greater. Unequal crossing over between adjacent duplications is the basis for diGeorge/venocardiofacial disorder, Williams syndrome, and several other diseases. This class of diseases has been termed "genomic disorders" [89].

In fact, unequal crossing over, also referred to as "nonallelic homologous recombination," can give rise to numerous, recognizable microdeletion and microduplication syndromes and can significantly contribute to genome plasticity (see also Sect. 3.5.5).

6.2.6.5 Intrachromosomal Unequal Crossing Over

With structure-homologous but not position-homologous genes, such as those found in multigene families (Sect. 3.5.5), unequal crossing over becomes possible not only between homologous chromosomes but also between sister chromatids (intrachromosomal unequal crossing over). Theoretical considerations have shown that this process could have played a role in molecular evolution [41].

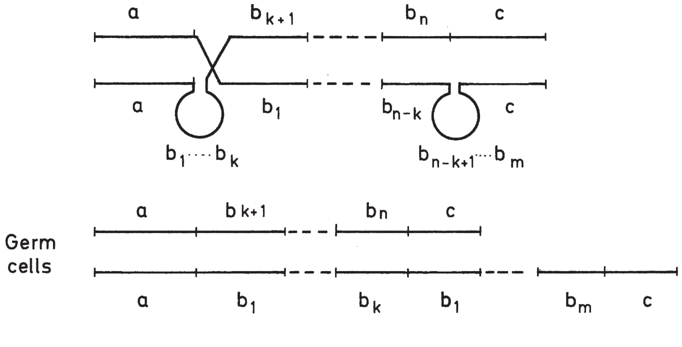

Fig. 6.14 The consequence of unequal crossing over. In subsequent generations chromosomes with (theoretically) unlimited numbers of a alleles may be formed. Unequal crossing over between any of them may lead to still larger (or still shorter) haplotypes. b_1 . . . b_k . . . b_n refer to homologous genes

6.3 Conclusions

A few years after the rediscovery of Mendel's laws early in the twentieth century the first exception to Mendel's third law (independent segregation) was discovered: genes located sufficiently close to one other on the same chromosomes often segregate together – they are linked. The frequency of recombination increases with increasing distance between these genes. Genes on the same chromosomes but located far apart from each other, however, may even segregate independently if the distance between them is greater – these are syntenic, but not linked. A great number of genetic markers are available for localizing human genes, and statistical methods for assessing linkage in the human genome and determining the distance between gene loci have been developed. Methods from cell, biochemical, and molecular genetics have helped in localizing genes to specific chromosomes and chromosome segments and led to the molecular isolation of these genes. Such techniques make it possible to localize genes for both normal and abnormal traits and to define the nature of such genes by positional cloning. The identification of genes involved in susceptibilities to common diseases with complex causes by linkage studies remains a major challenge. However, the advent of a human genome sequence, comparative sequence, and a haplotype map, as well as large-scale association studies comparing normal subjects with patients, are leading to some progress in molecular understanding of complex disease.

While genes involved in the same biochemical pathways are seldom located close together, some clusters of closely linked genes exist that have related functions; the genes of the major histocompatibility complex, for example, have been analyzed particularly thoroughly.

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Oligogenic Disease

7

Jon F. Robinson and Nicholas Katsanis

Abstract One of the primary goals of human and medical genetics is to assign predictive value to the genotype – that is to say, to use genetic information to assist in the diagnosis and management of disease. Recent work, originating primarily from disorders thought to be traditionally inherited in a Mendelian fashion, have blurred the boundaries between allele causality in monogenic and complex disease. Studies on genetic variation in disease are now revealing that essentially no disorder is transmitted solely in a Mendelian fashion; rather there are always multiple genetic and environmental factors that cause or modulate a disease phenotype. The focus of this chapter, *oligogenic disorders,* a term describing diseases caused by, or modulated by, a few genes, can provide a conceptual bridge between diseases classically considered monogenic and the poorly understood polygenic or complex disorders.

The inheritance of alleles generally follows Mendelian laws of segregation and independent assortment. However, this axiom does not necessarily hold true when the segregation of disease traits is considered. Mendelian inheritance is founded on the notion that a trait (not exclusively a disease phenotype) is transmitted through a single locus; however, even in the most classic monogenic disorders the 1:1 or 3:1 Mendelian ratio of dominant to recessive phenotypes, respectively, cannot explain the breadth of phenotypic variation found in a clinical setting. Although environment also plays a part, new research is showing that a large amount of the phenotypic variation in "Mendelian" disorders is due to genetic interaction of several genes (Nat Rev Genet 3:779–789, 2002). In that context, most, if not all, disorders should be considered multifactorial; and the main reason they are Mendelized is that the majority of the phenotype can be attributed to variation/mutations at a single locus.

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Contents

One of the primary goals of human and medical genetics is to assign predictive value to the genotype – that is to say, to use genetic information to assist in the diagnosis and management of disease. This includes both Mendelian traits, where interpretation of the relationship between a pathogenic mutation and its phenotypic consequence has been considered more straightforward, and complex traits, where determining the contribution of alleles to the predisposition to disease is far more challenging.

Recent work, originating primarily from disorders thought to be traditionally inherited in a Mendelian fashion, have blurred the boundaries between allele causality in monogenic and complex disease. Studies on genetic variation in disease are now revealing that essentially no disorder is transmitted solely in a Mendelian fashion; rather there are always multiple genetic and environmental factors that cause or modulate a disease phenotype. The focus of this chapter, *oligogenic disorders*, a term describing diseases caused by, or modulated by, a few genes, can provide a conceptual bridge between diseases classically considered monogenic and the poorly understood polygenic or complex disorders.

The inheritance of alleles generally follows Mendelian laws of segregation and independent assortment. However, this axiom does not necessarily hold true when the segregation of disease traits is considered. Mendelian inheritance is founded on the notion that a trait (not exclusively a disease phenotype) is transmitted through a single locus; however, even in the most classic monogenic disorders the 1:1 or 3:1 Mendelian ratio of dominant to recessive phenotypes, respectively, cannot explain the breadth of phenotypic variation found in a clinical setting. Although environment also plays a part, new research is showing that a large amount of the phenotypic variation in “Mendelian” disorders is due to genetic interaction of several genes [5]. In that context, most, if not all, disorders should be considered multifactorial; and the main reason they are Mendelized is that the majority of the phenotype can be attributed to variation/mutations at a single locus.

7.1 The Limitations of Mendelian Concepts

The transition from monogenic to oligogenic models highlights some of the limitations of concepts generated originally to facilitate the identification of pathogenic mutations in Mendelian disease. That is not to say the concepts of monogenic disorders were invalid, especially since they have been instrumental to our progress towards solving a significant proportion of the mutational load in human genetic disease. Nonetheless, an unavoidable side effect is that they have introduced a bias towards monogenic disorders, which will now have to be first recognized and then ameliorated.

Consider a traditional positional cloning approach to identifying a disease locus and the underlying allele(s) under a monogenic model. The three major genetic tools at our disposal are: (a) inheritance data in families; (b) the predicted effect of a change on the target gene and its protein, often coupled to the evolutionarily predicted tolerance for such a genetic lesion; and (c) the prevalence of a candidate disease allele in the general population.

A simple example of linkage analysis highlights some of the biases introduced under an a priori expectation of monogenic disease transmission. In a family in which a trait segregates under an autosomal recessive model (based on both the vertical pattern of inheritance, i.e., transmission, and the horizontal pattern of inheritance, i.e., recurrence risk), affected individuals are expected to carry homozygous or *compound heterozygous* – two different mutant alleles, at the causal locus, whereas normal individuals either have one or no pathogenic mutations (Fig. 7.1). In oligogenic disorders, however, homozygous mutations at the locus of interest may be present in both normal and affected individuals, which, if sufficiently prevalent in the pedigree, might actually mask a statistically significant linkage peak.

Similarly, recognizing the detrimental effect of an allele to the gene and its product is also biased towards a Mendelian paradigm. The majority of mutations reported to date for monogenic disorders are more severe, such as deletions that remove parts of the transcript or cause frameshifts, nonsense mutations, splice site changes or missense codons of highly conserved amino acid sites of a protein. By contrast, in oligogenic disease one can expect that there are several genes contributing to the phenotype, with the average effect of each mutation being less severe in comparison. This in turn poses the risk of misinterpreting true pathogenic alleles as benign variants. Indeed, even in diseases well recognized as monogenic, there are examples of additional alleles within the same locus that can affect the penetrance of the phenotype, even if their predicted effect (from sequence analysis) is at best dubious. For example, a study on erythropoietic protoporphyria (OMIM: 177,000), an autosomal dominant disorder of incomplete penetrance, demonstrated how disease manifestation requires a deleterious mutation at one allele of the *FECH* gene and a common, low-expression polymorphism of the other [38].

Because most deleterious mutations in monogenic disease have more severe effects on gene/protein function and cell physiology, they are often under negative selection (with a few notable exceptions, such as the hemoglobin sickle cell variant, common in sub-Saharan Africa, which protects against *Plasmodium falciparum* infection of malaria). As such, they are not expected to be in Hardy–Weinberg equilibrium in the population, which typically translates to their absence from a cohort of normal individuals. However, because mutations contributing to oligogenic traits are often expected to be milder and, in some instances, are not sufficient for pathogenesis, they can be found at equilibrium in the general population, which in turn has the potential to mask them as common polymorphisms of no pathogenic potential. This makes discerning these genetic lesions more difficult, because the more common a genetic variant is, the more difficult it becomes to justify its contribution to a particular phenotype. These issues are elaborated on later in the chapter, but overall, as we move from studying monogenic to oligogenic disease it becomes significantly more difficult to establish causal associative relationships between single alleles and the phenotype.

Several disorders have been highlighted to help illustrate how factors such as genetic heterogeneity, genetic modifiers, and oligogenicity can cause deviations from the Mendelian paradigm.

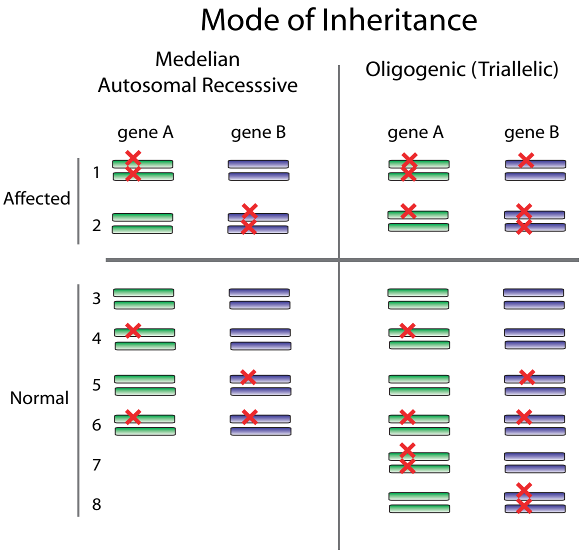

Fig. 7.1 Mendelian vs oligogenic (triallelic) inheritance. Under a Mendelian autosomal recessive model of inheritance, mutations in both alleles of a gene are required to manifest the disease phenotype. In triallelic inheritance, deleterious mutations of three alleles (two at one locus and one at another) are required for disease manifestation. In other forms of oligogenic inheritance single mutations in two different genes can genetically interact to manifest disease (not shown)

7.2 PKU and Hyperphenylalaninemia: Genetic Heterogeneity

Pronounced phenotypic variability in a disease commonly considered monogenic calls into question whether mutations at a single gene are sufficient to account for the variability of the disease phenotype, or whether the involvement of multiple genes should be suspected. This is exemplified by phenylketonuria (PKU; OMIM: 261,600), one of the first genetic disorders for which a biochemical defect was found before the advent of familial genetic analysis. In PKU, mutations in the hepatic (liver) enzyme phenylalanine hydroxylase (PAH) prevent the conversion of the essential amino acid phenylalanine to tyrosine [48]. Prenatal diagnosis of hyperphenylalaninemia in the 1960s provided the opportunity for early treatment; however, ~1% of patients did not respond well to the traditional therapy [5]. This suggested that other genetic factors could be involved, and in 1983 the mapping and cloning of the PAH gene revealed substantial *allelic heterogeneity* [94] – multiple alleles causing the same trait or disease. Allelic heterogeneity can result in variable phenotypes and severity of the disease depending on the severity of mutation in the locus. The discovery of other loci such as *biopterin-synthetase*, also leading to hyperphenylalaninemia (OMIM: 264,070) proved *locus heterogeneity* [16] – multiple loci contributing to a trait or disease – was another reason for the variable response to the traditional therapy. PKU and hyperphenylalaninemia are mostly inherited in a monogenic fashion; however, studies showing extensive phenotypic variability in patients with identical genotypes exemplify how other genetic or environmental factors modulate the phenotypic spectrum.

7.3 Cystic Fibrosis: Genetic Modifiers

Common genetic variation found within functional regions of the genome can often modify the outcome of even the most severe monogenic disorders. Cystic fibrosis (CF; OMIM: 602,421), an autosomal recessive disorder caused by deleterious mutations in the *CFTR* gene (cystic fibrosis transmembrane regulator), is often considered an excellent example of monogenic inheritance. Nearly all patients with classic forms of CF have mutations in the *CFTR* gene, with ~70% of those having the same pathogenic allele, ΔF508 (OMIM: 602,421) [52]. Although homozygous mutations in *CFTR* are almost completely penetrant, there is wide phenotypic variability, especially with regard to the severity of the pulmonary phenotype. Allelic heterogeneity can explain some of this variation, but there are several cases in which patients carrying the same alleles show different pulmonary phenotypes [21]. Environment, which is also a factor, does not explain all the variation, suggesting that there are contributing *genetic modifiers* – genes that modulate the severity of disease but are not necessarily causal. Recent association studies correlating the severity of a particular CFTR phenotype with alleles from candidate genes have found: low-expressing MBL (mannose-binding lectin, OMIM: 154,545), HLA (human leukocyte antigen; OMIM: 142,857) class II, TNFA (tumor necrosis factor-α; OMIM: 191,160) and TGFB1 (transforming growth factor-B1; OMIM: 190,180) all potentially modulate the pulmonary phenotype [5].

It is important to note that many of these modifiers are *polymorphisms*, i.e., genetic variants present in at least 1% of the population, highlighting the point that "polymorphism" and "neutral variant" are not synonymous. Therefore, the severity of a disease is largely dependent upon the presence or absence of certain common variants whose deleterious contribution is context dependent. The effects of these common variants, most of which are functional single nucleotide polymorphisms (SNPs) but can also include copy number variants (CNVs), Mb-sized deletions and duplications, largely go unnoticed in a healthy individual. However, under conditions of cellular or organismal stress that is established by mutations at a primary locus, such as in an individual with homozygous CFTR mutations, the effects of these polymorphisms become unmasked and can be protective or detrimental. For example, in patients with CF, certain *NOS1* (OMIM 163,731) polymorphisms that cause high NO production are associated with a slower decline in lung function [20]. It is thus important always to consider the effects of a disease-causing locus in the context of a person's entire genetic background.

7.4 Lessons Learned from Established Oligogenic Disorders

Even though *epistatic interactions* – genetic interactions between different genes in which the effect of one gene is modulated by one or more genes – between alleles at different loci are expected to be common contributors to the modulation of penetrance and expressivity, there remains a relative paucity of such examples in human genetic disease, especially when considering the fact that more than 1,000 human phenotypes have now been associated with a genetic lesion. This is probably reflective of both the bias towards Mendelizing disease phenotypes, as well as of the innate difficulty of establishing such phenomena. Nonetheless, the development of oligogenic disease models, coupled to higher sequencing capacity and improved computational and experimental protocols for investigating the potential effect of alleles on gene function, are unmasking a rapidly expanding list of oligogenic traits.

7.4.1 Bardet–Biedl Syndrome

Bardet–Biedl syndrome (BBS; OMIM 209,900) is a genetically heterogeneous disorder caused by recessive mutations in 1 or 2 of at least 12 different genes [51, 84]. It is also a clinically heterogeneous or *pleiotropic* disorder**,** with primary features including mental retardation, postaxial polydactyly, postnatal obesity, hypogenitalism, renal abnormalities, and progressive retinal dystrophy [10]. BBS also exhibits striking phenotypic variability both within and between families, which strongly suggested the presence of epistatic loci.

The oligogenic mode of inheritance now established for BBS illustrates the blurred boundaries between monogenic and multifactorial traits. In some families, homozygous or compound heterozygous recessive mutations at a single BBS locus seem to be sufficient to elicit the disease phenotype [68]. However, there is significant evidence showing that in some families there is a more complex digenic inheritance that requires at least three mutant alleles across two bona fide BBS genes (two at one locus and one at another) [51]. This is exemplified by the pedigree of a US family of European descent (Fig. 7.2), in which haplotype analysis predicted through identity-by-descent that the affected proband and unaffected sibling would carry two mutations in the *BBS2* locus [11]. Identity-by-descent refers to two alleles of the same locus deriving from a common ancestral chromosome and is usually proven by the presence of identical haplotype markers surrounding the gene of interest shared between the ancestor and descendant (i.e., parent and child). The affected sibling was also shown to have an additional mutation in another gene, *BBS6,* which was absent from the unaffected sibling, and the unaffected father was shown to be heterozygous for pathogenic mutations in both *BBS2* and *BBS6* genes (OMIM 209,900).

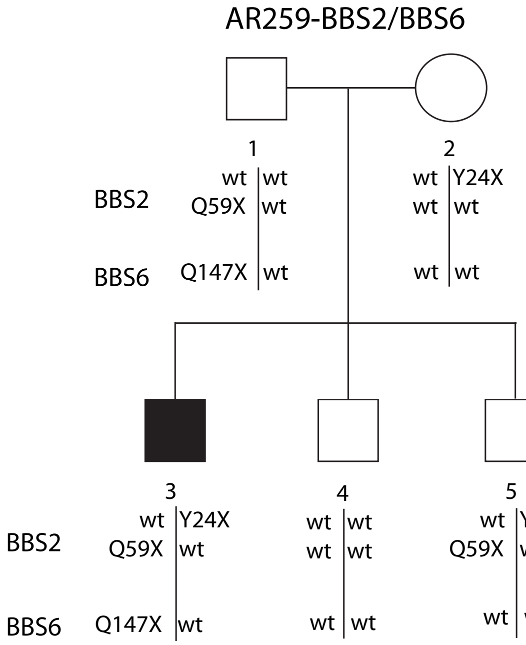

Fig. 7.2 BBS pedigree (triallelic) inheritance. In family AR259, both the affected proband (*3*) and an unaffected sib (*4*) contain compound heterozygous nonsense mutations in BBS2, suggesting other genetic factors were contributing to disease. Sequencing of BBS6 revealed a nonsense mutation in the affected proband that was inherited from the unaffected father but missing in the sibling with BBS2 mutations, suggesting that "triallelic" mutations are required for disease manifestation

This suggested that at least three mutations (two in *BBS2* and one in *BBS6*) were required for manifestation of the BBS phenotype. This type of mutant allele distribution has been observed in many BBS families; however, the genetic contribution of the third allele has been variable. In some families, such as that described in Fig. 7.2, the third BBS mutation can be best described as causal or a modifier of the penetrance of BBS. In other families, however, the third mutation likely acts as a genetic modifier, affecting the severity of the disease (i.e., exacerbating the clinical phenotypes).

7.4.2 Determining Oligogenicity

Recent studies on cohorts of patients with mutations in one of the two major BBS loci, *BBS1* or *BBS10* found approximately 13.3 and 18%, respectively, of patients had potentially three mutations (two at one locus and one at another) [12, 84]. Determining whether these alleles are pathogenic modifiers of penetrance, modifiers of disease severity, or simply benign missense variants can be done by systematically demonstrating three lines of evidence: (1) the presence of unaffected individuals carrying the same two mutations at the locus as affected individuals with three mutations; (2) the carrier frequency of the primary mutant allele is higher than would be expected under a Mendelian recessive model of inheritance. Modification of severity can simply be shown if (3) the allelic variant is correlated with more severe forms of disease. Evidence for oligogenicity in *BBS1* patients came from demonstration of the first two lines of evidence [12] even though a third mutation in another BBS gene had not been found. In a separate study on *BBS10*, the lack of unaffected individuals with homozygous or compound heterozygous *BBS10* mutations suggested that the third allelic variant at a second BBS locus (*BBS1*, *BBS4*, *BBS6*, or *BBS7*) were either severity modifiers or benign variants [84].

7.4.3 Cortisone Reductase Deficiency

Although BBS was one of the first disorders with documented oligogenic inheritance, a number of phenotypes have been described since for which similar genetic models apply. One interesting example is cortisone reductase deficiency (CRD; OMIM 604,931), a disorder characterized by excess of adrenocorticotropin-mediated androgen and phenotypes similar to polycystic ovary syndrome due to inability to convert cortisone to cortisol [29] 11B-HSD (OMIM 218,030) catalyzes interconversion of cortisone to cortisol, making it a natural candidate for harboring mutations that cause CRD. Interestingly, in one study, in patients affected with CRD all mutations were found to localize in intron 3 and none in the six exons of 11B-HSD [29]. Biochemical analysis, in the form of a luciferase reporter assay, showed that the mutant forms of 11B-HSD had 2.5 times lower transcriptional activity than the wild-type gene, suggesting that intron 3 acts as a transcriptional enhancer of 11B-HSD expression. Although 11B-HSD was clearly implicated in the disease, it could not fully explain the CRD phenotype, because 25% of unaffected controls were heterozygous and 3% were homozygous for the intron 3 mutations. In BBS, the presence of additional genes, mutations in each of which caused the same phenotype, were the catalyst for the establishment of oligogenicity. In CRD, analysis of the biochemical pathway was the route that provided suitable functional candidates. One key observation focused on the requirement of NADPH for 11B-HSD oxo-reductase activity, which in turn led to the sequencing of hexose-6-phosphate dehydrogenase (H6PDH; OMIM 138,090), an enzyme of the pentose phosphate pathway known to generate NADPH [29]. All affected individuals were found to be either homozygous or heterozygous for variants in exon 5 of *H6PDH*, in addition to having mutations in the gene encoding 11B-HSD. Biochemical analysis subsequently demonstrated that this variant had less than 50% the H6PHD activity of wild type. Triallelic inheritance of CRD was then hypothesized because all affected individuals had homozygous mutations in one gene and heterozygous mutations in the other. In support of this model was the presence of unaffected individuals who were solely homozygous for the *H6PDH* variants, solely homozygous for *11B-HSD* mutations, or *doubly heterozygous* – heterozygous for mutations at two different loci – for both *H6PDH* and *11B-HSD* mutations [29]. The suggested model of pathogenicity is noninteracting, nonallelic noncomplementation (see Sect. 7.4.4).

7.4.4 Hemochromatosis

An emerging trend from the earlier examples is that in oligogenic disorders candidate genes for contributing both causal and modifying alleles are often found in the same pathway as the predominant disease locus. A clear example of this can be seen in hereditary hemochromatosis (HH; OMIM 235,200), a typically adult-onset, genetically heterogeneous disorder characterized by abnormal iron deposition in tissues due to increase in duodenal iron absorption and release of iron from macrophages [54]. HH was originally classified as a strictly monogenic autosomal recessive disorder, but recent studies have discovered an autosomal dominant inheritance as well as autosomal recessive oligogenic transmission [54]. A founder mutation in *HFE* (OMIM 235,200), C282Y is the predominant cause of HH as it is homozygous in 64–100% of patients [62] and its iron overload phenotypes have been recapitulated in mouse models [54]. Allelic heterogeneity has been demonstrated by genotype–phenotype correlation studies after the discovery of another common variant with a 2% frequency of homozygosity in European countries causing a histidine-to-aspartate (H63D) substitution in HFE [42]. C282Y/H63D compound heterozygotes represent a large majority, between 74 and 100%, of non-C282Y homozygous patients and are found to have milder iron overload phenotypes [62]. This effect of a common variant modulating the risk for a disease may be a recurring theme in oligogenic disease (see also the RET example in the next section).

Locus heterogeneity has been demonstrated by the presence of rarer, non-HFE-related forms of hemochromatosis due to mutations in genes that encode transferrin receptor 2 (TFR2; OMIM 604,720), ferroportin 1 (FPN1, OMIM: 604,653), hepicidin antimicrobial peptide (*HAMP*, OMIM: 606,464), and hemojuvelin (HJV, OMIM: 608,374) [42, 54]. Discovery of these other forms, some of which have been associated with early ages of onset and increased severity of disease has led to a classification of hemochromatosis into four different types [75]. However, similar to BBS, where each implicated gene is related by a common system or pathway (the primary cilium), all the genes implicated in hemochromatosis are suggested to modulate HAMP transcriptional response to iron [54]. Interestingly, many of these genes also interact genetically to cause or to modulate hemochromatosis; for example, recent studies have correlated younger age of onset and increased severity of disease in homozygous HFE C282Y patients, with additional heterozygous mutations in one or two of the genes involved in juvenile hemochromatosis, *HJV* and *HAMP* [47, 55]. In the oligogenic, or more specifically digenic inheritance, double heterozygous mutations in *HFE* and *HAMP* are required for disease manifestation [63]. For this reason, hemochromatosis exemplifies two key paradigms regarding identity and relationships between genetic modifiers and causative genes in oligogenic inheritance: (1) causative genes of a disease are often good candidates for genetic modifiers or contributors in digenic inheritance; (2) genes implicated in an oligogenic disorder are likely involved in the same or similar/converging pathway, suggesting that, not surprisingly, it is the total genetic load in any given physiological system that determines the extent and rate of progression of clinical manifestations.

7.4.5 *Hirschsprung Disease*

One difficulty when considering oligogenic traits (and modifying alleles in general) is that the epistatic loci can contribute alleles that are sometimes found at high frequency in the general population. This is not surprising, since if all modifying alleles were rare, then epistasis would be infrequent and the recurrence risk in oligogenic traits would potentially be much lower (see subsequent sections for a detailed discussion on recurrence risks). Nonetheless, the effect of common alleles can pose a major challenge and the genetic analysis tools are sometimes insufficient to establish a causal/modifying relationship between the allele and the phenotype.

A prominent example of how a common functional polymorphism can have a significant effect on the penetrance of an oligogenic disorder is seen in Hirschsprung disease (HSCR, OMIM: 142,623) [31]. HSCR is a congenital disorder in which the enteric ganglia are absent along variable regions of the intestine. There are two general modes of inheritance: (1) the most prevalent, a recessive multifactorial mode, characteristic in short segment HSCR is associated with mutations in PMX2B [13] (OMIM: 603,851), and linked to three loci on chromosomes 3p21, 10q11 (most likely *RET*), and 19q12 [33] in different individuals; (2) an autosomal dominant mode characteristic in long segment HSCR that usually results from severe mutations in the *RET* proto-oncogene [30] (OMIM: 164,761), or mutations in seven other genes, including *EDNRB* (OMIM: 131,244), *ECE1* (OMIM: 600,423), *EDN3* (OMIM: 131,243), *GDNF* (OMIM: 600,837), *NRTN* (OMIM: 602,018), *SOX10* (OMIM: 602,229), and *ZFH1B* (OMIM: 605,802) [31]. Under 30% of patients have mutations in one of these eight genes, suggesting the existence of additional HSCR genes, or undiscovered mutations in the known genes, the latter highlighted by the observation that 91% L-HSCR [17] and 88% of S-HSCR [33] families show linkage to the *RET* locus, yet pathogenic coding *RET* alleles have been found in only ~50% [4]. Recently, a common sex-dependent variant in a *RET* enhancer was shown to have a significant contribution to HSCR risk, potentially accounting for 10–20 times the variation of susceptibility in all other known RET mutations [31]. This polymorphic variant, which was shown by luciferase reporter assays to reduce the RET enhancer activity six- to eight-fold, was originally missed in the *RET* mutational screens because it is located in a conserved noncoding region, RET intron 1, underscoring the importance of including conserved noncoding regions in mutational screens for oligogenic diseases. What is particularly informative in the context of oligogenic models is the high prevalence of this deleterious allele in the general population, with an allele frequency of 0.25 in Europe and 0.45 in Asia, postulated to result from an unknown selective advantage in heterozygotes [31]. This phenomenon is particularly relevant to both oligogenic and polygenic disorders, where many of the contributing genetic factors are in fact common polymorphisms instead of rare mutations. It is conceivable that deleterious polymorphisms are maintained at a high frequency in the population owing to a delicate balance between advantageous and detrimental gene–gene and gene–environment interactions. For example, certain alleles at one locus might not have a differential effect on a particular trait, but in a perturbed state resulting from exposures in the environment or deleterious alleles at other loci these alleles now confer different effects on the trait – a phenomenon referred to as cryptic genetic variation [35].

Overall, these four disorders illustrate how, within the same disease, one can find evidence of monogenic inheritance, effects of genetic modifiers, or oligogenic inheritance; in some ways, the distinction among these terms is somewhat arbitrary. The variability in the mode of inheritance among individuals is ultimately governed by interactions between an individual's genetic background and his environment. Hence, individuals respond to diet, drugs, exercise, climate, infections, and disease differently as a result of common genetic variation, which can take the form of single nucleotide changes, large-scale deletions, duplications, or inversions [78]. A comprehensive list of oligogenic disorders and their implicated genes can be found in Table 7.1.

Table 7.1 Oligogenic disorders

Syndrome or trait	OMIM number	Primary locus	Secondary locus	Effect	Reference
Alagille syndrome	118,450	*Jag1*	*Notch2*	Digenic	[5]
Alzheimer's disease	104,300	*APP*	*TGFB1*	Severity modifier	[5]
Antley–Bixler syndrome	207,410	*FGFR2*	*CYP21* *(postulated)*	Digenic/severity modifier	[73]
Autosomal dominant exudative vitreoretinopathy	133,780	*FZD4*	*LRP5*	Synergistic	[72]
Autosomal dominant nosyndromic deafness	601,842	*DFNA12*	*DFNA2*	Digenic/additive	[8]
Autosomal-dominant glaucoma	137,750	*MYOC*	*CYP1B1*	Onset/severity modifier	[5]
Bardet–Biedl syndrome	209,900	*BBS6/BBS2*	*BBS2/BBS6*	Digenic "triallelic"	[5]
	209,900	*BBS2/BBS4*	*BBS4/BBS2*	Digenic "triallelic"	[5]
Bartter syndrome type 4	602,522	*CLCNKA*	*CLCNKB*	Digenic	[77]
Becker muscular dystrophy	159,991	*DMD*	*MYF6*	Severity modifier	[5]
Breast and ovarian cancer	113,705	*BRCA1*	*HRAS1*	Penetrance/risk	[5]
Breast cancer	175,100	*BRCA*	*APC*	Modifier/risk	[5]
Congenital disorder of glycosylation type 1a	212,065	*PMM2*	*ALG6*	Severity modifier	[5]
Cortisone reductase deficiency	604,931	*HSD11B1/H6PD*	*H6PD/HSD11B1*	Digenic "triallelic"	[29]
Cystic fibrosis	603,855	*CFTR*	*CFM1*	Severity modifier	[5]
Cystinuria type A/B	220,100	*SLC7A9*	*SLC3A1*	Expressivity	[5]
Deafness: nonsyndromic recessive deafness (DFNB1)	22,090	*GJB2*	*GJB6*	Digenic	[25]
Emery–Dreifuss muscular dystrophy	181,350	*LMNA*	*DESMIN*	Digenic/synergistic	[67]
Epilepsy with febrile seizures plus	604,233	*SCN2A*	*postulated*	Digenic/severity modifier	[45]
Familial adenomatous polyposis	175,100	*APC*	*Mom1, Cox2, cPLA2*	Protective	[5]
Familial amyotrophic lateral sclerosis	147,450	*SOD1*	*CNTF*	Severity modifier	[5]
Familial hypercholesterolemia	143,890	*ARH*	*13q22-q32*	Digenic	[5]
Familial Mediterranean fever	249,100	*MEFV*	*SAA1*	Pleiotropy	[5]
Familial porphyria cutanea tarda	176,100	*UROD*	*HFE*	Digenic	[61]

(continued)

Table 7.1 (continued)

Syndrome or trait	OMIM number	Primary locus	Secondary locus	Effect	Reference
Familial thrombophilia	188,050	*Protein C*	*Factor V*	Digenic	[34]
Finnish congenital nephrosis	256,300	*NPHS1*	*NPHS2*	Triallelic, severity modifier	[5]
Hemochromatosis	602,390	*HFE*	*HAMP*	Digenic	[63]
Hereditary deafness	108,733	*PMCA2*	*CDH23*	Digenic/severity modifier	[32]
Hereditary deafness	108,733	*CDH23*	*PCDH1 5*	Digenic	[96]
Hirschsprung disease	142,623	*RET*	*NRTN*	Digenic, severity modifier	[28]
Holoprosencephaly	142,623	*RET*	*GDNF*	Digenic	[5]
Hypertension	142623	*RET*	*3p21; 19p12?*	Penetrance/risk	[5]
	236,100	*SHH*	*TGIF*	Digenic	[69]
	145,500	*ATP1A1*	*NKCC2*	Digenic/synergistic	[36]
Idiopathic hypogonadotropic hypogonadism	146,110	*GNRHR*	*FGFR1*	Triallelic/severity modifier	[71]
Junctional epidermolysis bullosa	226,700	*FGFR1*	*NELF*	Digenic/severity modifier	[5]
Kallman syndrome	308,700	*COL17A1*	*LAMB3*	Triallelic/severity modifier	[27]
		KAL1	*PROKR2*	Digenic	
Late-onset Fuchs corneal dystrophy	610158	*FCD1*	*postulated*	Digenic/onset modifier	[88]
Long QT syndrome	152427	*KCNQ1*	*KCNH2*	Digenic	[14]
Maternally inherited deafness	152427	*KCNH2*	*SCN5A*	Digenic	[64]
Melanoma	152427	*KCNE2*	*SCN5A*	Digenic	[64]
	561000	*12 S Ribosomal*	*D8S277*	Penetrance	[5]
	600160	*CDKN2A*	*MC1R*	Penetrance/risk	[5]
Midventricular hypertrophic cardiomyopathy	192600	*MYLK2*	*MYH7*	Digenic/additive	[24]
Myopathy	610099	*8p22-q11*	*12q13-q22*	Digenic	[43]
Osteoporosis	166710	*ERS2*	*NRIP1*	Digenic/nonsynergistic	[65]
Parkinson disease	168600	*ERS2*	*ESR1*	Digenic/nonsynergistic	[22]
Pheochromocytoma	171300	*PRKN*	*LRRK2*	Digenic	[89]
		PINK1	*DJ-1*	Digenic/synergistic	
Progressive external ophthalmoplegia	157640	*2cen*	*16p13*	Digenic	[23]
		POLG	*Twinkle*	Digenic	[92]
Pseudohypoaldosteronism	264350	*MR*	*ENaC*	Digenic	[2]
Refractory auto-inflammatory syndrome	142680	*TNFRSF1A*	*CIAS1*	Digenic/severity modifier	[91]
Retinitis pigmentosa	180721	*RDS*	*ROM1*	Digenic	[5]
Rett syndrome	312750	*MECP2*	*postulated*	Digenic/severity modifier	[74]
Spina bifida occulta (mouse)	182940	*PAX1*	*PDGFRA*	Digenic	[44]
Spinal muscular dystrophy	603011	*PAX1*	*E2A*	Digenic	[49]
Split hand/foot malformation with long bone deficiency	119100	*SMN1*	*H4F5*	Candidate severity modifier	[5]
		SHFLD1 (1q42.2-q43)	*SHFLD2 (6q14.2)*	Digenic	[70]
Type I von Willebrand disease	601628	*VWF*	*ABO blood group*	Penetrance	[5]
Type II diabetes mellitus	125853	*VWF*	*Galgt2*	Modifier	[5]
Type II diabetes mellitus	125853	*PPARG*	*PPP1R3A*	Digenic/additive	[76]
		TCF1	*SHP*	Digenic/severity modifier	[90]
Usher syndrome type III	276092	*INSR (mouse)*	*IRS1 (mouse)*	Digenic	[19]
		USH3	*MYO7A*	Severity modifier	[1]
Van der Woude syndrome	604547	*VWS*	*17p11.2*	Penetrance	[5]
Waardenburg syndrome type II and ocular albinism	103470	*MITF*	*TYR*	Digenic	[5]

7.5 Establishing Oligogenicity: Concepts and Methods

As discussed earlier in this chapter, there are biases in molecular tools and methodology against discovery of genetic factors involved in oligogenic diseases. These biases, however, can be overcome by adopting a methodology that does not ignore mutations with subtle effects, conserved noncoding sequences, variants in Hardy–Weinberg equilibrium, and linkage tests that consider multiple genes in the analysis. There are primarily four lines of evidence that suggest oliogenicity in a disease: (1) poor phenotype-genotype correlations, (2) phenotypic differences in animal models that are dependent on genetic background, (3) identification of a disease trait that is not transmitted in a Mendelian fashion, and (4) establishment of linkage within the same family to more than one locus.

7.5.1 Heritability

The classic Mendelian model used to discover genetic diseases is anchored in the assumption that the traits or clinical features of a disease are transmitted by a molecular defect at a single locus. Inaccuracy of this model is thus evident when there is large phenotypic variability between individuals that have identical alleles at the implicated locus. One example is two individualswith homozygous ΔF508 mutations but drastically different clinical outcomes of CF. To establish oligogenicity, it is necessary to determine whether some of the phenotypic variation is in fact due to genetic variation and not simply environment, or in other words determine the *heritability* – the proportion of phenotypic variation that is due to genetic variation. If one were to simply consider the phenotypic variance (var[P]) as the sum of the genetic variance (var[G])and environmental variance (var[E]) then heritability could be estimated as: $H^2 = \text{var}[G]/\text{var}[P]$ [86]. Heritability is difficult to measure, but the most common method of measurement is to use twin studies in which the phenotypic variability of monozygotic twins (MZ; genetically identical) and dizigotic twins (DZ; ~50% of alleles shared) is compared. Since variance in environment is generally considered small in this situation and MZ twins share twice as many genes as DZ twins, heritability is approximately twice the difference in correlation between MZ and DZ twins: [$H^2=2(R_{MZ}–R_{DZ})$] [40, 79]. Although heritability can be due to allelic heterogeneity at a single locus, high values suggest that there are multiple genes (locus heterogeneity) which contribute to the phenotypic variation; the heritability in HSCR, for example, is nearly 100%. In addition to high heritability values and poor genotype–phenotype correlation phenotypic variation in animal models can also suggest oligogenicity.

7.5.2 Mouse Models of Oligogenic Inheritance: Familial Adenomatous Polyposis

Mouse models have been powerful tools for recapitulating human phenotypes under a specific, fixed genetic background. This provides a significant advantage when studying a disease with phenotypic variability, because variability attributable to environment can be differentiated from that caused by the genetic background. By keeping the genetic background constant, through successive backcrosses of the mutant to an inbred strain, the genetic heterogeneity between individual mutant mice is virtually eliminated, helping to isolate the phenotypic effects of a single mutant locus. The presence of modifier loci can then be established if large phenotypic variation is present when comparing the mutant phenotype on different genetic backgrounds (backcrossing to two different inbred strains). One such example is elegantly demonstrated in a mouse model of familial adenomatous polyposis (FAP;OMIM 175,100), in which numerous adenomatous polyps develop in the colon and can eventually progress to carcinomas. FAP is caused by dominant mutations in the adenomatous polyposis coli (*APC*) gene [9, 82], which is mutated in the mouse model *Min* (multiple intestinal neoplasia) on chromosome 18 [87]. It was noticed that in *Min*/+ mice the number of intestinal neoplasms was heavily affected by genetic background: the *Min*/+ mice of the parent congenic strain *C57BL/6 J-Min*/+ have an average of 29 tumors, whereas crosses between these mice and the AKR strain resulted in the *Min*/+ F1 progeny having an average of six tumors [66]. This phenotypic variation seen in different mouse genetic backgrounds is similar to what is observed in humans where the same mutations in the APC gene can give rise to profound phenotypic differences even among family members.

For example, in some families the number of colonic polyps in affected individuals can range from very few to over 100 [56]. Even more interesting is that individual family members with the same APC mutation can manifest with different types of cancer from colonic polyps, gastric polyps, or nongastrointestinal neoplasms such as, osteomas, sarcomas, and carcinomas [81]. These phenotypic differences between family members are probably due to a combination of differences in genetic background (most likely functional polymorphisms modifying disease severity) and environment; however, it is difficult to differentiate between these two effects in human pedigrees. In contrast, the ability to perform multiple backcrosses and select for genetic and phenotypic markers make mice excellent tools for mapping genetic modifiers. This is illustrated in a subsequent study in which the DNA of the 110 *Min/+* mice from a backcross between two different strains [(AKR×B6−Min)×B6 backcross] were sequenced for 75 simple sequence length polymorphisms (SSLPs) distributed throughout the genome and then analyzed with respect to the quantitative phenotype (number of polyps) [26]. This resulted in the discovery of a dominant modifier that reduced the number of polyps, termed *Mom1* (modifier of Min) on chromosome 4, in a region of synteny to human chromosome 1p36-35. The allelic variation for *Mom1* was suggested to be responsible for approximately 50% of the genetic contribution to phenotypic variation based on assumed allelic differences between mouse strains. A further study found that a candidate gene, *Pla2g2a*, encoding a secretory phosphatase, mapped to the *Mom1* region and had 100% concordance between allele type and tumor susceptibility [59]. Nevertheless, causality of this gene was not proven until a transgenic line expressing *Pla2g2a* (OMIM: 172,411) recapitulated the tumor-resistant effects of the *Mom1* locus in a *Min* mouse line [20]. Interestingly, a later study also identified another locus, *Mom2* (modifier of *Min2*), on chromosome 18, which confers stronger resistance to *Min*-induced tumors than the *Mom1* locus [79].

7.5.3 Multigenic Models

As stated earlier in the chapter, deviations from a Mendelian paradigm noted when looking at disease transmission in familial pedigrees or mouse models can suggest oligogenic models. If a mutation at one locus requires a genetic interaction with a mutation at a second locus to elicit the disease phenotype, then it can easily be overlooked in certain pedigrees if a multigenic model of inheritance is not considered. This is because the mutant allele will be present in both normal and affected individuals (the affected ones carry a mutation at a second locus). In cases where the second locus is unknown, the first locus can be implicated in the disease by showing that the mutant allele segregates more often with the affected individuals than would be expected by its population frequency. If several different loci are later implicated individually with the phenotype it is imperative to go back and resequence the entire cohort – meaning both normal and affected subjects, for mutations in one or more of the implicated loci. As in the case of BBS and other oligogenic diseases, many of the affected patients with known mutations at one locus were found to harbor mutations at a second locus later shown to be implicated in the disease.

7.5.4 Linkage Analysis

Linkage analysis is a powerful tool for identifying loci involved in [the] transmission of a disease. In regard to oligogenic disorders, however, traditional linkage approaches must be modified must be modified to account for multiple segregating loci. A key example can be found in Hirschsprung disease (HSCR). In one study, *parametric linkage analysis* (a linkage test with assumptions such as mode of transmission, penetrance, or allele frequencies) and *nonparametric analysis* (NPL; a linkage test without models or assumptions on trait distribution) were first carried out in a traditional manner on 12 L-HSCR families by comparing segregation of polymorphic microsatellite markers in affected and unaffected subjects [17]. Weak evidence of linkage to chromosome 9q31 was observed (Lod score of 1.51, NPL score of 3.8), in addition to the expected strong linkage to *RET* on chromosome 10q11 (Lod score of 9.2; NPL score of 11.2). Subsequent sequencing and mutational analysis identified a diverse group of mutations at the *RET* locus in 11 of 12 families, reaffirming that *RET* is the major locus in L-HSCR. To circumvent the "monogenic bias" of traditional linkage tests, the approach was then modified by dividing the families into two groups, accepting the hypothesis that weaker *RET* mutations require a second locus to manifest the disease phenotype: group I were *RET* linked with severe nonsense and missense *RET* mutations, and group II were *RET* linked without missense and nonsense mutations. (Note: The introns were not sequenced in this study, suggesting that group II most probably has noncoding *RET* mutations). The reanalysis showed no linkage of group I (Lod = 0, NPL = 0.4), but significant linkage of group II (Lod = 4.8, NPL = 5.3), to 9q31, suggesting that mild *RET* mutations and allelic variants of an unknown locus on chromosome 9 are required for disease pathogenesis in the *RET*-linked families lacking exonic mutations. This study signifies that severity of mutations at the major contributing locus often determines whether additional mutations are required for disease manifestation.

7.6 Molecular Mechanisms of Oligogenic Disorders

Although there are now several examples of genetically determined oligogenic disorders (Table 7.1), few of these studies have functionally characterized the genes involved, resulting in a poor understanding of the underlying molecular mechanisms of oligogenicity. The difficult question to answer is how two mutant alleles at two different loci can act in conjunction to cause or exacerbate the same phenotype, a phenomenon known as *nonallelic noncomplementation*. Although nonallelic noncomplementation has been established in various organisms and physiological processes, such as transcriptional regulation and cytoskeletal motility [95], there are few mechanistic examples for alleles implicated in human genetic disease.

Since mutations and allelic variation contributing to disease ultimately effect expression, function, and/or interaction of proteins (with exceptions attributable to noncoding RNA), understanding protein–protein interaction in the cell is essential to elucidating these oligogenic mechanisms. There are two main models that explain nonallelic noncomplementation: the dosage model and the *poison model*. In the case of the dosage model, a concomitant decrease in dosage (expression or function) of two different interacting proteins is necessary to cause a pathogenic phenotype (Figs. 7.3, and 7.4). One example is found in *Drosophila melanogaster*, where null mutations in a ligand, *slit*, which is required for neuronal migration, fail to complement null alleles of its receptor, robo [18, 53]. By contrast, the poison model posits that a mutant protein disrupts or "poisons" a multimeric protein complex to which it normally binds, but not enough to cause visible pathogenesis (Fig. 7.5). This is most likely, because there are sufficient remaining functional complexes. A second mutation in a different protein of the same complex then lowers the functionality of the multimer beyond a certain threshold, which results in a visible pathogenic phenotype. Therefore, the poison model differs from the dosage model in that at least one mutation does not cause just a reduction of functionality, but a "poisoning" of the complex via sequestration of other components or a novel but disruptive interaction [60, 95]. An example can be seen in *Drosophila* and yeast, where altered α-tubulins act as "poisons" through disruption of microtuble polymerization or sequestering β-tubulin [5]. In humans, a good example of the poison model may also be seen in retinitis pigmentosa (RP), where the mechanisms of digenic inheritance caused by mutations in *ROM1* and *RDS* are well understood. It has been shown that these two proteins first form homodimers, which then combine to form a heterotetramer [37]. Mutations in RDS disrupt the proper formation and function of RDS–RDS homodimers and an additional null mutation in *ROM1* further exacerbates the effect on the tetrameric complex [58]. This digenic interaction is believed to be the cause of photoreceptor degeneration in RP.

In another oligogenic model termed *noninteracting noncomplementation*, mutations in two noninteracting proteins can also give rise to an oligogenic disorder, as seen in the example of cortisone reductase deficiency. In this example, H6PDH and 11β-HSD1 proteins are not thought to interact, but act at different stages of a common pathway – endolumeal H6PDH regenerates NADPH in the endoplasmic reticulum required for the oxo-reductase activity of 11β-HSD1 [29]. Each mutated protein thus contributes quantitatively to the dysfunction of the pathway resulting in an additive reduction of 11β-HSD1 oxo-reductase activity beyond a certain threshold that results in a disease phenotype (Fig. 7.6). Another mechanism of oligogenicity of two non-interacting proteins can be found when they have redundant functionality (Fig. 7.7). An example can be seen between Mitf and Tfe3, two members of the Mitf–Tfe family of basic helix-loop-helix-leucine zipper transcription factors that have redundant roles

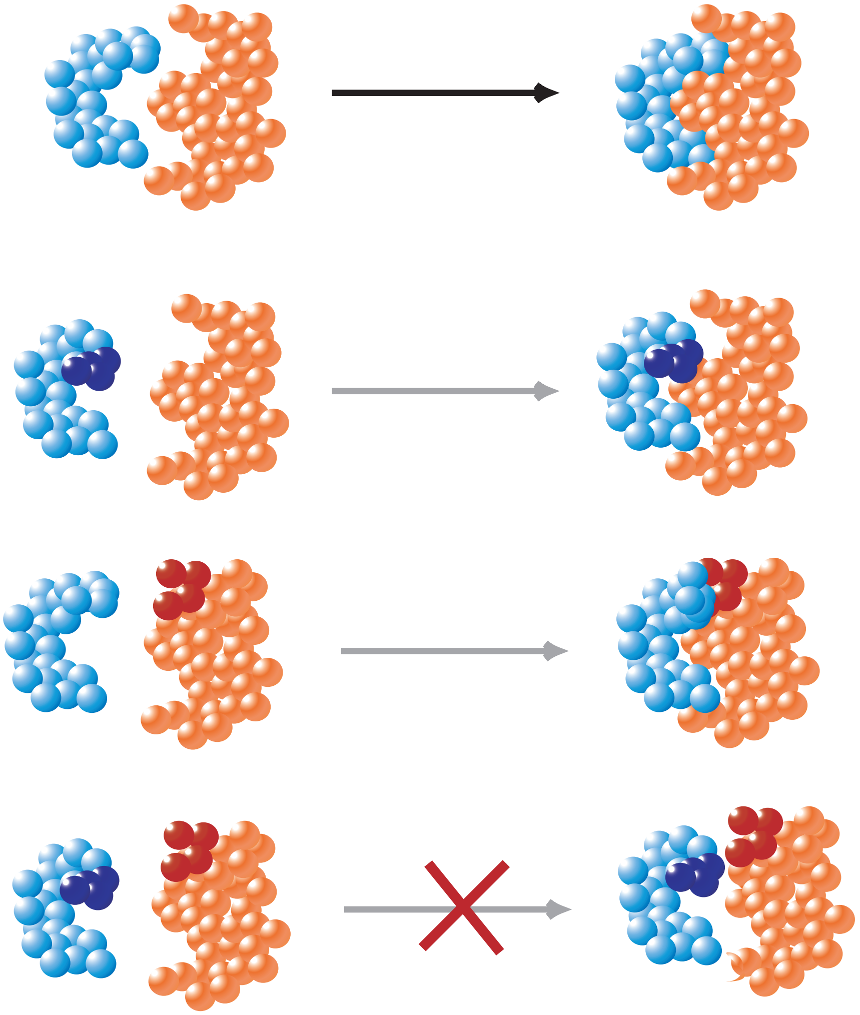

Fig. 7.3 *Dosage model 1:* direct interaction. Mutations at one locus (mutated proteins are indicated by *darker balls*) affect binding but are not enough to disrupt the formation of the complex between the two proteins. Mutations in both loci, however, prevent complex formation or functionality below a critical threshold that results in the disease phenotype

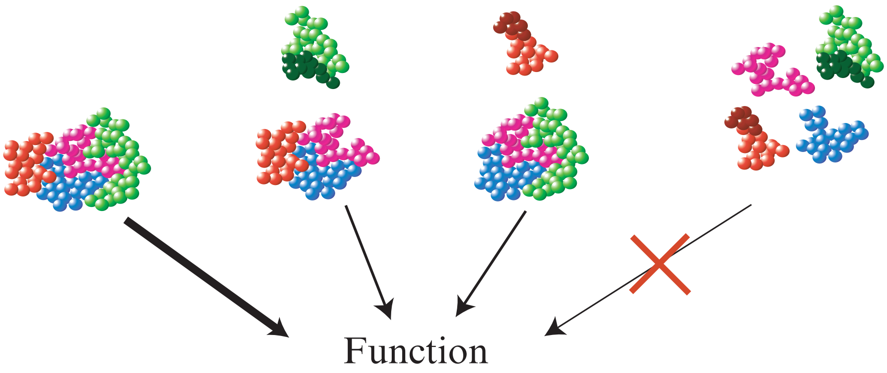

Fig. 7.4 *Dosage model 2:* complex. In a complex between four different proteins, mutations at one locus prevent binding of one protein component and lower (*thinner arrows*), but do not eliminate complex functionality. An additional mutation in another protein that does not directly bind the first one prevents formation or functionality of the entire complex

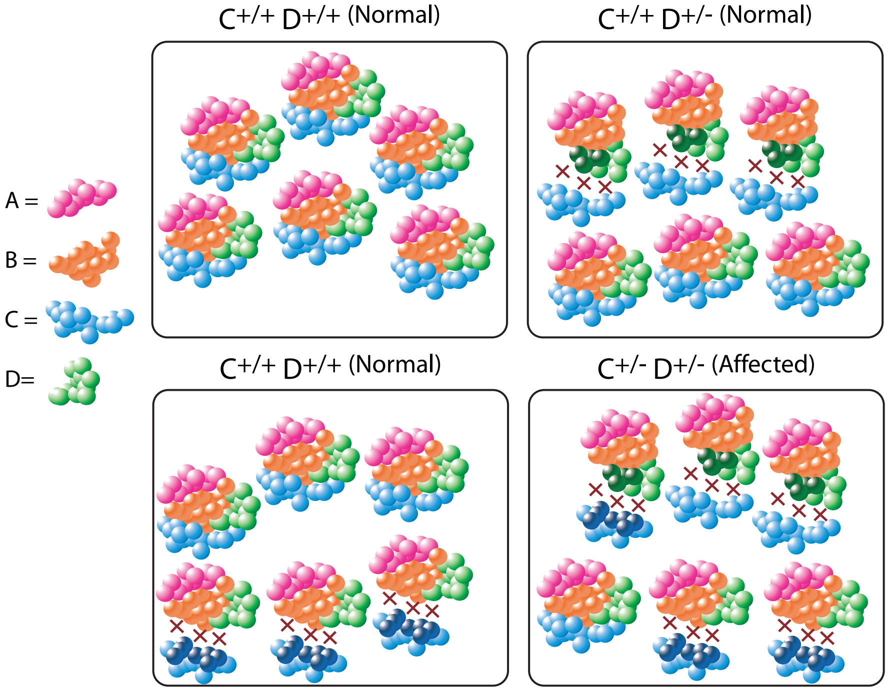

Fig. 7.5 *Poison model.* Mutations in protein C "poison" the complex by binding incorrectly and blocking the binding of another member; however, there are sufficient functional units to prevent a clinical phenotype. Another mutation (or mutations) in protein D disrupt(s) more units of the complex, resulting in very few functional complexes and a disease phenotype

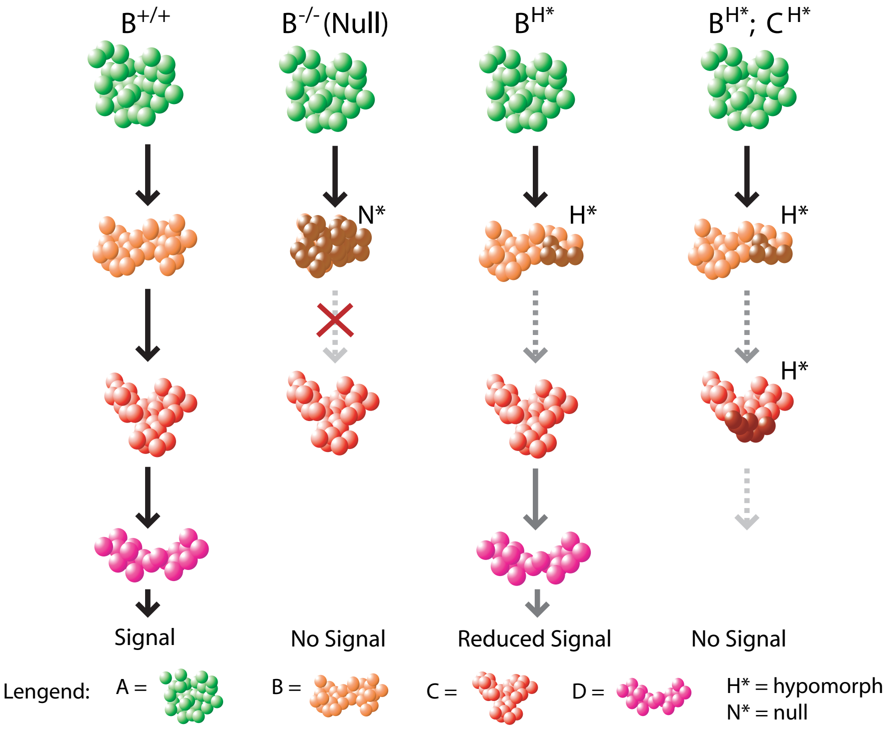

Fig. 7.6 *Noninteracting hypomorphs.* Null alleles of any protein in this pathway (*A–D*) cause a loss of signal (a null mutation in *B* that results in loss of signal in the second column). Hypomorphic (*H**) mutations in *B* result in slightly reduced signal that does not cause a phenotype. However, a second hypomorphic mutation in a downstream protein *C* creates an additive loss of signal that has the same effect as the null mutation

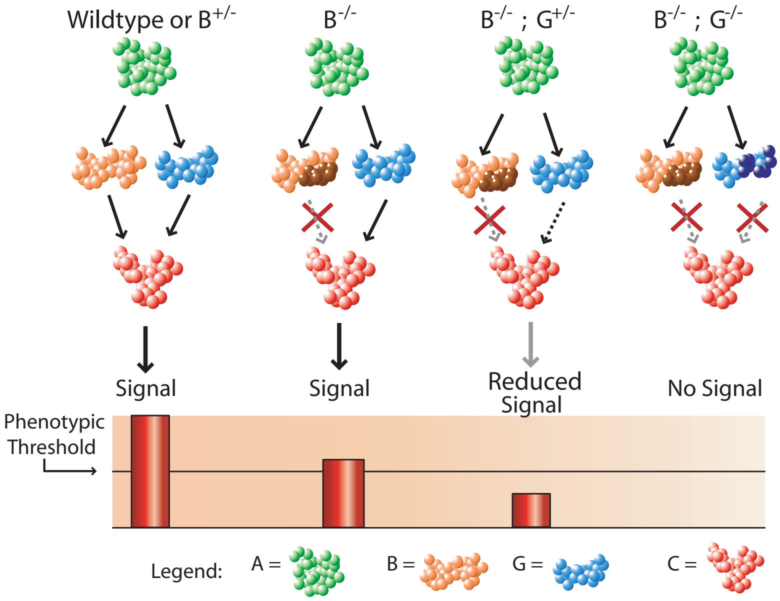

Fig. 7.7 *Noninteracting redundant functionality.* In this signal transduction pathway proteins *B* and *G* have redundant functionality. Homozygous mutations in protein B do not reduce signaling beyond a phenotypic threshold owing to protein *G*'s functional redundancy. However, heterozygous mutations in protein *G* in addition to homozygous loss of function of protein *B* reduces the signal below the critical threshold, resulting in disease

in osteoclast development. In one study, osteoclasts were found to have no abnormalities in *Mitf* or *Tfe3* null mice; however, the combined loss of the two genes resulted in severe osteoporosis [83]. Although Mitf and Tfe3 do interact to form heterodimers, this interaction was shown not to be essential for function or osteoclast development. In contrast, eliminating both the Mitf and Tfe3 homodimers, and thus their individual functions, resulted in the osteoporosis phenotype.

7.7 Modular or Systems Biology

Modular or systems biology is the study of molecular interactions or pathways as a particular cellular system with a discrete function, rather than a focus on the individual functions of proteins. A common theme illustrated by the examples of oligogenic disorders described previously is that the molecular basis of disease usually involves a particular pathway and its individual components. These pathways and their constituents can be better characterized as functional "modules," an evolving concept first described by Hartwell et al., which proposes the existence of discrete subsystems of biological organization. Modules are composed of many different types of molecules, and each module has a discrete function that results from highly regulated interaction of their components: protein, DNA, RNA, and/or small molecules. Oligogenic disorders may result from disruption of two or more components of a particular module. The concept of modules also suggests that both different disorders with similar clinical manifestations and locus heterogeneity within a single disorder probably result from mutations disrupting different components of a single molecular system. For example, cortisone reductase deficiency and polycystic ovary syndrome (PCOS), both disorders of a particular system regulating adrenal steroidogenesis, are due to greater conversion of cortisol to cortisone and impaired biosynthesis of cortisol, respectively, both of which cause adrenal androgen excess and PCOS or PCOS-like phenotypes in women [15, 93]. The four types of hemochromatosis, disorders of abnormal iron deposition, can be characterized into a system or module regulating heme biosynthesis. Extending this line of thought, it is notable that patients with familial porphyria cutanea tarda (OMIM 176,100), a disorder of reduced activity of uroporphyrinogen decarboxylase (UROD), the fifth enzyme in the heme biosynthetic pathway, are also associated with HFE mutations found in hemochromotosis patients [61].

Functional modules, by definition, have a particular function that can be distinguished from other modules through either spatial localization or biochemical specificity. This is exemplified in ciliopathies such as nephronophthisis (OMIM 256,100), Bardet–Biedl syndrome (209,900), Alstrom syndrome (203,800), Meckel–Gruber syndrome (OMIM: 249,000), Joubert syndrome (213,300) [77], and polycystic kidney disease (173,900), where mutated genes are all components of a particular module, viz. the primary cilium [6]. In keeping with the view of the cilium as a functional module, mutations in different ciliary or basal body genes can result in ciliopathies with overlapping clinical manifestations, such as RP, obesity, polydactyly, renal cysts, and diabetes [6]. In addition, different ciliopathies can arise from mutations in the same gene, the severity of which determines the spectrum of clinical manifestations and thus the disease. For example milder mutations in *BBS6* are associated with McKusick–Kaufman syndrome, whereas more severe mutations (or mutations combined with alleles in other BBS genes), lead to BBS [50].

Functional modules are not necessarily rigid or fixed structures, and any given constituent may belong to different modules, which might offer one explanation as to why mutations in the same gene can sometimes lead to unrelated phenotypes. An example is *RET*, which encodes a receptor tyrosine kinase and is expressed in cell lineages derived from neural crests [46]. *RET* is involved in molecular systems that regulate cell proliferation, migration, differentiation, and survival during embryogenesis [3]. Different mutations in *RET* can give rise not only to HCSR, as described earlier, but also to multiple endocrine neoplasia type IIA or type IIB (*MEN2*) [41], or familial medullary thyroid carcinoma (FTC; OMIM 188,470) [39]. Germline missense mutations resulting in constitutively active *RET* can cause the different subclinical cancer types, depending on the tissues affected. There is large phenotypic variability, even between the different cancer types, which can range from medullary thyroid carcinoma, a tumor of the adrenal medulla, and hyperparathyroidism in MEN2A (OMIM: 171,400) [41], to a more complex clinical phenotype including ganglioneuromas on the tongue, lips, and eyelids, intestinal ganglioneuronas, thickened cornea nerves and a marfanoid habitus in MEN2B (OMIM 162,300). HCSR results from mutations that disrupt *RET* function, resulting in a loss of enteric ganglia. This large phenotypic variability resulting from mutations in one gene suggests that the "module-specific" functions of *RET* can be separated temporally (period of development), spatially (tissue or cell type), and/or through chemical specificity (expression of tissue specific ligands or cofactors that interact with *RET*).

The concept of modules or molecular systems in the context of disease can be important in the search for modifier alleles. The involvement of a modular component in a particular disease suggests that mutations disrupting other components of that module may also contribute to the same disease or related disorders. The advantages of having a modular or systems approach in understanding disease are illustrated in a recent BBS study that used the hypothesis that the disruption of the primary cilium or its constituents is the sole basis for BBS and led to the discovery of a BBS modifier, MGC1203 (OMIM 209,900) [7]. Accepting the hypothesis that undiscovered genes involved in BBS would potentially interact with known BBS genes, several yeast two-hybrid screens were performed and identified more than 60 candidates. Since BBS is a ciliopathy, presence of these candidate genes was then screened for in the ciliary proteome – a database of purported ciliary localizing proteins [57]. This resulted in the identification of MGC1203 as the only protein present in both data sets. Further characterization through combined use of biochemical, genetic, and in vivo tools identified MGC1203 as a modifier that has an epistatic effect on BBS mutations, with some allelic variants resulting in greater disease severity. This study underscores the importance of using a variety of molecular and genetic tools under a paradigm of "modularity" of disease components in order to dissect the potentially weak contribution of genes in an oligogenic disorder.

7.8 Conclusions

The study of oligogenic disorders, diseases caused or modulated by a few genes, is elucidating new principles of methodology that are necessary to overcome the inherent limitations of current methods towards classification of monogenic disease. The discovery of genetic modifiers, compounded by the poor genotype–phenotype correlations in even the most quintessential monogenic disorders, is highlighting the vague lines between what should be considered monogenic or oligogenic. In fact, evidence of both modes of inheritance is sometimes found within the same clinically classified disorder.

The highlighted examples of oligogenic disorders suggest that the individual genetic factors are often confined to a similar pathway or module. The alleles of these genes contributing to disease, however, are not necessarily rare mutations, but can be common polymorphisms that only elicit an effect in a particular physiological setting. To disclose these often weak genetic interactions new methodology must be applied, such as considering heritability, using mouse models of varying genetic backgrounds, and modifying traditional linkage approaches. Overall, the future of understanding complex disease lies in analyzing genetic factors from a systems biology viewpoint under the premise of modularity of these factors within the same or between similar disorders.

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Formal Genetics of Humans: Multifactorial Inheritance and Common Diseases

8

Andrew G. Clark

Abstract The study of the genetics of complex traits is made complicated by the fact that the traits themselves are influenced by an interplay of many genes with many environmental factors. In this chapter the historical concepts of quantitative genetics, including additive variance and heritability, will be developed to underscore how important it is to understand that the root of the problem is to explain how genes contribute to the variance in a trait. With molecular genetic markers, such as SNPs, it is possible to test whether there are differences in the measured phenotype among the genotypes at the genetic marker, and this serves as a crude test of association. Many interesting challenges arise when such a test is expanded to 1 million markers spanning the entire chromosome, a design known as a genome-wide association study (GWAS). Complications due to population stratification, admixture, genotype x environment interaction, epistasis, and rare alleles are all considered. Methods that test association by use of excess of allele sharing in siblings (affected sib methods) or other relatives, or by excess cotransmission of alleles and a disease state (transmission disequilibrium test) have their own set of advantages and disadvantages. The chapter closes with some considerations of why the powerful methods presented here nevertheless leave much of the genetic variance in complex traits unexplained.

Content

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8.1 Genetic Analysis of Complex Traits

A primary goal of genetic analysis is to understand the causal relationships that connect the observed variation in phenotypes to the underlying genetic variation in the population. The simplest case was that observed by Gregor Mendel, where a single gene with two different co-dominant alleles presents a one-to-one correspondence between genotype and phenotype. In this situation, the ability to predict offspring ratios from any given parental phenotypes is very good. Most human traits do not follow these simple rules of transmission, but instead have a more complex association between genotype and phenotype. We become convinced that there is at least some genetic aspect to the transmission, because there is *familial resemblance*. These traits aggregate in families, but do not segregate like a single Mendelian gene. Such traits include stature and body proportions, facial features, skin color, and blood pressure. Many diseases may have a complex nexus of causes, but often the liability may differ between individuals and may be genetic in origin. In earlier years, a Mendelian framework was often superimposed naively on such data, with no testing of the formal requirements for simple modes of inheritance. We will show in this chapter that a fruitful way to approach the genetics of complex traits is to fit the data on individual genotypes and phenotypes to specific models that consider different ways in which the genetic variation may be causing the phenotypic variation. One outcome of this kind of model fitting is to map the genes responsible for the variation. But by the very nature of complex traits, there is also a role of environmental effects on the traits, and the observation that different genotypes respond differentially to environmental pressures means that the inferences about the genotype-phenotype association depends on the environmental context. Let us first consider some basic principles about variation at the phenotypic and genetic levels.

8.1.1 Variation in Phenotypic Traits

A fundamental idea to focus on in considering complex traits is that the primary feature that is of importance is among-individual *variation*. Nearly every trait shows some level of variation among individuals, from overall body size measurements, to the most minute features, such as fingerprints. Most biochemical traits also display variation, including the levels of many components of the blood (cholesterol, hemoglobin) and ranging up to the activities of metabolic enzymes in the liver. It is only because there is variation among individuals that there is an opportunity to identify underlying genes that themselves harbor genetic variation in the form of differences in DNA sequences. These gene variations in turn may mediate the phenotypic variation. Just because we can identify mouse mutants in orthologous genes that have profound effects on a particular phenotype, this does not guarantee that the population will harbor natural polymorphisms in that gene, which in turn will influence trait variability. Similarly, the genes that appear to be most responsible for variation in a trait may play a part in the mechanism for that aspect of biology that seems totally peripheral, or in many cases one has no clue why gene X influences trait Y. This level of decoupling of genetic and phenotypic variation may seem unnerving at first, but for many attributes of profound medical importance there is important phenotypic variability (such as susceptibility to atherosclerosis) and excellent understanding of relevant pathways, but relatively great uncertainty about the causes of variation.

8.1.2 Familial Resemblance and Heritability

Before we make the leap from phenotypic variation to seeking to find the genes responsible for that variation, there is one other attribute of the trait that is of vital importance. The trait might actually not have any genetic variation responsible for the phenotypic variation, but may instead be driven entirely by environmental influences, such as diet or exercise levels. Fortunately there is a rich history of study of the problem of detecting a role of genes in complex traits simply by asking whether the degree of resemblance among relatives is elevated above what one would see by chance.

A fundamental idea in quantitative genetics is that variability in a trait can be partitioned into components that contribute to that variability. We seek to explain the variability in the phenotypic measures in terms of both genetic and environmental factors. Environment is considered as a sort of trash-bin term to encompass all nongenetic factors that influence the phenotypic value. The simplest statement of a model is that the phenotypic value of an individual is composed of the sum of the genotypic value plus the environmental value:

$$\mathrm{P} = \mathrm{G} + \mathrm{E}$$

where P = phenotypic value, G = genotypic value, and E = environmental value.

The phenotypic values of all individuals in a population have a mean and a variance around this mean. The variance is distinguished from other measures of variability by one mathematical property: different variances can be added to give a total variance and, conversely, a total phenotypic variance V_{P} can be broken down into its components, such as the genotypic variance V_{G} and the environmental variance V_{E}:

$$V_{\mathrm{P}} = V_{\mathrm{G}} + V_{\mathrm{E}}$$

The idea that the sum of normally distributed factors yields a normal distribution whose variance is the sum of the variances of the components is true in the limit with many factors, and is a central idea in statistics (indeed, it is called the Central Limit Theorem) (Fig. 8.1).

However, the addition rule for variances applies only if genotypic and environmental values are independent of each other, i.e., when they are not correlated. If there is a correlation between the two, the covariance of G and E must be added:

$$V_{\mathrm{P}} = V_{\mathrm{G}} + V_{\mathrm{E}} + 2\,\mathrm{Cov}_{\mathrm{GE}}$$

Let us take an example from the area of genetics that first introduced these concepts – agricultural studies. It is normal practice in dairy husbandry to feed cows according to their milk yield. Cows that produce more milk are given more food. Such correlations of genetic and environmental factors tend to inflate the variance. Whether human societies present environmental perks to individuals in a way that is correlated with genetic proclivity is open to discussion. In any event, any such correlation between genetic and environmental variation should be identified, as it can cause serious problems in the modeling if it is ignored.

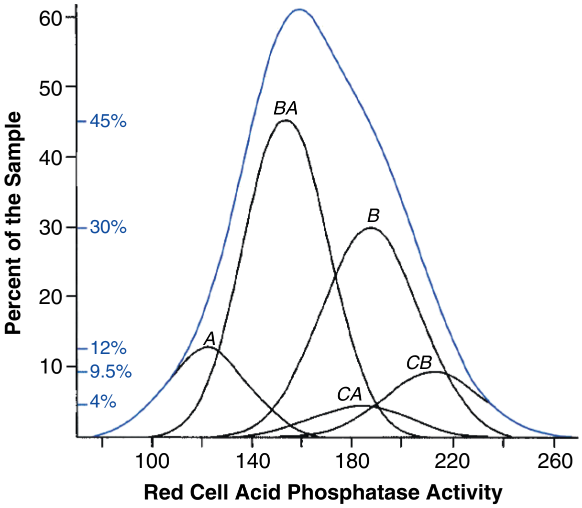

Fig. 8.1 The population distribution of acid phosphatase activity. The bell-shaped curve of total enzyme level (acid phosphatase) may be the sum of enzyme activities for acid phosphatase for genotypes of several polymorphic alleles, each having different overlapping acid phosphatase activity. Most phenotypes have a distribution in a population that results from summing over heterogeneous collections of genotypes (from Harris et al. 1968 Ann N Y Acad Sci. 151:232–242)

Another assumption is that specific differences in environments have the same effect on the various genotypes. When this is not so, there is an interaction between genotype and environment, giving an additional component to the variance V_{GE}. A prime example of genotype by environment interaction occurs with adverse reactions to drugs by a subset of individuals with a susceptible genotype. In the laboratory, where multiple replicate experiments may be run with identical genotypes of plants or animals, genotype × environment interaction is measured by testing the same genotypes across a range of environments.

The genotypic value V_{G} can be subdivided into several components: an additive component (V_{A}) and a component (V_{D}) measuring the deviation attributable to dominance and epistasis (V_{I}) from the expectation derived from the additive model. The dominance variance is contributed by heterozygotes (Aa) that are not exactly intermediate in value between the corresponding homozygotes (*aa* and *AA*). The variance contributed by epistasis refers to the action of genes that affect the expression of other genes. Hence, the concept of additive variance does not imply the assumption of purely additive action of the genes involved. Even the action of genes showing dominance or epistasis tends to have an additive component. The whole genotypic variance can be written:

Phenotypic variance	Genetic variance	Environmental variance	Genetic × environmental covariance
$V_P =$	$V_A + V_D + V_I$	$+ V_E$	$+ \text{Cov}_{GE}$

To estimate these various components of variance, one measures the phenotypes of individuals that have different known relationships to one another. There are simple algebraic relationships between the correlations of phenotypic measures among relatives and these components of variance. The one that we will focus on is the relationship between parents and offspring. Sir Francis Galton observed a nearly linear relationship between points that represent family groups plotted as follows. Define the *x*-axis as the average of the two parents' phenotypes (also called the midparent), and the *y*-axis as the average of the offspring phenotype. If each point represents a nuclear family, then in a population, such points will fall along a line whose slope is called the "heritability." If the slope is 1.0, this would mean that the average of the offspring is always equal to the average of the parents, and so the resemblance is perfect. More typically, the slope might be about 0.5, meaning that for every increase by a factor of 2 in the phenotype of the midparent, the offspring mean would increase by 1. Galton called this line through the scatter of points (Fig. 8.2) a "regression" line, because the offspring tend to be less deviant from the population mean than do the parents. As Galton put it, the offspring *regress* toward the population mean.

The heritability, as calculated by the midparent-offspring regression can also be written as:

$$h^2 = \frac{V_A}{V_P}$$

When it is written in this way, it is clear that heritability can be considered as the proportion of the total phenotypic variance that is explained by additive genetic effects. This expression varies between 0 and 1, and many morphological traits, such as height, have a heritability in the range of 0.7–0.8, implying that the bulk of the variance is genetic in origin. This very same term, often called the narrow sense heritability, has also been used by plant and animal breeders to predict the outcome of artificial selection for such economically useful traits as milk production in cows and egg laying in chickens. A high proportion of additive genetic variance implies that the trait will respond rapidly to selection. Heritability of many complex disorders, such as diabetes, is more in the range of 30–50%, implying that there clearly is a genetic component, but that this only explains part of the variation in disease risk.

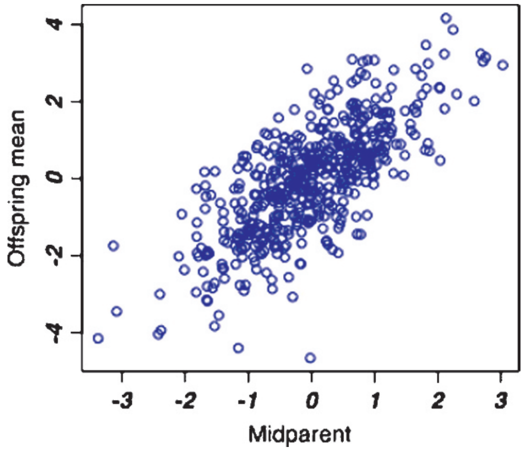

Fig. 8.2 The parent-offspring regression. The *x*-axis plots the average phenotypic measure of the two parents (the midparent) and the *y*-axis is the average phenotype of the offspring from each couple. Thus, each *point* on the plot represents a single nuclear family. The slope of the regression line through these points is the narrow-sense heritability. Francis Galton constructed many such scatter-plots, and inferred the degree of familial resemblance in this way

It is worth stating carefully some of the properties of heritability:

(a) Heritability is a ratio. A ratio changes when either the numerator or the denominator changes. There is an increase in h^2 when the numerator (V_G, genotypic, or V_A, additive, variance) increases, or when the denominator (V_P, phenotypic variance) decreases. We could also say that a reduction in environmental variability will actually increase the heritability!

(b) The estimation of heritability is based on theoretical correlations between relatives. These correlations are valid only for random mating. Assortative mating leads to other correlations and, unless taken into consideration, produces systematic errors in the estimation of h^2. The correlations resulting from assortative mating were first calculated by Fisher [7]. These correlations can be used for adjustment of h^2.

(c) An estimation of h^2 is strictly valid only when the assumption is made that covariance and interaction between genotypic and environmental values are 0.

Correlations between relatives do not prove that there is genetic variability; they may also be caused by common environmental influences within families. In animal breeding, where the environment can be controlled, this factor might either be neglected or quantified. In humans, this is almost impossible. One of the major areas of research in human complex trait genetics today is to develop better automated methods for measuring differences in the environments that individuals have experienced. For example, many chemical exposures can be assessed by directly measuring residues in the bloodstream.

8.1.3 The Special Case of Twins

The use of twins has been much more popular in the past as a means for understanding genetic transmission of traits and diseases, but twin studies remain an excellent tool for developing concepts of genetic transmission. Identical or monozygotic (MZ) twins represent a wonderful experiment in nature (Fig. 8.3), since their genetic identity implies that differences between MZ

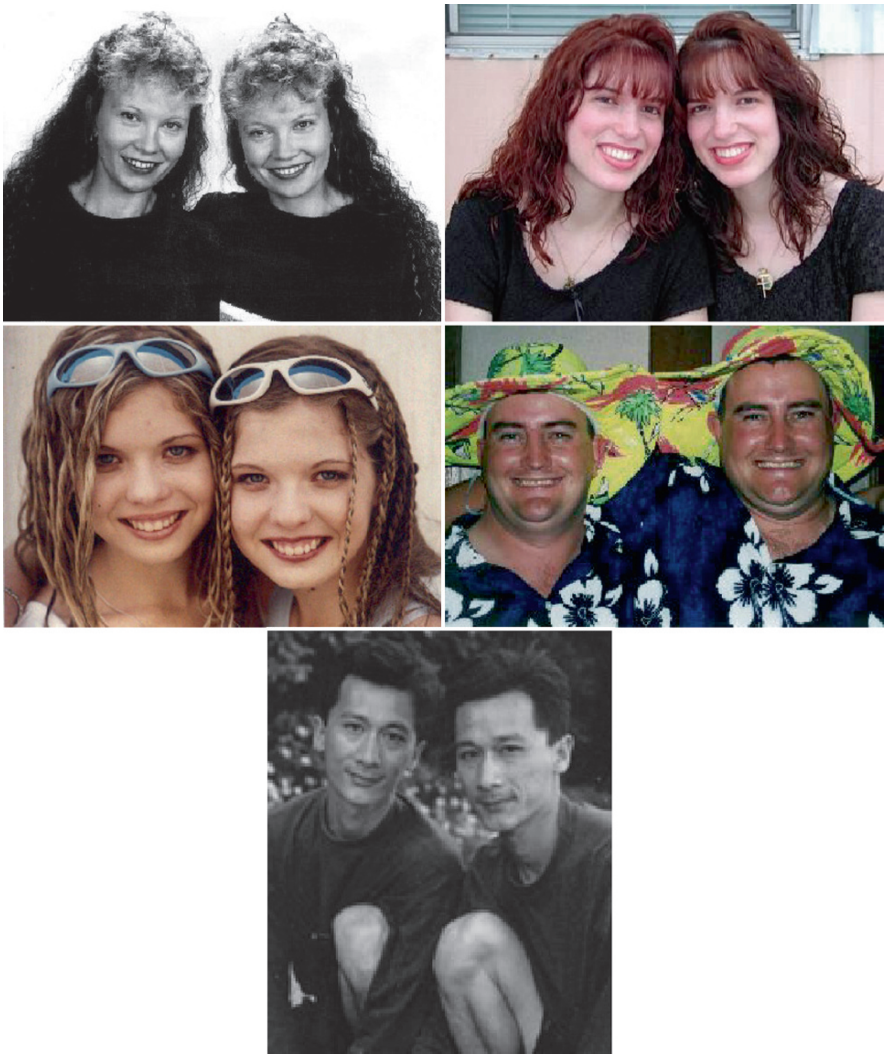

Fig. 8.3 Monozygotic twins have long fascinated human geneticists, and the questions about their biology change with advancing technologies. Initially interest focused on gross morphological similarities that were easy to measure. Now these questions center on issues of differential epigenetic modifications, differences in somatic mutations, altered patterns of X-inactivation, and similarities in brain activity as measured by functional MRI

twins must be due to accumulated perturbations of the environment [15]. In discussions on methods of quantitative genetics the use of twin data for quantitative assessment of the degree of genetic determination has been mentioned repeatedly. Indeed, twin investigations have played a major role in the history of human genetics. Especially in the field of behavior genetics, much of our current understanding is based on twin data. Therefore critical assessment of the twin method, its advantages, and limitations, is well motivated.

The twin method for assessing heritability is based on the biological observation that MZ twins originate from splitting of one zygote into two identical clones. It follows that any phenotypic differences between MZ twins must be largely caused by environmental influences. Somatic mutations may arise that generate differences between MZ twins, and efforts to quantify differences in somatic mutations between twin pairs using modern genomics technologies are under way in several laboratories. Environmental differences may manifest themselves by altering the epigenetic states of chromosomal regions, and an active area of research is to quantify the magnitude of differences in DNA methylation and histone acetylation between MZ twin pairs [2].

The degree of phenotypic similarity between MZ twins can be contrasted to the similarity between dizygotic (DZ) twins. Assuming that DZ twins are influenced by the same environmental differences but have only one-half of their genes in common by descent, the greater degree of resemblance of MZ twins provides a kind of measure of heritability. This heritability, however, is not the same as the parent-offspring regression approach mentioned above. Instead, the heritability one gets from twins is *broad-sense heritability*:

$$h_B{}^2 = \frac{V_G}{V_P}$$

where V_G and V_P refer to the total genotypic and phenotypic variance, respectively. This broad-sense heritability can be estimated from MZ and DZ twin pairs by calculating the average correlation between pairs of MZ twins (r_{MZ}) and the average correlation between pairs of DZ twins (r_{DZ}). The broad sense heritability is then $h_{\mathrm{B}}{}^2=2(r_{\mathrm{MZ}}-r_{\mathrm{DZ}})$. It takes some algebra to show exactly why this is so, and it is of course true only when there is no shared environment effect. If there is a shared environment effect and it is measurable, one can adjust the heritability downward using another formula.

The above model for estimating heritability from twins makes some key assumptions about the biology that deserve to be considered carefully. In particular, twins have a unique shared environment that nontwins do not, and one has to worry whether that shared time in utero may influence their degree of resemblance. Because they have shared nutrition and environmental stresses, this shared environment might be expected to inflate the resemblance of twins. Whether the resemblance is augmented more in MZ twins than DZ twin depends on the details of how the environment is experienced (e.g., in one chorion or in two chorions).

One appreciates the effect of the uterine environment simply by examining medical attributes of twins and nontwins. Twins suffer from a higher frequency of abnormalities during pregnancy and at birth. Their lower birthweight can be attributed only partly to the shorter duration of gestation. The stillbirth rate and infant mortality in early life are considerably higher in multiple births than in single ones; in later years, twins run a higher risk than nontwins of becoming mentally retarded, which is presumably at least partly due to complications during pregnancy and at birth. Even the mean IQ of both MZ and DZ twins is slightly lower than that of control populations.

Some features of twins result in a higher chance that they *differ* in traits. X-inactivation in females occurs at the division of the zygote after X-inactivation (and is a fairly disruptive process). It therefore may happen that all cells in which a certain X-linked gene has been inactivated end up in one twin, while all the cells with active X chromosomes are found in the co-twin. This phenomenon leads to clinical expression of X-linked traits (such as Duchenne muscular dystrophy or color blindness) in only one member of a female twin pair that is heterozygous for the X-linked trait. A striking example is that two of the MZ Dionne quintuplets were color blind! The roles of X-inactivation and of intrauterine effects on epigenetic modifications are two of the many processes that occur during development and result in altered resemblance between twins. Thus, twins remain a fascination for geneticists, although simple calculation of heritability based on twin resemblance is clearly fraught with problems.

8.1.4 Embedding a Single Measured Gene Influencing a Continuous Trait

Consider a trait that has important medical consequences, where the trait has continuous phenotypic variation but we also know about an underlying mechanism for the trait, and we have managed to identify a gene whose variation influences the trait. As an illustration, consider the example of warfarin dose and the *VKORC1* polymorphism. Warfarin is an important anticoagulant drug that is used for heart disease patients and other circumstances where it is important to "thin" the blood to prevent thrombosis (clotting). The problem with warfarin has been that it has a narrow range of dose within which it is effective – too low a dose and it fails to delay clotting time, but at too high a dose it leads to hemorrhagic complications. For each patient there is a period where the optimal dose for that patient must be determined by approximate testing of coagulation status. To make matters worse, there is wide variability among individuals in the best therapeutic dose.

Rieder et al. [17] did a retrospective study on a large cohort of individuals who had been on warfarin therapy. These individuals had been through the battery of tests to determine their correct warfarin dose, and this was the phenotype being considered. The target of warfarin is the vitamin K epoxide reductase complex 1 (*VKORC1*), and a first guess might be that there could be polymorphism in this gene that requires different doses of warfarin for effectiveness. The study was a stunning success, finding mean differences in optimal warfarin dose across genotypes.

Other studies had associated warfarin dose with the cytochrome P450 2C9 (*CYP2C9*) gene, and this raises the question of whether there might be other genes elsewhere in the genome that also contribute to variation in optimal warfarin dose. Cooper et al. [3] did a scan of 181 European warfarin users and a replication sample of 374 individuals. They tested 550,000 SNPs and found that *VKORC1* had by far the strongest association ($P=6.2\times10^{-13}$) and that a SNP in *Cyp2C9* has moderate significance ($P<10^{-4}$). Because none of the other SNPs attained significance in this study, the conclusion was that common SNPs with large effects on optimal warfarin dose are unlikely to be discovered outside of *VKORC1* and *CYP2C9*. In Sect. 8.1.5 we will see how to partition the variance in a trait, and to determine what fraction of the total variance in a phenotype is attributable to one or two major genes.

8.1.5 A Model for Variance Partitioning

The preceding section showed how the continuously varying phenotype can be thought of as having multiple causal factors that determine the phenotype. If one is lucky and has a handle on one of those factors, it is possible to determine what fraction of the total variation is explained by that one factor. Let us consider a model that seeks to explain variation in continuous traits as the sum of the effects over many loci. We can further assume that, as in the above section, we have a handle on one of the loci. Among a collection of individuals whose genotype is *aa*, we can define the mean phenotype as $-a$. For the *Aa* heterozygotes, let the mean phenotype be *d*, and for the *AA* homozygote, let the mean be $+a$. If the frequencies of the *A* and *a* alleles are *p* and *q*, an the population is in Hardy–Weinberg equilibrium, then the mean phenotype for the whole population is:

$$p^2a + 2pqd + q^2(-a) = a(p-q) + 2pqd$$

If we were to plot the phenotypes for these three genotypic classes on the *y*-axis, and label the *x*-axis with the genotypes *aa*, *Aa*, and *AA* at coordinates 0, 1, and 2 (think of this as measuring 0, 1, and 2 copies of the *A* allele), then a regression through these points has many useful attributes. The increase in phenotype for each addition of an *A* allele is the "average effect of an allelic substitution" and has the value $\alpha = a + d(q\text{-}p)$. The *y*-axis values for the points on the regression line are $-2p\alpha$, $2pqd$, and $2q\alpha$. These are the "breeding values," a term from classic animal breeding analysis. They give the value of each genotype if the allelic substitutions were purely additive. But because there is dominance, we can calculate the deviation of each observed phenotype from this regression line fit (like a residual in a regression). These are the dominance deviations, and they are $-2p^2d$, $2pdq$ and $-2q^2d$, respectively.

From the breeding values and the dominance deviations we can calculate two important attributes of this trait. The additive genetic variance is the variance in breeding values. This is the sum of the squared deviations from the mean, weighted by the population frequencies or:

$$\begin{aligned} V_A &= p^2(2q\alpha)^2 + 2pq[(q-p)\alpha]^2 \\ &\quad + q^2(-2p\alpha)^2 = 2pq[a + d(q-p)]^2 \end{aligned}$$

Recall that one of the definitions of narrow-sense heritability is the additive genetic variance divided by the phenotypic variance. This formula for the additive variance makes it clear that the additive genetic variance depends on allele frequencies, and it drops to zero with either $p=0$ or $p=1$. The variance in dominance deviations is the dominance variance. This is:

$$V_D = p^2(2q^2d)^2 + 2pq(2pdq)^2 + q^2(2p^2d)^2 = (2pqd)^2$$

The above formulae show clearly how the variance components are impacted by allele frequencies, and how heritability itself also varies with allele frequencies. The important point to remember about these measures of quantitative genetics is that these are parameters of a model, and the numbers have meaning only so far as the model explains the data. In many circumstances in plant and animal breeding for agricultural purposes, we have excellent data demonstrating the utility of the models. Human quantitative genetics cannot assess whether the model fits nearly as thoroughly, both because the environment is less well controlled and because the only crosses that can be observed are those drawn from a large, essentially randomly mating population.

In the absence of epistasis or genotype × environment interaction, the total genetic variance is the simple sum of the additive variance and dominance variance: $V_G = V_A + V_D$. This splitting of a variance into two parts is called variance partitioning, and a key part of modern quantitative genetics is to partition variance into components that have biological meaning. For example, in the *VKORC1* example, the total genetic variance in optimal warfarin dose can be partitioned into a component of variance attributable to the *VKORC1* gene, and another component that accounts for the rest of the genome. For further development of the models for partitioning quantitative genetic variation, see [6].

8.1.6 Relating the Model to Data

When there is a measured genotype that it is suspected is involved in a trait, the above model suggests a straightforward way to test what the effects of that gene on the phenotype are. We have to emphasize that this test is valid under the assumption that the effects across genes are additive. If this assumption is not right, then the inferred effects of the measured gene will not be valid – and the estimates can either spuriously overestimate the effects or underestimate them. Thus, a lot hinges on the validity of the assumption of additive effects.

First we bin the individuals in the population into the three bins based on their genotypes at the measured locus. If we plot them as in Fig. 8.4, it can be seen that one way to test the null hypothesis of no effect of this gene would be to perform a linear regression and test whether the regression coefficient (the slope) differs from zero. A nonzero slope indicates that the gene has an additive effect on this trait. A really wonderful aspect of this approach to the problem is that the slope is proportional to the additive effect contributed by this locus. Similarly, if the data are plotted with two *x*-values, where genotypes *AA* and *aa* are on the left, and *Aa* on the right, then the regression through these points will have zero slope if the heterozygotes are intermediate between the two homozygous classes. This would be true if there were zero dominance. So the estimator for the dominance effect is simply the regression coefficient obtained from the data when arranged in this way.

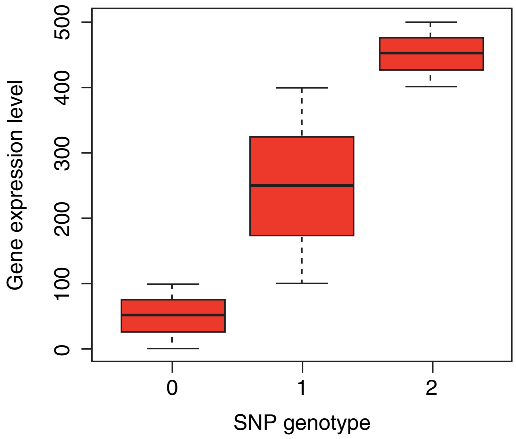

Fig. 8.4 Association of a single SNP with a continuous trait can be assessed by the regression plot depicted here: the *x*-axis has discrete elements for each genotype at one locus, and the *y*-axis is the continuously variable phenotypic measure. The slope of the regression line through these points yields the additive component of variance. If the genotype at this one SNP has no effect on the phenotype, this slope will be zero. The test of significance for whether the regression coefficient is greater than zero is the formal statistic test for whether this SNP shows an association with the phenotype

8.1.7 Mendelian Diseases Are Not Simple

While it is possible to trace the transmission of simple Mendelian traits and show that the trait is consistent with a major gene that is transmitted in the same way as smooth vs wrinkled peas, humans are so acutely aware of subtle phenotypic differences that the full spectrum of phenotypes associated with a major gene is almost never simple. And the departures from the pure Mendelian pattern are not always subtle. There are cases of individuals who are homozygous for a disease-causing allele, but are nevertheless perfectly healthy. Is this an example of a variant allele with reduced penetrance? Or, as is more often the case, are there other genes in the genome conferring a modifying influence, virtually suppressing the disease phenotype in these individuals? Mendelian disorders are not simply composed of two alleles, one healthy and one diseased; rather, a multitude of mutations that knock out function can result in disease, and there is also typically a series of alleles in the healthy group. In this case we have a mutation-selection balance between a fully functional gene and a rainbow series of alleles of reduced function. Heterozygotes may have even more intermediate phenotypes. It should be clear how this presents a situation where, despite the primary role of one major gene, there is nevertheless a continuous spectrum of disease severity in the population.

8.2 Genetic Polymorphism and Disease

Much of our understanding about the genetic basis of complex chronic diseases is based on our knowledge of Mendelian disorders, coupled with experiences in quantitative genetics of agricultural and laboratory organisms. We see that the complex disorders aggregate in families but do not segregate as Mendelian genes do, and so the inevitable conclusion is that the genetic basis involves many genes. In order to find those genes and to better understand the transmission of the disorder, we must construct a model for the genetic architecture. There may, for example, be a single major gene that accounts for most of the disease risk, but a series of modifier genes may temper the expression of this major gene. Or there may be ten genes, each of which is equally important in determining the trait. The frequency of the high-risk alleles may be very low, which may happen if there is natural selection driving them to low frequency in a mutation-selection balance, or they may have more intermediate frequency if they have little influence on reproductive fitness. In the next sections we will examine properties of polymorphisms in human genes and their impact on complex diseases.

8.2.1 Finding Genes Underlying a Complex Trait

In the preceding sections of this chapter we saw the consequences of a gene that has an effect on a trait and how its effect is added to the mix of effects of other genes and environment to add to the among-individual variability in the trait. Now imagine the situation in which you have no information about any of the underlying genes. The only data you have are the measurements of phenotypes of many individuals. You can determine that the trait has a heritable component because of the fact that relatives have correlated phenotypes. It is also clear that, if you did have measurements of the genotypes of a gene that happened to influence the trait (let the genotypes be *AA*, *Aa*, and *aa*), you might be able to see this from the fact that the phenotypes of these three genotypic groups might be different. The challenge is to identify an efficient way to find such genes.

8.2.2 Limitations of Pedigree Analysis

Probably the first approach one would consider for mapping the genes that underlie variation in a trait would be linkage analysis using pedigrees. This is a fundamental approach in human genetics, and it has a long history of success. As soon as one suspects a genetic basis for a syndrome, one has a collection of cases, and so by acquiring DNA samples from relatives, it becomes possible to test the linkage of the syndrome to anonymous marker loci throughout the genome. Methods for performing linkage analysis are provided elsewhere in this text, but there are several attributes of

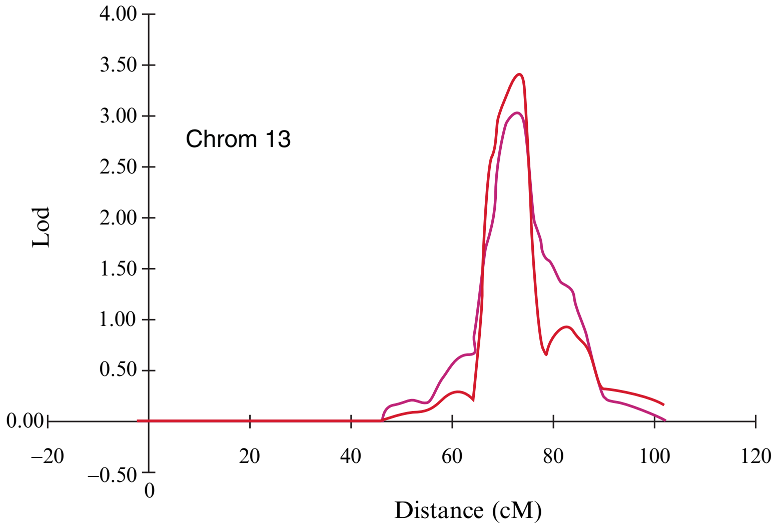

Fig. 8.5 Linkage analysis in pedigrees produces log-odds (LOD) plots like this one. In this example, there were 396 people in 22 families that were identified as having bipolar disorder in at least two members per family (these are called multiplex families). As described in the text, a model is fitted that provides the LOD score, representing the likelihood of obtaining such data given linkage at each position sliding along the chromosome. The salient feature to note is that the width of the LOD peak is nearly 20 cM or 20 Mbp across. This implies that there is relatively little confidence in the location, apart from there being a gene somewhere within that 20-Mbp region. (After [4])

linkage analysis that especially pertain to finding genes for complex traits that render linkage analysis somewhat less than ideal. First, if multiple genes are involved in a trait, the transmission pattern in a pedigree may be highly complex, and we may fail to detect the impact of any single marker through its marginal effect on risk. Even more serious is the fact that the resolution, in terms of accuracy of pinpointing the location of a gene on the genome, is limited by a combination of the sample size, the number of markers, and the number of meiotic exchanges represented by the pedigree. Typical pedigree studies have a mapping resolution of no better than 10 or 20 cM (centiMorgans), which is equivalent to approximately 10–20 Mbp of DNA sequence. This span typically encompasses dozens of human genes, and so one is left with a particularly challenging fine-mapping problem (Fig. 8.5).

8.2.3 A Prevailing Model: Common Disease Common Variants

For genetic association to be found by linkage disequilibrium, a fundamental constraint is that the rare allele must be relatively common (greater than about 10%) or the power to detect the association will be very low. Given that this approach can only find relatively common alleles, one can ask just how badly association mapping works for rare alleles. After all, common alleles, all else being equal, will contribute more to the total population variance in the trait, and will hence have a greater population attributable risk (defined elsewhere).What then are the prospects that common diseases will be caused by these relatively common alleles? Some Mendelian disorders can provide useful insight. If the disease is associated with a change in the environment, such as presence of malarial parasites, then alleles that may cause a disease (sickle cell anemia) may be driven to high frequency by the presence of a worse disease (malaria) against which they confer resistance. This kind of counterselection results in a heterozygote advantage, and any disease associated with alleles showing heterozygote advantage, either now or in the recent past, would be expected to have common alleles. The rapid expansion of the human population and the fact that many human populations have gone through population bottlenecks can also drive deleterious alleles to relatively high frequencies by drift and founder effects. In short, it was plausible that many diseases might have relatively common alleles as an underlying genetic cause. But these arguments do not make it particularly convincing that most complex diseases would be driven mostly by common alleles.

In the end it seems clear that successful identification of the common alleles causing disease would be the most desirable place to begin, since they likely harbor more of the population risk, and diagnostic tests that identify these tests are likely to identify more at-risk individuals than would tests for very rare alleles [1]. Now that more than 300 genome-wide association studies have been completed (http://www.genome.gov/gwastudies/), we can see that in no case was a very large portion of the total variance explained by the associated SNPs. While there are many success stories of finding well-replicated associations between disorders and common SNPs, the effect sizes of those SNPs are all very small. That the common SNPs do not explain much of the variation in risk does not imply that the Common Disease Common Variant Hypothesis is totally in error, however, because it is possible that the variance explained is eroded by the fact that we are looking at effects of marker SNPs, and perhaps not the actual SNPs causing the variation in risk. But the fact that so little of the variance in risk is explained is unfortunate, and it suggests that myriad rare alleles of larger effect might contribute a substantial portion of disease risk in humans.

8.2.4 Affected Sib-pairs

For a brief period in the 1990s, the affected sib-pairs method was very popular, and it met with some success in mapping genes for some traits (more than 600 papers applying affected sib methods appear in PubMed; see [20] for a review of methods). The basic idea is that because full pedigrees are time consuming, expensive, and difficult to collect, one could collect the single kind of relative best matched for age and environment, namely siblings. The principle behind mapping with affected sib-pairs is to score genetic markers throughout the genome in a collection of sibs, and then to scan the genotype data to identify regions of the genome that show an excess of genetic identity between the sibling pairs.

To make sense of affected sib-pair methods we need the concept of *Identity By Descent*. Two alleles sampled from either two individuals or the same individual are said to be identical by descent if they can be traced back to a single ancestor. If two parents have genotypes A_1A_2 and A_3A_4, then a pair of siblings may both be A_1A_3, in which case they share two alleles that are IBD, or they may be A_1A_3 and A_1A_4, in which case they share one allele IBD. Finally, the two siblings may be A_1A_3 and A_2A_4, in which case they share zero alleles IBD. If you consider all possibilities, you find that ¼ of the time they share two alleles IBD, ½ the time they are expected to share one allele IBD, and ¼ of the time they are expected to share no alleles IBD. In table form it looks like this:

	Count of alleles IBD		
	0	1	2
Observed	n_0	n_1	n_2
Expected	$n/4$	$n/2$	$n/4$

where $n = n_0 + n_1 + n_2$ is the total count of sib-pairs in the study. The test of association is to perform a simple Chi-square test. If the null hypothesis is rejected, and if there is an excess count of those sharing one and two alleles, then this SNP shows a positive association with the disorder. It is not so easy to explain the case when the null hypothesis is rejected with an excess of cases sharing zero alleles. It does not imply that the SNP has protective effects. There are many extensions of this simple affected sib-pair test, including use of LOD scoring, application to continuously varying traits, and application to cases where other circumstances result in an empirical deviation from ¼ : ½ : ¼ for the expected allele sharing.

The basic idea of affected sib-pair mapping is to find regions of the genome where affected sibs have an elevated chance of sharing more alleles than this null model. The LOD score equivalent to the Chi-square can be plotted for each SNP as one scans along the chromosome, resulting in plots remarkably like the LOD score plots from full pedigree mapping efforts. Affected sib-pair methods retain the advantage in being much faster and easier to collect than full pedigrees.

8.2.5 Transmission Disequilibrium Test

The problem of hidden population stratification was seen as a serious limitation of direct association testing, because any such stratification could result in false-positive test results that would be difficult to identify without a full independent replication study. The Transmission Disequilibrium Test (TDT) is one of the simplest designs that is immune to the problem of population stratification. Since it was first introduced by Spielman et al. [21], there have been dozens of extensions to allow a similar test approach to apply to other scenarios. We will focus on just the simplest application, since it shows why the test works so well.

Suppose our sample consists of trios, each of parents and an affected offspring. The essence of the TDT is to ask whether the two alleles at a heterozygous SNP are transmitted at a 50:50 ratio to the affected offspring. If the SNP is linked to a mutant allele at a disease-causing gene, then the transmission will be distorted. The test is essentially a Chi-square test for the co-transmission of the SNP and the disease state. If the count of trios where the *A* allele is transmitted is n_A, and the count of trios where the *a* allele is transmitted is n_a, then the Mendelian expectation is that each count would be $(n_A+n_a)/2$, so that the Chi-square is

$$X^2 = \frac{\left[n_A - \left(\frac{n_A+n_a}{2}\right)\right]^2 + \left[n_a - \left(\frac{n_A+n_a}{2}\right)\right]^2}{\left(\frac{n_A+n_a}{2}\right)} = \frac{(n_A-n_a)^2}{(n_A+n_a)}$$

This remarkably simple test has many positive attributes, not the least of which is the virtual immunity to distortions caused by population stratification. Its simplicity and robustness explain in part why it has been applied in nearly 1,200 published studies in human genetics.

8.2.6 Full-Genome Association Testing

In a major paradigm-shifting paper, Risch and Merikangas [18] pointed out the statistical limitations for mapping by determining linkage in pedigrees and carefully showed how we might be able to map in humans purely by association testing. This approach would work if there was relatively little linkage disequilibrium (LD) between SNPs or other genetic variants that are far apart along the chromosome. The hope was that a signature of high LD between a marker and a disease would indicate that the disease had to have risk factors mapping close to the SNP. This strongly motivated the quest for better understanding of LD across the human genome, and eventually led to completion of the human HapMap project [19]. The HapMap project provided us with a map of some 8 million markers and information on the pattern of LD across them in three human population samples. It also stimulated commercial entities to develop methods for genotyping those SNPs with high accuracy and low cost (see Sect. 8.3.2).

Risch and Merikangas [18] made the case for genome-wide association testing by showing that for a given sample size, one could have a greater probability of detecting association (higher power) by doing an association study than by doing a pedigree study. They considered a range of allele frequencies and genotypic relative risks for the disease-causing alleles, and several scenarios for the markers to be scored. It is impressive to see how accurately they foresaw the problems of testing 1,000,000 markers, estimating that a significance level of a $=5\times10^{-8}$ would be needed to have a low probability of false positives. In Table 8.1,

Table 8.1 Sample sizes needed to detect a gene that elevates the risk of a complex disease under different assumptions of frequencies, genotypic relative risks, and testing approaches. (from Risch and Merikangas [18])

					Association			
Linkage					Singletons		SibPairs	
Genotypic risk ratio (γ)	Frequency of disease allele A (*p*)	Probability of allele sharing (*Y*)	No. of families required (*N*)	Probability of transmitting diseases allele A (*P*(tr-A)	Proportion of heterozgous parents (Het)	(*N*)	(Het)	(*N*)
4.0	0.01	0.52	4260	0.800	0.048	1098	0.112	235
	0.10	0.597	185	0.800	0.346	150	0.537	48
	0.50	0.576	297	0.800	0.5	103	0.424	61
	0.80	0.529	2013	0.800	0.235	222	0.163	161
2.0	0.01	0.502	296.71	0.667	0.029	5823	0.043	1970
	0.10	0.518	5382	0.667	0.245	695	0.323	264
	0.50	0.526	2498	0,667	0.5	340	0.474	180
	0.80	0.512	11,917	0.667	0.267	640	0.217	394
1.5	0.01	0.501	4,620.807	0.600	0.025	19,320	0.031	7776
	0.10	0.505	67,816	0.600	0.197	2216	0.253	941
	0.50	0.51	17,997	0.600	0.5	949	0.49	484
	0.80	0.505	67,816	0.600	0.286	1663	0.253	941

From [18], the paper that convinced the human genetics community that by scoring genotypes and phenotypes in direct association tests we ought to be able to identify genetic variants responsible for disease. The genotypic risk ratio (γ) is the ratio of risk of genotypes AA:aa reproduced from their paper, you can see the massive reduction in sample size needed in an association study relative to a pedigree study for the same chance of finding a disease gene.

Note that association testing works by demonstrating a statistical correlation between allelic states of an anonymous marker and a putative risk-elevating locus. This approach is quite distinct from linkage-based mapping methods. The latter rely on identification of recombination events within the sample, and noting that two genes are closely linked if there are relatively few such recombination events. Because linkage methods rely on counting recombination events, the resolution comes from having a large number of such events. Even the largest pedigrees might have only a few thousand recombination events, and this limits the resolution and the statistical confidence in map distances obtained in linkage studies. Association studies seem to depend solely on the statistical correlation of allelic states, but behind this test is the idea that the correlations arise from a combination of low rates of recombination in the ancestral history of the variation and from random genetic drift. Genes that are far apart will have allelic states randomized relative to one another by recombination over a few generations. If the genes are close together, drift can generate LD, and recombination will be very slow to erode it, so at equilibrium there is a tendency for tightly linked genes to display LD.

8.3 LD Mapping and Genome-Wide Association Studies

8.3.1 Theory and How It Works: HapMap and Genome-Wide LD

The basic principle behind LD mapping, also called association mapping, rests on a few key assumptions. Suppose a population is in a state of near equilibrium, with relatively little mixing through migration, so that the resulting genetic variation in the population is in Hardy–Weinberg proportions. In a population that has a steady rain of mutations, there will be a balance between the input of variation by mutation and its loss by random genetic drift. Some of the mutations have a deleterious effect; other mutations have no measurable effect; and very rarely some will be advantageous.

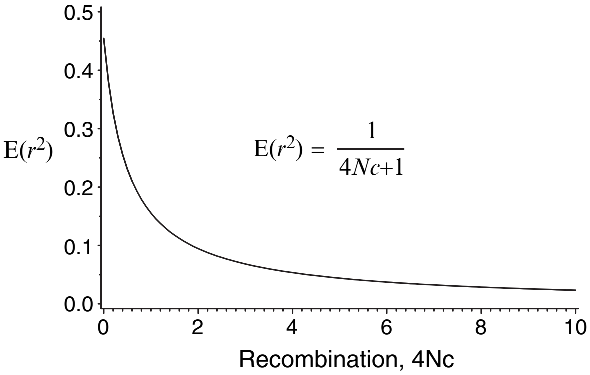

Fig. 8.6 Under the population genetic model, in which there is a balance between mutation, neutral drift, and recombination, there arises an equilibrium level of linkage disequilibrium (LD) as is plotted here. LD is here measured as the correlation coefficient r^2, as described in the text. The theory says that the expected value of r^2, or $E(r^2) = 1/(4N_e c + 1)$, where N_e is the effective population size, and c is the recombination rate. Note that the terms appear as the product $N_e c$, so that one expects the same LD if one doubles the population size and halves the recombination rate. The theory shows that there is a strong inverse relation between $4N_e c$ and LD

Because there is recombination occurring in each generation, the statistical association between mutant alleles will tend to erode over time; however, the effect of random drift is to keep the LD from completely decaying to zero. Instead, there is a balance between mutation, drift, and recombination that produces a steady state level of LD. An approximate relation at steady state is $E(r^2) = 1/(1 + 4N_e c)$, indicating that the expected linkage disequilibrium as measured by r^2 is a simple function related inversely to a term with $4N_e c$, where N_e is the effective population size and c is the recombination rate [14, 22]. According to this theory, one would get the same LD if one halved the recombination rate and doubled the population size, so long as $4N_e c$ is kept the same (Fig. 8.6).

Empirically, the data on human LD support the idea of association mapping very well. In particular, one does find SNPs that are in strong pairwise LD, but basically this only happens if the SNPs are in close physical proximity along the genome (Fig. 8.7). When a pair of SNPs is farther apart than 100 kb or so, they only very rarely have strong LD. This means that a strong association between a disease and an SNP provides fairly convincing evidence that a gene associated with elevating disease risk must reside near the marker SNP.

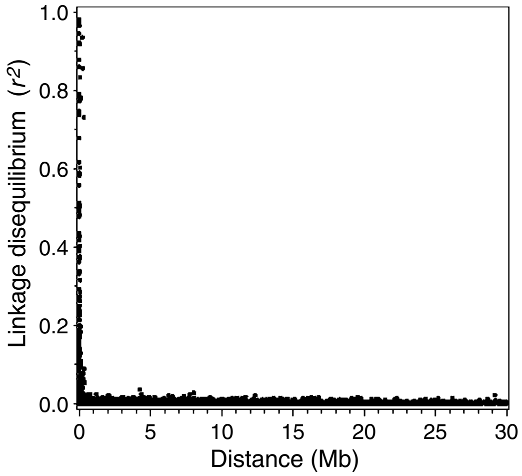

Fig. 8.7 A plot of the pairwise LD for a collection of SNPs from an early SNP study. In this study, the SNP genotypes were determined at several thousand SNPs in a few hundred people, and for each pair of SNPs it was possible to calculate the distance between them (for pairs on the same chromosome) and the level of LD between that SNP pair, showing clearly that SNPs that are far apart almost never have appreciable LD

According to this theory, if one imagines that there are SNPs responsible for disease, then there ought to be a statistical association between case/control status and the genotypes at nearby SNPs. Table 8.2 shows the steps for a genome-wide association test. The quality checking step is particularly vital, because despite the impressive gains in genotyping technologies, artifacts always creep into these studies and any slight perturbation from perfect genotyping calls can and usually does result in false-positive calls. Nearly every GWAS study had a moment of amazement when so many positive signals of association were seen, only for the number to dwindle as quality testing revealed more and more to be artifactual.

8.3.2 Technology: The Fantastic Drop in Genotyping Costs

One cannot overstate the importance of developments in the technology for large-scale molecular biology in accelerating the rate of discovery in human genetics. This is nowhere more true than in the area of genome-wide association testing. As recently as 2002, it cost about 1 U.S. dollar to score the genotype of an individual at one targeted nucleotide in the genome. Just 5 years later, one could score 1 million SNPs for $ 400, a 2,500-fold reduction in cost. This came about through development of mass manufacture of high-quality microarrays and methods to label and hybridize DNA to these arrays that gave highly accurate genotype calls. Competition among multiple manufacturers for competing technologies probably helped to drive the costs down as they drove speed and accuracy up. The next frontier is whole-genome sequencing at costs comparable to those of a CAT scan, and the human genetics community seems to have a consensus that this will happen within the next few years. Returning to the problem of mapping genetic variants that are associated with risk of complex diseases, even if we had complete DNA sequences of all the individuals in the case-control GWAS studies, many of the barriers to identification of genes responsible for inflated risk would still be there.

Table 8.2 Steps for a genome-wide association study

1. Identify the sample. Should be from a homogeneous population. Clearly defined cases and controls matched for gender and age.
2. Score the genotypes. Today this is almost universally done by applying standard commercial SNP genotyping chips from Affymetrix or Illumina.
3. Quality checking. It is necessary to take the genotype calls through rigorous testing for Hardy–Weinberg departures, spurious heterogeneity across runs, clustering of artifacts with cases, etc. Generally poor-quality DNA means removing some individuals, and some SNPs need to be removed.
4. Perform first-pass statistical inference**.** Nearly everyone starts with single-SNP tests, such as the Cochran–Armitage trends test.
5. Double-check all positives. The vast and overwhelming majority of positive hits seen at the first pass are errors of some sort. Disbelieve them until you fail to prove that they are errors.
6. Perform validation study. Standard practice is to repeat the study in another population to see that the same result is repeated.
7. Perform additional statistical inference. One can check for genotype x environment and epistatic effects, although the power will be low.

8.3.3 Case-Control Studies

Despite the fact that complex disorders are intrinsically embedded in likely interactions with environmental factors, the easiest design to begin genome-wide studies that identify genes associated with the disease is the case-control design. Because these tests entail examination of so many SNPs (typically 500,000 or 1 million SNPs), it is necessary to have large sample sizes so that the *P*-values of tests are sufficiently small, even when effect sizes are moderate, for the statistical tests to retain significance in the face of so many simultaneous tests. For example, the Wellcome Trust Case Control Consortium examined 2,000 cases for each of seven different disorders, and these were each contrasted against 3,000 controls [25]. With a complex disorder it becomes necessary to dichotomize individuals into these two bins, and it is crucial that this be done rigorously and homogeneously across the study., Other variables, such as sex, age, diet, etc. must either be randomized, controlled (e.g., by examining one sex only), or done as matched cases and controls, where the matching is for as many of these ancillary variables as possible. But case-control studies have a solid place in the history of medical research, and the simplicity of their design and ready access to samples stratified in this simple way means they are likely to continue to be useful. In addition, the first-pass statistical tests are very simple indeed.

8.3.4 Statistical Inference with Genome-Wide Studies

If the individuals in the study are placed into discrete bins of ;cases' and "controls," then the simplest way to consider the data is as a 3×2 table:

	AA	*Aa*	*aa*
Cases	n_{11}	n_{12}	n_{13}
Controls	n_{21}	n_{21}	n_{22}

It is legitimate to perform a 3×2 contingency Chi-square test on these data, provided the cell counts are sufficiently large (above 5 or so). For many SNPs one finds that the rare homozygous class has only a few observations, and in these cases one has to be careful about the aberrant behavior of the test statistic with small cell counts. One common way to solve the problem of small cell counts is to perform a permutation test to estimate the probability of a more extreme table. Another approach is to pool cells (e.g., the rarest genotype class, or column, could be pooled with the heterozygotes, yielding a 2×2 table).

Because the three genotypes are not totally independent categories, but rather there is an underlying order to them, a test more appropriate than the 3×2 contingency Chi-square is the Cochran–Armitage trend test. This test assumes that there is a linear trend in the phenotypes as one progresses from *AA* to *Aa* and *aa*, and obtains an asymptotically Chi-square test statistic under this model. Its primary advantage is in statistical power, because it effectively saves a degree of freedom. Just as for the contingency Chi-square, the significance test for the Cochran–Armitage trend test can be based on a permutation, and this allows it to be used even when cell counts are small. One needs to have *P*-values below 10^{-6} to attain significance across the whole study, and the Wellcome Trust Case Control Consortium was successful in achieving this for more than 80 SNPs across the seven disorders they mapped by GWAS (Fig. 8.8).

The genome-wide SNP chips are not successful at producing a reliable genotype call for every SNP in every individual, and the resulting missing data can be a challenge for analysis. One of the interesting features of dense SNP data is that because nearby SNPs are in LD, when one SNP call is missing, there is often some ability to predict the value of the missing genotype by use of the flanking SNPs. This "guessing the missing data" is known in statistics as *imputation* [8, 11]. While it sounds suspicious to fill in the missing data in this way, it is easy enough to test how well it works – simply take a large data set, blind yourself to some of the known genotype calls, and determine whether the imputation procedure gets the correct genotype call. When this is done, the misclassification error rate can be as low as 1%. With genome-wide SNP chips, whose density is one SNP every 3 kb on average, the imputation error rate varies with population but is typically less than 3%. Depending on the analysis, this can make a big difference. For the Wellcome Trust case-control study, use of imputed genotype calls often produced SNPs whose association *P*-values were more significant than the nonimputed SNPs.

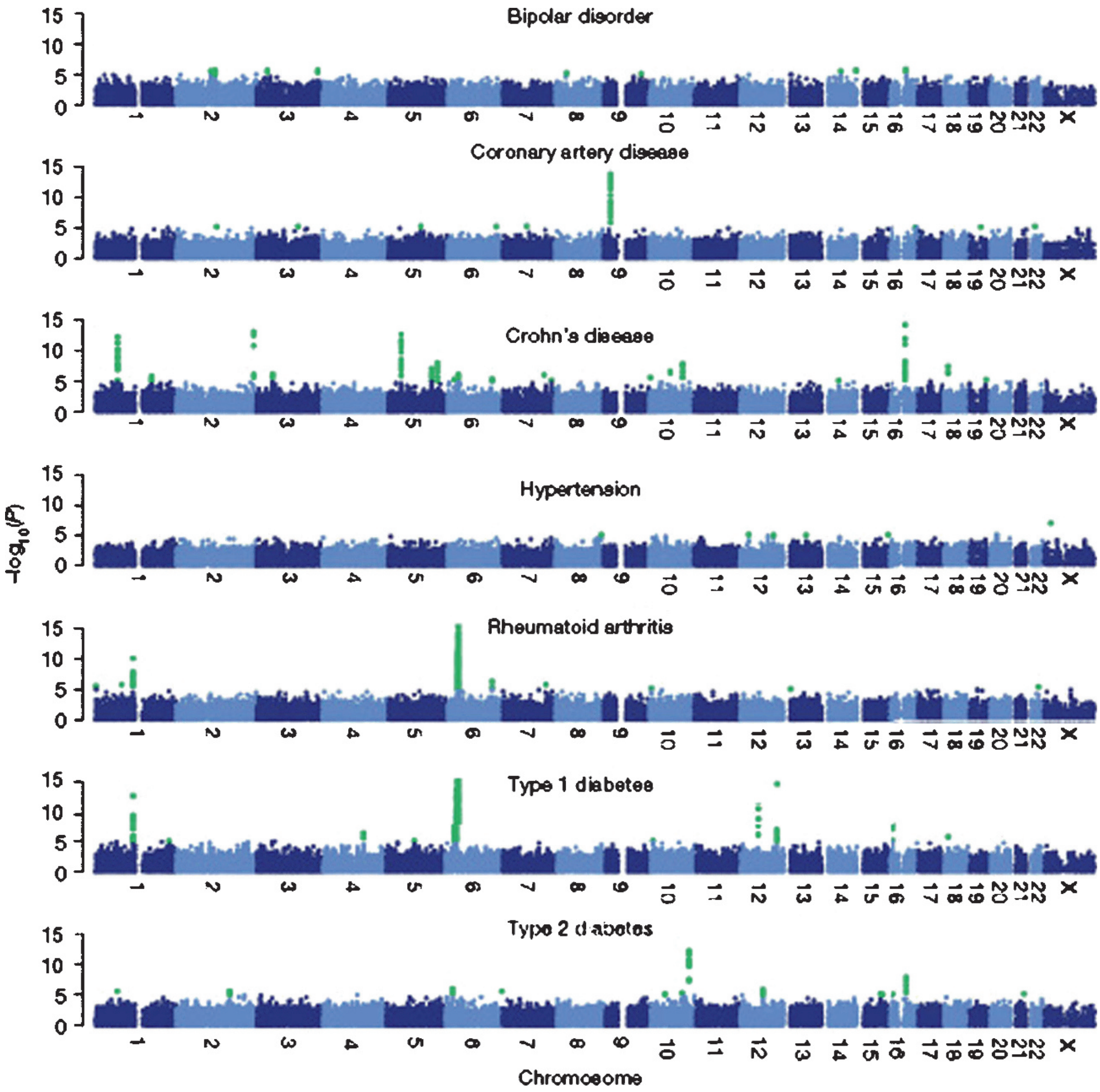

Fig. 8.8 Results observed by the Wellcome Trust Case Control Consortium in a large multi-disorder genome-wide association study. This study examined seven different complex disorders and performed genome-wide association tests for all traits using a common panel of healthy control individuals. Each of these plots (such plots are sometimes called Manhattan plots) shows the results of all 500,000 significance tests for association between each of the 500,000 SNPs and the specified disease. The *y*-axis of each plot is $-\log_{10}$ (*P*-value), so that a value of 6 implies a *P*-value of 10^{-6} (such an event would be seen by chance alone once out of every 1 million trials)

8.3.5 Replication and Validation

A problem with performing 500,000 tests at once is that one expects that 25,000 will be "significant" at $P<0.05$ by pure chance. Even when stringent criteria are applied to control for the false-positive rate, such as Bonferroni correction or use of False Discovery Rate, it is inevitable that if one places all the tests in rank order from the lowest to the highest *P*-value, that in amongst the significant tests at the lowest *P*-value range, there will be many tests that are spuriously considered positive. It is felt that the only way around this problem, to distinguish false positives from true posi-

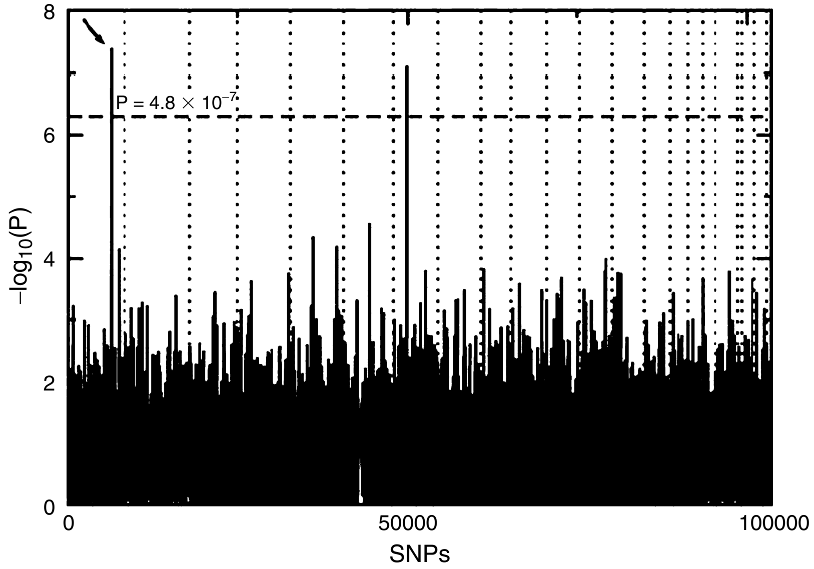

Fig. 8.9 A plot similar to that in Fig. 8.8, showing the outstandingly strong signal from the association of macular degeneration with complement factor H. The *dashed line* is the Bonferroni critical value for $P < 0.05$, implying that any point above this line would be expected to occur by chance only 1 out of 20 times even after doing the 100,000 tests. (After [9])

tives, is to "replicate" the study. The word "replicate" is placed in quotes because of course there is no way to truly replicate a human study. Each individual is unique and each set of environmental circumstances is unique. At best, a second study on a similar but independent second population sample might identify overlapping sets of genomic regions harboring variation associated with disease risk. If so, this does indeed lend support to the initial positive result. The rub is that the second population is not identical, and the differences in genotypic and environmental composition between the two studies may in fact account for the difference between the results. That is, it may truly be a positive in the first study and not in the second. For now we hope that this is relatively rare, and are forced to rely on replication as a signature of real and repeatable effects.

8.3.6 Age-Related Macular Degeneration and Complement Factor H

In the early days, when the human genetics community was coming to grips with the idea that genome-wide association studies might actually work, Klein et al. [9] published a paper that showed that it could work far better than anyone could have hoped. The disorder was age-dependent macular degeneration, and they applied a simple case-control design. What was remarkable about the study was that they genotyped only 116,204 SNPs (using one of the early commercial chips) in a ridiculously small sample of 96 cases and 50 controls. To have a test that remains significant in the face of 116,204 tests would require an odds ratio of something like 6.0, and in fact, this is just what they found (Fig. 8.9). The positive hit was in the gene for complement factor H, and the result immediately sent the AMD community scrambling to understand the role of this immunity factor in macular degeneration risk.

8.4 Admixture Mapping and Population Stratification

8.4.1 How to Quantify Admixture

Before considering how to use admixture for mapping purposes, first consider how one might try to determine the degree of admixture of an individual's genome, and whether it is possible to infer which alleles came from which population. If one could identify the "parental" populations from which the admixed population derives, then the first thing to do is to estimate allele frequencies in the parental and admixed populations. In the extreme example where the allele frequencies are 0 and 1 in the parentals, it is easy to see that the allele frequency in the admixed population directly gives an estimate of the proportion of the alleles derived from the second population. If instead the allele frequencies in the two parental populations are p_1 and p_2, and the frequency in the admixed population is p_a, then the admixture proportion, a, giving the proportion of the alleles derived from the second population, is:

$$\alpha = \frac{|\, p_a - p_1 \,|}{|\, p_2 - p_2 \,|}$$

It turns out this is a maximum-likelihood estimator for this simple single gene case. The situation gets more interesting when we have genome-wide data. For each region of the genome it is possible to estimate the proportion derived from each parental population, but what we really want is to identify for each individual the population of origin of that individual's two alleles. This is much easier with runs of SNP alleles along the chromosome, or haplotype segments. Based on the frequencies in the two parental populations, there are methods that produce reasonably accurate calls of the stretches of the genotype derived from each parental population. One effective approach applies a Markov hidden Markov model to the genotype data [22].

8.4.2 Using Admixture for Mapping

If two different populations have differing risk of a complex disorder, and there is an admixed population that also manifests the disorder, if one could identify regions of the genome derived from each population for each admixed individual, then a means of mapping might be to look for an association between disease status and population-of-origin of genomic segments. These methods are still being refined, but they appear to be very promising, especially in populations with variation in the degree of mixing of the two genomes [23]. It is good to have large blocks of unrecombined chromosomal segments to attain power, but more finely diced genomic regions are needed in order to map with fine resolution. Also, the method works best when the parental populations are well defined, and when there are only two parental populations that are widely separated from each other historically (to maximize allele frequency differences).

A reasonable target for admixture mapping methods are diseases that differ in incidence between the two parental populations. End-stage kidney disease has a lifetime incidence of about 1.5% in Europeans and about 7.5% in African Americans. At the outset we do not know whether there is a genetic basis for this, but admixture mapping could in principle identify genetic factors if they exist. One particular form of end-stage kidney disease that shows strong familial clustering is focal segmental glomerulosclerosis (FSGS). Relative to Europeans, African Americans have a fourfold increased risk for FSGS and an 18- to 50-fold increased risk for HIV-1-associated FSGS. For this reason, Kopp et al. [10] identified 190 African-American cases and 222 controls for FSGS, obtained genome-wide SNP data and applied admixture mapping. On chromosome 22 they found a region with a LOD score of 9.1, implying that African ancestry for this chromosomal region inflated the risk of FSGS by more than ninefold. Subsequent genotyping of additional SNPs in additional samples narrowed the mapping to the gene *MYH9*. The precise mutation(s) responsible for the elevated risk of African alleles are still not known, but this success and the relative ease of application of admixture mapping in studies of African American population samples, make it likely that we will see many future successes in its application.

8.4.3 The Perils of Population Stratification

Many complex disorders display a wide range of incidences across different human populations. At the outset we cannot say whether the difference in incidence is due to a difference in gene frequencies or whether differences in environmental exposures account for the variation in disease risk. Sometimes a population will face a change in an environmental factor, and then the role of environment can become starkly clear. For example, the increase in saturated fat consumption in the diet of Chinese, especially in large cities, is being accompanied by a sharp increase in cardiovascular disease [24]. The increase in protein content of the diet in post-World War II Japan was accompanied by an astonishing increase in the average stature of that population. But in addition to such clear environmental effects, many genes have allele frequencies that differ among populations, and whenever we try to do association tests when there are differences in disease incidence and allele frequencies, we must be wary of a serious artifact.

Suppose two populations have disease incidences of 4% and 20%. These two populations have been isolated geographically for thousands of years, and many alleles differ in frequency. Suppose one particular gene has allele frequencies of 0.10 and 0.30 in the two populations. Now imagine that there was a large influx of individuals from the second population into the first population, and the population sample consists of a 50/50 mix of individuals from the two populations, but investigators were unable to keep track of the ancestral origin of each individual. The population sample contains hidden stratification of these two population groups. The allele frequency in the sample would be $(0.10+0.20)/2=0.15$, and the disease incidence would likewise be the average of the two populations or 12%. But, assuming that there is zero association between this gene and the disease, the table of genotype and phenotype frequencies would be:

	AA	*Aa*	*aa*
Diseased	16	144	320
Healthy	76	856	2,580

This table was constructed by calculating the Hardy–Weinberg proportions in each population (frequencies of 0.01, 0.18, and 0.81 in one population and 0.04, 0.32, and 0.64 in the other), taking the average frequencies across the two populations for each genotypic class, and then calculating the disease incidence for each genotype. The Chi-square test of heterogeneity is $\chi^2=10.53$, for which $P<0.005$. We have generated an association that appears significant purely due to the fact that the population with the higher disease incidence happened by chance to have a higher allele frequency for this SNP. In fact, for any SNP having an allele frequency difference of sufficient magnitude between the two populations, there will be this same kind of spurious association. This is why it is so crucial to avoid hidden population stratification in association testing.

8.4.4 How to Correct for Hidden Population Stratification

Fortunately, there are ways to identify the problem of hidden population stratification that allow some degree of correction of the false positives it causes. First note that a mixture of two populations having different allele frequencies results in genotype frequencies that depart from Hardy–Weinberg proportions. The easiest way to see this is to imagine populations with allele frequencies 0 and 1. A mixture of the two would give 50% *AA*, 0% *Aa*, and 50% *aa* individuals. The allele frequency is 50%, but there is a massive deficit of heterozygotes (or excess of homozygotes). One way to tease apart the sample into its original populations is to try to find clusters of individuals each of which form a Hardy–Weinberg population. This is the basic idea behind the program STRUCTURE, which is widely used in heterogeneous population samples to try to understand its partitioning into units [16].

Another approach, first used in 1978 by L.L. Cavalli-Sforza's group [12], is to apply a principal components analysis to the genotype data. This is a multivariate statistical procedure that identifies linear combinations of the SNPs that explain the most among-individual variability (arbitrarily number coded as, for example 0, 1, and 2 for the three genotypes). Generally there are multiple orthogonal sets of "axes" or vectors of SNPs that are needed to describe the variation. What PCA does is provide the weightings for each SNP and each such principal component. In the end, one can simply plot these principal components for each individual, and to the extent that individuals are more genetically similar to each other, they will fall closer together in these plots. If there are separate clusters of individuals, as there might be if there were discrete populations, these would appear as clusters in the PCA plot. Recently this method was applied to a sample of some 7,000 individuals from Europe genotyped at 500,000 SNPs [13], and the plot of the first two principal components produces an astonishingly good reproduction of the geographic map of Europe (Fig. 8.10). What does this imply? Just that there is a measurable isolation by distance among Europeans, and that historically people have tended to marry and settle down not far from their birth place.

To use PCA for association testing, one could identify the discrete clusters and use this as a covariate in the analysis, trying to explain as much of the variance in disease risk by population of origin first, and then explaining the remainder with the allele frequencies. Alternatively, one could directly use the principal components loadings as cofactors in the association analysis. This is an area of active research, and some of the newer approaches for dealing with genetic ancestry

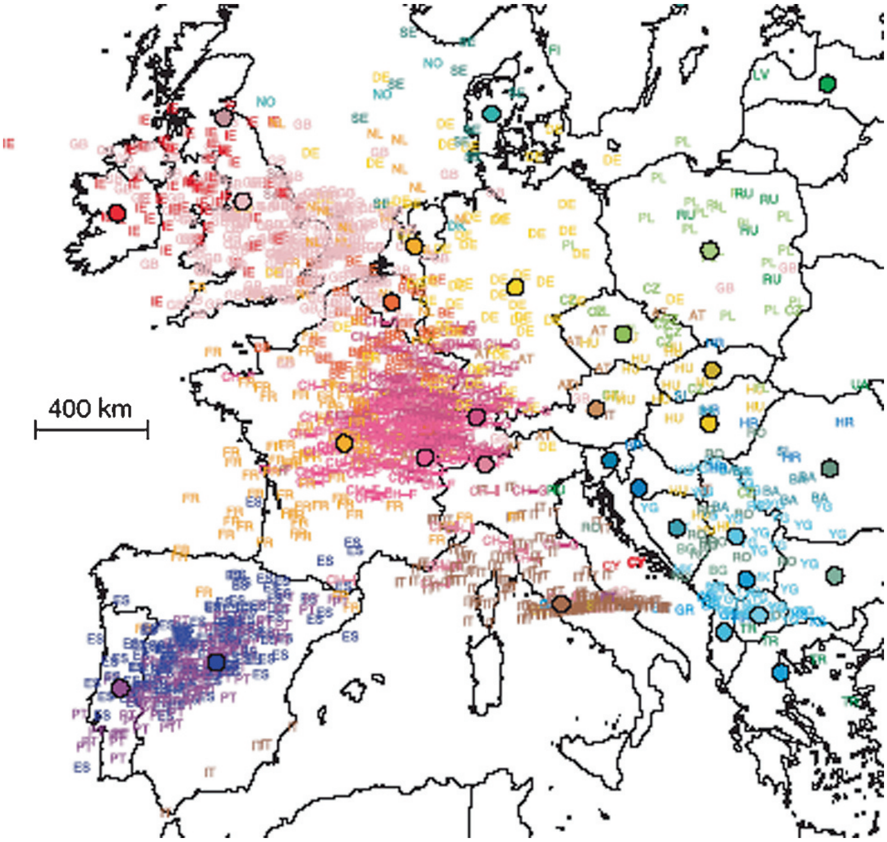

Fig. 8.10 The principal components plot from a study of 500,000 SNPs across a European sample of nearly 7,000 individuals. (From [13]). The raw genotype data were analyzed by Principal Components Analysis to try to find collections of SNPs that explain the most variance. A Principal Component is a combination of weightings of a subset of SNPs, and so after the PCA is run, each individual has a value for each principal component (PC1, PC2, PC3, etc.). If one plots a point (*x, y*) for the values (PC1, PC2) for each individual, one gets a plot like that shown. Note the impressive correspondence to the map of Europe, indicating that simple geographic distance is well correlated with the degree of genetic difference between individuals living that distance apart

and population structure in association studies are presented in Chap. 20 in this volume.

8.5 Complications

The models that we have presented up to now were purposely simplified so that the principle concepts would be clear. We assumed that the effects of many genes were additive, and proceeded to fit real data to this model without particularly questioning whether the model was correct. In fact, several factors can contribute to departures from this simple additive model, and many people think that these departures are virtually ubiquitous. Departures from additivity do not bring to a halt hopes of finding genes that act on complex traits, but they do make the problem more challenging.

8.5.1 Genotype by Environment Interaction

One of the challenges of studying the genetics of complex traits in humans is that we can never measure the same genotype in more than one controlled environment. Monozygotic twins at least give us some idea of the impact that different environments may have as a zygote undergoes development and eventually manifests mature phenotypes. With model organisms, where it is possible to produce many individuals with the same genotype, a very simple experiment produces a profoundly important result. The experiment is to simply rear the set of genotypes in two or more environments. Figure 8.11 shows an example of one such experiment, where a set of *Drosophila* lines were reared at two different temperatures, and body mass was measured in the resulting adult flies. As you can see, some lines gain weight

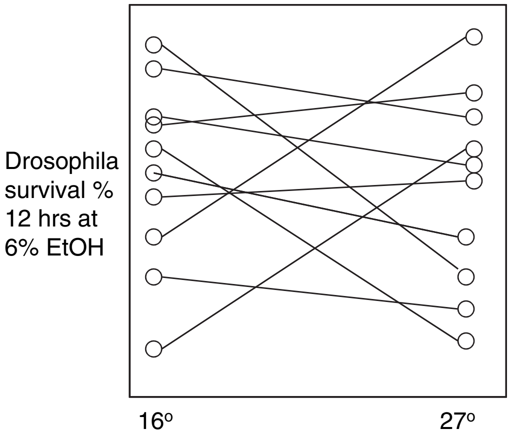

Fig. 8.11 A typical study of genotype × environment interaction obtained from model organism studies where the same genotypes can be reared in two or more environments. This kind of design nearly ubiquitously shows crossing of the mean phenotype lines, indicating a nonlinear effect of the environment attributable to a genotype × environment background. (Data from Kristi Montooth)

when moving from the low to the higher temperature, and other lines do the opposite.

Whenever the lines connecting the mean phenotypes across a range of environments cross, as they do in Fig. 8.11, this is a form of genotype × environment (G × E) interaction. More formally, we could set up an analysis of variance of these data, where the two factors are genotype and environment, and the interaction term in the analysis of variance would quantify the degree of G × E interaction. The impressive feature of this simple experiment is that whenever an experiment of this sort is done having any power at all, the observation of significant G × E is nearly universal.

Human examples of G × E interaction are a bit harder to find, but this is only because one has to define genotypes by particular targeted subsets of SNPs. A clear example of human G × E comes from drug responses. The particular example of *VKORC1* and warfarin response is a prime example. The phenotype of clotting time shows a strong interaction between genotypes at *VKORC1* and the environment of having taken warfarin. Given the ubiquity of G × E in animal and plant studies, one might expect that a differential response to drugs, varying with the genotype of the patient, is probably also nearly universal.

A particularly good example of a genotype by environment is seen in α_1-antitrypsin. The antiproteolytic activity of human serum was detected in 1897, and in 1900 Landsteiner showed this activity to be located in the albumin fraction. Antiproteolytic activity is measured by hydrolysis of artificial substrates by trypsin in the presence of the serum to be tested. The concentration of antiproteolytic activity increases quickly, for example with bacterial infection, after injection of typhoid vaccine, and during pregnancy. Interindividual differences in levels of antiproteolytic activity in the blood were first observed in 1963. A simple recessive mode of inheritance was proposed for low α_1-antitrypsin levels. Many different alleles have been discovered that vary widely in their activity levels. The gene is located on 14q31-32; it spans 10.2 kb, and has five exons. Two variants, Z and S, are especially important because the α_1-antitrypsin level is appreciably reduced relative to the common M type.

Subcutaneous injection of typhoid vaccine and diethylstilbestrol leads to a 100 % increase in activity of subjects with the MM type. Heterozygotes of the MZ type show a moderate increase, whereas in homozygotes of the ZZ type hardly any increase is seen. Many studies have shown that the rate of obstructive pulmonary disease in these ZZ individuals is at least 15 times the rate in the general population. Among ZZ homozygotes only 70–80% develop obstructive emphysema, and in heterozygotes the frequency is much lower. When a patient is exposed to recurrent bronchial irritation, such as that caused by smoking or frequent infections, these enzymes cause digestive damage to the lungs. Tobacco smoking enhances the danger of bronchial infections and hastens the progress of the disease. Once we are in the era of widespread genotyping for medical diagnostics, individuals who are found to be ZZ homozygotes and possibly ZM heterozygotes ought to get extra guidance regarding their exceptional risk of COPD, especially if they smoke.

The a_1-antitrypsin polymorphism is an example in which there is a subset of genotypes with heightened environmental sensitivity. The associated diseased condition can be thought of as one of reduced penetrance, and that penetrance is increased by an environmental trigger. The genetics of COPD appears to be complex, but for individuals with the ZZ genotype of a_1-antitrypsin, the disease is practically Mendelian. This is one of the more hopeful situations motivating the study of genotype × environment interactions – many diseases that we think of as complex and unpredictable may prove to have a simple gene of large effect whose otherwise low penetrance is triggered by an identified environmental factor. Such situations are also highly sought after because they provide a means whereby early genotypic analysis may result in an ability to give advice about environmental hazards that could greatly impact disease prevalence.

8.5.2 Epistasis

In the context of complex traits, epistasis is the situation when the risk of the disorder departs from an additive effect across two or more risk-elevating SNPs. Table 8.3 makes the situation clear. If one locus has marginal phenotypes (means across all other factors) of a_1, a_2, and a_3, and the other locus has marginal phenotypes of b_1, b_2, and b_3, then the two-locus genotypes have phenotypes that might fit the additive pattern as depicted in Table 8.3. Any departure from this additivity is an example of epistasis. One extreme example is where all the genotypes in the table have one phenotype, but the *aabb* genotype in the lower right corner has a radically different genotype. Consider two parallel pathways, where the organism requires the product of one or the other pathway, and the *aa* genotype knocks out one pathway, and the *bb* genotype knocks out the other. In this case, all the genotypes except *aabb* would get the required product, but the *aabb* doubly homozygous mutant would fail in both pathways and would produce the extreme phenotype. This kind of epistasis is rampant in model organisms, but when we try to test for it in human complex traits, it is not so easy to find. The reason is primarily due to the greatly reduce statistical power to detect such interaction effects. Given this low statistical power, it is premature to conclude that epistasis is not very prevalent in humans.

Table 8.3 Two-locus genotypes and additive genotypic effects[a]

	BB	*Bb*	*bb*
AA	a_1+b_1	a_1+b_2	a_1+b_3
Aa	a_2+b_1	a_2+b_2	a_2+b_3
aa	a_3+b_1	a_3+b_2	a_3+b_3

[a] Define (a_1, a_2, a_3) as the effect of genotypes *AA*, *Aa*, and *aa* on the phenotype, and (b_1, b_2, b_3) as the effects of genotype *BB*, *Bb*, and *bb*, then the matrix below gives the expected genotypic effects for the nine pairwise genotype combinations assuming that the two loci have additive effects. These genotypic effects would be equivalent to the measured phenotypes in the environmental effect is zero

It has been argued that epistasis is especially likely to be found for phenotypes that are closely related to molecular function. The argument is that molecular biology is loaded with intermolecular interactions, and so if there is polymorphism in pairs of molecules that interact in some key pathway, then it is all the more likely that those variants may display an interaction in disease risk. Following this reasoning, Dimas et al. [5] examined pairs of SNPs for possible interactions in driving transcript abundance. They used the genome-wide expression data generated by the Sanger Centre in the 210 cell lines from the unrelated individuals whose genotypes were scored in the HapMap study. Reasoning that coding SNPs might be compensated for by flanking SNPs, they specifically looked for coding-flanking SNP pairs that influenced transcript abundance in nonadditive ways. After identifying nonsynonymous SNPs that affect expression and flanking SNPs that also affect expression, they performed an ANOVA test for each SNP pair to detect main effects and pairwise interactions. At a significance level of $P<0.001$ they expected 331 such interactions by chance, but observed 412. In this set were several cases of strong and highly significant interactions. Although the final conclusion does not overwhelmingly suggest that pairwise interactions are rampant in the human genome, the test had relatively low power given the small sample sizes. As our ability to apply tests of epistasis to larger samples targeted at specific pathways improves, it does seem likely that epistatic interaction among human genetic variants will be seen to play as important a role as has been found in genetic model organisms.

8.6 Missing Heritability: Why is so Little Variance Explained by GWAS Results?

One of the more surprising results from the genome-wide association studies has been that they uniformly find only SNPs of very small effect, and that even the sum of the effects of all the SNP associations that are found only explains a small proportion of the total genetic variance. This implies that if one has the SNP genotype for all the SNPs that impact a trait, one still has rather poor ability to predict the phenotype. This is surprising in light of the density of SNP genotypes obtained (one every 3 kb on average) and the large sample sizes (in some studies in excess of 30,000). The most dramatic example of this poor prediction ability is the case of body height (stature). The heritability of stature in humans is approximately 80%, making it one of the more strongly heritable complex phenotypes that we know. Despite this, even the top 20 SNPs found to be associated with stature explain less than 5% of the variance. Because we know from the heritability studies that there are genetic factors explaining the familial resemblance, this problem is sometimes called "missing heritability"; or, by analogy with dark matter in astrophysics, it is also called "dark heritability."

There are several reasons why a GWAS study may fail to explain more of the genetic variance in a complex trait. First, the SNPs that are used as markers are not expected to be the causal factors that drive the phenotype, but instead are correlated with the trait-affecting SNPs. This indirect association would erode the prediction power. Second, the SNPs that are used as markers are only quite common, because they were chosen from the HapMap studies, which specifically sought to catalog common SNPs. If much of the variance in traits is driven by rare SNPs, the correlation between the SNP markers that were used and these rare SNPs could be quite low. Third, it is clear that the complex traits that are studied include an environmental component, and if there are genotype × environment interactions (G×E), each SNP genotype will be averaged across all the environments, so that its effect would appear to be eroded compared with an SNP that had no such G×E interaction. Soon we hope to have the means to directly test for G×E interactions, but the primary challenge that must be tackled is to have accurate and meaningful measurements of the environment. Fourth, the statistical models have only made use of single SNPs at a time, and the trait may instead be driven by interactions among SNPs, or epistasis. It is also possible that there are other sources of heterogeneity, including epigenetic differences among individuals.

8.7 Concluding Remarks

The human genetics community is striving to improve methods for identification of genes that underlie complex genetic disorders and to understand how the effects of genes combine to produce inflated risk of disease. As part of the effort to better understand the role of rare alleles, the 1000 Genomes Project (www.1000genomes.org) was launched to provide the stimulus to accelerate the development of sequencing technologies that reduce the cost while increasing the speed and accuracy of whole-genome resequencing methods. Statistical methods need to be developed that accommodate the known complexities that may connect variation at the genotypic and phenotypic levels. While we can have confidence that methods of genome-wide association testing based on full genome sequence will be developed and improved in the near future, the prediction of an individual's disease risk given only his or her genome sequence may never attain useful accuracy (apart from extreme alleles that are nearly deterministic for some disorders, such as Mendelian disorders), especially if the disorder is heavily impacted by stochastic environmental factors, or by complex interactions between genotype and environment. But prediction of individual risk could make an enormous difference to public health, especially if environmental amelioration of that risk were possible, and so the drive to maximize prediction accuracy will motivate work in this area for years to come.

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8.1 Lessons from the Genome-Wide Association Studies for Complex Multifactorial Disorders and Traits

Jacques S. Beckmann and Stylianos E. Antonarakis

Abstract Genome-wide association studies (GWAS) between common sequence variation and phenotypic variation were recently performed for a large number of human phenotypes. This was possible due to the discovery of the common variation in human populations, the development of technologies for large-scale and inexpensive genotyping, and the collection of very large number of well-phenotyped samples. GWAS were successful in identifying low risk alleles in candidate genes or loci. More importantly, these studies disclosed unexpected molecular pathways for different common, multifactorial disorders and traits, thereby providing new working hypotheses. Yet, the current clinical utility of these findings remains limited.

Contents

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The recent discovery of the common variation of genomes from different human populations (57, 58) and the availability of technical platforms for massive and low-cost genotyping of hundreds of thousands of polymorphic markers in very large number of samples has made genome-wide association (GWA) studies possible for numerous human complex multifactorial phenotypes (disorders and traits). The latter are complex traits, which are influenced by distinct combinations of multiple genetic and nongenetic factors, contributing together to a graded phenotype.

The results of the first series of GWA studies are very recent, since most of them have been published in the last 18 months (see Table 8.1.1 and Fig. 8.1.1 for the phenotypes studied); several important lessons have emerged from these studies, and a short summary of those is discussed in this addendum.

The prevailing hypothesis underlying this quest for genetic variation contributing to complex traits was that these diseases are essentially caused by a number of common disease variants, each with small to modest effects (the common disease–common variant hypothesis, (32, 51). One of the few examples of an association of a genetic variation with a disease phenotype in

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Table 8.1.1 Alphabetic list of phenotypes studied with GWAS (http://www.genome.gov/GWAStudies, June 1 2009 (20))

Addiction
Adiponectin levels
Age-related macular degeneration
Aging traits
AIDS progression
Alzheimer's disease
Amyotrophic lateral sclerosis
Anthropometric traits
Anti-cyclic citrullianted peptide antibody
APOE*e4 carriers with late-onset Alzheimer disease
Asthma
Asthma (toluene diisocyanate-induced)
Atopic dermatitis
Atrial fibrillation
Atrial fibrillation/atrial flutter
Attention deficit hyperactivity disorder
Attention deficit hyperactivity disorder (time to onset)
Attention deficit hyperactivity disorder and conduct disorder
Attention deficit hyperactivity disorder symptoms (interaction)
Autism
Basal cell carcinoma (cutaneous)
Behcet's disease
Bilirubin levels
Biochemical measures
Biomedical quantitative traits
Bipolar disorder
Black vs blond hair color
Black vs red hair color
Blond vs brown hair color
Blood lipid traits
Blood pressure
Blue vs brown eyes
Blue vs green eyes
Body mass (lean)
Body mass index
Bone mineral density
Bone mineral density (hip)
Bone mineral density (spine)
Brain imaging in schizophrenia (interaction)
Brain lesion load
Breast cancer
Burning and freckling
Celiac disease
Cholesterol, total
Chronic lymphocytic leukemia
Chronic obstructive pulmonary disease
Cognitive test performance
Colorectal cancer
Conduct disorder (interaction)
Coronary artery calcification
Coronary artery disease
Coronary disease
Coronary spasm in women
C-reactive protein
Creutzfeldt-Jakob disease
Crohn's disease
Crohn's disease and sarcoidosis (combined)
Cystatin C
Cystic fibrosis severity
Diabetes-related insulin traits
Diabetic nephropathy
Diastolic blood pressure
Echocardiographic traits
Electrocardiographic conduction measures
Electrocardiographic traits
End-stage renal disease
Environmental confusion in the home
Episodic memory
Essential tremor
Exercise treadmill test traits
Exfoliation glaucoma
Factor VII
Fasting plasma glucose
F-cell distribution
Fetal hemoglobin levels
Folate pathway vitamins
Freckles
Gallstones
General cognitive ability
HDL cholesterol
Hearing impairment
Heart failure
Heart rate variability traits
Height
Hemostatic factors and hematological phenotypes
Hepatitis B
Hip bone size
Hip geometry
Hirschsprung's disease
HIV1 viral setpoint
Hyperactive-impulsive symptoms
Hypertension
Hypertension (young onset)
Idiopathic pulmonary fibrosis
Inattentive symptoms
Incident diabetes
Inflammatory bowel disease
Inflammatory bowel syndrome
Insulin response
Intracranial aneurysm
Iris color
Ischemic stroke
Juvenile idiopathic arthritis
Kawasaki disease
Knee osteoarthritis
Late-onset Alzheimer disease
LDL cholesterol
Lung adenocarcinoma
Lung cancer
Lupus
Major CVD
Major depressive disorder
Male pattern baldness
Mean forced vital capacity from two exams
Mean platelet volume
Melanoma
Memory performance
Menarche (age at onset)

(continued)

Table 8.1.1 (continued)

Menarche and menopause (age at onset)
Menopause (age at onset)
Methamphetamine dependence
Morbidity-free survival
Multiple sclerosis
Multiple sclerosis (age of onset)
Multiple sclerosis (severity)
Myeloproliferative neoplasms
Myocardial infarction
Myocardial infarction (early onset)
Myopathy
Narcolepsy
Neuroblastoma
Neuroblastoma (high-risk)
Neuroticism
Nicotine dependence
Nonsyndromic cleft lip with or without cleft palate
Normalized brain volume
Obesity
Obesity (early-onset extreme)
Obesity-related traits
Osteonecrosis of the jaw
Other metabolic traits
Other pulmonary function traits
Other subclinical atherosclerosis traits
Otosclerosis
Pain
Panic disorder
Parkinson disease (familial)
Parkinson's disease
Personality dimensions
Plasma carotenoid and tocopherol levels
Plasma eosinophil count
Plasma level of vitamin B12
Plasma levels of liver enzymes
Plasma levels of polyunsaturated fatty acids
Plasma Lp (a) levels
Progressive supranuclear palsy
Prostate cancer
Protein quantitative trait loci
Psoriasis
Pulmonary function measures
QT interval
QT interval prolongation
Recombination rate (females)
Recombination rate (males)
Red vs non-red hair color
Renal function and chronic kidney disease
Reponse to lithium treatment in bipolar disorder
Response to diuretic therapy
Response to iloperidone treatment (PANSS-T score)
Response to iloperidone treatment (QT prolongation)
Response to interferon beta therapy
Response to ximelagatran treatment
Restless legs syndrome
Rheumatoid arthritis
Sarcoidosis
Schizophrenia
Select biomarker traits
Serum bilirubin levels
Serum IgE levels
Serum markers of iron status
Serum metabolites
Serum urate
Serum uric acid
Skin pigmentation by reflectance spectroscopy
Skin sensitivity to sun
Sleep duration
Sleepiness
Smoking behavior
Smoking cessation
Soluble ICAM-1
Stroke
Subarachnoid aneurysmal hemorrhage
Successful cognitive aging
Systemic lupus erythematosus
Systemic lupus erythematosus in women
Systolic blood pressure
Tanning
Telomere length
Thyroid cancer
Thyroid-stimulating hormone
Tonometry
TP53 carriage
Treatment response for acute lymphoblastic leukemia
Treatment response to TNF antagonists
Triglycerides
Type 1 diabetes
Type 2 diabetes
Type 2 diabetes and 6 quantitative traits
Ulcerative colitis
Urinary albumin excretion
Urinary bladder cancer
Venous thromboembolism
Volumetric brain MRI
Waist circumference and related phenotypes
Waist circumference traits
Warfarin maintenance dose
Weight
Wet age-related macular degeneration
YKL-40 levels

the pre-high-throughput genotyping era was that of the *ApoE* variants and late-onset Alzheimer disease. In this particularly unusual case, a common haplotype of two nonsynonymous codon variants in the *ApoE* gene was strongly associated with Alzheimer disease. The risk allele (*ApoE4* defined by R112 and R158) in homozygosity has repeatedly been shown to confer a 15-fold risk of Alzheimer disease relative to all other allelic combinations (8, 53). After the introduction of high-throughput genotyping, several reports described the strong association of the complement regulatory gene factor H in age-related macular degeneration (ARMD) (11, 17, 19, 28). The successful identification

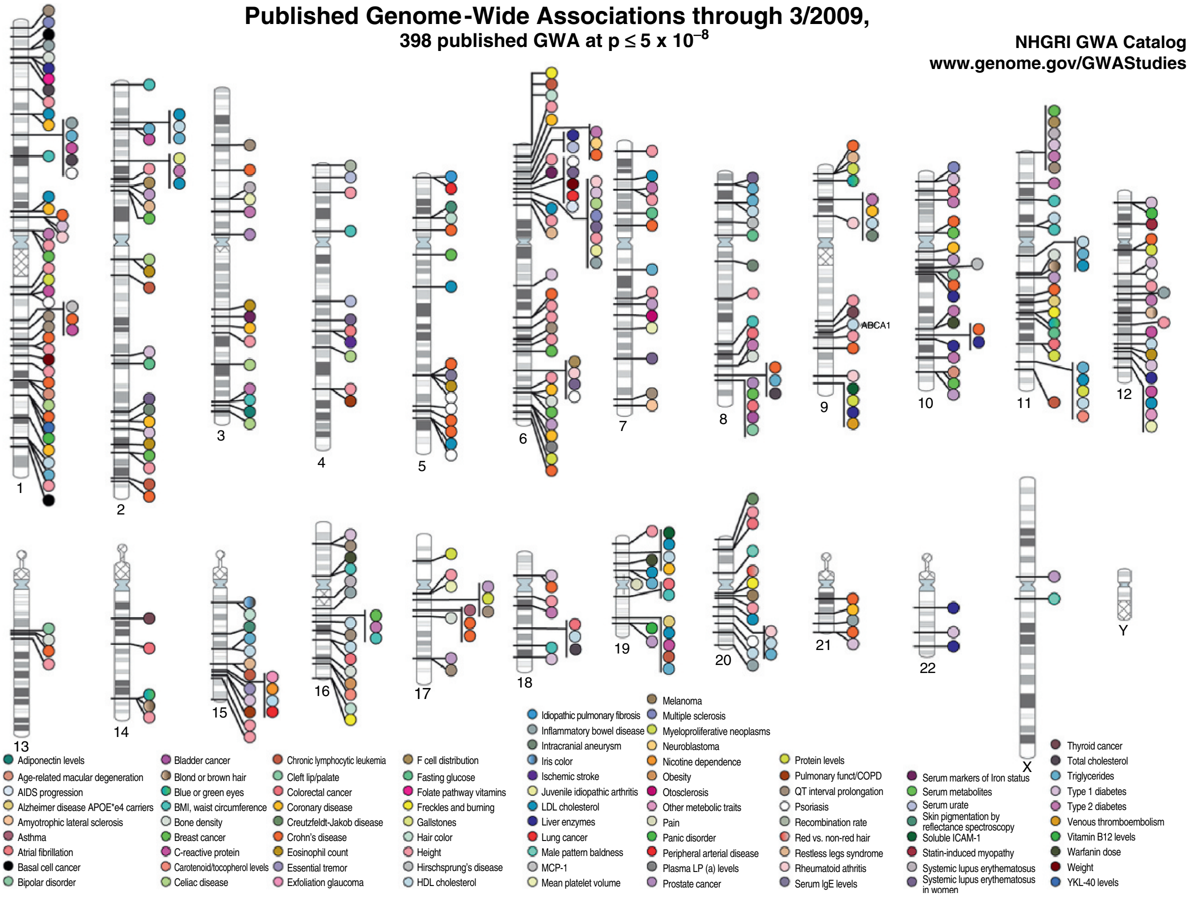

Fig. 8.1.1 Schematic representation of the published genome-wide associations; each *colored dot* represents an associated genomic locus for a given phenotype. (Reproduced from http://www.genome.gov/GWAStudies (20))

of a major locus involved in the etiology of ARMD, a complex phenotype, was heralded as a prelude to a rich harvest of numerous additional strong genetic risk factors for a wide variety of phenotypes. Thus, it took only a couple more years for this genome-wide association study (GWAS) approach to become widespread, and in the following years rapidly growing numbers of such studies were published, mostly in high-impact-factor journals reporting some successes in the quest for susceptibility factors for numerous phenotypes. The GWAS are usually done as follows (Fig. 8.1.2): DNA samples are collected from patients and well-matched controls (for dichotomous traits) or from a population for quantitative traits. Genotypes for several thousand to a million common SNPs are determined in all of these samples. Because of linkage disequilibrium (LD), these SNPs have been selected to extract approximately 80% of the genetic information. Thus, a subset of SNPs – called tagging SNPs – are genotyped, and because of LD the genotypes of nearby SNPs can be inferred, a process referred to as imputation. For each SNP (genotyped or imputed) a p-value is calculated for association of its alleles with the dichotomous or the quantitative trait. Statistical correction for multiple testing is applied to ensure detection of significant SNP-trait associations only. Odds ratios (OR) or relative risk (RR) estimates for the risk alleles are calculated from the data.

Today, it is widely recognized that initial mapping assignments require validation of the GWAS findings in independent cohorts (6, 23, 45). These steps are often accompanied or followed by high-resolution mapping of the associated genomic intervals (Fig. 8.1.2).

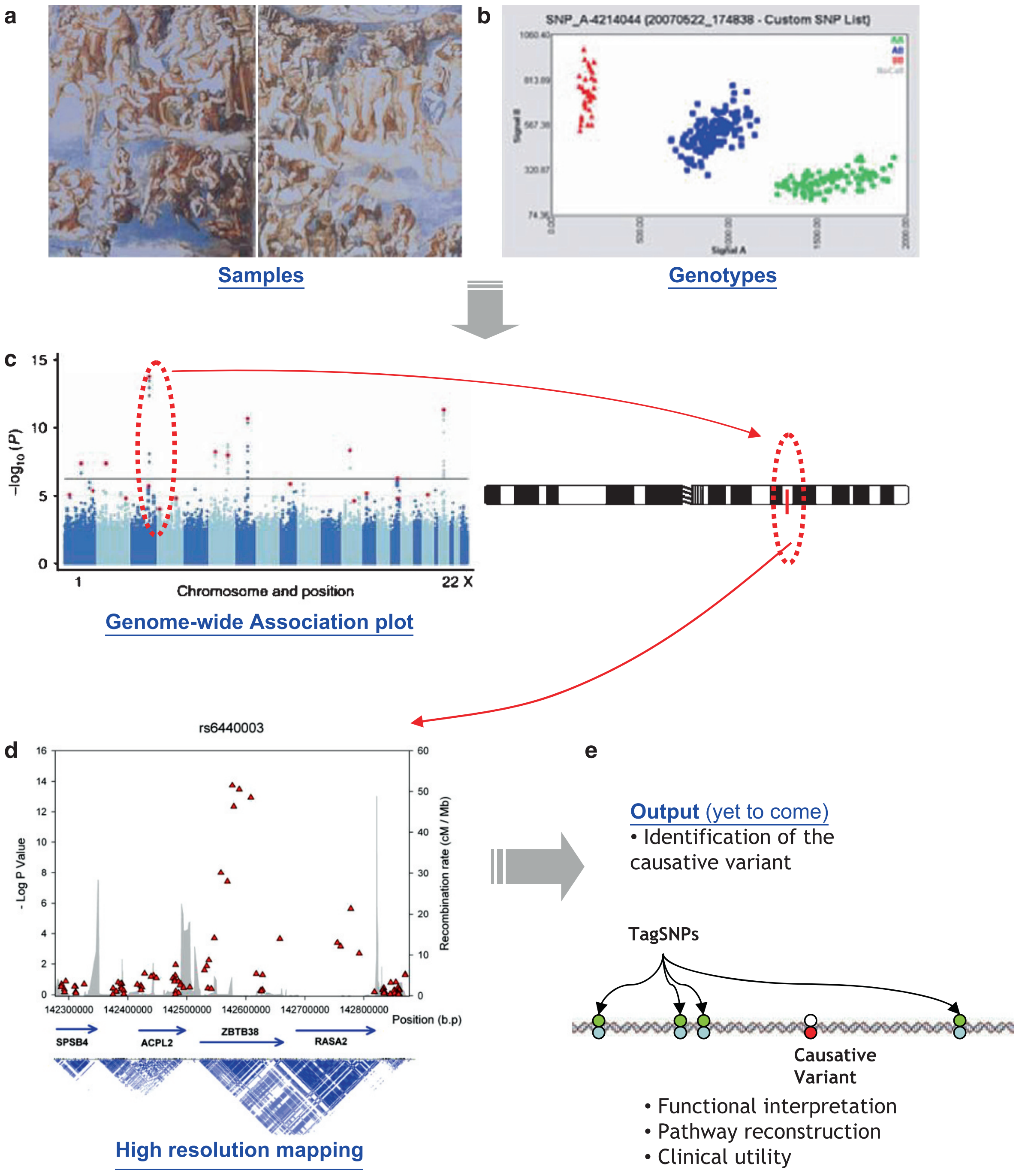

Fig. 8.1.2 Schematic representation of the different steps involved in GWA studies. The starting points for any GWAS are carefully phenotyped (population or case/control) cohorts from which DNA is collected from each participating individual. (**a**) Genotypes for several thousand to a million common SNPs are determined in all of these samples. (**b**) For each SNP (genotyped or imputed) a *p*-value is calculated for association of its alleles with the dichotomous or the quantitative trait. (**c**) The significance levels of the corresponding SNP-phenotype associations are plotted with the SNP order reflecting their corresponding locations along the chromosomes (from 1 to X): so-called Manhattan plots. (**d**) Close-up of about 0.5-Mb-long selected region on chromosome 3 (after (66)) for which SNP significance levels exceed a predefined threshold (*horizontal line* in **C**): *red triangles* represent the significance values of each SNP; *blue arrows* denote the genes falling in this interval and their orientation; *gray curve* shows the inferred recombination rate across this interval; *blue triangles* at the *bottom* show the extent of LD among the SNPs shown (*darker blue* strong LD; *lighter blue* weak LD). This region is subjected to fine mapping and/or replication in an independent validation cohort. (**e**) Once validated and size-restricted, the corresponding candidate intervals are subjected to a variety of analyses, including deep sequencing, and functional assays, in an attempt to eventually identify the causative variant(s) and its/their functional consequences and possibly to translate the resultant findings into clinical and public health utility

8.1.1 Lessons from Current GWAS

Altogether, over 200 GWAS studies have now been published, accounting for the statistical association and subsequent validation between a considerable number of genomic regions defined by SNP variation (more than 500 by June 2009; http://www.hugenavigator.net; http://www.genome.gov/GWAStudies and (20)) and common complex traits (see Table 8.1.1 and Fig. 8.1.1 for selected examples). The database at the NIH as of 06/04/09 includes 334 publications and 1,553 SNPs associated with complex traits. Among the latter there are several strong SNP-phenotype associations with *p*-values of 10^{-100} or less. These strong associations are in the vicinity of or involve the following genes: *UGT1A1* and serum bilirubin levels (10^{-324}); *VKORC1* and warfarin maintenance dose (10^{-181}); HLA and type 1 diabetes (10^{-129}); *SLC2A9* and serum urate (10^{-168}); *HLA-drb1* and rheumatoid arthritis (10^{-186}); *IRF4*, *HERC2*, and *MC1R* and hair color (10^{-127}, 10^{-103}, and 10^{-142} respectively); and *OCA2* and eye color (10^{-241}).

It should be remembered that these findings represent associated genetic markers that merely point out genomic regions, and the associated markers or regions are usually not the causal etiologic variants and do not point out genes causally responsible for the increased or decreased risk for these phenotypes. One thing of note, however, is that some of these loci include genes coding for known drug targets (e.g., *HMGCR* and statins (26, 69)), and nearly one in five of these association hits map in or near genes known to be mutated in Mendelian syndromes (21) that include the associated phenotype as part of the disease spectrum. For example, markers around the *MC4R* gene have been associated with early-onset obesity (12) or *IFHI1* in type 1 diabetes (46); pathogenic mutations in these genes have been shown to cause, respectively, "monogenic" obesity and type 1 diabetes. Complex SNP-phenotype associations and single-gene disorders may thus be part of a continuous etiologic spectrum on genetic diseases. The elucidation of the causal factors can thus provide an enriched list of candidate genes for both parts of this spectrum (2, 65). Furthermore, what applies to single-gene disorders, i.e., extensive allelic heterogeneity, locus heterogeneity, and incomplete penetrance, is likely to apply equally to common disorders. Thus the study of additional rare Mendelian disorders (beyond the 2,500 for which the molecular basis is known as of June 1 2009, http://www.ncbi.nlm.nih.gov/Omim/mimstats.html), will continue to assist the search for the causative variation on complex common phenotypes (2, 65).

8.1.2 Genomic Topography of Trait-Associated Variants

It is informative to examine the nature and genomic localization of associated SNPs, even if the latter are most likely only proxies to the causal etiologic variant. A recent analysis of the published validated SNP-trait associations revealed that 43% of these associations map to intergenic regions; the rest fall in gene coding regions, 45% in introns; 9% result in a nonsynonymous amino acid change, 2% map to 5′ or 3′ UTRs, and another 2% result in synonymous changes (20).

At first approximation, these initial observations suggest that genomic variants in noncoding genomic regions are likely to substantially contribute to the risk for complex common phenotypes. For these loci, the road from a validated statistical association to the identification of the etiologic variant and of its functional basis is still long, cumbersome, and uncertain (e.g., (43)). However, they also indicate that many associated SNPs are enriched in coding regions. These findings provide some enthusiasm for the identification of the functional causative variant(s) and of the molecular pathway(s) involved in the pathogenesis of the phenotypic trait. Nonsynonymous coding SNPs, which a priori are expected to be more deleterious, are significantly enriched in the associated regions. The corresponding SNPs and genes thus represent interesting candidates for further follow-up studies. A similar trend of enrichment was also seen for SNPs mapping in promoter regions (20).

8.1.3 How Important Is the Identified Genetic Contribution to the Variance of the Traits Studied?

Unfortunately for the overwhelming majority of studies, the OR for the risk allele of each associated SNP to the phenotypic trait is relatively low; in other words, most of the associated common variation explains only a small fraction of the phenotypic variance. Indeed, even after the study of large to huge cohorts (in some extreme cases with more than 30,000 patients and an equal number of controls), with over half a million SNPs for most examined traits, we have only uncovered what can be metaphorically expressed as “the tip of the iceberg”: each genomic region identified contributes a modest effect, and collectively all associated regions for a given trait explain only a small fraction (5–10%) of the observed phenotypic variation attributed to genetic elements. With the notable exception of the HLA region on chromosome 6p, most risk factors confer on average a 20% increase in risk (or a RR of disease susceptibility or OR of 1.2). Thus, after the completion of the majority of the GWAS in a large number of appropriate samples most of the risk-contributing genetic variation remains elusive ((1, 14, 21, 43), etc.).

Let us illustrate this with adult human height, a good example of a complex prototypic trait that should be amenable to genetic dissection. Height, a clearly polygenic trait, has several attributes that make it an optimal target for genetic investigations: it has a strong genetic component (heritability in the order of 85–90%): it is easily and accurately measurable, so that height data are available for large cohorts; it is stable over most of adult life and shows an almost normal population distribution; though subject to environmental influences (as evidenced by the height differences in successive generations (7))), the latter play a small overall role. Thus, all these elements combined would suggest that the genetics of height should be easier to elucidate than that of other traits (such as hypertension or type 2 diabetes, T2D). The GWAS harvest was successful. Over 45 “height” loci were identified and confirmed, including genes known to be mutated in Mendelian syndromes of abnormal skeletal growth (e.g., (65)). But cumulatively all these loci explain less than 10% of the genetic variance (i.e., the rest is still unknown), the gene with the strongest effect accounting only for 0.6 cm per contributing allele (15, 34, 55, 65, 66). The situation prevailing in other studies is analogous. *FTO*, for instance, is the locus that has the largest effect on body weight so far, yet it accounts at most for 1% of the overall phenotypic variance (e.g., (4, 37, 70)). Even for the hard-to-dissect hypertension phenotype (59), a first set of genome-wide significant hits was recently obtained, with each susceptibility allele contributing less than 1 mmHg of systolic blood pressure (36, 47). However, for blood pressure, variations in the order of 2 mmHg may explain a substantial burden of cardiovascular disease at population level (56), so that the cumulative effect of multiple genetic variants might still be of public health relevance even if they had low predictive power at the individual level.

We are thus left with an as yet unsolved enigma: where is the rest of the missing heritability (39)? It is of interest to note that methods used to estimate heritability assume that there are no gene-environment interactions, which may be an unreasonable assumption. Where indeed have all the height variants gone, or is it that our premises and hopes are wrong? Numerous scenarios have been put forward to explain the relatively modest contribution of the common variation to the risk of polygenic, complex phenotypes (see, e.g., (1, 13, 14, 21, 29, 44, 61)). These include (1) incomplete marker coverage, and thus the possibility that important areas of the genome have not been examined; (2) allelic heterogeneity at a given locus (see, e.g., (10, 18, 40, 41, 49); (3) the contribution of rare variants (50, 67, 71), including structural (CNVs and smaller) variants (3) as risk factors for complex disorders; initial studies in autism (e.g., (42, 54, 68)) or schizophrenia partially support this possibility (e.g., (64, 72)); (4) epistatic interactions, i.e., the contribution of the combination of alleles at two or more loci to the risk for a given trait; (5) gene–environment interactions or different environmental exposures of subpopulations; (6) epigenetic modifications, such as methylation or transgenerational RNA (i.e., RNA transmitted through the sperm or egg), which might account for a parent-of-origin effect that would be lost under current GWAS investigations; and possibly (7) overestimation of heritability (39). Future studies, including the detection of all variants by high-throughput sequencing, may clarify these issues in the years to come.

8.1.4 Predictive Power and Clinical Utility of the Trait-Associated Variants

As a result of the modest effects contributed by each of the risk alleles tagged by the genotyped SNPs, their risk profiles (individually or even collectively) and the ensuing predictive power are still small. In comparison to the usual clinical factors, estimates relying on known genetic factors are poor predictors of risk, and only marginally improved prognosis (see, e.g., (38)). Thus, their clinical utility is so far limited. At present, this precludes acceptable and useful individual genotypic discrimination, personalized prognosis, and intervention (1, 9, 31, 65), except possibly in the field of pharmacogenetics, as illustrated by the warfarin example (5). It is a matter of current debate whether we will ever be able to provide effective preventive medicine statements based on the genetic profiling of individuals (see (14, 21, 29)). In spite of the current skepticism and the absence of validation of the proposed prediction algorithms, others have already taken the opportunity to venture into direct-to-consumer predictive genetic tests (http://ghr.nlm.nih.gov/handbook/testing/directtoconsumer). The controversies around these possibilities and the tendency for the control of genetic testing to shift from the clinical professionals into the hands of consumers (27) are new challenges for predictive medicine (24, 25, 30, 61). Hence, it is essential that consumers and health providers be made aware of the potentials, but also of the limitations and pitfalls, of many of the promises associated with genetic tests for complex traits. Proper (public and professional) education is thus a necessity, as is adequate genetic counseling of all those seeking to learn more about themselves by knowing their genetic variability profiles.

8.1.5 Pathophysiological Dissection of Complex Traits

Yet even if the associated SNPs do not accurately predict common traits, many of the newly identified loci highlight molecular pathways, including some not previously known to be involved in the corresponding pathophysiological processes (13, 21, 62). GWAS studies of Crohn disease revealed the central role of autophagy and bacterial defense in this disease process (48, 52) in an unbiased/unsupervised fashion, thereby opening new possibilities for translational research. Genes involved in signaling pathways, chromatin, the extracellular matrix or cell cycle, and cancer, among other matters, are implicated in height (22, 65); in T2D we encounter genes involved in pancreatic beta-cell formation and function as well as in pathways affecting glucose levels and obesity (13); furthermore, in obesity, we encounter genes highly expressed in the brain, and particularly in the hypothalamus, and possibly involved in weight regulation (60, 70). Pathway involvement is thus an important finding of GWAS studies and could provide potential new candidate genes, thus enabling further targeted investigations and a new way to categorize diseases.

It is also interesting to note that different GWAS identified common/shared risk SNPs for more than one common disorder revealing either similar or shared pathogenic mechanisms or coincident mapping of etiologic variants (Fig. 8.1.3). In many instances these co-occurrences are not surprising (e.g., that of Crohn disease and T2D and that of prostate, colorectal, or breast cancer), while in others the co-occurrence is unexpected (e.g., for a *TCF2* gene variant in prostate cancer and T2D) (13, 16, 33, 43). Some of the identified variants may have pleiotropic effects, being protective for one disease and a susceptibility factor for another (e.g., *TCF2*; see also Fig. 8.1.3). Discovering functional risk variants for one trait could thus help studies of other traits and diseases.

The discovery of genomic risk loci for complex phenotypes could also help to subclassify these phenotypes, in a similar way to the subclassification of similar phenotypes for Mendelian disorders (2). This may facilitate the study of the pathophysiology of certain phenotypes or identify individuals at risk for adverse reactions to certain medications.

8.1.6 Concluding Remarks

GWAS, based on the hypothesis that there are common risk alleles for common complex phenotypes, have provided a large number of very low risk alleles that are much less impressive than the risk conferred by the well-known HLA system known for several decades. On the other hand, some important risk alleles for a few disorders have been identified (e.g., late-onset macular degeneration (11, 17, 19, 28)); a few unknown regulators of quantitative traits have also been identified (e.g., *BCL11A* and fetal hemoglobin (35, 63)). The results yielded so far by GWAS provide a wealth of hypotheses for identification of molecular pathways involved in the complex phenotypes. However, the majority of the risk alleles identified provide only a very small risk to the phenotypes studied. For most of the studied traits, clinical utility thus remains to be demonstrated. This may be acutely true for those pleiotropic variants that, depending on the disease considered,

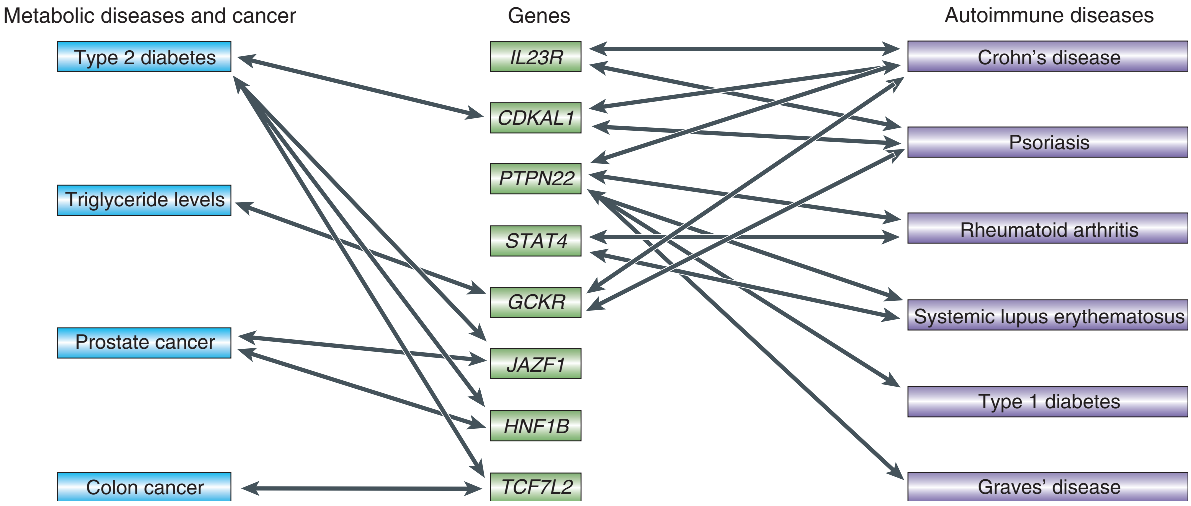

Fig. 8.1.3 Overlap of genetic risk factor loci for common diseases. A surprising finding of genome-wide association studies is that loci can be associated with the risk of developing two or more diseases, probably sharing common molecular causes. For some loci, distinct risk alleles are associated with different diseases or, in other instances, the same alleles can be protective for one disease and confer susceptibility to another. Eight loci are shown here for illustrative purposes with their impact on autoimmune, metabolic diseases or cancer. (After (13))

could assume either a protective or a predisposing role. It seems thus that the predictive value of the risk alleles is doubtful for a given individual. Most of the loci that contribute to the heritability of the common traits remain to be identified.

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Epigenetics

9

Bernhard Horsthemke

Abstract According to its founder, Conrad Hal Waddington, epigenetics studies the causal interactions between genes and their products, which bring the phenotype into being. Waddington was the first to recognize that the developing organism is a nonlinear dynamic system and that development proceeds through a time-ordered set of epigenetic states, which are mitotically stable, but potentially reversible. The metastability of epigenetic states explains why developmental processes are buffered against minor changes in genotype and environment (a phenomenon called canalization), yet one genotype can give rise to more than one phenotype (phenotypic plasticity). Among several epigenetic inheritance systems, chromatin marking is the one that has received most attention from modern molecular biology. It has been recognized that tissue-specific gene expression patterns can be mitotically stable, because the genome is parceled into chromatin states that allow or repress use of the genetic information (permissive and repressive chromatin, respectively). Permissive and repressive chromatin states are characterized by specific patterns of DNA methylation, histone modification, and chromatin configuration. Within a cell, most often both alleles of a gene are either active or inactive. However, there are several examples where the two alleles of a gene, although identical in sequence, are functionally different. The difference can be parent-of-origin specific (as a result of genomic imprinting) or random (X inactivation and allelic exclusion). Epigenetic variation, occurring at random or induced by the environment, can lead to phenotypic variation and, in its most extreme form, to disease. In general, epigenetic states are cleared between generations. It is a matter of debate to what extent epigenetic states can be transmitted through the mammalian germline from one generation to the next.

Contents

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9.1 History, Definition, and Scope

The term "epigenetics" was coined in the 1940s by the British scientist Conrad Hal Waddington. "Epigenetics" is a neologism which combines the words "epigenesis" (embryonic development) and "genetics." Before Waddington, embryology and genetics had been separate disciplines. Embryologists used chemical reagents and surgical techniques to study development, but could not explain the similarity between parents and offspring. Geneticists studied the behavior of genes during inheritance, but could not explain the development of a particular phenotype. Waddington abrogated the distinction between embryology and genetics. He defined epigenetics as: "[T]he branch of biology which studies the causal interactions between genes and their products which bring the phenotype into being" [35]. His concept also led him to expand the classic model of the genotype–phenotype distinction by including the "epigenotype":

$$\begin{aligned}&\text{Genotype} + \text{Epigenotype} + \text{Environment}\\&= \text{Particular Phenotype}\end{aligned}$$

According to Waddington the epigenotype is the "[S]et of organizers and organizing relations" involved in creating a particular phenotype during development. As the prefix "epi-" implies, this set of organizers and organizing relations operates *on* or *over* the genes.

A fundamental characteristic of the epigenotype is that it is relatively autonomous. It is not strictly determined by the genotype, but also by the environment and subject to random variation. Furthermore, it is relatively stable, but not as stable as the genotype. As a consequence, one genotype can have alternative epigenotypes and hence phenotypes (a phenomenon called phenotypic plasticity), and observed inheritance patterns may deviate from expected Mendelian inheritance patterns. In fact, epigenetics does not only investigate the question of how the genotype brings the phenotype into being, but also why the phenotype often does not correlate with the genotype. Inbred animals and monozygotic twins can be discordant for a certain phenotype because of epigenetic variation.

A very illuminating example of phenotypic plasticity is the bee. In principle, larvae with identical genotypes develop into bees with identical phenotypes. This is because developmental processes are canalized. However, larvae fed with royal jelly develop into queens. Thus, under certain environmental conditions, development follows a different trajectory and leads to a different phenotype.

The field of epigenetics did not see very much activity until the late 1980s, when molecular mechanisms controlling gene activity in higher eukaryotes and the inheritance of cell phenotypes (cellular memory) began to be unraveled. Most importantly, several studies had shown that the methylation of certain cytosine residues within the DNA could control gene expression and that these patterns could be inherited from cell to cell [16]. Concomitant with these studies and the advent of complete genome sequences, the term epigenetics was narrowed down to mean the study of cell-heritable changes in gene activity that are not based on differences in the DNA sequence. To date, most researchers equate epigenetics with the study of chromatin-marking systems (DNA methylation, histone modification, and chromatin remodeling). Although it can be argued that such a narrow definition excludes other important epigenetic inheritance systems, such as steady state systems (e.g., metabolic feedback loops) and structural inheritance systems (e.g., prion-induced protein refolding), the current focus on chromatin-marking systems has greatly advanced our understanding of development and variation in higher organisms.

Although chromatin changes also play a fundamental part in transcriptional control, epigenetics should not be equated with the study of transcriptional control. While the latter deals with the factors and mechanisms involved in switching gene activity on or off, epigenetics deals with the variation and cellular inheritance of gene activity states. It is their relative stability that matters in epigenetics.

9.2 Chromatin-Marking Systems

Although all cells of an organism – with a few rare exceptions – have the same genome, dividing fibroblasts give rise to new fibroblasts only and dividing hepatoblasts give rise to new hepatoblasts only. The tissue-specific gene expression patterns that characterize the fibroblast, hepatoblast, and other cells of the body, are cell heritable, because the genome is parceled into metastable chromatin states that allow or repress the use of the genetic information (permissive and repressive chromatin, respectively). Permissive

Table 9.1 Epigenetic diseases

Disease	Locus	Clinical findings
Diseases caused by a mutation in a gene encoding an epigenetic factor		
ICF syndrome	*DNMT3B*	Immunodeficiency, centromere instability and facial anomalies; recurrent infections
Rett syndrome	*MECP2*	Loss of acquired skills, microcephaly, mental retardation, autistic features, seizures, hand stereotypies, gait apraxia
Rubinstein-Taybi syndrome	*CREBBP*	Mental retardation, downslanting palpebral fissures, hypoplastic alae nasi, broad thumbs and toes
9q34 subtelomeric deletion syndrome	*EHMT1*	Mental retardation, hypotonia, brachycephaly, flat face with hypertelorism, synophrys, anteverted nares, tented upper lip, everted lower lip, prognathism, conotruncal heart defects, behavioral problems
Coffin–Lowry syndrome	*RSK2*	Mental retardation, full lower lip, soft hands with tapering fingers, pectus carinatum, scoliosis
X-linked alpha thalassemia mental retardation	*ATRX*	Mental retardation, tented upper lip, abnormal genitalia, alpha thalassemia
Diseases caused by an epimutation at a specific gene locus		
Prader–Willi syndrome	*MAGEL2, NDN, C15orf2, SNRPN, snoRNA genes*	Neonatal muscular hypotonia, feeding difficulties in infancy, hyperphagia and obesity starting in early childhood, hypogonadism, short stature, small hands and feet, sleep apnea, behavioral problems, mild to moderate mental retardation
Angelman syndrome	*UBE3A*	Microcephalus, ataxia, absence of speech, abnormal EEG pattern, severe mental retardation, frequent laughing
Beckwith–Wiedemann syndrome	*IGF2*, *CDKN1C*	High birth weight, hypoglycemia, macroglossia, exomphalos, increased risk of Wilms' tumor
Silver–Russell syndrome	*IGF2* and others	Pre- and postnatal growth retardation
Fragile X mental retardation syndrome	*FMR1*	Mental retardation, macroorchidism, long face, large ears, and prominent jaw, behavioral problems
Facioscapulohumeral muscular dystrophy	*D4Z4*	Progressive muscular weakness affecting the face and the scapulae followed by the foot dorsiflexors and the hip girdle

and repressive chromatin states are characterized by specific patterns of DNA methylation, histone modification, and chromatin configuration. Several recognizable syndromes are caused by mutations in genes that encode chromatin-marking proteins (Table 9.1).

9.2.1 DNA Methylation

DNA methylation in the mammalian genome refers to the methylation of cytosine (5-methyl-cytosine, m^5C) within a CpG dinucleotide. CpG is a palindromic sequence, and typically the cytosines in both strands are methylated. It is estimated that approximately 80% of C residues within CpG dinucleotides are methylated. As m^5C can undergo spontaneous deamination to thymine, methylated CpG dinucleotides are hot spots for mutation and are slowly eliminated during evolution. Therefore, in large parts of the mammalian genome, CpG dinucleotides occur at a much lower frequency than expected from the relative frequency of C and G residues. Certain "islands" of 0.5–5 kb, however, are GC rich and have the expected frequency of CpG dinucleotides [2]. In general, these CpG islands are unmethylated and overlap the promoter and exon 1 of a gene. It is likely that the binding of transcription factors or the process of transcription protects them from methylation.

Several techniques have been developed to determine DNA methylation patterns. The gold standard is the sequencing of sodium bisulfite-treated genomic DNA [13]. Sodium bisulfite converts C, but not m^5C, into uracil. During PCR, thymine replaces uracil. The PCR products are then sequenced directly or after subcloning into a plasmid vector. The presence of a CpG dinucleotide in the final sequence indicates that the cytosine residue was originally methylated. A TpG dinucleotide that is not present in the untreated DNA indicates the presence of an unmethylated cytosine in the genomic DNA. Cytosines outside CpG dinucleotides are always unmethylated and should present as thymines if the bisulfite conversion has been complete.

9.2.1.1 DNA Methyltransferases

5-Methyl-cytosine is not incorporated into the DNA by the DNA polymerase, but is the result of posttranscriptional modification of cytosine by DNA methyltransferases. Three active DNA methyltransferases have been identified in human cells: DNMT1, DNMT3A, and DNMT3B (Fig. 9.1). In addition, there is one putative DNA methyltransferase (DNMT2) and a protein that is highly similar to DNMT3A and B, but devoid of catalytic activity (DNMT3L). All methyltransferases use *S*-adenosyl-methionine as a methyl donor. DNMT1 is ubiquitously expressed and is a maintenance methyltransferase, which methylates hemi-methylated CpG dinucleotides in the nascent DNA strand after replication (Fig. 9.1). It is essential for maintaining DNA methylation patterns in proliferating cells. DNMT3A and DNMT3B are regulated during development. They carry out de novo methylation and thus establish new DNA methylation patterns (Fig. 9.1). DNMT3L cooperates with DNMT3A and DNMT3B to establish methylation imprints (see below). The activity and function of DNMT2 remains undefined.

Targeted mutations of the murine orthologs of the *DNMT1*, *DNMT3A*, and *DNMT3B* genes lead to methylation defects and pre- or post-natal lethality. Targeted mutations in the murine ortholog of *DNMT3L* impair imprinting and gametogenesis. In humans, mutations of the *DNMT3B* gene cause autosomal recessive ICF syndrome (*i*mmunodeficiency, *c*entromere instability, and *f*acial anomalies; Table 9.1). Centromere instability correlates with severe hypomethylation of the satellite DNA.

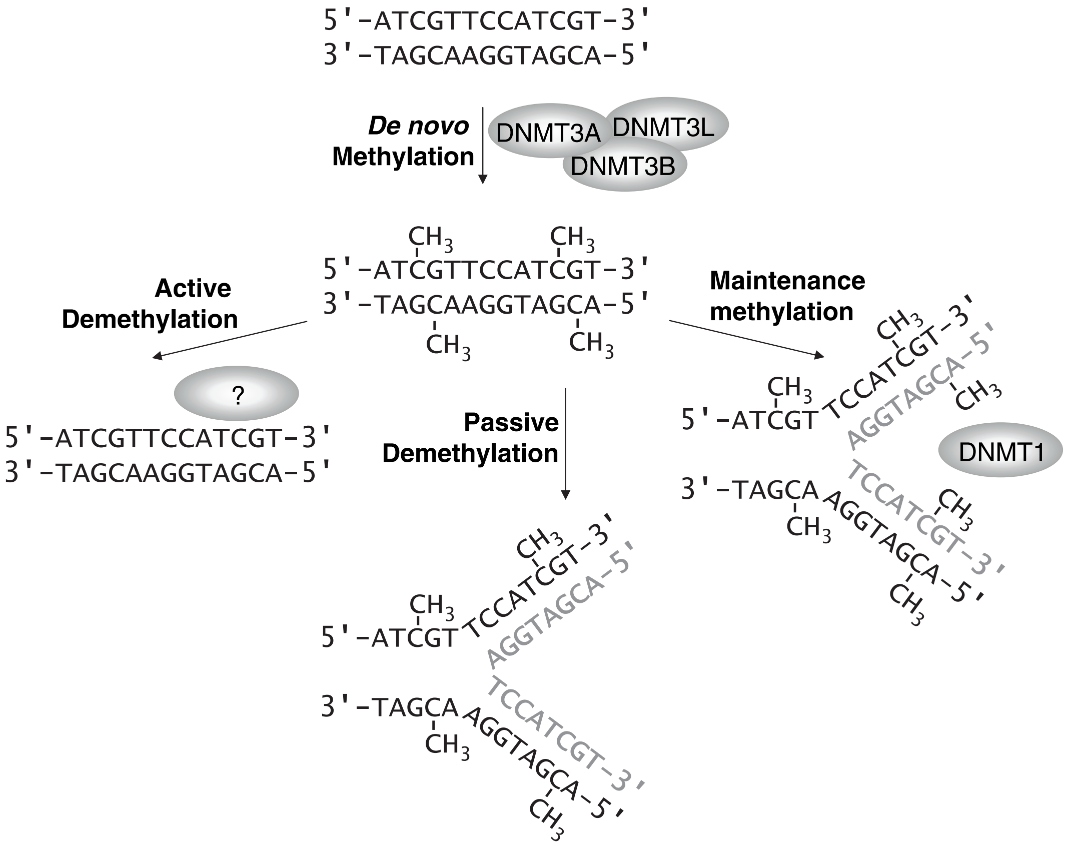

Fig. 9.1 DNA methylation. For details see text

At present, no bona fide DNA demethylase has been identified, although the existence of such an enzyme is likely. Shortly after fertilization, the sperm DNA is actively demethylated by the oocyte. Although there is no convincing evidence for an enzyme that removes the methyl group from m^{5}C, DNA repair enzymes can excise m^{5}C and replace it by C. Passive demethylation occurs by DNA replication in the absence of DNMT1 (Fig. 9.1). In cell culture, passive DNA demethylation can be achieved with DNMT1 inhibitors. The most frequently used compound is 5-aza-cytidine.

9.2.1.2 Methyl-Cytosine-Binding Proteins

Mammalian cells contain several proteins that bind to single m^{5}Cs or clusters of neighboring m^{5}Cs. At present, six methyl-CpG-binding proteins have been identified: MECP2, MBD1, MBD2, MBD3, MBD4, and KAISO. MECP2 contains a methyl-CpG-binding domain (MBD) and a transcription repression domain (TRD). MECP2, MBD1, and MBD2 function as transcription repressors. MBD4 is a DNA glycolase and is involved in DNA mismatch repair. KAISO lacks an MBD domain and binds methylated CGCG through its zinc-finger domain. The CpG-binding proteins recruit chromatin-remodeling and transcription factor complexes to methylated DNA regions in the genome. MECP2, for example, recruits the SIN3A corepressor complex, which contains a histone deacetylase, and sets up repressive chromatin. MBD2 forms a complex with the NuRD complex, which contains an ATP binding-dependent protein as well as a histone deacetylase. This complex represses methylated promoters. These examples also highlight the synergy between the three chromatin-marking systems (see also below).

Targeted mutations in the murine orthologs of the MBD proteins are associated with prenatal lethality (*Mbd3*$^{-/-}$) or neurological and behavioral defects (*Mecp2*$^{-/-}$ and *Mbd2*$^{-/-}$). In humans, *MECP2* mutations are associated with Rett syndrome, which is an X-linked dominant neurodevelopmental disorder. Girls with Rett syndrome have apparently normal development throughout the first 6 months of life, before they lose previously acquired skills (for more clinical details see Table 9.1). Initially it was believed that the loss of MECP2 led to widespread loss of gene repression. This, however, does not appear to be the case. To date, only two genes (*BDNF* and *DLX5*) have been identified as target genes.

9.2.2 Histone Modification

Within the nucleus, the DNA is packaged into chromosomes. Nucleosomes are the basic structural unit of a chromosome. The nucleosome core particle consists of a complex of eight histone proteins – two molecules each of histones H2A, H2B, H3, and H4 – and 146 base pairs of double-stranded DNA wrapped around the histone octamer. Each nucleosome core particle is separated from the next by approximately 80 bp of DNA and the linker histone H1. To a first approximation, the core histones are globular molecules, from which the flexible N-terminus protrudes. The side chains of lysine, arginine, and serine residues within the N-terminal tail of the histones are subject to extensive posttranslational modification (Fig. 9.2). The modifications include

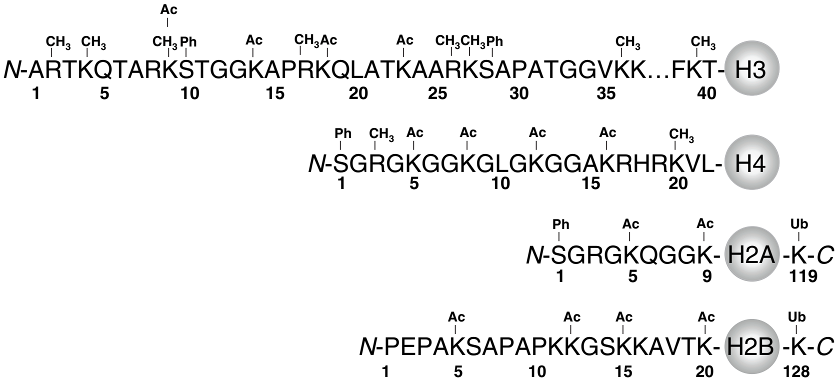
Ac
CH3 CH3 CH3 Ph Ac CH3 Ac Ac CH3 CH3 Ph CH3 CH3
N-ARTKQTARKSTGGKAPRKQLATKAARKSAPATGGVKK...FKT- H3
1 5 10 15 20 25 30 35 40
Ph CH3 Ac Ac Ac Ac CH3
N-SGRGKGGKGLGKGGAKRHRKVL- H4
1 5 10 15 20
Ph Ac Ac Ub
N-SGRGKQGGK- H2A -K-C
1 5 9 119
Ac Ac Ac Ac Ub
N-PEPAKSAPAPKKGSKKAVTK- H2B -K-C
1 5 10 15 20 128

Fig. 9.2 Histone modifications. The amino acid sequences of the N-terminal and C-terminal tails of histones H3, H4, H2A, and H2B are given in the one-letter code. Possible posttranslational modifications of the amino acid side chains of arginine (*R*), lysine (*K*), and serine (*S*) are indicated above the sequence (*CH3* methylation, *Ac* acetylation, *Ph* phosphorylation, *Ub* ubiquitinylation)

the acetylation, ubiquitinylation, and mono-, di-, and trimethylation of lysine, the mono- and dimethylation of arginine, and the phosphorylation of serine. Furthermore, histone ubiquinylation, histone sumoylation, and poly-ADP-ribosylation have been described. The pattern of modification determines the accessibility of promoter and enhancers to transcription factors and thus gene activity.

Many different combinations of histone modifications are possible, providing the cell with a wealth of different possibilities to regulate gene activity. Trimethylation of Lys4 of H3 (H3K4) and acetylation of Lys9 of H3 (H3K9), for example, have been associated with active gene expression, whereas trimethylation of H3K9 and H3K27 has been associated with transcriptional silencing.

The pattern of histone modification at a locus of interest is determined by chromatin immuno-precipitation (ChIP). Living cells are treated with formaldehyde to crosslink DNA and proteins. The chromatin is then fragmented by ultrasound and antibodies are used to precipitate the DNA-protein complexes that contain histones with a specific modification. After reversal of the crosslink, PCR is used to amplify the DNA region of interest and the fold enrichment is calculated. For each modification, a separate experiment has to be done. Instead of PCR, the DNA can also be analyzed by hybridization to microarrays containing oligonucleotides from the region of interest (ChIP on chip) or by massive parallel sequencing (ChIPseq).

9.2.2.1 Histone Acetylation

The degree of histone acetylation, i.e., the acetylation of a lysine side chain, is determined by the activity of histone acetylases (HATs) and histone deacetylases (HDACs). Several transcription factors and co-activators have intrinsic HAT activity. There are three HAT families in humans (the GNAT, MYST, and CBP/p300 families), and more than a dozen other proteins with HAT activity. The acetylation of a lysine residue changes the charge of the histone molecule and leads to an "open," transcriptionally permissive chromatin configuration.

Several mutations affecting HAT genes have been identified in humans. Mutations of the *CREBBP* gene, which encodes the CBP protein, cause Rubinstein–Taybi syndrome (Table 9.1). A fusion of the *CREBBP* and *MOZ* (*MYST3*) genes, resulting from a t(8;16)(p11;p13) translocation, plays a causal role in the development of a subtype of acute myeloid leukemia.

There are at least 18 HDACs in humans, which fall into three classes. Class I and II HDACs have a catalytic domain which includes a zinc ion. Class III HDACs use NAD$^+$ as a co-factor. Histone deacetylation leads to a "closed," repressive chromatin configuration. As mentioned above, methyl-CpG-binding proteins can recruit HDACs to repress gene transcription.

Histone deacetylation can be inhibited by small compounds such as trichostatin A (TSA). TSA treatment of cells can lead to reactivation of silenced genes. Several *HDAC* genes have been knocked out in mice. A targeted mutation of the murine ortholog of *HDAC1*, for example, leads to intrauterine growth retardation and early embryonic lethality. In humans, *HDAC2* mutations have been found in certain types of cancer.

9.2.2.2 Histone Methylation

In contrast to the acetylation of lysine residues, the methylation of lysine residues does not change the charge of the histone molecule. It does, however, change its basicity and hydrophobicity. Furthermore, histone methylation is considered to be more stable than histone acetylation and thus more relevant for epigenetic inheritance. The degree of histone methylation is determined by the activity of histone methyltransferases (HMTs) and histone demethylases (HDMs).

Like DNA methyltransferases, HMTs use *S*-adenosyl-methionine as a methyl donor. The catalytic domain of lysine HMTs consists of approximately 130 amino acids and is called the SET domain. The acronym is derived from three drosophila proteins [*s*u(var)3-9, *e*nhancer-of zest, *t*rithorax]. At present, more than 300 proteins with a SET domain have been identified. The proteins also have bromo- and chromodomains. It is not clear how mono-, di-, and trimethylation of lysine residues are regulated.

The HMTs differ in their specificity. SUV39, G9A and EHMT1, for example, preferentially methylate H3K9. A targeted mutation of the murine *Suv39* gene leads to loss of H3K9 methylation in heterochromatin and chromosome defects. In G9a$^{-/-}$ mice, loss of H3K9 methylation in euchromatin leads to early embryonic lethality. Haploinsufficiency for *EHMT1* in humans is responsible for the clinical findings associated with the 9q34 subtelomeric deletion syndrome (Table 9.1).

In addition to lysine residues, arginine residues can be methylated. This reaction is catalyzed by protein arginine methyltransferases. At present, seven such enzymes are known in mammals. Similar to the lysine HMTs, the arginine HMTs differ in their specificity.

Only recently, several HDMs have been identified, for example the amine oxidase AOF2 (also called lysine-specific demethylase 1, LSD1) and the peptidylarginine-deiminase 4 (PADI4).

9.2.2.3 Histone Phosphorylation and Other Histone Modifications

The phosporylation and dephosphorylation of serin residues within histone molecules is catalyzed by kinases and phosphatases, respectively. Mutations in the *RSK2* gene, which encodes a ribosomal S6 kinase, causes Coffin-Lowry syndrome (Table 9.1). The kinase has several functions, one of which is the phosophorylation of histone H3.

In addition to acetylation, methylation, and phosphorylation, histone ubiquitinylation, histone sumoylation, and poly-ADP-ribosylation have been observed. Ubiquitin is a key component of target protein degradation. The ubiquinylation of histones, however, does not appear to be a marker for degradation, but for active chromatin. Sumoylation appears to have the opposite effect on transcription. SUMO is a *s*mall *u*biquitin-related *mo*difier. Poly-ADP-ribosylation is found in active chromatin.

9.2.3 Chromatin Remodeling

A number of studies have identified protein complexes which bind to DNA, hydrolyze ATP, and use the energy to change the relative position of the nucleosome with respect to the DNA. Chromatin remodeling can make DNA-binding sites accessible or inaccessible to transcription factors (Fig. 9.3). The best characterized complex is SWI/SNF, which contains a number of different proteins. It is of particular relevance to epigenetics that the change in chromatin structure brought about by chromatin remodeling factors persists even after the complex has dissociated from the chromatin.

Loss of function of a chromatin remodeling factor can impair gene expression and cause disease. Mutations in the *ATRX* gene, for example, which encodes a member of the SWI/SNF family, lead to X-linked alpha thalassemia mental retardation (ATRX) syndrome. ATRX is a developmental disorder characterized by mental retardation, dysmorphism, and reduced expression of the α-globin genes (Table 9.1).

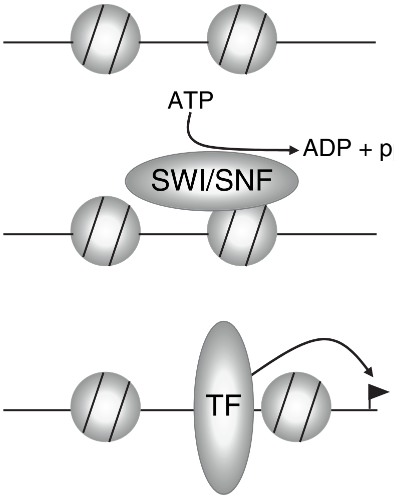

Fig. 9.3 Chromatin remodeling. The DNA (*black line*) is wrapped around nucleosomes (*gray balls*). In an energy-dependent mechanism, the chromatin remodeling complex SWI/SNF changes the relative position of the nucleosomes, so that a transcription factor (*TF*) can bind to its target sequence and activate gene transcription (*arrowhead*)

9.2.4 Synergistic Relations Between the Different Chromatin-Marking Systems

The different chromatin-marking systems cooperate with each other to set, spread, and maintain chromatin states. In some instances, DNA methylation depends on histone methylation, while in other instances the reverse is true. One possible scenario is shown in Fig. 9.4. H3K9 methylation also depends on deacetylation, because this lysine residue can be either acetylated or methylated. Furthermore, histone phosphorylation cooperates with histone acetylation in generating transcriptionally active chromatin. In this case and in other cases, a modification of one amino acid side chain can inhibit or enhance the modification of an amino acid side chain on the same or even a neighboring histone molecule. In addition, many other nonhistone proteins,

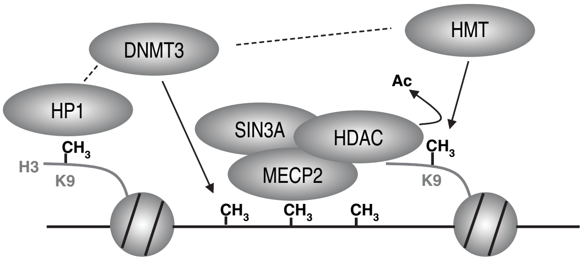

Fig. 9.4 Interaction between different chromatin-marking proteins. One possible scenario is shown. The heterochromatin protein HP1 binds to the methylated lysine residue K9 of histone H3 and triggers DNA methylation (through DNMT3) and histone methylation (through one of several HMTs). The methylcytosine-binding protein MECP2 recruits the co-repressor SIN3A and a histone deacetylase (HDAC)

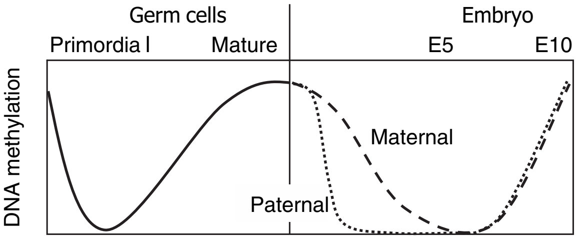

Fig. 9.5 Epigenetic reprogramming during gametogenesis and embryogenesis. DNA methylation is erased in primordial germ cells and newly established during gametogenesis. After fertilization (*vertical line*), the paternal genome is actively demethylated by the oocyte. The maternal genome looses methylation through a passive mechanism. At the blastula stage, de novo methylation starts

e.g., heterochromatic protein 1 (HP1), can be involved. HP1 is methyl-lysine-binding protein localized at heterochromatin sites, where it mediates gene silencing.

The chromatin-marking system is exceedingly complex, although in principle there are only two basic states: permissive and repressive chromatin. So why are there so many different combinations of DNA and histone modifications? It appears that different modification patterns determine the degree of stability of a chromatin state. In some instances, the chromatin state survives many mitotic and even meiotic cell divisions. In other instances, e.g., in embryonic stem cells, certain chromosomal regions are in a semi-permissive state, i.e., the genes in these regions are silent in the stem cells, but easily activated upon differentiation.

9.3 Specific Epigenetic Phenomena

During development, the genome undergoes stage- and tissue-specific epigenetic reprogramming. As a consequence, only a specific set of genes is active in any given cell at any given developmental stage. The most extensive epigenetic reprogramming occurs during gametogenesis and early embryogenesis, when the epigenetic states of almost all loci are erased and newly established. This is most impressive in mice (Fig. 9.5). The clearing of the epigenetic state between generations appears to be necessary to provide a clean state on which differentiation and development can occur.

Within a few hours after fertilization, the paternal genome is actively demethylated by the oocyte [22]. The enzymes involved in this process are still unknown. The maternal genome is protected against this active demethylation, but loses most of its methylation during subsequent rounds of cell division by passive demethylation (see Fig. 9.1).

During development, the developmental potential of the nuclear genome is more and more restricted. The zygote and early blastomeres are totipotent: they can give rise to all embryonic and extraembryonic tissues. Embryonic stem cells can give rise to all embryonic tissues, but not to extraembryonic tissues; they are pluripotent. The developmental potential of tissue-specific stem cells is restricted to certain cell lineages. Hematopoietic stem cells in the bone marrow stem cells, for example, can give rise to all the types of both the myeloid and lymphoid lineages, but not to other cells of the body, at least not under normal conditions. However, cell biologists are learning more and more to extend the developmental potential of such cells. It appears that the epigenetic states of the zygote, embryonic stem cells, tissue-specific stem cells, and terminally differentiated states differ in their relative stability.

The cloning of animals by transfer of somatic nuclei into enucleated oocytes requires clearing of the epigenetic state of the donor nucleus and restoration of cellular totipotency [38]. This process is inefficient and error prone, so that the success rate is rather low. Furthermore, viable offspring often have an unusually large birth weight (“large offspring syndrome”) and severe organ malformations. Nevertheless, the successful generation of cloned animals proves that development does not involve genetic changes, but reversible epigenetic changes.

At a given developmental stage, similar cells have similar epigenotypes and therefore similar phenotypes. However, there are interesting exceptions to this rule. One is position-effect variegation in *Drosophila*, which was described first by Muller [25]. He had observed that flies with a certain inversion on the X chromosome had red-white mosaic eyes, although all cells had the same genotype (Fig. 9.6). The inversion placed the *white* gene next to pericentric heterochromatin. Normally, the *white* gene is expressed in every cell of the adult *Drosophila* eye, resulting in a red eye phenotype. In the mutant flies, the *white* gene is expressed in some cells in the eyes and silenced in others. Silencing is due to spreading of the heterochromatin into the *white* locus. Variegation can be suppressed or enhanced by numerous *trans*-acting mutations. The identification of the mutated genes has led to the discovery of important chromatin-marking proteins. The gene affected in the suppressor mutation Su(var)39, for example, encodes a H3K9 histone methyltransferase. The human ortholog (SUV39) has been mentioned above.

Within a cell, most often both alleles of an autosomal gene are either active or inactive. However, there are several examples where the two alleles of a gene, although identical in sequence, are functionally different. The difference can be parent-of-origin specific (as a result of genomic imprinting) or random (X inactivation and allelic exclusion).

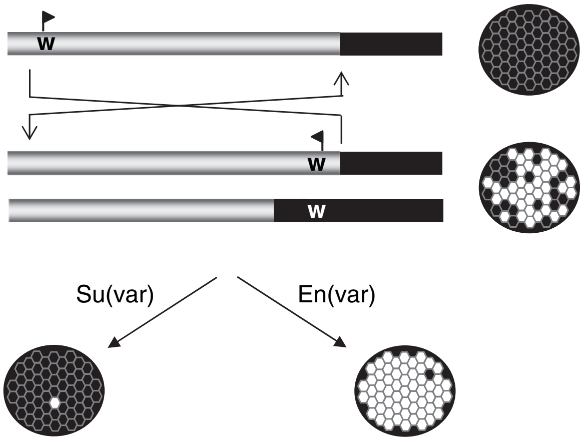

Fig. 9.6 Position effect variegation in *Drosophila*. The diagram shows the *white* gene (*w*) on the X chromosome. Gene transcription is indicated by the *triangular flag*. Pericentromeric heterochromatin is shown in *gray*. A wild-type fly has red eyes (*top right*). A mutant fly has mosaic eyes (*below*). *Trans*-acting mutations can suppress or enhance the spreading of heterochromatin (*bottom left and right*). *Su(var)* suppressor of variegation; *En(var)* enhancer of variegation

9.3.1 Genomic Imprinting

In placental mammals (eutheria) approximately 100 genes are expressed from either the paternal or the maternal allele only. These genes are subject to genomic imprinting, which is an epigenetic process by which the male and the female germline each confer a sex-specific mark (imprint) on certain chromosomal regions. As a consequence, the paternal and the maternal genome are functionally nonequivalent and both are required for normal embryonic development. This was first shown by nuclear transplantation experiments in mice [23,33], but it is also observed in humans. Occasionally, the haploid genome of an unfertilized egg undergoes duplication. Although such an egg has 46 chromosomes, it does not develop into a normal embryo, but into a benign ovarian teratoma, which contains tissues from all three germ layers, but no trophoblast. Similarly, an egg that has been fertilized by two spermatozoa but has lost its maternal genome does not undergo normal development; it develops into a hydatidiform mole, which is degenerated trophoblast tissue.

The nonequivalence of the maternal and paternal genomes is also obvious from uniparental disomies. Uniparental disomy refers to the presence of two copies of a chromosome (or part of a chromosome) from one parent and none from the other [8]. Uniparental disomy per se does not interfere with genomic imprinting or cause clinical problems. However, if the affected chromosome pair carries an imprinted gene, both alleles of this gene will be inactive or active, depending on the parental origin of the chromosomes. The presence of zero or two active copies of an imprinted gene interferes with normal development and growth.

The parental copies of imprinted regions differ with respect to DNA methylation, histone modification and, consequently, gene expression. Despite the identification of parent of origin-specific chromatin differences of imprinted chromatin regions, the nature of the primary imprint is still a matter of debate.

Genomic imprints are erased in primordial germ cells, newly established during later stages of germ cell development, and stably inherited through somatic cell divisions during postzygotic development (Fig. 9.7a).

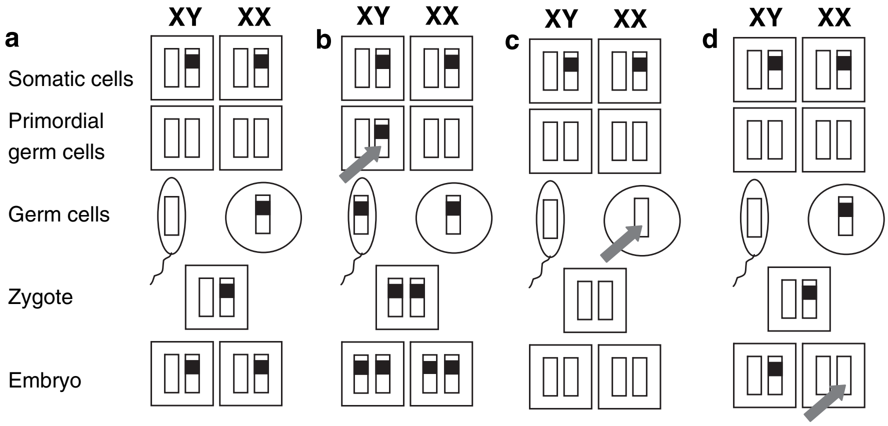

Fig. 9.7 Genomic imprinting. (**a**) Imprints are erased in the primordial germ cells, newly established during gametogenesis and maintained in the zygote and embryo. Imprinting defects can result from an error in imprint erasure (**b**), imprint establishment (**c**) or imprint maintenance (**d**). For clarity, only one pair of homologous chromosomes (*open bars*) and a maternal imprint (*black box*) are shown

They survive the global waves of DNA demethylation and remethylation during early embryonic development (see above), although it is not clear what protects them. In somatic cells, the imprint is read by the transcription machinery and used to regulate parent of origin-specific gene expression so that only the paternal or the maternal allele of a susceptible gene is active.

Many imprinted genes are involved in regulating resource acquisition of the embryo and fetus. In fact, it has been proposed that imprinting co-evolved with the placenta. In eutherian mammals, the fetus grows at the expense of the mother. As proposed by the genetic conflict theory [37], the paternal genome is "interested" in extracting as many resources from the mother as possible. This is because a male can spread his genes through many different females. By contrast, maternally inherited genes protect the mother from being exhausted by the fetus, because a female can spread her genes only through multiple pregnancies.

Imprinted genes are not randomly distributed in the genome, but tend to occur in clusters. In humans, imprinted gene clusters have been found on chromosomes 6, 7, 11, 14, and 15. The clustering of imprinted genes suggests that the primary control of imprinting is not at the single gene level, but at the chromosome domain level. Indeed, several clusters have been found to contain a *cis*-acting imprinting center (IC) which controls imprint establishment and imprint maintenance [4].

The proximal long arm of human chromosome 15 (15q11-q13) contains a cluster of imprinted genes which are affected in the Prader–Willi syndrome (PWS) and the Angelman syndrome (AS; Fig. 9.8, Table 9.1) Paternal-only expression of *MKRN3*, *NDN*, *SNRPN*, and (possibly) *MAGEL2* is associated with differential DNA methylation. Whereas the promoter/exon 1 regions of these genes are unmethylated on the expressing paternal chromosome, the silent maternal alleles are methylated. In addition to DNA methylation, the parental copies of these genes also differ in histone modification.

The *SNRPN* gene encodes two proteins, SNURF and SNRPN, serves as a host for 79 C/D small nucleolar (sno) RNA genes, and overlaps, in an antisense orientation, the *UBE3A* gene. The snoRNAs are encoded within introns of the *SNRPN* gene. They are expressed from the paternal allele only, because they are processed from the paternally expressed *SNRPN* sense/ *UBE3A* antisense transcript during the splice process. Thus, imprinted expression of the snoRNAs is indirectly regulated through *SNRPN* methylation. Unlike other C/D box snoRNAs, the snoRNAs encoded within the *SNRPN* locus do not serve a guide RNAs for 2'-*O*-ribose methylation of nucleotides in rRNA. Their function remains to be determined.

A ~4-Mb de novo interstitial deletion of the paternal chromosome 15 [del(15)(q11-q13)pat], which includes the entire imprinted domain plus several nonimprinted genes, is found in the majority (~70%) of patients with PWS. The second most common genetic abnormality in PWS (~30%) is a maternal uniparental disomy 15 [upd(15)mat], which most often arises from maternal

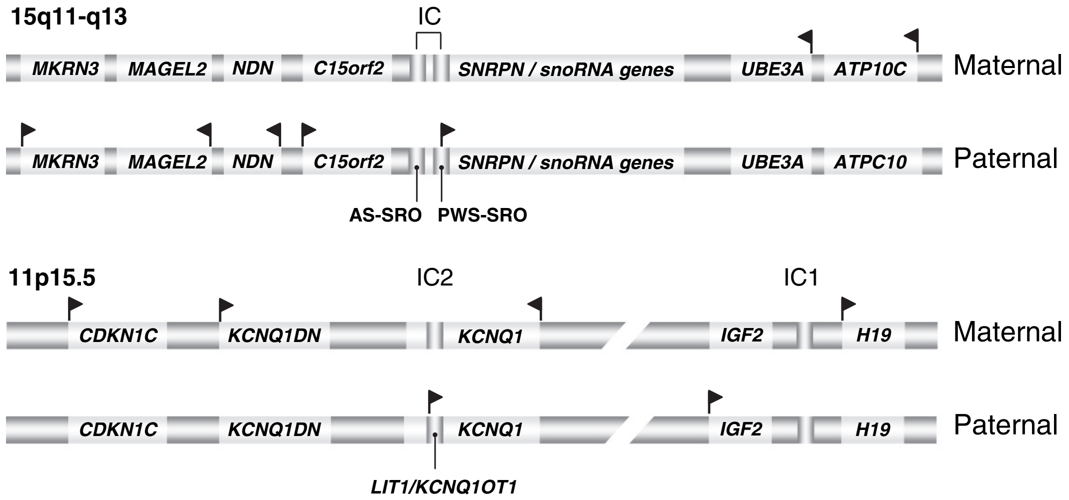

Fig. 9.8 Imprinted chromosome domains. For clarity, the maps (from centromere, *left*, to telomere, *right*) are not drawn to scale, and not all genes are shown. Several genes show imprinted expression in some tissues only. Gene transcription is indicated by *triangular flags* (*IC* imprinting center)

meiotic nondisjunction followed by mitotic loss of the paternal chromosome 15 after fertilization. A few patients with PWS have apparently normal chromosomes 15 of biparental inheritance, but the paternal chromosome carries a maternal imprint (imprinting defect). All three lesions lead to the lack of expression of imprinted genes that are active on the paternal chromosome only. They can easily be detected by DNA methylation analysis. Patients with PWS lack unmethylated alleles of several loci within 15q11-q13.

In contrast to the paternally active genes, the maternally active *UBE3A* gene lacks differential DNA methylation. Another striking difference is that imprinted *UBE3A* expression is tissue-specific. At present it is unclear how tissue-specific imprinting of *UBE3A* is regulated, but the paternally expressed *SNRPN* sense/*UBE3A* antisense transcript may be involved in silencing the paternal *UBE3A* allele.

The loss of function of the *UBE3A* gene leads to AS. Similar to PWS, the major lesion in AS is a common large deletion of 15q11-q13 (~70%), but in AS the deletion is on the maternal chromosome. AS can also result from upd(15)pat (~1% of cases), which most often arises from the postzygotic duplication of a zygote carrying only a paternal chromosome 15, or the lack of a maternal imprint on the maternal chromosome (imprinting defect; ~4% of cases). All three lesions can be detected by DNA methylation analysis. The patients lack a methylated allele of maternally methylated loci within 15q11-q13. A few percent of patients have a mutation in the maternal *UBE3A* gene, and some 10% of patients suspected of having AS have a genetic defect of unknown nature. The latter two classes of patients cannot be detected by methylation analysis.

Imprinting in 15q11-q13 is under the control of a bipartite imprinting center, which overlaps the promoter/exon 1 region of *SNRPN*. The imprinting center (IC) was identified by the mapping of small deletions in patients with an aberrant imprint [4]. An element within the smallest region of deletion overlap in patients with AS (called the AS-SRO) is necessary for establishment of the maternal imprint in the female germline. A deletion of this element prevents maternal imprinting of the mutated chromosome. A child inheriting this chromosome will develop AS, because the maternal *SNRPN* allele is unmethylated and expressed, and the maternal *UBE3A* allele is silenced. Since deletions of the AS-SRO element affect maternal imprinting only, they are silently transmitted through the paternal germline. This explains why in some families only few and distantly related individuals are affected (Fig. 9.9).

An element within the smallest region of deletion overlap in patients with PWS (called the PWS-SRO) is necessary for the postzygotic maintenance of the paternal imprint. A paternally derived deletion of this element leads to an epigenetic state in 15q11-q13 that

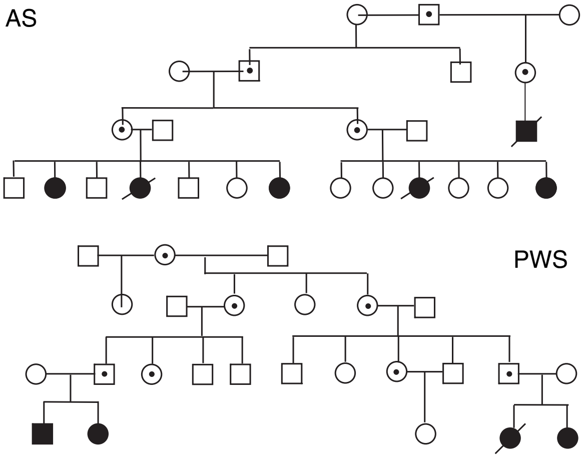

Fig. 9.9 Segregation of imprinting center deletions. Unaffected carriers are indicated by a *dot*. Note that a deletion of the AS-SRO element of the 15q IC in the Angelman syndrome family (*top*) is benign when transmitted through a male. In the female germline it prevents the establishment of the maternal imprint. A deletion of the PWS-SRO of the 15q IC in the Prader–Willi syndrome family (*bottom*) is benign when transmitted through a female. After transmission through a male, the deletion chromosome acquires a maternal methylation pattern during early embryogenesis

resembles the maternal imprint. A child with such a chromosome will develop PWS, because all paternally expressed genes are silent. Since deletions of the PWS-SRO element affect the paternal imprint only, they are silently transmitted through the maternal germline (Fig. 9.9).

Another cluster of imprinted genes with relevance to human disease is located on 11p15.5 (Fig. 9.8). It is affected in patients with Beckwith–Wiedemann syndrome (BWS) and some patients with Silver-Russell syndrome (SRS) (Table 9.1). BWS is caused by overexpression of the paternally active *IGF2* gene and silencing of the maternally expressed *H19* gene or by silencing or mutational inactivation of the maternally active *CDKN1C* gene. These genes map to the short arm of chromosome 11, but are controlled by two different ICs, *IGF2/H19* IC (IC1), and *LIT1/KCNQ1OT1* (IC2), which controls imprinting of *CDKN1C*.

IC1 contains several binding sites for the CCCTC-binding factor (CTCF), which is a multifunctional protein [15]. As a consequence of genomic imprinting, the binding sites on the paternal chromosome (as well as the *H19* promoter) are methylated, while they are unmethylated on the maternal chromosome. Binding of CTCF to the unmethylated IC1 on the maternal chromosome isolates the *IGF2* gene from two enhancers, which it shares with *H19* (Fig. 9.10 a). As a consequence, *IGF2* is silent, whereas *H19* is active. CTCF cannot bind to the methylated IC1 on the paternal chromosome. Therefore, the paternal *IGF2* allele is active, whereas the maternal *H19* allele is inactive.

The function of the IC2 is rather different. It is located within the *KCNQ1* gene and serves as a promoter for an antisense gene (*LIT1/KCNQ1OT1*). The IC2 is methylated on the maternal chromosome and unmethylated on the paternal chromosome. As a consequence, *LIT1/KCNQ1OT1* is expressed only from the paternal chromosome. By an unknown mechanism, *LIT1/KCNQ1OT1* transcription silences *cis* expression of *CDKN1C*.

9.3.2 X Inactivation

In female mammals, one X chromosome is inactivated to ensure that levels of X-linked genes are equal between XY males and XX females. This form of dosage compensation takes place during early development and, once established, is maintained through many cell divisions. In rodent preembryos, X inactivation is initially imprinted, in that the paternal X (X_p) is inactivated. In the inner cell mass of the blastocyst, X_p is reactivated, and then each cell makes a random choice with regard to X inactivation. In extraembryonic tissues, X_p remains inactivated. X inactivation is initiated at a master control locus in Xq13, the X inactivation center (Xic). The Xic is also involved in "counting," by which the cell recognizes whether it has one, two, or more X chromosomes, and "choice," by which the cell determines which X will remain active. Any supernumerary X chromosome will be inactivated.

The Xic harbors the *Xist* gene (X-inactive specific transcript), which is required for the initiation of X inactivation [3]. The gene encodes a 17-kb untranslated RNA. The RNA is transcribed from and coats the X chromosome that has been selected to become inactivated. Within one or two cell cycles gene silencing occurs along the length of this chromosome. The association of *Xist* RNA with chromatin is mediated by sequences that are functionally redundant and dispersed throughout the transcript. The silencing requires

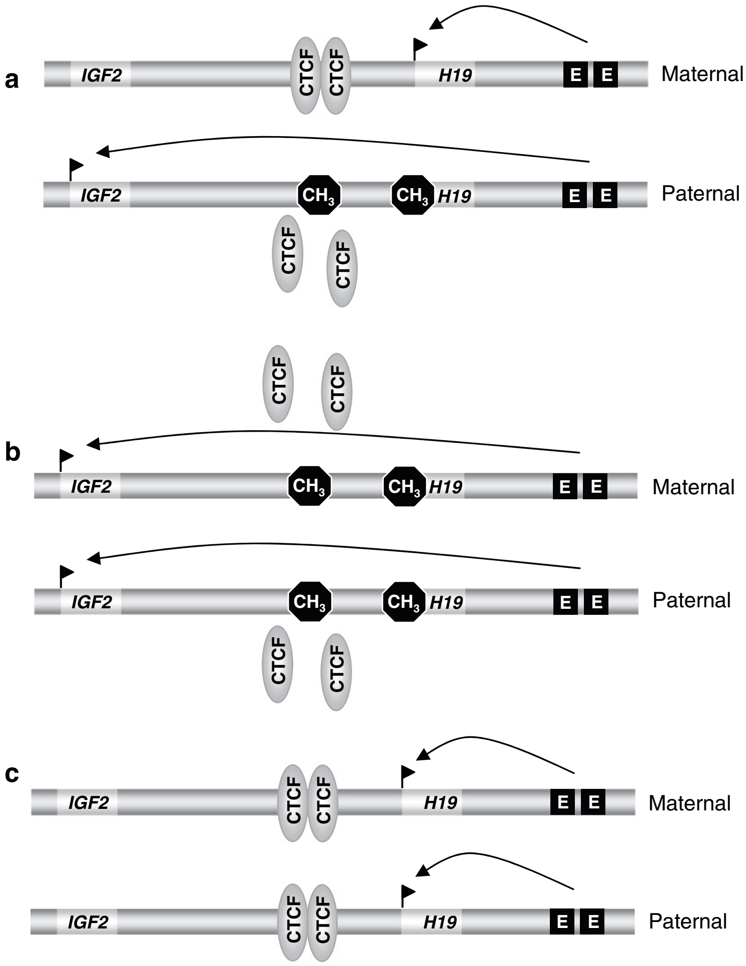

Fig. 9.10 Enhancer competition model. *IGF2* and *H19* share two enhancers (*E*) telomeric to *H19*. Transcription is indicated by the *triangular flags*. (**a**) Normal situation; (**b**) imprinting defect (aberrant methylation of the maternal allele) leading to Beckwith-Wiedemann syndrome; (**c**) imprinting defect (lack of paternal methylation) leading to Silver-Russell syndrome. For details see text

a conserved region at the 5' end. After the initiation of silencing, *Xist* becomes dispensable.

The X-chromosomal genes are inactivated by extensive histone modifications: hypoacetylation of H3 and H4, dimethylation of H3K9, trimethylation of H3K27, and hypomethylation of H3K9. Polycomb group proteins and associated chromatin-modifying enzymes (Eed and Enx1, the latter being a H3K9 and K27 HMT) are mediators of the transition from the reversible inactive, Xist-dependent phase of inactivation, to the irreversible, Xist-independent phase. Later, the incorporation of the histone variant macroH2A and DNA methylation play an important part in maintaining the inactive state.

Although X inactivation in humans is random, not all females have an equal proportion of cells in which X_p and X_m, respectively, are inactivated. There are at least two reasons for this observation. First, the choice appears to be influenced by genetic variation with the Xic, resulting in the preferential inactivation of a certain X chromosome. In the extreme, X inactivation may be heavily skewed. X Inactivation skewing can lead to phenotypic expression of an X-linked recessive trait in a female if the mutation is on the active X chromosome.

Second, if an X chromosome carries a mutation that confers some disadvantage on cell proliferation, cells that have chosen this chromosome to be active will be selected against during the growth or adult life of the female. In fact, it has been observed that the skewing of X inactivation can increase during the life of a woman.

Although X inactivation is rather stable, the chromatin marks are in principle reversible and can be lost during aging. In fact, there is ample evidence for this. Reactivation of the inactive X during aging can have deleterious effects if the reactivated gene carries a mutation.

9.3.3 Allelic Exclusion in the Olfactory System

Although there are more than 1,000 odorant receptor (*OR*) genes in the genome, they are expressed in a mutually exclusive and monoallelic manner in olfactory sensory neurons. The one neuron-one receptor rule is essential for the brain to determine which odorant is present in the environment. Similar to the expression of antigen receptor genes in lymphocytes, DNA rearrangements have long been regarded as a possible mechanism for the allelic exclusion of the *OR* genes. However, mice cloned from the nuclei of mature olfactory sensory neurons expressed the full repertoire of ORs [7,21]. These experiments suggest that *OR* choice and maintenance are epigenetic.

According to a current model, the *OR* genes are initially silent, but in a semipermissive state [31]. By a stochastic and presumably inefficient process, on average a single *OR* allele is activated per cell. At this stage the choice is unstable, but a functional OR protein may mediate a feedback stabilization that commits the cells to the receptor. This commitment involves full activation of the selected *OR* gene by changing its chromatin state from semipermissive to permissive, and repression of all the other *OR* genes by changing their chromatin state from semipermissive to repressive.

It is unclear why *OR* gene expression must be monoallelic. It is possible that this pattern of expression is a consequence rather than a requirement of singular gene choice. A stochastic choice process as described above is more likely to select individual alleles than two copies of the same gene. However, it is also possible that monoallelism is important for some biological process unrelated to *OR* choice.

9.4 Epigenetic Variation and Disease

Epigenetic states are set by developmental processes, but subject to natural variation. Epigenetic variation can result from genetic variation, environmental variation, and errors of the epigenetic machinery. In most cases, all three sources of epigenetic variation play a part, although the relative contribution of each source may vary. It should also be noted that genetic variation, environmental variation, and errors of the epigenetic machinery are not independent of each other. Genetic variants of chromatin-marking proteins, for example, can be associated with an increased error rate of the epigenetic machinery, and the error rate may further be affected by environmental variation.

Normal epigenetic variation is likely to contribute to the phenotypic range of normal individuals. Rare aberrant epigenetic states are called epimutations and have an important role in several diseases [17].

In some cases there is a one-to-one correspondence between genotype and epigenotype (obligatory epigenetic variation) [30] (Fig. 9.11 a). This is seen in certain diseases where a *cis*-acting DNA mutation always leads

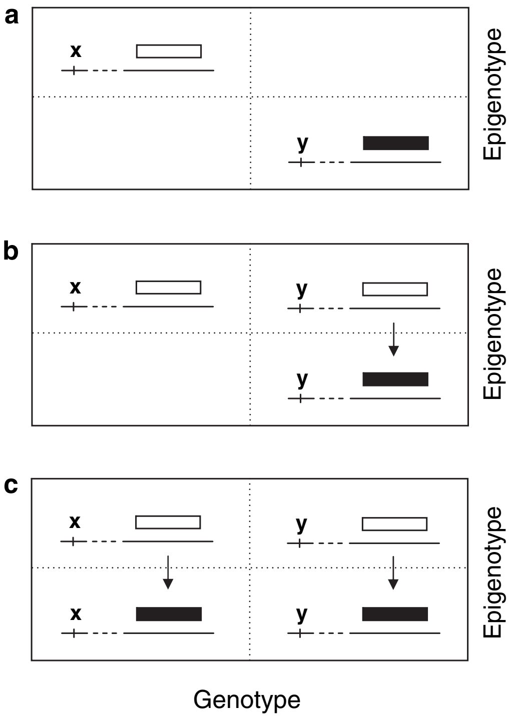

Fig. 9.11 (**a–c**) Three classes of epigenetic variation. The range of autonomy of epigenetic variation in relationship to genotypic context is expressed in three genotype x epigenotype matrices. The *horizontal axis* distinguishes between two genotypes that are represented by alleles *x* and *y*. At a genomic location either in *cis* or in *trans* (*dashed line*), two alternative epigenetic states are depicted as either *open* or *filled boxes*. A In this scenario, the epigenotype of the locus is strictly determined by genotype; so, the epigenotype is an obligatory phenotype of the alternative genotypes. (**b**) The matrix illustrates facilitated epigenetic variation (epiallele formation: *open box–filled box*) that can occur, in a probabilistic manner, only in the context of genotype y. (**c**) In this matrix, stochastic events generate alternative epialleles at some finite frequency regardless of the genotype. (Reprinted from [30], by permission from Macmillan Publishers Ltd: *Nature Reviews Genetics,* copyright 2006)

to an aberrant epigenetic state (secondary epimutation). In other cases, the epigenotype is completely independent of the genotype (pure epigenetic variation; Fig. 9.11 c). This is seen in diseases where an epimutation has occurred without any change in the DNA sequence (primary epimutation), although certain genotypes may increase the risk of such a primary epimutation (facilitated epigenetic variation; Fig. 9.11 b). In practice, it is often impossible to distinguish between pure and facilitated epigenetic variation, because the underlying genotypes are not completely known.

9.4.1 Obligatory Epigenetic Variation

There are at least two monogenic disorders in which an epimutation represents the major pathogenetic mechanism. These are fragile X mental retardation syndrome (FMR1) and facioscapulohumeral muscular dystrophy (FSHD) (Table 9.1). FMR1 is an X-linked dominant disease caused by the expansion of an unstable trinucleotide repeat (CGG) within exon 1 of the *FMR1* gene. It is one of the most common causes of mental retardation. The number of repeats varies in the human population. Repeats with more than 58 copies are unstable and can expand to several hundred copies during proliferation of the diploid oogonia in the fetal ovary. After fertilization of an oocyte carrying an expanded *FMR1* allele, the CGG repeat and *FMR1* promoter are methylated. DNA methylation, histone deacetylation, and the establishment of repressive chromatin in this region silence the *FMR1* gene.

FSHD is an autosomal dominant disorder that has been linked to a 3.3-kb, -tandemly repeated sequence (*D4Z4*) in the subtelomeric region of the long arm of chromosome 4. In normal individuals the number of *D4Z4* repeats varies between 11 and 150 units, whereas FSHD patients have fewer than 11 repeats. It has been proposed that deletion of *D4Z4* is associated with an open chromatin structure in 4q35 and the inappropriate expression of several genes within this region, but the mechanism is still not clear.

Imprinting defects resulting from the deletion of a *cis*-acting imprinting center (IC) are another example of an epimutation resulting from a DNA sequence change. Deletions of the 15q IC impair the imprint in the Prader–Willi/Angelman syndrome region (see Sect. 9.3.1). Approximately 50% of these deletions are familial mutations. Two families are shown in Fig. 9.9. IC deletions have also been described in rare patients with BWS.

A unique epimutation affecting the α-globin gene *HBA2* has been described by Tufarelli et al. [34]. The authors studied an individual with an inherited form of α-thalassemia who has a deletion that results in a truncated, widely expressed gene (*LUC7L*) becoming juxtaposed to the structurally normal α-globin gene *HBA2*. Although it retains all of its local and remote *cis*-regulatory elements, expression of *HBA2* is silenced. *LUC7L* is transcribed from the opposite strand to the α-globin genes. In the patient, RNA transcripts from the truncated copy of *LUC7L* (missing the last three exons) extend into the *HBA2* CpG island, thus generating an antisense transcript with respect to *HBA2*. Antisense RNA transcription appears to mediate methylation of the *HBA2* CpG island during early development and silencing of *HBA2* expression. There are also several examples of chromosomal translocations affecting the epigenetic state of genes adjacent to the breakpoints. This is the case in particular in translocations involving the X chromosome. A very instructive case was published by Jones et al. [20]. The authors studied a male patient with an unbalanced X;13 translocation [46,XY,+der(X;13)(q10;q10),-13] and bilateral retinoblastoma. The patient has an extra copy of Xq, but no signs of Klinefelter syndrome. DNA replication and methylation studies suggested that the extra copy of Xq, which is attached to the long arm of one chromosome 13, was inactivated and that inactivation had spread to chromosome 13 and silenced the *RB1* gene in 13q14. This epimutation is equivalent to a constitutional *RB1* mutation and explains the development of bilateral tumors in this patient.

9.4.2 Pure Epigenetic Variation

As monozygotic twins are assumed to have the same genomic DNA sequence, any phenotypic difference should be attributable to environmental or epigenetic variation. Preliminary studies on global and locus-specific DNA methylation and histone methylation in monozygotic twins suggest that the patterns are highly similar at birth, but less similar in older twins [11]. The differences may be the result of environmental variation and/or random errors of the epigenetic machinery, and these differences appear to accumulate with age. At present it is not clear to what extent epigenetic differences in monozygotic twins explain discordance in disease phenotypes, although several examples are known. DNA methylation analysis of the *AXIN1* locus in monozygotic twins discordant for a caudal duplication anomaly revealed increased methylation in the affected twin [27]. Another example is the Beckwith–Wiedemann syndrome. Interestingly, there is an excess of monozygotic twins among patients with BWS, and almost all of them are female and discordant for the disease. All of the patients with a healthy twin sib are hypomethylated at the maternal *LIT1/KCNQ1OT1* locus. This epigenetic difference is most probably due to a failure of imprint maintenance in a cell of the early embryo. As a consequence, *LIT1/KCNQ1OT1* is expressed from both chromosomes and both alleles of *CDKN1C* are silenced. This defect may then increase the risk of twinning. As suggested by Weksberg et al. [36], it is possible that a group of cells carrying a postzygotic *LIT1/KCNQ1OT1* imprint maintenance defect could preferentially increase the proliferation rate of this group of cells beyond that of normal cells, thereby generating asymmetry of the entire cell mass and increasing the chance of separation of the epigenetically distinct cell clones.

BWS can also be caused by an imprinting defect affecting the *IGF2/H19* IC (Fig. 9.10 b). Methylation of the maternal *CTCF* binding sites and *H19* promoter leads to activation of the maternal *IGF2* allele, two doses of IGF2, and overgrowth. Loss of methylation on the paternal chromosome allows binding of CTCF to the IC. As a consequence, the paternal *IGF2* allele is isolated from the enhancers and silenced (Fig. 9.10 c). Lack of IGF2 results in pre- and postnatal growth retardation and is one cause of Silver-Russell syndrome (Table 9.1).

In genetic syndromes involving imprinted genes the majority of imprinting defects are primary epimutations. They cannot only occur during imprinting maintenance, but can also result from an error of imprint erasure in primordial germ cells or imprint establishment during later stages of gametogenesis. In PWS patients with an imprinting defect not caused by an IC mutation the affected chromosome is always derived from the paternal grandmother [5]. This finding suggests that the (grand)maternal imprint has not been erased in the paternal germline (Fig. 9.7b). In contrast to PWS, imprinting defects in AS result from an error in imprint establishment or an error in postzygotic imprint maintenance (Fig. 9.7c, d). At least 30% of AS patients with a primary imprinting defect are somatic mosaics, and there appears to be some correlation between the percentage of abnormally imprinted cells and the severity of the disease. It is likely that the role of mosaic imprinting defects on chromosome 15 in mental retardation is severely underestimated.

Primary epimutations also have an important role in cancer. In 1983 Feinberg and Vogelstein discovered altered DNA methylation in cancer cells [9]. Subsequently, these authors and others demonstrated that hypomethylation can lead to inappropriate activation of oncogenes. In 1986 Baylin et al. identified hypermethylation of the calcitonin gene in human lung cancers and lymphomas [1], but the role of these changes in tumor development was unknown. Soon after the discovery of the first tumor suppressor gene (the retinoblastoma gene *RB1*), it was found that the *RB1* promoter is methylated in a subset of retinoblastomas [14], suggesting that tumor-suppressor silencing can also occur by way of an epigenetic pathway. Methylation of tumor-suppressor genes has now been found in virtually all tumors, and the field of cancer epigenetics is growing rapidly.

In general, tumor-predisposing epimutations are found only in premalignant or malignant cells. In some instances, however, the epimutation has also been detected in normal somatic cells. Some individuals have constitutional loss of imprinting at the *IGF2* locus [6]. These individuals appear to be at an increased risk for colon cancer.

Primary epimutations also appear to play a part in cardiovascular disease. In a similar way to tumors, atherosclerotic lesions are characterized by global DNA hypomethylation and local DNA hypermethylation. These similarities should not surprise us, because a key step in the atherogenetic process is the proliferation and migration of smooth muscle cells. Once within the intima, the phenotype of the smooth muscle cells switches from contractile to "dedifferentiated." It has been suggested that methylation of estrogen receptor-α gene (*ESR1*) could contribute to these processes [39].

Whereas secondary epimutations result from DNA mutations, it is less clear what triggers primary epimutations. Primary epimutations probably represent errors in the establishment or maintenance of an epigenetic state. An interesting model for tumor-suppressor methylation has been proposed by Clark and colleagues [32]. According to this model, a combination of transient gene silencing and methylation seeding leads to recruitment of the methyl-CpG-binding protein MBD2, histone deacetylase, and DNA methyltransferase. This then leads to the spreading of DNA and histone methylation, and consequently to the establishment of silent chromatin.

The spontaneous epimutation rate can be modified by environmental variation. Cellular levels of the methyl donor *S*-adenosyl-methionine, for example, depend—to a certain degree—on the levels of folic acid, choline, and vitamin B taken up from nutrition. Changes in DNA methylation by folate levels have been observed in various types of cancers and also in animal models. After folate washout in patients with hyperhomocysteinemia, Ingrosso et al. observed biallelic expression of *H19* [18], which normally is expressed from the maternal allele. After folate treatment, they observed a shift back to monoallelic expression.

Since patients with a primary epimutation do not have a DNA sequence mutation, the epimutation should in principle be reversible. As mentioned in the paragraph on chromatin-marking, 5-aza-cytidin and trichostatin A can be used to change DNA methylation and histone acetylation patterns, respectively. Other drugs are being developed to modify histone methylation and phosphorylation patterns. Although these drugs are not locus specific, they appear to have some beneficial effect on cancer development, and clinical trials with these drugs are under way.

9.4.3 Facilitated Epigenetic Variation

Obligatory and pure epigenetic variation are the two extremes of the relation between genotype and epigenotype. In most instances, the genotype has some effect on the epigenotype. For example, certain DNA sequence variants appear to be more susceptible to epimutations than others. There is tentative evidence for a genetic predisposition to epimutations in the Beckwith–Wiedemann syndrome region [26]. Four single nucleotide polymorphisms (SNPs) in a differentially methylation region of the *IGF2* gene occur in 3 out of 16 possible haplotypes: The frequency of one haplotype was significantly higher and that of another haplotype significantly lower in BWS patients than in controls.

Similarly, there appears to be a certain haplotype of the 15q IC that increases the risk of a sporadic imprinting defect leading to Angelman syndrome [40]. The increased risk could be attributed to the alleles of two polymorphisms. It is likely that the IC contains a binding site for a *trans*-acting factor involved in establishing the maternal imprint and that this factor binds with different efficiency to the different alleles. This may increase the risk that the maternal imprint is not, or not completely, established. Genetic variation may also influence epigenetic states in trans, e.g., by affecting proteins that are directly or indirectly involved in the establishment or maintenance of chromatin marks. 5,10-Methylenetetrahydrofolate reductase (MTHFR) is a key enzyme of the one-carbon atom metabolism, which provides DNA and HMTs with the methyl-group donor *S*-adenosyl-methionine. A frequent variant of the *MTHFR* gene (677C>T) encodes a thermolabile protein with reduced enzymatic activity. Homozygosity for the 677C>T variant is associated with reduced global DNA methylation, but only in the presence of low folate levels [12]. This is a good example of the interaction between genetic variation and environmental variation in affecting epigenetic variation. The interaction appears to lead to a shortage of intracellular levels of *S*-adenosyl-methionine, so that postreplicative, hemimethylated DNA is inefficiently methylated by the maintenance methyltransferase DNMT1. As a consequence, methylation is lost during successive rounds of DNA replication. A shortage of *S*-adenosyl-methionine also appears to affect the establishment of the maternal methylation imprint by the de novo methyltransferases DNMT3A and DNMT3B, because mothers who are homozygous for the *MTHFR* 677C>T variant are at increased risk of conceiving a child with an imprinting defect on chromosome 15, which leads to Angelman syndrome [40].

9.5 Transgenerational Epigenetic Inheritance and Evolution

While epigenetic variation in somatic cells has been well documented, there are few data available on epigenetic variation in the human germline. A study on human sperm revealed DNA methylation differences between and within the germlines of normal males [10]. The largest degree of variation was detected within the CpG islands and pericentromeric satellite DNA. It is not clear, however, whether the observed patterns can be transmitted across generations. The inheritance of epigenetic states through mitotic cell divisions of an organism is relatively faithful, but major reprogramming events during gametogenesis and early embryogenesis usually prevent the inheritance of epigenetic states between generations. However, there appear to be exceptions to this rule. At least in plants, epigenetic states have been shown to be transmitted unchanged through the germline. A good example is that of paramutations, which were first described in maize. Paramutations result from an interaction between the two alleles of a gene: at loci susceptible to paramutations a silent allele can silence its active homolog. Paramutations do not involve a change in DNA sequence, but a change in chromatin structure, which is meiotically stable. Phenotypes that are subject to paramutations show deviations from Mendelian inheritance.

Evidence for transgenerational inheritance in mammals is scanty. The best-studied examples are two mouse lines carrying a retrotransposon upstream of a gene, the *agouti viable yellow* (A^{vy}) [24] and the *axin-fused* ($Axin^{Fu}$) mice [29]. In both cases, the transcriptional activity is under the control of a promoter within the retrotransposon. In A^{vy} mice, retrotransposon driven ectopic expression of the *agouti* gene leads to yellow coat color, obesity, diabetes, and increased tumor risk. Methylation of the promoter prevents ectopic gene expression and results in pseudo-agouti coat color. In addition to yellow and pseudo-agouti mice, mottled mice are observed. These mice are somatic mosaics for ectopic *agouti* expression. The phenotype of the mice can be modified by maternal nutrition during pregnancy, specifically by folate supplements. The A^{vy} mice show that genetically identical organisms can have different phenotypes. Furthermore, the phenotypes can be passed on to subsequent generations, although the penetrance is incomplete. For example, yellow dams have more yellow offspring than pseudo-agouti dams have. This suggests that the epigenetic state is not always cleared between generations. Surprisingly, however, the retrotransposon is demethylated during early embryogenesis. Thus, DNA methylation cannot be the inherited mark, and other possibilities, such as histone modifications, have to be considered.

The A^{vy} and $Axin^{Fu}$ mice are rare variants involving a retrotransposon, and it is not clear whether epigenetic states of normal mammalian genes can be transmitted through the germline. In humans there is no direct evidence for transgenerational epigenetic inheritance, although epidemiological studies suggest that it might occur. One such study found that paternal grandfathers' food supply during their prepubertal slow growth phase was linked to the mortality risk ratio of their grandsons [28]. Indirect evidence comes from the study of patients with PWS and an imprinting defect not caused by an IC mutation. As mentioned above, the affected chromosome is always derived from the paternal grandmother, suggesting that the (grand)maternal imprint was not erased in the paternal germline and is transmitted to the child [5].

A few multigenerational families with an epimutation have been described in the literature, but it is not possible in these cases to distinguish between transmission of an epimutation and transmission of a genetic variant predisposing to an epimutation in each generation.

Despite the lack of direct evidence for transgenerational epigenetic inheritance in mammals, especially in humans, it is of great interest for the theory of evolution. Germline transmission of epigenetic states would allow for the inheritance of acquired traits and thus provide a basis for adaptive evolution [19]. Because of random or environmentally induced epigenetic variation, new epigenetic variants appear in the population. If they are transmitted through the germline and are selectively favorable, they will accumulate in the population and allow the genotypes to occupy a new adaptive zone while maintaining the old one. Under continued selection pressure, the favorable epigenetic variants will be fixed by genetic changes, which can be transmitted to the offspring in a more stable way. Genetic fixation of epigenetic changes may also underlie speciation.

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Human Gene Mutation: Mechanisms and Consequences

10

Stylianos E. Antonarakis and David N. Cooper

Abstract A wide variety of different types of pathogenic mutation occur in the human genome, with many diverse mechanisms responsible for their generation. These types of mutation include single base-pair substitutions in coding, regulatory and splicing-relevant regions of human genes, and also micro-deletions, micro-insertions, duplications, repeat expansions, combined micro-insertions/deletions ("indels"), inversions, gross deletions and insertions, and complex rearrangements. A major goal of molecular genetic medicine is to be able to predict the nature of the clinical phenotype through ascertainment of the genotype. However, the extent to which this is feasible in medical genetics is very much disease, gene, and mutation dependent. The study of mutations in human genes is nevertheless of paramount importance for our understanding of the pathophysiology of inherited disorders and for optimizing diagnostic testing, as well as in guiding the design of new therapeutic approaches.

Contents

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10.1 Introduction

A wide variety of different types of pathogenic mutation occur in the human genome, with many diverse mechanisms being responsible for their generation. These types of mutation include single base-pair substitutions in coding, regulatory and splicing-relevant regions of human genes, and also micro-deletions, micro-insertions, duplications, repeat expansions, combined micro-insertions/deletions ("indels"), inversions, gross deletions and insertions, and complex rearrangements. A major goal of molecular genetic medicine is to be able to predict the nature of the clinical phenotype through ascertainment of the genotype. However, the extent to which this is feasible in medical genetics is very much disease, gene, and mutation dependent. The study of mutations in human genes is nevertheless of paramount importance for our understanding of the pathophysiology of inherited disorders and for optimizing diagnostic testing, as well as in guiding the design of new therapeutic approaches.

The first description of the exact molecular defect in a human disease (sickle cell mutation, a substitution

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from Glu to Val at the 6th codon of the β-globin gene) was identified by Ingram in 1956, who found that the difference between hemoglobin A and hemoglobin S lies in a single tryptic peptide (158). His analysis was made possible by methods developed by Sanger for determining the structure of insulin and Edman's stepwise degradation of peptides. Since then, continuous advances have potentiated the identification of numerous disease-related genes and the discovery of thousands of underlying pathologic lesions. Single base-pair substitutions (68%) and micro-deletions (16.4%) are the most frequently encountered mutations in the human genome, the remainder comprising an assortment of micro-insertions (6.6%), indels (1.5%), gross deletions (5.6%), gross insertions and duplications (1.0%), inversions, repeat expansions (0.23%), and complex rearrangements (0.8%). Characterized mutations occur not only in coding sequences, but also in promoter regions, splice junctions, introns and untranslated regions, and any other functional region of the genome. Mutations can interfere with any stage in the pathway of expression from gene activation to synthesis and secretion of the mature protein product. This chapter attempts to provide an overview of the nature of mutations causing human genetic disease and then considers their consequences for the clinical phenotype. The interested reader is also referred to the third edition of this work for an in-depth discussion of mutation rates and factors influencing the generation of mutations. Two online databases contain information on disease-related (pathogenic) mutations: *Mendelian Inheritance in Man* (http://www.ncbi.nlm.nih.gov/Omim/) and the *Human Gene Mutation Database* (http://www.hgmd.org).

10.2 Neutral Variation/DNA Polymorphisms

The term *polymorphism* has been defined (336) as a "Mendelian trait that exists in the population in at least two phenotypes, neither of which occurs at a frequency of less than 1%." Polymorphisms are not rare. Indeed, there is enormous variation in the DNA sequences of any two randomly chosen human haploid genomes. Clearly, not all variations within a gene result in the abnormal expression of protein products. Indeed, single nucleotide substitutions/polymorphisms (SNP) occur in 1/~600–1,200 nucleotides in intervening sequences and flanking DNA (13, 75, 103, 244, 285, 342). These substitutions represent the most common forms of DNA polymorphism that can be used as markers for specific regions of the human genome. Similarly, some single nucleotide substitutions in the coding regions of genes may also be normal (nonpathogenic) polymorphic variants even if they result in nonsynonymous substitutions of the polypeptide product (247). For example, there are three common forms of the β-globin (*HBB*) gene on chromosome 11p. These forms differ at five nucleotides, one of which lies within the first exon of the gene and results in a synonymous codon. The average human gene contains >120 biallelic polymorphisms, 46 of which occur with a frequency >5% and 5 within the coding region (78).

Some polymorphisms entail the alteration of an encoded amino acid, e.g., the Lewis *Le* alleles of the *FUT3* gene (245), whereas others may introduce a stop codon that serves to inactivate the gene in question, e.g., the secretor *se* allele of the *FUT2* gene present in 20% of the population (176). However, not all polymorphisms are SNPs. Examples of other types of gene-associated polymorphism in the human genome include triplet repeat copy number (e.g., in the *FMR1* gene; see Sect. 9.2.1.3), gross gene deletion (e.g., *GSTM1* and *GSTT1*) (273), gene duplication (e.g., *HBG2*) (318) intragenic duplication (e.g., *IVL*) (132), micro-insertion/deletion (e.g., *PAI1*) (84), indel (e.g., *APOE*) (120), gross insertion (e.g., the inserted *Alu* sequence in intron 16 of the *ACE* gene) (277), inversion (e.g., the 48 kb Xq28 inversion involving the *EMD* and *FLN1* genes) (307)) and gene fusion (e.g., between the *RCP* and *GCP* visual pigment genes) (241)). It can be seen that the mutational spectrum of polymorphisms in the human genome is qualitatively different to that underlying human disease; they may vary in terms of location and frequency but otherwise they display remarkable similarities indicative of the same underlying mutational mechanisms.

It is likely, however, that some SNPs, both frequent and rare, alter the risk for common complex human phenotypes. A public SNP database now contains more than 10 million entries (dbSNP; http://www.ncbi.nlm.nih.gov/SNP/index.html). An international project has recently been completed, termed the "HapMap project" (8, 68, 144), the goal of which was to define the patterns of common SNP genetic variation in a sample of 270 DNAs from individuals of European, African, Chinese, and Japanese origin (http://www.hapmap.org/). The data obtained from this project constitute approximately 2.8 million SNPs and are publicly available. The results of this project are likely to contribute significantly to our understanding of both common and rare human genetic disorders and traits.

Another form of polymorphic variation in our genome is the presence of variable numbers of tandem repeats (VNTRs). The repeat unit can be 10–60 nucleotides in length and many different alleles may exist at a given locus (164, 358). The combination of a VNTR and single nucleotide substitutions within the repeat unit results in an extremely high level of polymorphic variability that can be used as a unique bar-code to distinguish different individuals (165). The introduction of the polymerase chain reaction (PCR) (286) permitted the rapid detection and analysis of variation in short sequence repeats (SSR), e.g., $(GT)_n$ repeats (212, 347) . These are common polymorphisms that occur on average once every 50 kilobases (kb) of genomic DNA. The SSRs also display many alleles and the repeat unit can be two, three, four, five or more nucleotides. Poly(A) tracts may also be polymorphic, exhibiting variation in the number of A residues (101); many of these polymorphisms are localized at the ends of *Alu* repetitive elements. Another kind of polymorphism in the human genome involves the presence or absence of retrotransposons (i.e., *Alu* or LINE repetitive elements or pseudogenes) at specific locations (10, 71) . Furthermore, duplicational polymorphisms have also been reported for some human genes, e.g., *HBA1*, *PRB1-4*, *HBZ*, *CYP21/C4A/C4B* (39, 71).

The use of comparative genomic hybridization against BAC or oligonucleotide arrays has revealed extensive copy number polymorphisms/variation of sizable genomic regions (CNP or CNV) (156, 294, 300). Details of more than 1,400 such genomic variants may be found in the following databases: *Human Structural Variation Database*, http://paralogy.gs.washington.edu/structuralvariation; *Database of Genomic Variants*, http://projects.tcag.ca/variation. A CNV map of the human genome of the 270 "HapMap" individuals has revealed a total of 1,440 CNV such regions which cover some 360 megabases (12% of the genome) (274). The functional significance, if any, of the majority of these polymorphic variants is however unknown. Copy number variants may predispose to phenotypic variability. For example, it has recently been observed that copy number variation of the orthologous rat and human *Fcgr3/FCGR3B* genes is a determinant of susceptibility to immunologically mediated glomerulonephritis (5). Copy number variants in the *CCL3L1* and *DEFB4* genes have also been found to be associated with increased susceptibility to HIV infection and Crohn's disease, respectively (111, 126).

Deletional polymorphisms are also remarkably frequent in the human genome: a typical individual has been estimated to be hemizygous for some 30–50 deletions >5 kb, spanning >550 kb in total and encompassing >250 known or predicted genes (67, 222). Since such deletions appear to be in linkage disequilibrium with neighboring SNPs, we may surmise that they share a common evolutionary history (145).

Human DNA polymorphisms have proven extremely useful in developing linkage maps, for mapping monogenic and polygenic complex disorders, for determining the origin of aneuploidies and chromosomal abnormalities, for distinguishing normal from mutant chromosomes in genetic diagnoses, for performing forensic, paternity, and transplantation studies, for studying the evolution of the genome, the loss of heterozygosity in certain malignancies, the detection of uniparental disomy, the instability of the genome in certain tumors, recombination at the level of the genome, the study of allelic expression imbalance, and the development of haplotype maps of the genome. However, in studying the role of a candidate gene in a given disorder, it is imperative to distinguish between pathogenic mutations that cause a clinical phenotype and the polymorphic variability of the normal genome.

10.3 Disease-Causing Mutations

10.3.1 The Nature of Mutation

Figure 10.1a depicts the frequencies of the various mutation types responsible for molecularly characterized human genetic disorders, as recorded in the *Human Gene Mutation Database* (HGMD) (http://www.hgmd.org) and elsewhere (196, 312)). HGMD records each mutation *once* regardless of the number of independent occurrences of that lesion. Figure 10.1b shows the frequency of the first mutation per disease recorded in *Mendelian Inheritance in Man* (MIM) (http://www.ncbi.nlm.nih.gov/Omim) and by Antonarakis and McKusick (11). As of March 31, 2009, HGMD contained some 88,317 different mutations in 3,337 human genes, whereas MIM contained examples of allelic variants in 2,514 human genes.

10.3.1.1 Nucleotide Substitutions

Single nucleotide substitutions are the most frequent pathologic mutations in the human genome (Fig. 10.1). Most of these alterations occur during DNA replication, which is an accurate, yet error-prone, multistep process. The accuracy of DNA replication depends on the fidelity of the replicative step and the efficiency of the subsequent error correction mechanisms (214). Analysis of more than 7,000 missense and nonsense mutations associated with human disease has indicated that the most common nucleotide substitution for T (thymine) is to C (cytosine), for C it is to T, for A (adenine) it is to G (guanine) and for G it is to A (195). Transitions are therefore much more common than transversions. Some 61% of the missense/nonsense mutations currently logged in HGMD are transitions (T to C, C to T, A to G, G to A), whilst 39% are transversions (T to A or G, A to T or C, G to C or T, C to G or A).

Among single nucleotide substitutions there is one that clearly predominates, and it represents the most common type of mutational lesion: CpG dinucleotides mutate to TpG at a frequency that is about 5 times that of mutations in all other dinucleotides (15, 195, 361, 363). This substitution, which generates TG when it occurs on one DNA strand and CA ("CG to TG or CA rule") when it is on the other, is a major cause of human genetic disease. This phenomenon was first observed in the factor VIII (*F8*) gene in cases of hemophilia A (361), but it was soon noted in studies of many other genes (74). In hemophilia A, CG to TG or CA mutations account for 46% of point mutations in unrelated patients (14). In the HGMD (312) (http://www.hgmd.org), such mutations currently account for ~20% of the total number of missense/nonsense mutations. Among CpG dinucleotide mutations, transitions to TG or CA account for ~90% of substitutions. The mechanism of this common type of mutation appears to be methylation-mediated deamination of 5-methylcytosine (5mC). In eukaryotic genomes, 5mC occurs predominantly in CpG dinucleotides, most of which appear to be methylated (see (70) for review). 5mC then undergoes spontaneous nonenzymatic deamination to form thymine (Fig. 10.2). There is a bias in terms of the origin of CpG to TpG mutations: most occur in male germ cells

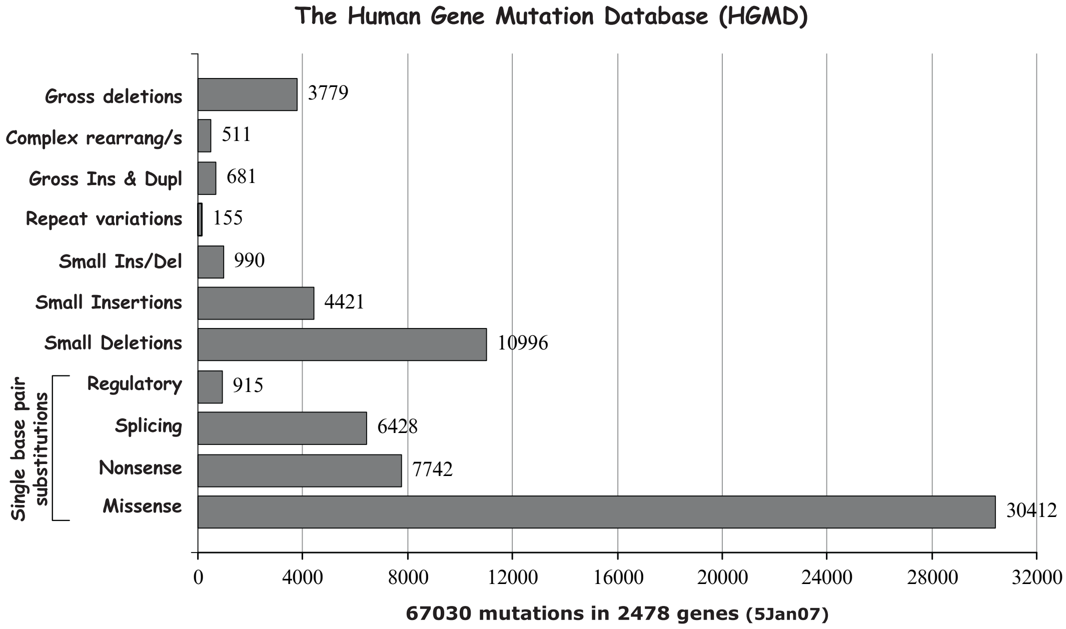

Fig. 10.1 Spectrum of different types of human gene mutations logged in the Human Gene Mutation Database as of January 2007

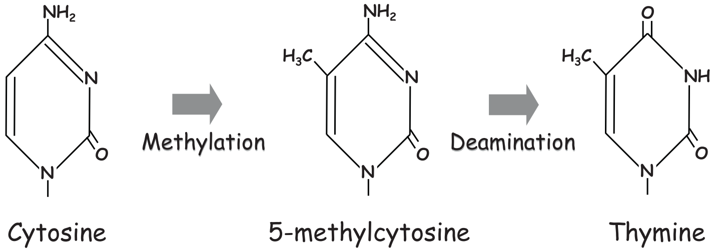

Fig. 10.2 Schematic representation of cytosine, 5'-methylocystosine, and thymine, and the chemical events involved in the mutational transformation of cytosine to thymine

(the male/female ratio is 7 to 1). One reason for this may be that sperm DNA is heavily methylated, whereas oocyte DNA is comparatively undermethylated (99). Another reason may be the considerably higher number of germline cell divisions in males than in females (154).

In a recent analysis, the average direct estimate of the combined rate of all mutations was 1.8×10^{-8} per nucleotide per generation. Single nucleotide substitutions were found to be approximately 25 times more common than all other mutations, whilst deletions were approximately three times as common as insertions; complex mutations were very rare, and the CpG context was found to increase substitution rates by an order of magnitude (185). Rates of different kinds of mutations were also found to be strongly correlated across different loci (185).

10.3.1.2 Micro-Deletions and Micro-Insertions

Deletions or insertions of a few nucleotides are also fairly common as a cause of human inherited disease. Most of these are less than 20 bp in length. Indeed, the majority of micro-deletions involve <5 nucleotides. In HGMD, the deletion of 1 bp accounts for 48% of small deletions, whilst an additional 30% involve 2 or 3 nucleotides. The majority of micro-deletions recorded (78%) result in an alteration of the reading frame. Most micro-deletions occur in regions that contain direct repeats of 2 bp or more. The most common length of direct repeat is 3 bp (48% of direct repeats associated with short deletions (15)). The most plausible mechanism for small deletions mediated by the presence of direct repeats is the slipped mispairing model (104) (Fig. 10.3). In addition, deletions of one or a few nucleotides frequently occur in runs of the same nucleotide, e.g., a poly(T) region (198). Finally, inverted repeats and "symmetric elements" are also frequently found in the immediate vicinity of micro-deletions (73, 289). Krawczak and Cooper (193) identified a consensus sequence – TG(A/G)(A/G)(G/T)(A/C) – which they claimed represented a deletion hotspot.

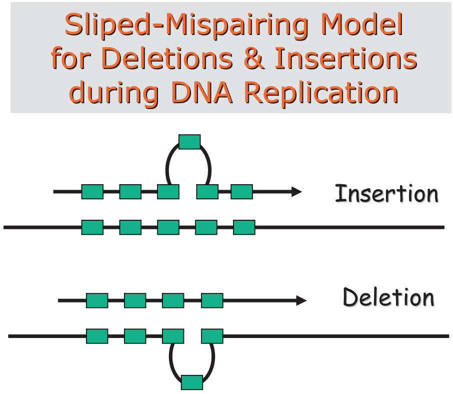

Fig. 10.3 Schematic representation of the slipped mispairing model for deletions and insertions during DNA replication

Micro-insertions (again up to 20 nucleotides) are rarer than micro-deletions; thus, in HGMD there are three times as many micro-deletions as micro-insertions (Fig. 10.1a). Nearly half of these involve the insertion of only 1 nucleotide (Fig. 10.4). As is the case with micro-deletions, most micro-insertions lead to alterations of the reading frame and are located in regions containing direct or inverted repeats or runs of the same nucleotide. Details of possible mechanisms of generation during replication can be found in elsewhere (72). There are as yet insufficient data available to estimate the frequency ratio of micro-insertions or micro-deletions in male or female germ cells. In the case of such lesions in factor VIII (*F8*) gene, 56% of micro-deletions/-insertions have been reported to occur in DNA regions harboring direct repeats or runs of the same nucleotide (14).

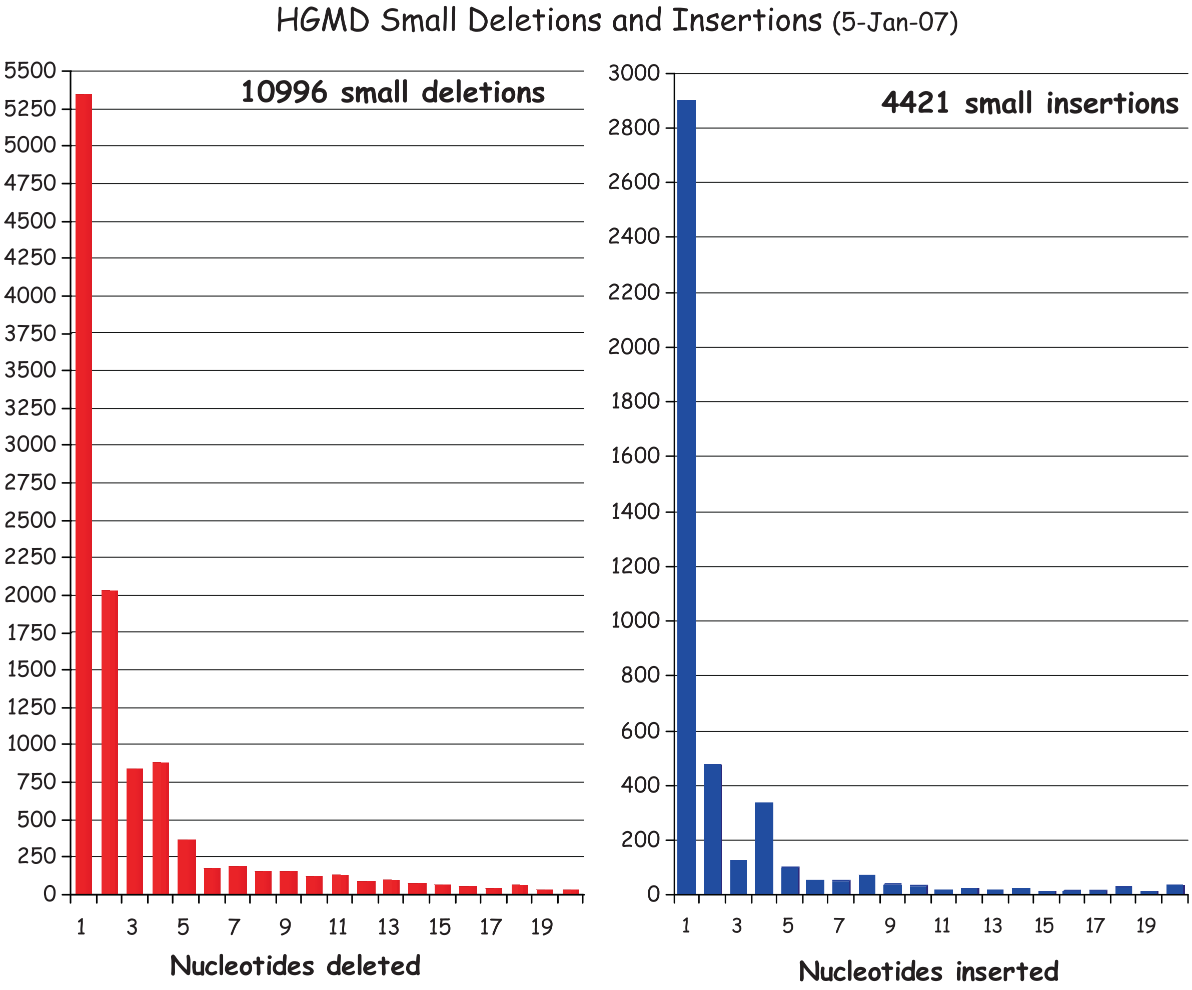

Fig. 10.4 Size distribution of short (<20 bp pathogenic human gene deletions and insertions (HGMD; http://www.hgmd.org, 5 January 2007)

HGMD data (3,767 micro-deletions and 1,960 micro-insertions) were used to perform a meta-analysis of micro-deletions and micro-insertions causing inherited disease, both defined as involving ≤20 bp DNA (23). A positive correlation was noted between the micro-deletion and micro-insertion frequencies for 564 genes for which both micro-deletions and micro-insertions have been reported. This is consistent with the view that the propensity of a given gene/sequence to undergo micro-deletion is related to its propensity to undergo micro-insertion. While micro-deletions and micro-insertions of 1 bp constitute, respectively, 48 and 66% of the corresponding totals, the relative frequency of the remaining lesions correlates negatively with the length of the DNA sequence deleted or inserted. Many micro-deletions and micro-insertions of >1 bp can potentially be explained in terms of slippage mutagenesis, involving the addition or removal of one copy of a mono-, di-, or trinucleotide tandem repeat. The frequency of in-frame 3 and 6 bp micro-insertions and micro-deletions was, however, found to be significantly lower than that of mutations of other lengths, suggesting that some of these in-frame lesions may not have come to clinical attention. Various sequence motifs were found to be overrepresented in the vicinity of both micro-insertions and micro-deletions, including the heptanucleotide CCCCCTG that shares homology with the complement of the 8-bp human minisatellite conserved sequence/

chi-like element (GCWGGWGG). The "indel hotspot" GTAAGT (and its complement ACTTAC) were also found to be overrepresented in the vicinity of both micro-insertions and micro-deletions, thereby providing a first example of a mutational hotspot that is common to different types of gene lesion. Other motifs overrepresented in the vicinity of micro-deletions and micro-insertions included DNA polymerase pause sites and topoisomerase cleavage sites. Several novel micro-deletion/micro-insertion hotspots were noted, and some of these exhibited sufficient similarity to one another to justify terming them "super-hotspot" motifs. Analysis of DNA sequence complexity also demonstrated that a combination of slipped mispairing mediated by direct repeats, and secondary structure formation promoted by symmetric elements, can account for the majority of micro-deletions and micro-insertions. Thus, micro-insertions and micro-deletions exhibit strong similarities in terms of the characteristics of their flanking DNA sequences, implying that they are generated by very similar underlying mechanisms.

A similar analysis of micro-deletions and micro-insertions in 19 human genes yielded evidence for an elevated micro-deletion rate at YYYTG and an elevated micro-insertion rate at TACCRC and ATMMGCC (186). Kondrashov and Rogozin (186) also found that ~45% of micro-deletions led to the removal of a repeated sequence, an event they termed "deduplication" in order to highlight the identity of the deleted sequence and the sequence abutting the site of deletion.

10.3.1.3 Expansion/Copy Number Variation of Trinucleotide (and Other) Repeat Sequences

Another mechanism of human gene mutation causing hereditary disease is the instability of certain trinucleotide repeats and their expansion in affected genes (44, 218, 282). A growing number of disorders (in excess of 150 are now recorded in HGMD), the majority of which involve neuromuscular tissues, have been found to be due to, or associated with, the expansion of repeat sequences; of these, 23 are expansions of triplet repeats. The first such disease was fragile X, a common cause of male mental retardation, which mapped to chromosome Xq27.3. Table 10.1 lists some examples of these disorders, which include Huntington disease, myotonic dystrophy, spinobulbar muscular atrophy, spinocerebellar ataxia 1, spinocerebellar ataxia 3 or Machado-Joseph disease, the fragile E site, and dentatorubral pallidoluysian atrophy. Genetic "anticipation" (the earlier onset and increasingly severe phenotype in successive generations) is a common phenomenon in these disorders (141). The trinucleotide involved is usually either CAG or CGG, but occasionally CTG, GCG, or GAA. It can be located in the 5' untranslated region (UTR), as in the case of the *FMR1* gene underlying fragile X, within the coding region (as in Huntington disease, SCA1, SCA3, and Kennedy disease) encoding poly(Gln), in an intron, as in Friedreich ataxia (*FXN*) and myotonic dystrophy type 2 (*ZNF9*), or in the 3' UTR, as in myotonic dystrophy type 1 (*DMPK*; Table 10.1, Fig. 10.5). The expansion of the triplet repeat either prevents its expression (329), results in a dominant gain–of–function mutation mediated by the longer poly(Gln) peptide (150), or alters the RNA processing of other genes (211, 288).

The trinucleotide repeats are usually polymorphic in human populations. Rarely, however, the number of trinucleotide repeats lies within a high-risk category that is termed "premutation." In such a case, the premutation exhibits a high probability of further expansion (instability) to yield disease-related alleles ("full mutation"). In fragile X, for example, the normal polymorphic alleles of the CGG repeat contain between 10 and 50 triplets, the premutation between 50 and 200, and the full mutation more than 200 triplets (119). Expansion of premutations to full mutations only occurs during female meiotic transmission. The probability of repeat expansion correlates with repeat copy number in the premutated allele. Since the premutation must precede the appearance of a full mutation, all mothers of affected children carry either a full mutation or a premutation (119). Premutation alleles may also be associated with late-onset movement disorders and premature ovarian failure (69, 163)

The precise mechanism of repeat expansion is unclear, although it is known that DNA polymerase progression is blocked by CTG and CGG repeats and the resultant idling of the polymerase could serve to catalyze slippage, leading to repeat expansion (171). In the case of spinocerebellar ataxia 1 (SCA1), interruption of the CAG repeat with a CAT unit is associated with more stable trinucleotide repeat (56). More details about these "dynamic mutations" can be found in the appropriate sections covering individual disorders, and have also been treated by Wells (348). Short

Table 10.1 Various examples of disorders of trinucleotide and other repeat expansions

	Disorder	Inheritance	Gene	Chr	OMIM#	Repeat	Normal	Mutant	Repeat	**Mutation**	**Parental**
									location	type	gender bias
1	Fragile X syndrome	XLD	*FMR1*	Xq27.3	309,550	CGG	6–52	60–200 premutation 230-1000 full mut	5’UTR	LOF, FraX	Maternal
2	Fragile E mental retardation	XLD	*FMR2*	Xq28	309,548	GCC	7–35	130–150 premutation 230–750 full mut	5’UTR	LOF, FraX	ND
3	Myotonic dystrophy	AD	*DMPK*	19q13	160,900	CTG	5–37	50–3,000	3’UTR	?Dom negative	Maternal
4	Spinobulbar muscular atrophy	XLR	*AR*	Xq13-21	313,700	CAG	11–33	38–66	Coding	GOF, LOF	ND
5	Huntington disease	AD	*HD*	4p16.3	143,100	CAG	6–39	36–121	Coding	GOF	Paternal
6	Dentatorubro-pallidoluysian atrophy	AD	*DRPLA*	12p13.31	125,370	CAG	6–35	51–88	Coding	GOF	Paternal
7	Spinocerebellar ataxia 1	AD	SCA1/*ATX1*	6p23	601,556	CAG	6–39	41–81	Coding	GOF	Paternal
8	Spinocerebellar ataxia 2	AD	SCA2/*ATX2*	12q24.1	601,517	CAG	14–31	35–64	Coding	GOF	Paternal
9	Spinocerebellar ataxia 3	AD	SCA3/*MJD1*	14q32.1	109,150	CAG	12–41	40–84	Coding	GOF	Paternal
10	Spinocerebellar ataxia 6/ Episodic ataxia type 2	AD	*CACNA1A*	19p13	601,011	CAG	7–18	20–23 EA2 21–27 SCA6	Coding	ND	ND
11	Spinocerebellar ataxia 7	AD	*SCA7*	3p12-13	164,500	CAG	7–17	38–130	Coding	GOF	Paternal
12	Friedreich ataxia	AR	*FRDA1*	9q13-21.1	229,300	GAA	6–34	80 premutation 112-1700 full mut	Intron 1	LOF, FraX	Maternal
13	Progressive myoclonus epilepsy 1	AR	*CSTB*	21q22.3	601,145	CCCCG-CCCCGCG	2–3	35–80	5’ flanking	LOF	Paternal
14	Synpolydactyly	AD	*HOXD13*	2q31-q32	142,989	(GCG)n(GCT) n(GCA)n	15	22–29	Coding	ND	??
15	Oculopharyngeal muscular dystrophy	AD	*PABP2*	14q11.2-q13	602,279	GCG	6	7–13	Coding	ND	??

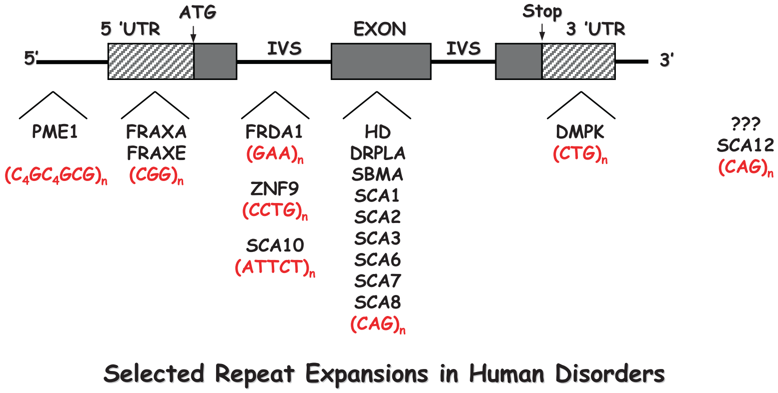

Fig. 10.5 Location of the repeat expansion in selected human disorders

expansions of GCG trinucleotide codons encoding Ala have been observed in the *HOXD13* gene causing dominant polydactyly, and in the *PABP2* gene causing oculopharyngeal muscular dystrophy (37, 237). These mutations may be due to unequal crossing-over rather than polymerase slippage. Generally speaking, it is likely that repeat instability is a consequence of the resolution of unusual secondary structure intermediates during DNA replication, repair and recombination (264).

A repeat expansion of 12 nucleotides (CCCCGCC-CCGCG) in the 5' flanking region of the *CSTB* gene causes one form of the recessive progressive myoclonus epilepsy (EPM1) (201). This indicates that repeat sequences other than trinucleotides can become expanded and cause human disorders. This particular expansion silences the *CSTB* gene, probably because it alters the spacing of transcription factor binding sites from each other and/or the transcriptional initiation site (202).

A tetranucleotide repeat expansion $(CCTG)_n$ in intron 1 of the *ZNF9* gene causes myotonic dystrophy type 2 (211). This expansion can be between 75 and 11,000 repeats in length. The expansion of the pentanucleotide repeat $(ATTCT)_n$ is responsible for the phenotype of spinocerebellar ataxia 10 (SCA10). The expansion occurs in intron 9 of the *SCA10* gene and can be up to 22.5 kb in length (221). Expansions of even longer repeats have been reported. In Usher syndrome type 1C, for example, there is an expansion of a 45-bp VNTR in intron 5 of the *USH1C* gene (9 tandem repeats instead of the usual less than 6 such repeats); this expansion has been predicted to inhibit transcription of the gene (332). There are also cases in which a large repeat expansion is not associated with a particular phenotype, e.g., the expansion of an AT-rich 33-mer repeat in the dictamycin-sensitive fragile site 16B (364).

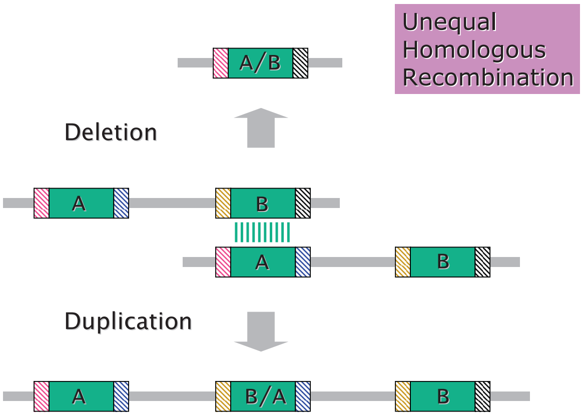

Fig. 10.6 Homologous unequal combination between similar regions of sequences A and B. The recombination events cause either deletions or duplications. In the case of a deletion, a hybrid sequence is generated, with the first part from sequence A and the second, from sequence B. The middle sequence in the duplication product is also a hybrid sequence, with the first part from sequence B and the second, from sequence A

10.3.1.4 Gross Deletions

Gross deletions are common causes of certain disorders and rare in others. In most of the X–linked disorders, for example, large deletions account for about 5% of molecular defects. In other disorders, however, such as steroid sulfatase deficiency, large deletions of the *STS* gene account for 84% of patients (24). The same is true for disorders such as Duchenne muscular dystrophy, growth hormone deficiency and α-thalassemia (92, 242, 335).

A considerable number of large deletions are probably generated by mispairing of homologous sequences and unequal recombination (Fig. 10.6). One of the best examples of homologous unequal recombination is the case of α-globin genes on chromosome 16p. As a result of a recent evolutionary duplication of the α-globin genes, extensive regions of sequence homology exist between the two closely linked α-genes. Unequal crossover results in either deletion of one α-gene or the creation of a fusion hybrid gene (106). The reciprocal product chromosomes carry three α-genes and are not associated with a clinical phenotype (127). Another example of a fusion gene resulting from an unequal crossover is the case of hemoglobin Lepore characterized by a hybrid gene between the δ- and β-globin genes on chromosome 11p (21). In the case of steroid sulfatase deficiency, the deletion can be as large as one megabase (Mb) (299). In Kallmann syndrome, translocation can occur as a result of unequal mispairing of X- and Y-homologous sequences (138).

A number of common genetic disorders are due to large deletions (or duplications) caused by unequal crossing-over of homologous sequences. Figure 10.7 depicts various examples, which include a 1.5-Mb deletion of 17p12 in hereditary neuropathy with liability to pressure palsies (HNPP) (276), deletion of 1.5 Mb of 17q11.2 in neurofibromatosis type 1 (98), deletion of 1.6 Mb of 7q11.23 in Williams syndrome (115), deletion of 5 Mb of 17p11.2 in Smith-Magenis syndrome (169), deletion of either 3 Mb or, more rarely, 1.5 Mb of 22q11 in DiGeorge and velo-cardio-facial syndrome (102, 298), and 4-Mb deletions of 15q in Prader-Willi and Angelman syndromes (54). A recurrent deletion of ~0.5 Mb of 17q21.3, which may be mediated by a common inversion polymorphism, has also been described (188, 301, 305, 311). For a review of chromosomal "duplicons," the low copy repeats that mediate deletions and duplications, see (168). It has been estimated that approximately 5% of the human genome is duplicated either intra- or inter-chromosomally (22). The large deletions or duplications (see below) due to duplicon crossover are also termed "genomic disorders." A recent review of such genomic disorders may be found in (303).

In many cases of large deletion, homologous unequal crossover occurs between repetitive elements such as *Alu* sequences. The *Alu* repeat is the most abundant repetitive element, with about 1.5×10^6 copies in the human genome (86, 203). The element is about 300 bp in length and consists of two similar regions separated by a short A-rich region. Unequal crossover can occur between *Alu* sequences oriented either in opposite directions or in the same direction.

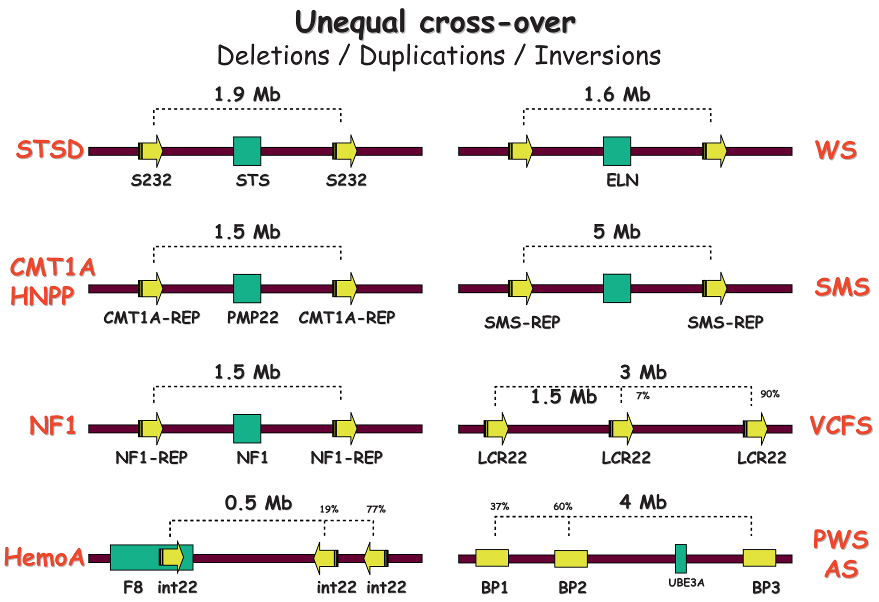

Fig. 10.7 Genes, duplicons, and diseases. Unequal crossover between homologous sequences (duplicons) produce either deletions or duplications of the DNA between the duplicons. The duplicons are shown by *arrows* or *clear boxes*. Genes included in the duplications/deletions are shown as *dark boxes* (*AS* Angelman syndrome *CMTA1* Charcot-Marie-Tooth type 1, *HemoA* hemophilia A, *HNPP* hereditary neuropathy with liability to pressure palsies, *NF1* neurofibromatosis 1, *PWS* Prader-Willi syndrome, *SMS* Smith-Magenis syndrome, *VCFS* velo-cardio-facial syndrome, *STSD* steroid sulfatase deficiency, *WS* Williams syndrome)

In addition, unequal crossings over have been noted between *Alu* elements and nonrepetitive DNA sequences without homology to *Alu*s. The best examples of *Alu-Alu* recombination occur in the genes encoding the low-density lipoprotein receptor (*LDLR*), which underlies familial hypercholesterolemia, and complement component 1 inhibitor (*C1I*) (205, 313)). All but one of the breakpoints associated with *LDLR* gene deletions occur within *Alu* repeats. By contrast, deletions in other *Alu*-rich genes (e.g., *GLA1*) do not necessarily involve *Alu* repetitive elements (189).

Nonhomologous (illegitimate) recombination occurs between two DNA sites that share minimal sequence homology of a few basepairs. This type of recombination during meiosis or alternatively, slipped mispairing during DNA replication mediated by short (2–8) nucleotide direct repeats flanking the deletions is a common finding in many instances of large gene deletions (281). Such deletions have been studied, for example, in hemophilia A; a compilation of 46 junctions from large deletions revealed that about 50% shared 2- to 6-bp homology at the breakpoint junction, as compared with only 17% in which the deletion was due to *Alu-Alu* recombination (356). Similar results have been reported from the intron 7 deletion hotspot in the Duchenne muscular dystrophy (*DMD*) gene; 8/9 deletion breakpoints examined were found to be flanked by DNA sequences with minimal homology (223).

It has also been proposed that alternative DNA conformations may trigger genomic rearrangements through recombination-repair activities. Distance measurements have indicated the significant proximity of alternating purine-pyrimidine and oligo(purine.pyrimidine) tracts to breakpoint junctions in 222 gross deletions and translocations, respectively, involved in human diseases. In 11 deletions analyzed, breakpoints could be explained by non-B DNA structure formation (20).

The Gross Rearrangement Breakpoint Database (GRaBD; http://www.uwcm.ac.uk/uwcm/mg/grabd/). This database was established primarily for the analysis of the sequence context of translocation and deletion breakpoints in a search for characteristics that might have rendered these sequences prone to rearrangement (3). GRaBD, which contains 397 germline and somatic DNA breakpoint junction sequences derived from 219 different rearrangements underlying human inherited disease and cancer, represents a large but not comprehensive collection of sequenced gross gene rearrangement breakpoint junctions. Analysis of these breakpoints has extended our understanding of illegitimate recombination by highlighting the importance of secondary structure formation between single-stranded DNA ends at breakpoint junctions. For example, potential secondary structure was noted between the 5’ flanking sequence of the first breakpoint and the 3’ flanking sequence of the second breakpoint in 49% of rearrangements, and between the 5’ flanking sequence of the second breakpoint and the 3’ flanking sequence of the first breakpoint in 36% of rearrangements (58). In addition, deletion breakpoints were found to be AT rich, whereas translocation breakpoints were GC rich. Alternating purine-pyrimidine sequences were found to be significantly overrepresented in the vicinity of deletion breakpoints, while polypyrimidine tracts were over-represented at translocation breakpoints (2).

10.3.1.5 Large Insertions (Via Retrotransposition)

A less common, but nevertheless still fascinating, mechanism of human gene mutation is the de novo insertion of repetitive elements via retrotransposition. The phenomenon was first observed in humans in the factor VIII (*F8*) gene in two unrelated de novo cases of severe hemophilia A (175). Truncated LINE (long interspersed) repetitive elements were introduced into exon 14 of the factor VIII (*F8*) gene, where they caused disruption of the reading frame. The inserted elements contained a poly(A) tract and caused a target site duplication of more than 12 nucleotides. Further analysis of these insertions revealed that, in one case, the inserted element was an exact but truncated copy of a full-length LINE element, with open reading frames found at chromosome 22q11 (97). The master source gene produces an mRNA that is probably reverse transcribed (possibly via a reverse transcriptase encoded by itself) and the double stranded nucleic acid is then reinserted into an A-rich region of the genome (Fig. 10.8). LINEs probably integrate into genomic DNA by a process called target-primed reverse transcription (251). The proposed mechanism of LINE retrotransposition is as follows: an active LINE is transcribed in the nucleus and is subsequently transported to, and translated in, the cytoplasm. The two LINE proteins, ORF1 and ORF2, complex with their encoding LINE transcript in ribonucleoprotein particles. The complex is then transported to recipient DNA sequences where target-primed

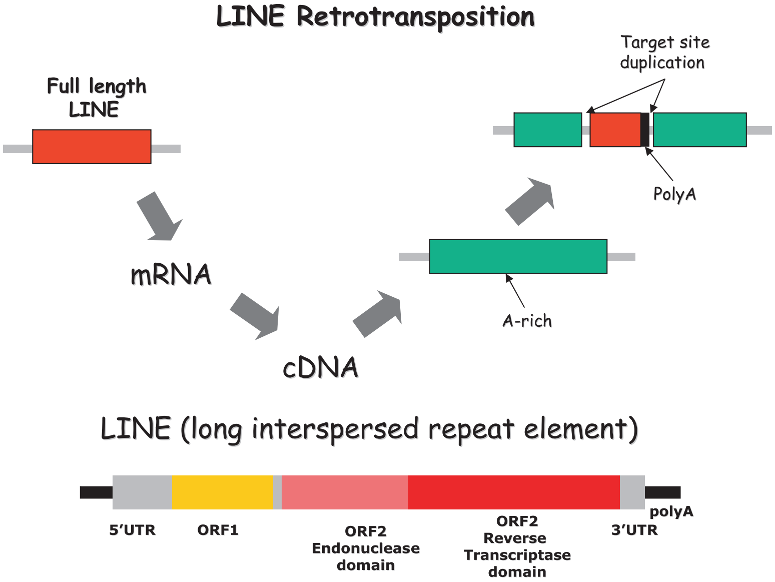

Fig. 10.8 Schematic representation of LINE retrotransposition. A master retrotransposon (full-length LINE from one chromosomal location is transcribed to double-stranded DNA and inserted into an adenine-rich region of another chromosomal location. The transposon has a poly(A) tail and produces a target site duplication

reverse transcription occurs. The new, integrated LINE copy is usually truncated at its 5' end. Over evolutionary time, L1s have shaped mammalian genomes through a number of different mechanisms. First, they have greatly expanded the genome both by their own retrotransposition and by providing the machinery necessary for the retrotransposition of other mobile elements, such as *Alu* sequences or SVA elements (49). Secondly, they have shuffled non-L1 sequence throughout the genome by a process termed transduction. Accidents of retrotransposition can cause disease and a number of such insertions have been reported to date (174, 251). It is noteworthy that insertions of these elements within introns of genes or flanking regions are probably not associated with disease, but instead represent rare, private polymorphisms (355).

Similar retrotranspositions that involve members of the *Alu* sequence family have also been reported in several genes (examples include *Alu* insertions into the *NF1* gene causing type 1 neurofibromatosis, into the factor IX (*F9*) gene causing hemophilia B, and into the cholinesterase (*BCHE*) gene in a case of acholinesterasemia) (238, 333, 341). It is likely that LINEs provide the molecular machinery necessary for the retrotransposition of *Alu*s. One study using mutation analysis of the *F9* gene has estimated the frequency of retrotransposition to be such that it occurs somewhere in the genome of about 1 in every 17 children born (208).

In an analysis of 199 unrelated families with proven mutations in *BTK* X-linked agammaglobulinemia, two families with retrotransposon insertions at exactly the same nucleotide within the coding region of the *BTK* gene have been identified. These insertions, of an SVA element and an *Alu*Y sequence, respectively, occurred 12 bp before the end of exon 9. Both had the typical hallmarks of a retrotransposon insertion, including target site duplication and a long poly A tail. The occurrence of two retrotransposon sequences at precisely the same site suggests that this site may be especially vulnerable to insertional mutagenesis (65).

Some 17% of a collection of gross insertions, all ≥276 bp in length, were due to LINE-1 (L1) retrotransposition involving different types of elements (L1 trans-driven *Alu*, L1 direct, and L1 trans-driven SVA) (49). A meta-analysis of 48 recent L1-mediated retrotranspositional events known to have caused human genetic disease revealed that 26 were L1 *trans*-driven *Alu* insertions, 15 were direct L1 insertions, four were L1 *trans*-driven SVA insertions, and three were associated with simple poly(A) insertions (52). The systematic study of these lesions, when combined with previous in vitro and genome-wide analyzes, allowed several conclusions regarding L1-mediated retrotransposition to be drawn: (a) ~25% of L1 insertions are associated with the 3' transduction of adjacent genomic sequences, (b) ~25% of the new L1 inserts are full length, (c) poly(A) tail length correlates inversely with the age of the element, and (d) the length of target site duplication in vivo is rarely longer than 20 bp. This analysis also suggested that some 10% of L1-mediated retrotranspositional events are associated with significant genomic deletions in humans.

Interestingly, Audrezet et al. (19) reported an indel in the *CFTR* gene that involved the insertion of a short 41-bp sequence with partial homology to a retrotranspositionally-competent LINE-1 element. These authors dubbed such insertions of ultra-short LINE-1 elements "hyphen elements."

10.3.1.6 Large Insertion of Repetitive and Other Elements

The insertion of non-retrotransposons, namely beta-satellite repeats, has been observed in the human genome. The insertion of 18 copies of the 68-bp monomer of the beta satellite repeat in exon 11 of the *TMPRSS3* gene on chromosome 21 caused one form of recessive nonsyndromic deafness, DFNB10 (293). This may have been mediated by invasion of the genomic DNA by a small polydispersed circular DNA (spcDNA).

A patient with a sporadic case of Pallister–Hall syndrome has been shown to have experienced a de novo nucleic acid transfer from the mitochondrial to the nuclear genome. This mutation, a 72-bp insertion into exon 14 of the *GLI3* gene, creates a premature stop codon and predicts a truncated protein product. Both the mechanism and the cause of the mitochondrial-nuclear transfer are however unknown (326). A second example of pathologic mitochondrial-nuclear sequence transfer has been subsequently (and retrospectively) identified in the *USH1C* gene, but appears to have arisen via a novel mechanism, "*trans*-replication slippage" (49).

Gross insertions (>20 bp) comprise < 1% of disease-causing mutations. In an attempt to study these insertions in a systematic way, 158 gross insertions ranging in size between 21 bp and approximately 10 kb were identified from the HGMD; their study has revealed extensive diversity in terms of the nature of the inserted DNA sequence and has provided new insights into the underlying mutational mechanisms (49). Some 70% of gross insertions were found to represent sequence duplications of different types (tandem, partial tandem, or complex). In the context of a 26-bp insertion into the *ERCC6* gene, Chen et al. also speculated as to whether they had found evidence for another mechanism of human genetic disease, involving the possible capture of DNA oligonucleotides (49).

10.3.1.7 Inversions

The most common inversion found to date is that associated with the *F8* gene, which occurs via intrachromosomal recombination mediated by a 9.5-kb sequence that is repeated three times in the last megabase of Xqter; once in intron 22 of the *F8* gene and twice about 400 kb telomeric to the first (200, 240) (Fig. 10.9). Most inversions, which are high-frequency independent recurring events, involve the distal sequence. The vast majority of inversions occur in male germ cells (280), perhaps because intrachromosomal recombination is inhibited by the presence of homologous X chromosomes (the male-to-female ratio was estimated

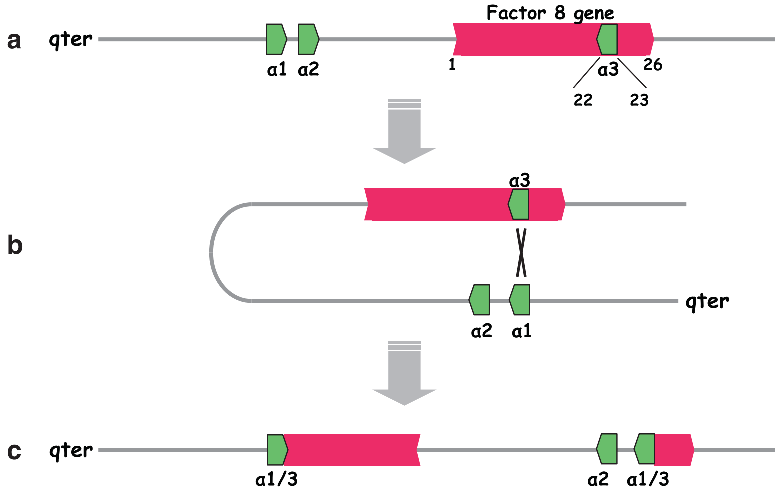

Fig. 10.9 A–C. Common inversion of the factor VIII (*F8*) gene in severe hemophilia A. (**a**) Schematic representation of the most distal 1 megabase of Xq. Regions α_1, α_2, and α_3 are 9.5-kb highly homologous DNA elements. The orientations of these sequences are shown by *arrows*. (**b**) Introchromosomal recombination between elements α_1 and α_3. (**c**) The crossover results in the inversion of exons 1 to 22 of the *F8* gene

to be about 300–1). Almost all mothers of persons with inversion hemophilia A are carriers of the abnormality. DNA diagnosis of the molecular lesion in severe hemophilia A has been greatly facilitated by the frequent occurrence of this common inversion of the *F8* gene (45% of individuals with severe hemophilia A). The frequency of de novo *F8* gene inversion has been estimated at 7.2×10^{-6} per gamete per generation. Another example of inversion has been described in the *IDS* gene (also on Xq) in about 13% of cases of Hunter syndrome (34). Inversions of DNA sequences have also been reported in the β-globin gene cluster on 11p and in the *APOA1-APOC3-APOA4* gene cluster on 11q (167, 172).

A meta-analysis of inversions of ≥5 bp but <1 kb has been performed by Chen et al. (51). Of the 21 mutations studied, 19 were found to be compatible with a model of intrachromosomal serial replication slippage in *trans* (SRStrans) mediated by short inverted repeats. Eighteen (one simple inversion, six inversions involving sequence replacement by upstream or downstream sequence, five inversions involving the partial reinsertion of removed sequence, and six inversions that occurred in a more complicated context) of these were found to be consistent with either two steps of intrachromosomal SRStrans or a combination of replication slippage in *cis* plus intrachromosomal SRStrans. The remaining lesion, a 31-kb segmental duplication associated with a small inversion in the *SLC3A1* gene, was explained in terms of a modified SRS model incorporating the concept of "break-induced replication." This study has therefore lent broad support to the idea that intrachromosomal SRStrans can account for a variety of complex gene rearrangements involving inversions.

10.3.1.8 Duplications

Duplications of whole genes or exons have contributed very significantly to the evolution of the human genome (71). Indeed, most gene clusters (e.g., β-globin, growth hormone, Hox) owe their origin to gene duplications that have occurred during vertebrate evolution. Furthermore, the presence of similar domains in proteins (e.g., immunoglobulin-like domains in many transmembrane proteins) are due to duplications of certain exons.

Occasionally, however, duplications may also be the cause of genetic disorders. The most frequent mechanism of duplication is homologous unequal crossover, as described for large deletions. In fact, most large duplications are generated as the reciprocal product of a deletion resulting from homologous unequal crossover. Duplications are less common, however, than their theoretically reciprocal deletions (see, e.g., (151), for the *DMD* gene). This may be due to the nonpathogenicity of a duplication (e.g., α-globin genes (127)), elimination of duplications as is the case for the *HPRT1* gene, or the fact that not all mechanisms that lead to deletions also produce duplications. A large and common duplication has been identified in cases of Charcot-Marie-Tooth disease type 1A (265). This duplication involves 1.5 Mb of DNA on chromosome 17p containing the peripheral myelin protein 22 (*PMP22*) gene. It results from homologous unequal crossover events between 24-kb repeats that flank the duplicated region. The reciprocal deletion product of this recombination event is responsible for a completely different clinical phenotype: hereditary neuropathy with liability to pressure palsies (Fig. 10.7). Another notable duplication of at least 500 kb that includes the *PLP1* gene is a frequent cause of Pelizaeus-Merzbacher disease (357). The pathogenetic mechanism of these duplications involves unequal crossing-over in meiosis mediated by "duplicons" in the genome (168).

The molecular defect in the majority of cases with ectrodactyly type SHFM3 on chromosome 10q24, is an approximately 0.5-Mb tandem duplication. The exact pathogenetic mechanism of this duplication is unknown (90).

Additional gene duplications causing recognizable syndromes include the *APP* duplication causing early-onset Alzheimer disease (283), the *SNCA* duplication and Parkinson disease (306), and the triplication of an ~605-kb segment containing the *PRSS1* gene in families with hereditary pancreatitis (206).

10.3.1.9 Gene Conversion

Gene conversion is the modification of one of two alleles by the other. It involves the nonreciprocal correction of an "acceptor" gene or DNA sequence by a "donor" sequence, which itself remains physically unchanged. In most known instances of gene conversion as a cause of human genetic disease, the functional gene has been wholly or partially converted to the sequence of a highly homologous and closely linked pseudogene, which therefore acts as the donor sequence. Probable examples include the genes for steroid

21-hydroxylase (*CYP21*) (327), polycystic kidney disease (*PKD1*) (345), neutrophil cytosolic factor p47-*phox* (*NCF1*) (130), immunoglobulin λ-like polypeptide 1 (*IGLL1*) (230), glucocerebrosidase (*GBA*) (108)), von Willebrand factor (*VWF*) (105), and phosphomannomutase (*PMM2*) (291). These gene/pseudogene pairs are all closely linked with the exception of the *VWF* gene (12p13) and its pseudogene (22q11-q13), and the *PMM2* gene (16p13) and its pseudogene (18p). Together, these two exceptions seem to establish a precedent for the occasional occurrence of gene conversion between unlinked loci in the human genome.

10.3.1.10 Insertion-Deletions (Indels)

A relatively rare type of mutation causing human genetic disease is the insertion-deletion, or *indel*, a complex lesion that appears to represent a combination of micro-deletion and micro-insertion. One example is provided by the 9 deleted base-pairs encoding codons 39–41 of the α2-globin (*HBA2*) gene that were replaced by eight inserted bases that served to duplicate the adjacent downstream sequence (250). Indels constitute a fairly infrequent type of lesion causing human genetic disease; some 1.5% of lesions in HGMD fall into this category.

Several indel hotspots have been noted in a meta-analysis of HGMD data on 211 different indels underlying genetic disease (57). A GTAAGT motif was found to be significantly overrepresented in the vicinity of the indels studied. The change in complexity consequent to a mutation was also found to be indicative of the type of repeat sequence involved in mediating the event, thereby providing clues as to the underlying mutational mechanism. The majority of indels (>90%) were explicable in terms of a two-step process involving established mutational mechanisms. Indels equivalent to double base-pair substitutions (22% of the total) were found to be mechanistically indistinguishable from the remainder and may therefore be regarded as a special type of indel.

10.3.1.11 Other Complex Defects

Complex mutational events that involve combined gross duplications, deletions, and/or insertions of DNA sequence have been not infrequently observed and together constitute ~1% of entries in HGMD. One example of this type of gene defect is a 10.9-kb deletion coupled with a 95-bp inversion in the factor IX (*F9*) gene causing hemophilia B (178). The molecular characterization of this type of lesion is often extremely complicated and in most cases the underlying mutational mechanisms could not be readily inferred.

Recently, however, a meta-analysis of 21 complex gene rearrangements derived from the HGMD revealed that all but one could be accounted for by a model of serial replication slippage, involving twin or multiple rounds of replication slippage (50). Thus, of the 20 complex gene rearrangements, 19 (seven simple double deletions, one triple deletion, two double mutational events comprising a simple deletion and a simple insertion, six simple indels that may constitute a novel and noncanonical class of gene conversion, and three complex indels) were compatible with the model of serial replication slippage in *cis*; by contrast, the remaining indel in the *MECP2* gene appears to have arisen via interchromosomal replication slippage in *trans*.

10.3.1.12 Molecular Misreading

Long runs of adenines (and perhaps other mononucleotides or dinucleotides) promote a phenomenon termed “molecular misreading,” by which DNA replication/RNA transcription and/or translation result in erroneous products with different numbers of (A)s derived from the original DNA sequence. In a family with hypobetalipoproteinemia, a deletion of one C in the A_5CA_3 coding sequence of the *APOB* gene results in a run of $(A)_8$. The patient, however, did not have severe disease, because some ApoB protein was made. This was the result of molecular misreading, in which ~10% of the resulting mRNAs contained $(A)_9$ instead of the expected $(A)_8$; this partially restored the reading frame, thereby templating the synthesis of low amounts of normal ApoB (210). Similarly, a family with mild to moderately severe hemophilia A with a deletion of one T within the coding A_8TA_2 sequence of the *F8* gene has been reported. The partial “correction” of the phenotype was due to restoration of the reading frame because of molecular misreading in which ~5% of the resulting RNAs contained $(A)_{11}$ instead of the expected $(A)_{10}$. In this family, there was also evidence for ribosomal frameshifting during translation of the mutant RNA (360).

Another example of this phenomenon was observed in the *APC* gene. A T-to-A transversion is present in the coding A_3TA_4 sequence of the *APC* gene in 6% of

Ashkenazi Jews, and in about 28% of Ashkenazim with a family history of colorectal cancer. This mutation creates a small hypermutable region, indirectly causing cancer predisposition because there are many somatic cells in which stretches of $(A)_9$ occur instead of the expected $(A)_8$; the $(A)_9$ results in frameshifting and a truncated dysfunctional APC (199). Interestingly, in the neurofibrillary tangles, neuritic plaques, and neuropil threads in the cerebral cortex of Alzheimer disease and Down syndrome, abnormal forms of β-amyloid precursor protein and ubiquitin B have been observed. These aberrant proteins were produced because of + 1 frameshifting that resulted from a deletion of AG in a sequence GAGAG that occurred in the coding regions of both genes (*APP* and *UBB*, respectively). This dinucleotide deletion was again the result of molecular misreading during transcription or posttranscriptional editing of RNA (330). This mechanism is likely to yield a considerable quantity of abnormal RNA molecules and protein products in somatic cells (259).

10.3.1.13 Germline Epimutations

Epimutations are modifications of DNA that constitute clonally heritable (yet potentially reversible) alterations in the transcriptional status of a gene that lead to the abnormal silencing of that gene. Epimutations are not mutations in the strictest sense of the word, since they do not alter the gene's nucleotide sequence. However, germline epimutations of the *MLH1* gene have been reported in individuals with multiple cancers (316) and in the *MLH1* and *MSH2* genes in hereditary nonpolyposis colorectal cancer (147). These heritable inactivating epimutations are characterized by mono-allelic hypermethylation of the *MLH1* gene and, to all intents and purposes, are functionally equivalent to conventional mutations.

10.3.1.14 Frequency of Disease-Producing Mutations

Mutation Frequency Within Genes. The frequency of different molecular defects is not the same for every gene and every disorder. It depends very largely on the DNA sequence characteristics of the gene in question (e.g., the presence of repeat units or homologous sequences) and the function of, and evolutionary constraints experienced by, its encoded protein (314). For some genes, deletions predominate; for others, one particular type of lesion such as an inversion may be especially common. Some genes exhibit mainly frameshifts and stop codons associated with a specific disorder, whereas others manifest mainly missense mutations for a given phenotype, or expansions of trinucleotide repeats.

Disease mutations are nonuniformly distributed within genes (229). Such mutations were found to be statistically overrepresented in conserved domains, and underrepresented in variable regions, even after allowing for the amino acid site variability of domains over long-term evolutionary history. This finding suggests that there is a nonadditive influence of amino acid site conservation on the observed intragenic distribution of disease mutations.

Mutation Frequency Within Human Populations. Population genetic considerations are also likely to be very important in determining why some mutations occur frequently, either within a patient cohort or in the population at large (see *Frequency of Inherited Disorders Database*, http://archive.uwcm.ac.uk/uwcm/mg/fidd/; *FINDbase*, http://www.findbase.org/). Selection, migration and genetic drift are all likely to play a part, as well as the mutation rate (114, 320, 365). Thus, the mutational spectrum of the *PAH* gene underlying phenylketonuria appears to result from a range of different factors including founder effect, range expansion and migration, genetic drift and possibly also heterozygote advantage (368). Selection can also serve to maintain deleterious mutations at high frequencies in particular populations by overdominant selection (heterozygote advantage). Good examples of this phenomenon are provided by a reduction in risk of severe malaria associated with female heterozygotes and male hemizygotes for mutations in the X-linked *G6PD* gene (232, 284), for individuals heterozygous for the β-globin (*HBB*) sickle cell mutation, Glu6Val (4), and for individuals heterozygous and homozygous for α^+-thalassemia (351). Intriguingly, however, the protection against malaria afforded by sickle cell disease and α^+-thalassemia when inherited individually is lost when the two conditions are co-inherited (350). Other possible examples of heterozygote advantage include an elevated cortisol response in heterozygous carriers of *CYP21A* mutations (352), higher values for hemoglobin, serum iron and transferrin saturation in women heterozygous for *HFE* gene mutations (82), resistance to prion infection conferred by a common prion protein (*PRNP*) polymorphism (225), resistance to severe sepsis in heterozygous carriers of the factor V Leiden polymorphism, Arg506Gln (177), and increased keratinocyte cell survival in individuals heterozygous for *GJB2* gene mutations (64). Resistance to cholera toxin (122), protection against bronchial asthma (292), and resistance to *Pseudomonas aeruginosa* infection (269) have all been mooted as possible bases for overdominant selection in heterozygous carriers of *CFTR* gene mutations. However, cystic fibrosis heterozygotes have been shown to secrete chloride at the same rate as individuals lacking *CFTR* gene mutations (149).

A number of genetic diseases are known to be particularly prevalent in Jewish populations (236, 252). The presence of four distinct lysosomal storage diseases at significant frequencies among Ashkenazi Jews has often been thought to provide evidence for a selective advantage accruing to heterozygotes in this population. However, evidence in support of the idea of genetic drift appears to be more compelling (117, 278).

Selection may also act at an extremely early stage to boost the frequency of some mutations that are deleterious at a later stage in development. Gain-of-function missense mutations in the fibroblast growth factor receptor 2 (*FGFR2*) gene responsible for Apert syndrome have been shown to confer a selective advantage on spermatogonial cells by promoting the clonal expansion of mutant cells (128, 129)

10.3.1.15 Chromosomal Distribution of Human Disease Genes

Human disease genes are characterized by the greater lengths of their encoded amino acid sequences, larger numbers of longer introns, broader ranges of tissue expression, and wider phylogenetic distributions (187, 216). Human disease genes are also known to be unevenly distributed between human chromosomes (48, 152). Furthermore, synonymous nucleotide substitutions appear to occur at a higher rate in human disease genes, a finding that may reflect increased mutation rates in the chromosomal regions in which disease genes are found (152). It may be that disease genes are more prevalent in genomic regions that experience elevated rates of mutation (55). Another possible explanation is that the disease gene set may contain a disproportionately lower number of genes expressed in the germline (152). This is because mutations in such genes might be expected to be more effectively repaired by transcription-coupled repair (transcription-coupled repair in the germline appears to account for the strand asymmetry that the human genome exhibits in terms of inherited mutations (133, 217). Strand asymmetries with respect to the mutation rate may, however, also arise through the influence of DNA replication origins (321) and recombination (153, 275).

10.3.1.16 Mutation Nomenclature

Some consistency in the way in which mutations are described is essential for the accurate and unambiguous reporting and curation of mutation data. The most recently published set of guidelines on how to describe mutational changes in human genes is to be found in den Dunnen and Antonarakis' work published in 2001 (91).

10.3.1.17 Mutations in Gene Evolution

Mutations in human gene pathology and evolution represent two sides of the same coin in that those same mutational mechanisms that have frequently been implicated in human pathology have also been involved in potentiating evolutionary change (71). Regardless of whether they are advantageous, disadvantageous, or neutral, these mutational changes and their putative underlying causal mechanisms are very similar. It is now clear that the gene has often been a dynamic entity over evolutionary time, and not a static one. Indeed, during vertebrate evolution, many genes have undergone gross rearrangement as a result of the action of a variety of mutational processes, including insertion, inversion, duplication, repeat expansion, translocation, and deletion. What links pathology and evolution is the underlying genomic architecture with its hitherto largely unexplored vocabulary of structural elements, and different types and patterns of repetitive DNA sequences (303). It can thus be seen that the mutational spectra of germline mutations responsible for inherited disease, somatic mutations underlying tumorigenesis, polymorphisms (either neutral or functionally significant), and differences between orthologous gene sequences exhibit remarkable similarities, implying that they are very likely to have causal mechanisms in common.

10.3.2 Consequences of Mutations

10.3.2.1 Mutations Affecting the Amino Acid Sequence of the Predicted Protein, but not Gene Expression

Many missense mutations (i.e., nucleotide substitutions that result in an amino acid substitution) cause hereditary disease in humans. Missense mutations are of importance for understanding the structure or function of a protein, since they usually occur in amino acid residues of structural or functional significance (228). Occasionally, however, not only is the mutated residue not conserved in mouse, but the substituting residue in humans is identical to its wild-type counterpart in the orthologous murine gene (123). It is thought that the most likely explanation for the majority of these cases of fixation of disease mutations in mice is *compensatory mutation*. This hypothesis holds that loss-of-function amino acid substitutions in a protein can be rescued by additional substitutions in the vicinity that compensate structurally for the original change.

It is sometimes difficult to establish a causative link between a missense mutation and a disease phenotype (76). The absence of the mutation in a large sample (usually 200 individuals) from the same ethnic group as the patient serves to exclude the possibility of a common polymorphism. Amino acid substitutions in evolutionarily conserved residues can also be good candidates for true pathogenicity (228). If the function of the protein is known, assessment of the effect of the missense mutation can be performed by in vitro mutagenesis and functional assay. Finally, the introduction of the mutation into an entire organism (e.g., into transgenic mice) and the study of its systemic effects provide one of the best means of assessing its contribution to a particular clinical phenotype. Amino acid substitutions can be shown to reduce or abolish the physiological function of a protein; for example, missense mutations have been identified in factor VIII that abolish thrombin cleavage, which is necessary for its activation (15), interfere with binding to other proteins, such as von Willebrand factor (143), or create or abolish *N*-glycosylation sites (9). In other proteins, mutations have been identified, e.g., in DNA binding domains, catalytic domains, transmembrane domains, ATP-binding regions, receptor-ligand contact sites, and phosphorylation or other chemical modification sites. Missense mutations may also affect protein folding, causing a dramatic change in secondary and tertiary structure such that the protein can no longer fulfill its physiological function.

A classic example of a missense mutation in the active site of an enzyme is provided by α1-antitrypsin Pittsburgh, found in an individual with a fatal bleeding disorder (253). The underlying mutation in the α1-antitrypsin (*SERPINA1*) gene substituted Arg for Met358 within the active site of the molecule. Substitution by Arg served to alter the substrate specificity of α1-antitrypsin by converting its "bait loop" (which is specific for elastase) to one that was specific for thrombin. In effect, the molecule lost its anti-elastase activity and became a serine protease inhibitor capable of inhibiting thrombin and factor Xa.

Mutations involving gains of glycosylation have generally been considered rare, and the pathogenic role of the new carbohydrate chains has never been formally established. Vogt et al. (337), however, identified three children with Mendelian susceptibility to mycobacterial disease who were homozygous with respect to a missense mutation in the *IFNGR2* gene that created a new *N*-glycosylation site in the IFNγR2 chain. The resulting additional carbohydrate moiety was found to be both necessary and sufficient to abolish the cellular response to IFNγ. From 10,047 HGMD mutations in 577 genes encoding proteins trafficked through the secretory pathway, 142 candidate missense mutations (~1.4%) in 77 genes (~13.3%) for potential gain of *N*-glycosylation were identified. Six mutant proteins were shown to bear new *N*-linked carbohydrate moieties. Thus, it may be that an unexpectedly high proportion of mutations causing human genetic disease do so via the creation of new *N*-glycosylation sites. Indeed, the pathogenic effects of these mutations may be a direct consequence of the addition of *N*-linked carbohydrate.

Missense mutations can result in disease by (1) elimination or reduction of the physiological activity/role of the protein; (2) gain of function by which the amino acid substitution creates new functional capabilities of the protein in biochemical and developmental processes in which the protein either does not participate or has a different role; (3) change of the target function of another protein, as in the case of the mutation in the protein C cleavage site at Arg 506 of coagulation factor V, which is associated with thrombophilia (30), or in the case of a mutation in the thrombin cleavage site of factor VIII that eliminates normal activation of factor VIII (16), or in the case of severe obesity from childhood and R236G in the human proopiomelanocortin (*POMC*) gene that disrupts the dibasic cleavage site between beta melanocyte-stimulating hormone (beta-MSH) and beta-endorphin (46); and (4) participation of the mutant polypeptide in protein complexes, which renders the entire complex abnormal or nonfunctional, as in the case of the triple helical structure of certain collagens in which incorporation of one abnormal collagen chain results in "protein suicide" or an abnormal structure that degrades rapidly (41).

Missense mutations have a multitude of different effects on protein structure and function including (a) introduction of larger residues within the hydrophobic protein core leading to adverse interactions between residues, (b) introduction of buried charged residues, (c) disruption of protein-protein interactions, (d) disruption of hydrogen bonding, (e) interference with DNA binding, (f) breakage of disulphide covalent linkages, (g) mutation of catalytic residues, (h) perturbation of metal binding, and (i) disruption of quaternary structure.

Without in-depth analytical studies, missense mutations are often difficult to distinguish from polymorphisms with little or no clinical significance. In the "post-genome era," a substantial amount of human genetic variation will become amenable to high-throughput analysis in the form of single nucleotide polymorphisms (SNPs), and many of these SNPs will directly influence the structure, function, or expression of genes and the RNAs/proteins they encode. Prior knowledge as to which SNPs are most likely to be clinically relevant would greatly enhance the power of studies that aim to identify disease genes through the genotypic screening of patients in both families and populations. Inclusion of structural/functional information could be especially important in the elucidation of multifactorial disease, where genetic heterogeneity and complex interactions between genes and environment have so far limited the success of genetic epidemiological studies (146). Recently, several predictive models have been developed that employ a number of different biophysical parameters to estimate the likely impact of an amino acid substitution on the structure and function of a protein (112, 315, 317, 340, 343). These models have been used to distinguish reasonably successfully between pathologic substitutions, functional polymorphisms, and neutral polymorphisms. Vitkup et al. (334) have concluded that mutations at arginine and glycine residues are together responsible for about 30% of cases of genetic disease, whereas random mutations at tryptophan and cysteine have the highest probability of causing disease.

10.3.2.2 Mutations Affecting Gene Expression

Mutations that do not result in amino acid substitution invariably affect gene expression, i.e., transcription, RNA processing and maturation, translation, or protein stability. Total or partial gene deletions, insertions, inversions, and other gross rearrangements obviously result in the loss of gene expression. These types of mutation are usually less frequent unless the genomic sequence environment of specific genes (e.g., presence of repeats) predisposes to such lesions. Disorders with high frequencies of gross rearrangements include α−thalassemia, Duchenne muscular dystrophy, steroid sulfatase deficiency, and hemophilia A. Some partial gene deletions that eliminate one or a few exons in frame result in milder clinical phenotypes because gene expression is not totally eliminated; the resulting protein may lack an amino acid domain that is not critical for its function (362).

10.3.2.3 Transcription (Promoter) Mutations

Mutations in known promoter motifs usually lead to reduced (or occasionally increased) mRNA levels. Such mutations have been studied in the TATA box of the β-globin (*HBB*) gene (12). Other nucleotide substitutions within DNA motifs that bind transcription factors include those located in the CACCC motif of the β−globin (*HBB*) gene influencing transcription factor EKLF binding (248, 266) , several motifs in the γ-globin (*HBG*) genes (63), the CCAAT motif of the *F9* gene influencing C/EBP binding (80), the SP1 motif of the *LDLR* gene promoter (183), the HNF-1 binding site in the *PROC* gene (29), and the binding site for the transcription factor Oct-1 in the lipoprotein lipase (*LPL*) gene (359). These few examples are only representative of a total of over 370 known promoter mutations listed in HGMD and causing human genetic disease. The importance of these mutants lies in the specific DNA sequences thereby implicated in binding to transcription factors. Although most of the known mutations reduce the levels of mRNA production, some substitutions actually increase it. Examples include various lesions in the promoters of the Gγ and Aγ globin (*HBG1* and *HBG2*) genes that cause hereditary persistence of fetal hemoglobin due to the inappropriate continuation of γ–globin gene expression into adult life (346). An increase in the distance of promoter elements from the transcriptional start site may also result in gene silencing. Such an example has been found in the promoter elements of the *CSTB* gene in progressive myoclonus epilepsy type 1 (EPM1) (202). Mutations that alter the transcriptional regulation of gene expression have been reviewed elsewhere (295).

The concomitant change in local DNA sequence complexity surrounding a substituted nucleotide is directly related to the likelihood of a regulatory mutation coming to clinical attention (196). This finding is consistent with the view that DNA sequence complexity is a critical determinant of gene regulatory function and may reflect the internal axial symmetry that frequently characterizes transcription factor binding sites.

Polymorphisms in the promoter region that are associated with differential levels of gene expression may predispose to common disorders. For example, a G>A single nucleotide polymorphism (SNP) at nucleotide-6 relative to the transcriptional initiation site of the angiotensin (*AGT*) gene influences the basal level of transcription and may predispose to essential hypertension (159). Listed in HGMD are in excess of 250 disease-associated promoter polymorphisms plus >170 functional promoter polymorphisms that significantly increase or decrease promoter activity but which have not yet been associated with a clinical phenotype.

10.3.2.4 mRNA Splicing Mutants

Single base-pair substitutions in splice junctions constitute at least 10% of all mutations causing human inherited disease. There are, however, a wide variety of mutations within both introns and exons that can affect normal RNA splicing (see (194) for review). The different mechanisms by which disruption of pre-mRNA splicing play a role in human disease were reviewed by Faustino and Cooper in 2003 (110). The most commonly found mutations occur in the invariant dinucleotides GT and AG found at the beginning and end of the donor (5') and acceptor (3') consensus splice sequences (see Fig. 10.10 for the consensus splice elements and Fig. 10.11 for the different kinds of RNA splicing abnormalities). Almost all of these mutations cause either exon skipping or cryptic splice site utilization, resulting in the severe reduction or absence of normally spliced mRNA. In addition, mutations in nucleotides +3, +4, +5, +6, -1 and –2 of the consensus donor splice site have frequently been observed (Fig. 10.12), with variable severity of the RNA splicing defect. Similarly, mutations in positions -3 and the polypyrimidine tract of the consensus acceptor splice site have been noted (Fig. 10.12). In the majority of these cases, some normal splicing occurs and the defect is not severe. Utilization of cryptic splice sites leads to the production of abnormal mature mRNA with premature stop codons or to the inclusion of additional amino acids after translation (see (15) for examples, and references cited therein).

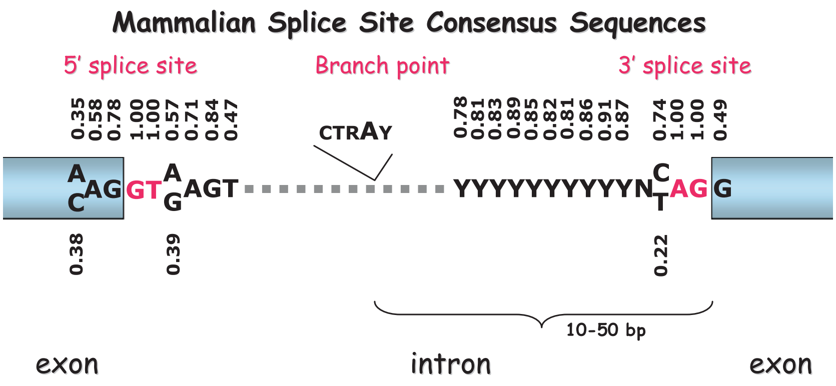

Fig. 10.10 Consensus sequences for the donor (5' splice) and acceptor (3' splice) sites and the branch point. *Numbers* above or below the nucleotides correspond to frequencies of a given nucleotide in a large number of mammalian splice site sequences. Note that the dinucleotides GT and AG (in *red*) at the beginning and end of the intron are invariant

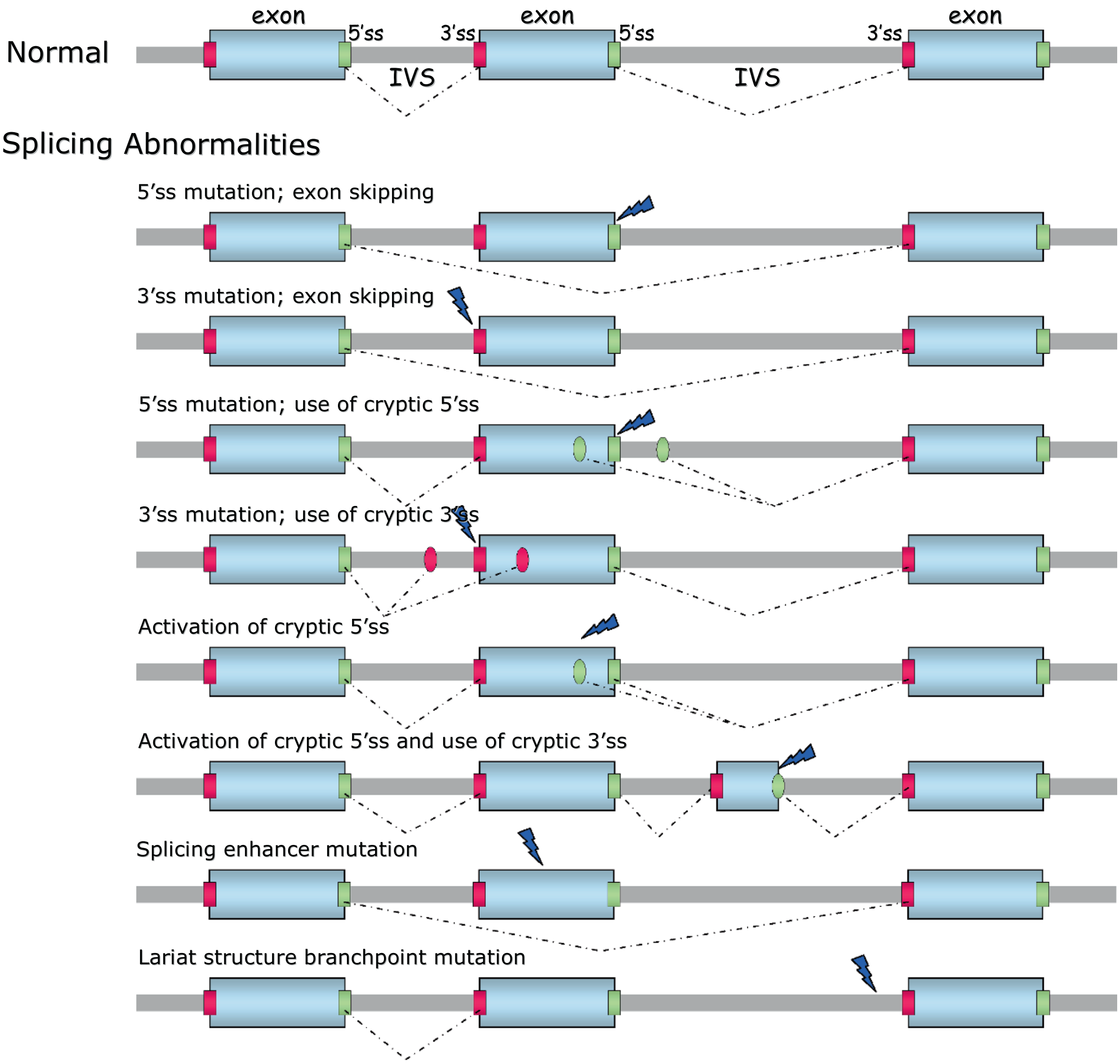
Normal
exon
5'ss
3'ss
exon
5'ss
3'ss
exon
IVS
IVS
Splicing Abnormalities
5'ss mutation; exon skipping
3'ss mutation; exon skipping
5'ss mutation; use of cryptic 5'ss
3'ss mutation; use of cryptic 3'ss
Activation of cryptic 5'ss
Activation of cryptic 5'ss and use of cryptic 3'ss
Splicing enhancer mutation
Lariat structure branchpoint mutation

Fig. 10.11 Examples of splicing abnormalities in introns of human genes. Exons are shown as *blue boxes*; introns, as *lines* between exons. *Green squares* denote the normal 5' (donor) splice sites; *red squares* represent the normal 3' (acceptor) splice sites. *Green* and *red circles* denote cryptic 5' and 3' splice sites, respectively. The *broken blue wedge* represents the site of mutation

Employing a neural network for splice site recognition, Krawczak et al. (197) performed a meta-analysis of 478 disease-associated splicing mutations, in 38 different genes, for which detailed laboratory-based mRNA phenotype assessment had been performed. Inspection of the ±50-bp DNA sequence context of the mutations revealed that exon skipping was the preferred phenotype when the immediate vicinity of the affected exon-intron junctions was devoid of alternative splice sites. By contrast, in the presence of at least one such motif, cryptic splice site utilization became more prevalent. This association was, however, confined to donor splice sites. Outside the obligate dinucleotide, the spatial distribution of pathological mutations was found to differ significantly from that of SNPs. Whereas disease-associated lesions clustered at positions -1 and +3 to +6 for donor sites and −3 for acceptor sites, SNPs were found to be almost evenly distributed over all sequence positions considered. When all putative missense mutations in the vicinity of

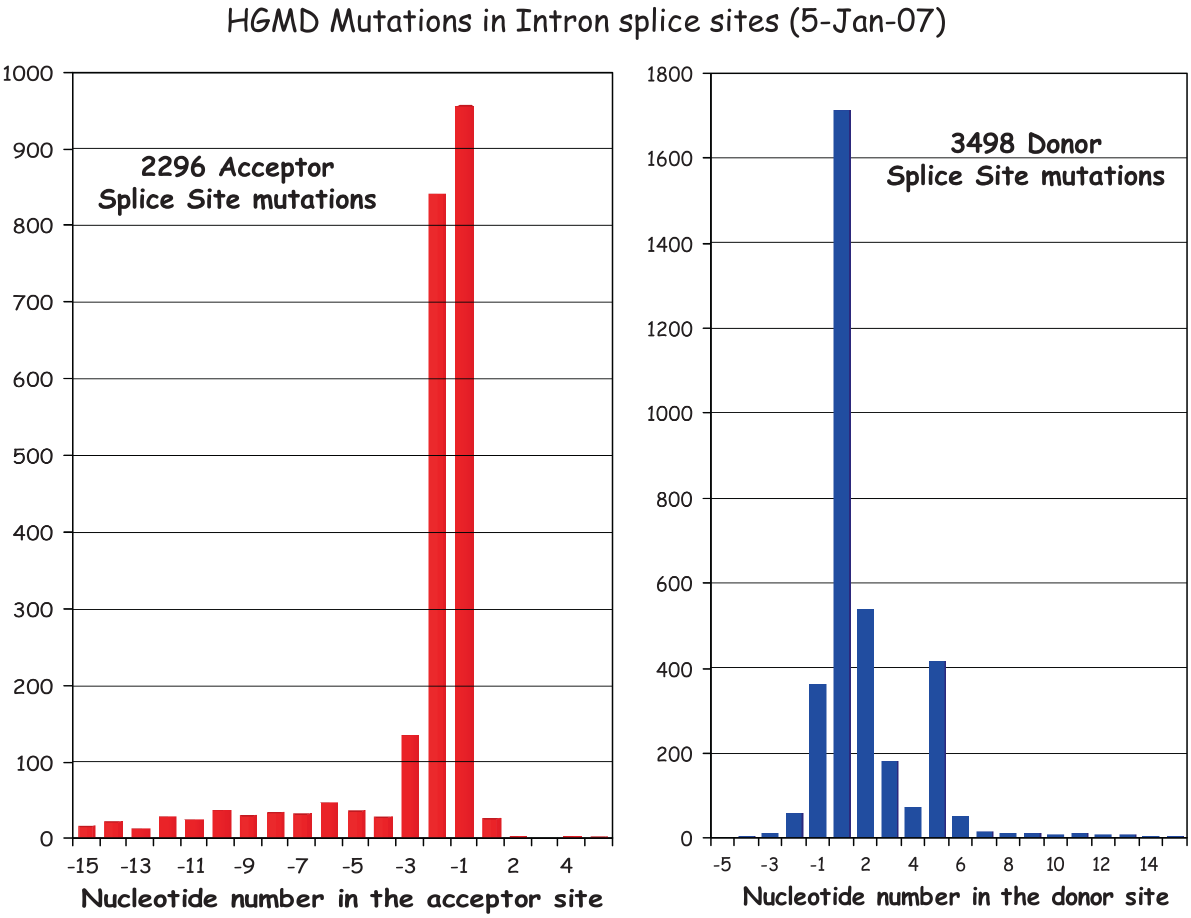

Fig. 10.12 Mutations in the consensus sequences of splice junctions recorded in the HGMD

splice sites were extracted from the HGMD for the 38 studied genes, a significantly higher proportion of changes at donor sites (37/152; 24.3%) than at acceptor splice sites (1/142; 0.7%) was found to reduce the neural network signal emitted by the respective splice site. It is estimated that some 1.6% of disease-causing missense substitutions in human genes are likely to affect the mRNA splicing phenotype.

Other kinds of mutation in introns include those that cause the activation of cryptic splice sites (by altering a sequence so as to make it more similar to an authentic consensus splice site) or by creation of new splice sites (323). In both instances, new intron splice patterns occur with consequent introduction of stop codons or abnormal peptides after translation. These mutations do not completely abolish normal splicing and are therefore not associated with the absence of normal mature mRNA. A mutation in a lariat structure branchpoint (302) has been found in the *L1CAM* gene in a patient with X-linked hydrocephalus (279). By contrast, another mutation in intron 5 of the type 2 neurofibromatosis (*NF2*) gene created a consensus branchpoint sequence and led to the activation of a cryptic exon (87).

Some 98.7% of all splice sites in human genes conform to consensus sequences that include the invariant dinucleotides GT and AG at the 5' and 3' ends of the introns, respectively (40). Noncanonical sequences (e.g., GA-AG, GC-AG, and AT-AC) do, however, occur at human splice junctions, albeit much less frequently (<0.02, 0.69, and 0.05%, respectively. Some of these noncanonical splice sites are nevertheless known to be utilized with high efficiency and may be conserved over quite long stretches of evolutionary time. Such sites have occasionally come to clinical attention when they have harbored mutations causing human inherited disease (304). Moreover, the utilization of a cryptic noncanonical donor splice site within exon 1 of the *HRPT2* gene in a case of familial isolated primary hyperparathyroidism as a consequence of a causative lesion in intron 1 of the gene has been reported. RNA isolated from EBV-transformed lymphoblastoid cell lines derived from the patients was utilized to demonstrate the consequences at the level of the mRNA phenotype (the loss of 30 bases from the mRNA transcript).

Single base-pair substitutions within "splicing enhancer" sequences may also perturb splicing by promoting exon skipping; examples include a mutation in intron 3 of the growth hormone (*GH1*) gene causing short stature (62) and a mutation in exon 5 of the adenosine deaminase (*ADA*) gene causing ADA deficiency (287). In patients with frontotemporal dementia with parkinsonism, three heterozygous mutations in a cluster of 4 nucleotides +13 to +16 of exon 10 of the microtubule-associated protein tau (*MAPT*) gene destabilized a potential stem-loop structure that is probably involved in regulating the alternative splicing of exon 10. This caused more frequent use of the 5' splice site and an increased proportion of tau transcripts that include exon 10. The increase in exon 10+ mRNA increased the proportion of tau protein containing four microtubule-binding repeats, which is consistent with the neuropathology described in families with this type of frontotemporal dementia (155). One mutation found in the *ATM* gene causing ataxia-telangiectasia, was a deletion of four nucleotides (GTAA) in intron 20 within an intron-splicing processing element (ISPE) that is complementary to U1 snRNA. This element mediates accurate intron processing and interacts specifically with U1 snRNP particles (256). Finally, the intronic prothrombin (*F2*) gene 19911A>G polymorphism influences splicing efficiency by altering a known functional pentamer CAGGG motif (338).

Some nonsense mutations cause skipping of one or more exons, presumably during pre-mRNA splicing in the nucleus; this phenomenon has been termed "nonsense-mediated altered splicing" (NAS) but its underlying mechanism is unclear. The first such mutation was described in the *FBN1* gene in Marfan syndrome (95). It is now recognized that any nucleotide substitution within exons (nonsense, missense or translationally silent synonymous point mutation) that disrupts a splicing enhancer or silencer (ESE enhancer splicing element; CERES composite exonic regulatory element of splicing) or creates an exon splicing silencer (ESS) may affect either the pattern or efficiency of mRNA splicing (32, 43, 47, 213) (Fig. 10.13). In exon 12 of the *CFTR* gene, about one quarter of synonymous variations result in exon skipping, and hence lead to the synthesis of an inactive CFTR protein (257). For a review on the effects of exonic variants in splicing, and additional examples of such pathogenic mutations, see (255). It has been estimated that pathogenic effects of ~20% of mutations in the *MSH2* gene result from missense mutations that disrupt ESE sites and perturb splicing. Similarly, the pathogenic effects of ~16% of missense mutations in the *MLH1* gene are thought to be ESE-related (131).

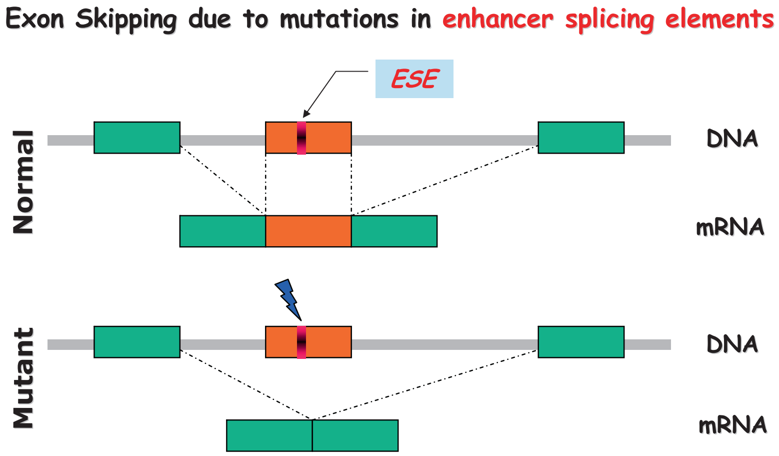
Exon Skipping due to mutations in enhancer splicing elements
ESE
Normal
DNA
mRNA
Mutant
DNA
mRNA

Fig. 10.13 Exon skipping attributable to nonsense, missense, and silent mutations in enhancer splicing elements (*ESE*). This element is shown as a darkened segment of the middle exon

Splice-mediated insertional inactivation involving an *Alu* repeat was first reported by Mitchell et al. in 1991 (231). Analysis of the ornithine δ-aminotransferase (*OAT*) mRNA of a patient with gyrate atrophy revealed a 142 nucleotide insertion at the junction of exons 3 and 4. An *Alu* sequence is normally present in intron 3 of the *OAT* gene, 150 bp downstream of exon 3. The *Alu* sequence found in the cDNA was identical to this one, except that the patient was homozygous for a C→G transversion in the right arm of the *Alu* repeat which served to create a new 5' splice site. This activated an upstream cryptic 3' splice site (the poly(T) complement of the *Alu* poly(A) tail followed by an AG dinucleotide) and a new "exon," containing the majority of the right arm of the *Alu* sequence, was recognized by the splicing apparatus and incorporated into the mRNA. The splice-mediated insertion of an *Alu* sequence in reverse orientation has also been reported in the *COL4A3* gene causing Alport syndrome (182).

A number of "deep intronic" mutations, at some considerable distance from splice sites and known splicing-related sequence elements, have been reported as a cause of human inherited disease (77, 140, 325). Such lesions often create novel splice sites thereby activating cryptic exons ('pseudoexons'). As mutational screening techniques improve, it is anticipated that an increasing number of such lesions will be identified which will turn out to have adverse effects on the mRNA splicing phenotype.

10.3.2.5 RNA Cleavage-Polyadenylation Mutants

A number of examples of RNA cleavage-polyadenylation mutations have now been described (53). Those reported occur in the sequence AAUAAA, which is 10–30 nucleotides upstream of the polyadenylation site and is important for the endonucleolytic cleavage and polyadenylation of the mRNA. Mutation in this sequence of the β-globin (*HBB*) gene results in mild thalassemia (249). In these cases, normal polyadenylation and cleavage occurs at a level about 10% of normal. Alternative AAUAAA sites downstream of the mutated one are used, resulting in larger mRNAs that are highly unstable. Other mutations near the poly(A) cleavage sequence may result in mRNA destabilization; one such mutation has been described 12 bp upstream of the AAUAAA sequence of the *HBB* gene in a patient with β-thalassemia (42).

The G>A mutation at the 3'-terminal nucleotide of the 3' untranslated region (UTR) of the *F2* (prothrombin) gene mRNA gives rise to an elevated prothrombin plasma level and represents a common genetic risk factor for the occurrence of thromboembolic events. This mutation creates an inefficient 3' end cleavage signal and represents a gain-of-function mutation, causing increased cleavage site recognition, increased 3' end processing, and increased mRNA accumulation and protein synthesis (124, 271) .

10.3.2.6 Mutations in miRNA-Binding Sites

Micro-RNAs (miRNAs) post-transcriptionally down-regulate gene expression by binding to complementary sequences on the 3' untranslated regions (UTRs) of their cognate mRNAs, thereby inducing either mRNA degradation or translational repression. Over 400 human miRNAs have so far been identified, but many more probably still remain to be discovered. These miRNAs are each likely to down-regulate a large number of different target mRNAs. Mutations in miRNA-binding sites could in principle cause disease, although in practice only one such lesion has so far been reported: a G A transition in a binding site for miR-189 within the 3' UTR of the *SLITRK1* gene of two apparently unrelated Tourette syndrome patients (1). Experimental confirmation of the functional effect of this mutation came from the demonstration that, in the presence of miRNA-189, in vitro constructs bearing the 3' UTR mutation served to increase repression of a reporter gene by comparison with the wild-type.

An instructive pathogenic mutation was recently found in an miRNA target site. A quantitative trait locus with a major effect on muscle mass of Texel sheep was mapped to a chromosome interval encompassing the myostatin (*GDF8*) gene. The *GDF8* allele of Texel sheep is characterized by a G-to-A transition in the 3' UTR that creates a target site for mir1 and mir206, miRNAs that are highly expressed in skeletal muscle. This causes translational inhibition of the myostatin gene and hence contributes to the muscular hypertrophy of Texel sheep (60). A further example of a functional miRNA target site variation involves an SNP in the 3'UTR of the human *AGTR1* gene; the variant allele is not down-regulated by miR155; remarkably, the variant allele has been associated with hypertension in numerous studies (296).

10.3.2.7 Cap Site Mutations

Transcription of the mRNA is initiated at the so-called cap site, which is protected from exonucleolytic degradation by the addition of α-methylguanine. An A-to-C transversion at the cap site of the β-globin (*HBB*) gene was found in a patient with β-thalassemia (354). It is not, however, clear whether this mutation causes reduced transcription or abnormal initiation of transcription since C is found in 6% of transcriptional initiation sites (190) (the most common nucleotide (76%) at position +1 is A). A functional (C/A) polymorphism of the transcriptional initiation site has been noted in the *APOH* gene; the rarer A allele displayed a carrier frequency of 0.12 and was associated with markedly reduced plasma β2 glycoprotein I (226).

10.3.2.8 Mutations in 5' Untranslated Regions

Sequence motifs in the 5' UTRs of genes are thought to play a role in controlling the translation of the encoding mRNA. The phenotypic effects of lesions in 5' UTRs and their clinical consequences have been reviewed (45). Mutations in the iron response element (IRE) in the 5' UTR of the ferritin (*FTH1*) gene interfere with the post-transcriptional regulation of ferritin synthesis by decreasing the affinity of IRE for IRE-binding protein (125). By contrast, decreases in the steady state level of β-globin (*HBB*) mRNA have been noted in association with a single base deletion at position +10, a G-to-A substitution at position +22, a C-to-G transversion at position +33, and a 4 bp deletion (AAAC) at position +(40-43) in the *HBB* 5' UTR (18, 148, 297).

10.3.2.9 Mutations in 3' Regulatory Regions

Sequences in the 3' regulatory regions (3' RRs) of genes are known to be involved in controlling mRNA cleavage/polyadenylation and determining mRNA stability, nuclear export, intracellular localization, and translational efficiency. Although such regions are rich in regulatory elements, relatively few pathologic mutations have been reported (53, 66). Although only ~0.2% of mutations currently logged in HGMD are located within 3' RRs, this is likely to represent a rather conservative estimate of their actual prevalence. A typical example is the G→A transition 69 nucleotides downstream of the polyadenylation site of the δ-globin (*HBD*) gene causing δ-thalassemia (233); the mutation occurs within a GATA motif and serves to increase the binding affinity of the sequence for erythroid-specific DNA binding protein.

In an attempt to study 3' RR mutations systematically, Chen et al. (53) collated 121 3' RR variants in 94 human genes including 17 mutations in the upstream core polyadenylation signal sequence (UCPAS), 79 in the upstream sequence (USS) between the translational termination codon and the UCPAS, 6 in the left arm of the 'spacer' sequence (LAS) between the UCPAS and the pre-mRNA cleavage site (CS), 3 in the right arm of the 'spacer' sequence (RAS) or downstream core polyadenylation signal sequence (DCPAS), and 7 in the downstream sequence (DSS) of the 3'-flanking region. All the UCPAS mutations and the rather unusual cases of *DMPK*, *SCA8*, *FCMD*, and *GLA* mutations were found to exert a significant effect on the mRNA phenotype, and the majority cause monogenic disease. By contrast, most of the remaining variants were polymorphisms, were found to exert a comparatively minor influence on mRNA expression, but may predispose to, protect from, or modify complex clinical phenotypes. The systematic study of these lesions permitted the identification of consistent patterns of secondary structural change that promise to allow the discrimination of nonfunctional USS variants from their functional counterparts.

10.3.2.10 Translational Initiation Mutations

Mutations in the ATG translational initiation codon have been reported in quite a wide variety of disorders (e.g., (270)). Instances of substitutions in all three nucleotides have been observed in β-thalassemia, Norrie disease, albinism, phenylketonuria, McArdle disease, and Albright osteodystrophy, among others. Indeed, a total of 251 mutations within ATG translational initiation codons are recorded in HGMD, representing ~0.6% of all missense and nonsense mutations. Almost invariably, the mutation leads to severe reduction of steady state mRNA levels similar to that associated with nonsense mutations. The mutant mRNA is presumably not translated. The first AUG codon occurs in the context of the so-called Kozak consensus sequence GCCA/GCCAUGG, which is thought to be recognized by the 40S ribosomal subunit (191). Mutations at the initiator methionine ATG may completely abolish translation; however, there are alternative possibilities, viz. utilization of the mutant ATG with much reduced efficiency or translational initiation at the next available ATG codon. A C/T polymorphism immediately 5' to the ATG codon within the Kozak sequence of the *CD40* gene is thought to influence translation efficiency (162).

Some diseases are caused by mutations that perturb the initiation step of translation by changing the context around the start AUG codon or introducing upstream AUG codons (see (192) for a review). The scanning mechanism provides a framework for understanding the effects of these changes in mRNAs. The scanning mechanism refers to the entry of the small ribosomal subunit at the (usually capped) 5' end of the mRNA and linear migration until an AUG codon is encountered. Mutational mechanisms such as: (a) reinitiation at an internal start codon (e.g., thrombopoietin, *TPO*); and (b) leaky scanning (as in the case of the *Rx/rax* gene underlying the mouse eyeless mutation) probably account for such cases.

Naturally occurring mutations in the GCCA/GCC<u>AUG</u>G motif include (for the numbering of the mutant nucleotide, the A of the AUG codon is +1; see references in (192)): (a) +4 G-to-A in the androgen receptor (*AR*) gene in a family with partial androgen insufficiency; (b) -1 C-to-T transition in the α-tocopherol transfer protein (*TTPA*) gene in a family with vitamin E deficiency; (c) a 2nt deletion causes an A-to-C change at position -3 of the α-globin gene (*HBA*) in a patient with α-thalassemia; (d) -3 A-to-T transversion in the mouse *Pax6* gene causes defects in eye development; (e) -3 G-to-C somatic mutation in the *BRCA1* gene in one case of highly aggressive sporadic breast cancer. It is not surprising that most of the naturally occurring mutations involve positions -3 and +4, the positions wherein experimentally induced mutations have the strongest effect.

10.3.2.11 Termination Codon Mutations

The classic example of a termination codon mutant is the case of the α_2-globin Constant Spring, with a mutation in the normal stop codon; this substitution leads to incorporation of an additional 31 amino acid residues in the α_2-globin polypeptide chain (59). The resulting protein is unstable and does not interact properly with the β–globin chains of hemoglobin. Some 81 mutations within Term codons are recorded in HGMD, representing ~0.2% of all missense/nonsense mutations.

10.3.2.12 Frameshift Mutations

A large number of frameshift mutations have been described in numerous disease-related genes. All lead to altered translational termination with abnormal polypeptide chains after the frameshifts; severe phenotypes are usually seen. Frameshifts occur with micro-deletions or micro-insertions and exon skipping. The mechanisms underlying these mutations were discussed earlier in this chapter.

10.3.2.13 Nonsense Mutations

Nonsense mutations obviously cause premature termination of translation and truncated polypeptides. Some 48% of nonsense mutations in HGMD are to codon TGA, with 28% being to TAA and 24%, to TAG. About 55% of the newly created TGA codons are CG-to-TG transitions resulting from the methylation-mediated deamination of 5mC described earlier. Many such mutations have been described in a large number of disease-related genes.

Nonsense mutations are usually associated with a reduction in the steady state level of cytoplasmic mRNA (28). This mechanism of "nonsense-mediated mRNA decay" (NMD) is responsible for the degradation of mRNAs that contain a premature termination codon at a position at least 50 nt upstream of an exon-exon boundary (219), but it is not universal (157). One or more parameters could be affected: the transcription rate, the efficiency of mRNA processing or transport to the cytoplasm, or mRNA stability.

Nonsense mutations account for at least 11% of all described gene lesions causing human inherited disease. In the majority of cases, the resulting disorders are recessive in nature as a consequence of the haploinsufficiency resulting from the NMD-induced absence of the truncated proteins (which ensures that such polypeptides do not interfere with the function of the wild-type protein). Nonsense mutations that do not elicit NMD can, however, give rise to a dominant negative condition (e.g., mutations in the *SOX10* gene causing Waardenburg-Shah syndrome (160)). Since, for NMD to be activated, the nonsense mutation must reside at least 50-55 nt upstream of an exon-exon boundary, it follows that the precise location of the nonsense mutation could be an important factor in predicting the pathogenicity of that lesion. By way of example, nonsense mutations within the last exon of the human β-globin (*HBB*) gene do not elicit NMD. As a consequence, the truncated β-globin product has near-normal abundance, fails to associate properly with α-globin, and hence gives rise to a dominantly inherited form of α-thalassemia (318). Different nonsense mutations within the same gene may thus be associated with different clinical phenotypes depending upon whether or not NMD is activated. Another example of this is provided by a nonsense mutation (Q37X) in the *DAX1* gene of an adrenal hypoplasia congenita patient; this lesion is associated with a milder clinical phenotype than expected on account of the expression of a partially functional, amino terminal-truncated DAX1 protein synthesized from an alternative in-frame translational start site at Met83 (254).

In practical terms, the observation of greatly reduced or absent cytoplasmic mRNA associated with nonsense mutations has important implications for mutation screening. Thus, attempts to obtain mRNA for RT-PCR and mutation detection may result in amplification of nucleic acid from only the non-nonsense mutation-bearing allele. Nonsense mutations in the factor VIII (*F8*) gene (hemophilia A) and fibrillin (*FBN1*) gene (Marfan syndrome) have been associated with the skipping of exons containing these mutations (95, 240), and this observation has now been extended to other genes; exon skipping is either complete or partial. The mechanism underlying this phenomenon is unknown although a number of intriguing models have been proposed (118).

10.3.2.14 Unstable Protein Mutants

Missense mutations can cause abnormal protein folding and are therefore associated with reduced expression owing to instability of the protein. Reviews of mutations that affect protein stability can be found in (6, 258). For proteins that circulate in body fluids, most mutations are associated with 'CRM-negative' status in which the amount of protein correlates with the amount of activity or "CRM-reduced" status in which the amount of activity is still lower than the amount of protein produced. Many such mutations have been seen in factor VIII causing mild/moderate hemophilia A (14).

The nature of the biophysical properties of amino acid substitutions in p53 that increase their likelihood of coming to clinical attention has been explored (239); these include solvent inaccessibility, the number of adverse steric interactions introduced and a reduction in H-bond number. This study was extended by modeling *in silico* all amino acid replacements that could potentially have arisen from an inherited single base-pair substitution in five human genes encoding arylsulfatase A (*ARSA*), antithrombin III (*SERPINC1*), protein C (*PROC*), phenylalanine hydroxylase (*PAH*), and transthyretin (*TTR*) (317). A total of 9,795 possible mutant structures were modeled and 20 different biophysical parameters assessed. Comparison with the HGMD-derived spectra of 469 clinically detected mutations indicated that several types of mutation-associated change affected protein function, including the energy difference between wild-type and mutant structures, solvent accessibility of the mutated residue, and distance from the binding/active site. These parameters are considered to be important in protein folding, which adds support to the view that many missense mutations come to clinical attention by virtue of their consequences for protein folding and stability (38, 135) .

10.3.2.15 Mutations in Remote Promoter Elements/Locus Control Regions

In the β-globin gene cluster, a regulatory region about 10 kb upstream of the ε-globin (*HBE*) gene has been identified that is capable of directing a high level of position-independent β-globin gene expression (137). This region, termed the locus control region (LCR), is thought to organize the entire 60-kb β-globin gene cluster into an active chromatin domain and to enhance the transcription of individual globin genes (310). A similar LCR is also present in the α-globin gene cluster and other gene clusters (339). Deletions of the LCR in the β-globin gene cluster result in silencing of the β-globin and other genes of the cluster, even though the genes themselves are intact (346). A particular 25-kb deletion, known as Hispanic γδβ-thalassemia, which deletes sequences 9.5–39 kb upstream of the ε-globin gene including the LCR, renders the β-globin gene 60 kb downstream of the deletion nonfunctional (100). This extraordinary effect of the deletion of the LCR is thought to be due to an altered (DNase I-resistant) state of chromatin associated with nonfunctional genes. Several other examples of similar deletions in the LCR of the α-globin gene cluster have been reported (209).

10.3.2.16 Cellular Consequences of Trinucleotide Repeat Expansions

Trinucleotide repeat expansion has been discussed earlier. In the case of fragile X, the $(CGG)_n$ repeat is located in the 5' UTR of the *FMR1* gene and its expansion to full mutation results in hypermethylation of the promoter region, loss of transcription, and hence silencing of the gene (344). Loss of the encoded protein, FMRP, which is thought to play a role in dendritic mRNA transport and translation, is responsible for the classical fragile X syndrome phenotype. Gene inactivation can also be caused by altering the spacing of promoter elements from the transcriptional start site as in the case of the 12mer repeat expansion in the *CSTB* gene (202).

When the trinucleotide repeat lies within the gene coding region, as in Huntington disease, its expansion results in an abnormal protein with a gain of function owing to the enlargement of the polyglutamine tract. Mutant huntingtin exerts its pathologic effects via abnormal protein aggregation, transcriptional dysregulation, mitochondrial dysfunction, excitotoxicity, and abnormal cellular trafficking, leading to neuronal loss particularly in the dorsal substratum (35).

Another example of a gain-of-function mutation is provided by the expansion of the CTG repeat in the 3' UTR of the *DMPK* gene causing type 1 myotonic dystrophy (DM1). This does not abolish transcription but rather causes nuclear retention of RNA transcripts leading to the transcriptional dysregulation of other genes (83). CTG expansion appears to lead to the sequestration of cellular RNA-binding proteins which in turn gives rise to the abnormal splicing of multiple transcripts. DM1 thus exemplifies a disease whose mechanistic basis lies at the RNA level.

10.3.2.17 Mutations Producing Inappropriate Gene Expression

Hereditary persistence of fetal hemoglobin (HPFH) and hereditary persistence of α-fetoprotein (HPAFP) are two clinical conditions that are prototypes for the inappropriate expression of γ-globin (*HBG1 and HBG2*) and α-fetoprotein (*AFP*) genes, respectively. Normally the levels of fetal hemoglobin (HbF; α2γ2) in adult life are very low, as there is a switch from fetal to adult hemoglobin during the perinatal period. Similarly, AFP is produced at a high level in fetal liver but declines rapidly after birth. In HPFH and HPAFP, however, the levels of HbF and AFP, respectively, are inappropriately high in adult life. This is often due to single nucleotide substitutions in the promoter regions of the *HBG2*, *HBG1*, or *AFP* genes. A considerable number of mutations that occur in the region -114 to -202 of the γ-globin genes have been characterized and presumably cause persistent expression of their corresponding genes (346). A similar situation has been observed with a -119 mutation in the *AFP* gene (224). These mutations occur within DNA-binding motifs for transcriptional regulators.

A very interesting mutational mechanism has been proposed for facioscapulohumeral muscular dystrophy (FSHD), an autosomal dominant myopathy. This disease is characterized by deletions of a tandem 3.3-kb repeat termed D4Z4 on chromosome 4q35. In the general population, the size of the D4Z4 repeat array may vary between 11 and 150 units, whereas FSHD patients carry fewer than 11 repeats (142). Partial deletion of D4Z4 leads to a local change in chromatin structure (267). As a consequence, genes expressed in muscle and located up to 3 Mb upstream of D4Z4 are inappropriately overexpressed. A multiprotein complex binds D4Z4 and appears to mediate the transcriptional repression of neighboring genes. The deletion of an integral number of D4Z4 repeats below a certain threshold reduces the number of bound repression complexes, and consequently decreases transcriptional repression of 4q35 genes including the *ANT1* gene, an excellent candidate for contributing to the pathogenesis of FSHD (121).

10.3.2.18 Position Effect in Human Disorders

In several instances, a DNA alteration is found well outside the putative gene that is primarily involved with a disease. Mutations acting by "positional effect" are those in which the transcription unit and minimal promoter of the gene remain intact but there is a nearby alteration that influences gene expression (180). These positional effect DNA lesions may involve distal promoter regions, enhancer/silencer elements, or changes in the local chromatin environment. The positional effect could be up to several megabases away from the gene of interest. The examples of the LCR in the β-globin gene cluster and the transcriptional repressor

D4Z4 in FSHD are provided elsewhere in this chapter. Most of the position effects are due to chromosomal rearrangements that frequently lead to alteration of the chromatin environment of the gene. Possible mechanisms that may lead to a positional effect include: (a) separation of the transcription unit from distant *cis*-regulatory elements by the rearrangement (enhancer removal results in gene silencing, whereas silencer removal results in inappropriate gene activation); (b) juxtaposition of the gene with an enhancer element from another part of the genome; (c) removal of an insulator or boundary element may also lead to inappropriate gene silencing; (d) enhancer competition of DNA sequences that were juxtaposed to the gene; (e) positional effect variegation in which the chromosomal rearrangement causes the juxtaposition of an euchromatic gene with a region of heterochromatin.

Some examples of positional effect mutations attributable to translocation breakpoints include genes *PAX6* in aniridia (109), *SOX9* in campomelic dysplasia (268, 331), *POU3F4* in X-linked deafness (88), *HOXD* complex in mesomelic dysplasia (308), *FOXL2* in blepharophimosis/ptosis/epicanthus inversus syndrome (BPES) (31, 79), and the *SHH* gene in preaxial polydactyly (207). In these cases, the translocation breakpoints may be in excess of a megabase away from the inappropriately expressed/silenced gene. Indeed, in one example of campomelic dysplasia, the breakpoint maps ~1.3 Mb downstream of the *SOX9* gene, making this the longest range position effect so far found (331). For a recent review of position effect mutations, see (181).

It is likely that, in the majority of cases, the position effect involves a highly conserved *cis*-acting regulatory element. These *conserved noncoding elements* (CNCs; also termed multiple-species conserved sequences (MCS), conserved non-genic sequences (CNGs); the most highly conserved are also called ultraconserved elements (UCEs)) comprise approximately 1–2% of the human genome and represent potential targets for pathogenic mutations (27, 33, 93, 94, 319). An example of such a lesion is provided by the 52-kb deletion of a large noncoding region downstream of the sclerostin (*SOST*) gene in patients with van Buchem disease, leading to altered expression of the *SOST* gene (215). The deletion disrupts a bone-specific enhancer element that drives *SOST* gene expression.

Pathogenic mutation may also occur in nonconserved elements that could become functional after the introduction of the mutant sequence. This pathogenetic mechanism has been described underlying a variant form of α-thalassemia. Affected individuals from Melanesia have a gain-of-function regulatory single-nucleotide polymorphism (rSNP) in a nongenic region between the α-globin genes and their upstream regulatory elements. The rSNP creates a new promoter-like element that interferes with the normal activation of all downstream α-like globin genes (85).

10.3.2.19 Position Effect by an Antisense RNA

An individual with an inherited α-thalassemia has been described who has a deletion that results in a truncated, widely expressed gene (*LUC7L*) becoming juxtaposed to a structurally normal α-globin (*HBA2*) gene. Although it retained all of its local and remote *cis*-regulatory elements, expression of the *HBA2* gene was nevertheless silenced and its CpG island became completely methylated at an early stage during development. The antisense RNA of the *LUC7L* gene appears to have been responsible for the silencing of the *HBA2* gene (324).

10.3.2.20 Abnormal Proteins Due to Fusion of Two Different Genes

The translation of fusion genes results in novel proteins with different or abnormal properties from their parent polypeptides. Fusion genes are either the result of (1) homologous unequal crossing-over, or (2) junction sequences at breakpoints of chromosomal translocations. Hemoglobin Lepore, a fusion of δ- and β-globin genes, is the prime example of the first mechanism. Other examples of abnormal fusion genes caused by unequal crossover include the case of glucocorticoid-suppressible hyperaldosteronism (GSH), an autosomal dominant form of hypertension, caused by oversecretion of aldosterone (262); some GSH patients have hybrid genes between *CYP11B1* and *CYP11B2,* two highly homologous cytochrome P450 genes on 8q22. The hybrid gene contains the regulatory elements of *CYP11B1,* expressed in the adrenal gland, and the 3’ coding region of *CYP11B2,* which is essential for aldosterone synthesis. Another example is the case of abnormalities of color vision resulting from fusion of the green and red color pigment (*RCP*, *GCP*) genes (239). Recombination between the Kallmann gene on Xp22.3 (*KALX*) and its homolog (*KALY*) at Yp11.21

results in a fusion gene that is transcriptionally inactive and is associated with Kallmann syndrome secondary to an X;Y translocation.

A growing number of hematologic malignancies are associated with abnormal fusion proteins, the genes of which are found at the breakpoints of chromosomal translocations. One of the first reported examples was the case of fusion of the *BCR* and *ABL* genes in the t(9;22) known as Philadelphia (Ph) chromosome in chronic myelogenous leukemia. The *BCR* gene is on chromosome 22 and the *ABL* gene is on chromosome 9; after the translocation junction, a fusion gene is created with the promoter elements of the *ABL* gene and the 3' half of the *BCR* gene (25). A new abnormal protein is detected in the leukemia cells, the abnormal function of which probably contributes to the malignant phenotype. Another example is the case of Ewing sarcoma (a solid tumor of bone) in which an 11;22 translocation results in a fusion of the *FLI1* gene on 11q24 with the *EWS* gene on 22q12 (89); for a classic review see (272)). Fusion genes can be readily identified by PCR and can serve either as diagnostic indicators for relapse in the disorders concerned or as indicators of the need for an alternative therapeutic regimen.

10.3.2.21 Mutations in Genes Involved in Mismatch Repair Associated with Genomic Instability in the Soma

The study of somatic mutation is extremely important both for the study of cancer (116) and other diseases such as paroxysmal nocturnal hemoglobinuria (107). Mutations that lead to abnormal or abolished function of genes encoding for proteins involved in DNA mismatch repair are of particular importance because they lead to accumulation of mutations throughout the genome. For example, some forms of hereditary nonpolyposis colon cancer (HNPCC), which may account for up to 10% of colon carcinoma, are due to mutations in genes such as *MSH2* or *MLH1* that encode mismatch repair proteins (113, 204, 260) . In families with mutations in these genes, the DNA of tumor tissue shows considerable instability as detected by the generation of new alleles for numerous DNA polymorphic markers (161). One of the genes affected by the genomic instability is that encoding the type II transforming growth factor-β (TGF-β) receptor (*TGFB2R*), which has a run of 10 adenines in its coding region. This run of As is altered, resulting in a frameshift and absence of the receptor, which in turn releases the cell from TGF-β-inhibitory effects and contributes to malignancy (220). The discovery and further study of genes of the mutation repair system will enhance our understanding both of germline and of somatic mutations.

To date, relatively few studies have attempted to compare germline and somatic mutational spectra for the same genes. This notwithstanding, the mutational mechanisms underlying single base-pair substitutions (290, 328), micro-deletions and micro-insertions (134, 166, 328), and even gross gene rearrangements (184, 246) often appear to exhibit similarities between the germline and the soma.

10.3.2.22 Mosaicism

Germline mosaicism is a relatively frequent mechanism of inherited disease and provides an explanation for the inheritance pattern in cases where multiple affected offspring are born to clinically and phenotypically normal parents (367). It arises through the occurrence of a mutation de novo in a germline cell or one of its precursors during the early embryonic development of the parent. Since mitotic divisions predominate in both spermatogenesis and oogenesis, most germline mutations are likely to be mitotic rather than meiotic in origin. *Somatic mosaicism* results from mutations occurring during mitotic cell divisions in the embryo with subsequent clonal expansion of the affected cells (139). The clinical effect of somatic mosaicism depends critically upon the developmental stage at which the mutation occurs. Thus, a mutation that occurs very early on in embryonic development is likely to affect many somatic tissues. By contrast, mutations occurring rather later may give rise to a phenotype that is confined to a single body region or even to a single organ. Somatic mosaicism arising at a very early embryonic stage can involve both somatic cells and germ cells. Such individuals (*gonosomal mosaics*) are at risk of having affected children.

10.3.2.23 Sex Differences in Mutation Rates

Sex differences in mutation rates may have a variety of different underlying causes. For *premeiotic mutations*, the single most important factors are likely to be the much higher number of cell divisions during spermatogenesis than oogenesis and the fact that the number of male germ cell divisions experienced is age dependent (81). However, the likelihood of a given mutation having originated in a particular parent is often dependent upon the nature of the mutation in question. In general, point mutations tend to display a paternal bias, arising during spermatogenesis, whilst gross deletions tend to occur predominantly in females, having originated during oogenesis (26, 136).

10.3.2.24 Concepts of Dominance and Recessiveness in Relation to the Underlying Mutations

A genetic character is held to be *dominant* if it is manifest in the heterozygous state and *recessive* if it is not. Thus, for a truly dominant condition, homozygotes should be clinically and phenotypically indistinguishable from heterozygotes (349). If this is not so, and the homozygote is more seriously affected, then the respective alleles may be regarded as *semi-dominant* (366).

In general, most recessive alleles are loss-of-function alleles and include gross gene deletions and rearrangements, frameshift mutations, nonsense mutations, etc. By contrast, dominant alleles are often associated with gain of function, resulting either from dominant negative mutations (which interfere with and hence abrogate the function of the wild-type allele) or from dominant positive mutations (which confer increased, constitutive, novel or toxic activity upon the mutant protein). Examples of dominant negative mutations are to be found in the *GH1* (61) and *KIT* (309) genes, whilst dominant positive mutations have been reported in the *PMP22* (263), *GNAS1* (7), *DMPK* (234), and *SERPINA1* (253) genes. It should be noted that loss-of-function mutations (e.g., *TERT* (17) and *RUNX2* (179)) can also be associated with dominantly inherited conditions in cases where a 50% reduction in the level of the protein product is sufficient to impede function.

For X-linked diseases it is probably inappropriate to use the terms dominant and recessive, since males are hemizygous and females often display variable expressivity of their heterozygous mutations owing to skewed X-inactivation or clonal expansion (96).

10.4 General Principles of Genotype-Phenotype Correlations

Several general principles have emerged as a result of the intensive study of causative mutations in genetic disorders. The following discussion highlights some of these principles. The reader is encouraged to use the Online Mendelian Inheritance in Man (OMIM) database at http://www3.ncbi.nlm.nih.gov/Omim for further information or for specific genes and clinical phenotypes. Wolf's review (353) provides an excellent guide to the complex issues inherent in the study of the relationship between mutant genotype and clinical phenotype.

Mutations in the Same Gene may be Responsible for More than One Disorder. There are many examples to illustrate the principle that mutations in a single gene can cause different and distinct clinical phenotypes ("allelic heterogeneity"). Historically, the first example is that of the β-globin (*HBB*) gene on 11pter. Mutations of this gene cause β-thalassemia, sickle cell disease, and methemoglobinemia. The *L1CAM* gene on Xq28 has been shown to be mutated in hydrocephalus and stenosis of aqueduct of Sylvius, MASA syndrome (*m*ental retardation, *a*phasia, shuffing gait, *a*dducted thumbs), and spastic paraplegia 1. The *COL1A2* gene on 7q21-q22 is involved in four different clinical forms of osteogenesis imperfecta (types II, III, IV, and atypical) and in Ehlers-Danlos syndrome type VII B. The fibroblast growth factor receptor 2 (*FGFR2*) gene is mutated in three different craniosynostosis syndromes, namely Pfeiffer, Crouzon, and Jackson-Weiss. The *COL2A1* gene is implicated in Stickler syndrome type 1, SED congenita, Kneist dysplasia, achondrogenesis-hypochondrogenesis type 2, precocious osteoarthritis, Wagner syndrome type 2, and SMED Strudwick type. In a survey of 1014 genes causing disorders in OMIM, 165 genes were associated with two disorders, 52 genes with three disorders, 24 genes with four disorders, and 19 genes with five or more disorders (11).

One Disorder May Be Caused by Mutations in More than One Gene. There are a plethora of similar clinical phenotypes caused by mutations in different genes. This observation, also known as "nonallelic" or "locus" heterogeneity, is well understood, thanks to linkage analyzes for genetic disorders and the search for mutations in different genes. Thus, tuberous sclerosis, a relatively common autosomal dominant disorder, is caused by lesions in at least two different loci: *TSC1* on 9q34 and *TSC2* on 16p13.3. Approximately 60% of TSC families show linkage to the *TSC2* locus and 40% to the *TSC1* locus. Hereditary nonpolyposis colon cancer has been associated with mutations in five different genes. *MLH1* on 3p, *MSH2* on 2p16, *PMS1* on 2q31-q33, *PMS2* on 7p22 and *MSH6* on 2p16. Retinitis pigmentosa has so far been associated with a total of 23 different genes, and the list is still growing. We expect that disorders of complex or polygenic phenotypes, such as hypertension, atherosclerosis, diabetes, schizophrenia, and manic-depressive illness, will be associated with a considerable number of genes scattered throughout the genome.

One and the Same Mutation May Give Rise to Different Clinical Phenotypes ("Polypheny"). The clinical phenotype does not only depend on the one mutation in the responsible gene; it can be modified by the action of any of the other ~25,000–30,000 genes in the genome (353). The environment can also have an important role in the full development of the clinical phenotype. The classic sickle cell disease mutation in the β-globin (*HBB*) gene (Glu6Val) may be associated with severe or mild sickle cell disease. The amelioration of the severe clinical phenotype in this case can be attributed to the increased expression of γ-globin genes and the presence of high levels of HbF. The genomic environment of the β-globin gene cluster may therefore modify the severity of sickle cell disease, as may genetic variation originating from other loci, e.g., the α-globin genes (73). Another example of this phenomenon has recently been provided by studies of certain craniosynostoses. Both Pfeiffer and Crouzon syndromes can be associated with the same C342Y or C342R mutations in the *FGFR2* gene.

The clinical phenotype associated with the D178N missense mutation in the prion protein (*PRNP*) gene is critically dependent upon the presence of the Met or Val 129 polymorphic allele to which it is coupled. When D178N lies in *cis* to the Met129 allele, fatal familial insomnia (FFI) results, whereas D178N coupled to the Val129 allele is associated with Creutzfeldt-Jakob disease (261). The Met/Val 129 polymorphism also exerts an effect in *trans* through the normal allele, since FFI is more severe and of longer duration in patients homozygous for either the Met or the Val allele.

One of the best examples of the contribution of the environment to the clinical phenotype of single gene disorders is that of phenylketonuria resulting from phenylalanine hydroxylase (PAH) deficiency. Individuals homozygous or compound heterozygous for mutations in the *PAH* gene develop severe mental handicap if fed a normal diet. However, the cognitive status remains normal if these individuals are fed with a special, "phenylalanine-free" diet.

Mutations in More than One Gene May Be Required to Express a Given Clinical Phenotype (Digenic Inheritance; Triallelic Inheritance). Digenic inheritance refers to clinical phenotypes caused by the co-inheritance of mutations in two unlinked genes. Thus one form of retinitis pigmentosa is due to the co-inheritance of mutations in the *RDS* gene on 6p *and* the *ROM* gene on 11q (170). Individuals with either one or the other mutation,do not suffer from the disease. In similar vein, digenic inheritance of mutations in the *MITF* and *TYR* genes has been reported as a cause of Waardenburg syndrome type 2 in conjunction with ocular albinism (235). This phenomenon may be common in polygenic disorders and in disorders with "low penetrance."

Triallelic inheritance refers to clinical phenotypes with apparent recessive mode of inheritance caused by the co-inheritance of three mutant alleles, two in one gene and one in another gene. An example of triallelic inheritance is provided by the Bardet–Biedl syndrome. There are pedigrees in which affected individuals have two mutant alleles in the *BBS6* gene and one mutant allele in the *BBS2* gene. Other pedigrees have two mutant alleles in the *BBS2* gene and one mutant allele in *BBS6* (173). This type of inheritance indicates that some forms of BBS have a complex pattern of inheritance. As above, this phenomenon may be relevant in polygenic disorders and in disorders with "low penetrance."

Different Mutations in the Same Gene May Give Rise to Distinct Dominant and Recessive Forms of the Same Disease. von Willebrand factor (vWF) deficiency is a relatively common monogenic disease of blood coagulation. Many mutations have been studied in the *VWF* gene on chromosome 12p. A proportion of mutations (usually deletions, nonsense codons, or frameshift mutations) cause vWF deficiency with a recessive mode of inheritance; other mutations (mostly missense substitutions), however, are associated with a dominant mode of inheritance of the vWF deficiency (243).

Whereas the majority of hitherto characterized growth hormone (*GH1*) gene lesions (including gross deletions and missense/nonsense mutations) that underlie familial short stature are inherited in autosomal recessive fashion, there is a group of intron 3 splicing mutations that are characterized by a dominant mode of inheritance (62). These lesions result in the in-frame skipping of exon 3 encoding 40 amino acids, including a Cys residue. The dominant negative nature of this mutation is thought to be explicable in terms of the participation of the resulting free unpaired cysteine residue in an illegitimate intermolecular disulfide linkage, leading to dimerization of the mutant molecule with a normal GH molecule and inhibition of GH secretion.

10.5 Why Study Mutation?

The sequencing of the human genome is now essentially complete and its annotation well under way. Full exploitation of the emerging data, specifically in relation to understanding the etiology of inherited disease and disease predisposition, is likely to be hampered by our ignorance of the basic processes underlying inter-individual, inter-population, and inter-species genetic diversity, however. At the population level, such an understanding is seen as essential for any meaningful interpretation of the prevalence/incidence patterns observed for diseases with a genetic basis. Within families, it is a prerequisite for being able to explain how inter-individual variation arises and how variable phenotypic expression can be associated with identical gene lesions. Thus, for human genome sequence data to be useful in the context of molecular medicine, they must eventually be related to the genetic variation underlying human inherited disease. To this end, the meta-analysis of pathological germline mutations in human genes should facilitate:

1. The assessment of the spectrum of known genetic variation underlying human inherited disease.
2. The identification of factors determining the propensity of DNA sequences to undergo germline mutation.
3. The optimization of mutational screening strategies.
4. Improvements in our ability to predict the clinical phenotype from knowledge of the mutant genotype.
5. The identification of disease states that exhibit incomplete mutational spectra, prompting the search for, and detection of, novel gene lesions associated with different clinical phenotypes (227).
6. Extrapolation toward the genetic basis of other, more complex traits and diseases (36).
7. Improvements in our understanding of the function of a given protein.
8. Meaningful comparison between the mechanisms of mutagenesis underlying both inherited and somatic disease.
9. Studies of human genetic diseases in their evolutionary context (303).

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Human Hemoglobin

11

George P. Patrinos and Stylianos E. Antonarakis

Abstract The hemoglobin molecule can be studied with greater facility than any other human protein. This is because blood can easily be taken from many individuals, hemoglobin is the principal protein of red blood cells, and its extraction does not require complicated biochemical methods. It is therefore not surprising that this protein is the most thoroughly studied and the globin genes the best analyzed in humans. Work on human hemoglobin began with the investigation of sickle cell disease (SCD) in 1910, and in 1925 Cooley and Lee first described a form of severe anemia associated with splenomegaly amd characteristic bone changes. This condition has been known since then as thalassemia – a term derived from the Greek word "*θαλασσα*" (thalassa=sea), referring to the Mediterranean Sea – or Cooley's anemia. Genetically oriented studies of human hemoglobins have proceeded apace, starting with the elucidation of the amino acid sequence and structure of the molecule in the 1960s. The hemoglobin system is currently a paradigm for the understanding of gene action at the molecular level. Hemoglobin research is comparable in human biochemical and molecular genetics to that of research on Drosophila and phage in basic genetics. Most concepts derived from hemoglobin research can be readily applied to other proteins, and it has been possible to teach many conceptual principles of human genetics by means of examples from the hemoglobin system. This chapter summarizes the main aspects of human hemoglobin research that has provided, overall, important insights into: (a) Protein structure and function, (b) Gene structure and expression, (c) Developmental gene regulation, (d) Long-range gene interactions and chromatin structure, (e) Gene evolution, (f) Genotype-phenotype relationships and phenotype modifiers, (g) Mechanisms of action of pathogenic mutations, (h) Polymorphisms and haplotype blocks, (i) Molecular diagnostics of inherited disorders, and (j) Gene therapy of monogenic disorders.

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11.1 Introduction

The hemoglobin molecule can be studied with greater facility than any other human protein. This is because blood can easily be taken from many individuals, hemoglobin is the principal protein of red blood cells, and its extraction does not require complicated biochemical methods. It is therefore not surprising that this protein is the most thoroughly studied and the globin genes the best analyzed in humans. Genetically oriented studies of human hemoglobins have proceeded apace, starting with the elucidation of the amino acid sequence and structure of the molecule in the 1960s. The hemoglobin system is currently a paradigm for the understanding of gene action at the molecular level. Hemoglobin research is comparable in human biochemical and molecular genetics to that of research on *Drosophila* and phage in basic genetics. Most concepts derived from hemoglobin research can be readily applied to other proteins, and it has been possible to teach many conceptual principles of human genetics by means of examples from the hemoglobin system. In particular, hemoglobin research has been instrumental in providing important insights into:

- Protein structure and function
- Gene structure and expression
- Developmental gene regulation
- Long-range gene interactions and chromatin structure
- Gene evolution
- Genotype–phenotype relationships and phenotype modifiers
- Mechanisms of action of pathogenic mutations
- Polymorphisms and haplotype blocks
- Molecular diagnostics of inherited disorders
- Gene therapy of monogenic disorders

11.2 Historical Perspectives

Work on human hemoglobin began with the investigation of sickle cell disease (SCD). In 1910, Herrick observed a peculiar sickle-shaped abnormality of red cell structure in an anemic African-American student [63]. It soon became apparent that this condition is fairly common among African Americans. Affected patients suffer from hemolytic anemia and recurrent episodes of abdominal and musculoskeletal pain. In 1923, Taliaferro and Huck recognized that the condition is hereditary [164]. In 1949 it was shown separately by Neel and Beet that patients with SCD are homozygous for a gene that causes, in heterozygosity, an innocuous condition known as sickle cell trait, which is found in about 8% of the African–American population [105]. In 1925, Cooley and Lee were the first to describe a form of severe anemia associated with splenomegaly and characteristic bone changes [25]. This condition has been known since then as thalassemia or Cooley's anemia [25]. Unlike β-thalassemia, however, the α-thalassemias have taken longer for us to understand, largely because for many years the genomic structure of the α-globin gene cluster, i.e., two α-globin gene copies per haploid genome in normal individuals, was not appreciated. The major clinical syndromes resulting from α-thalassemia (Hb H disease and the Hb Bart's hydrops fetalis syndrome) were first recognized in the mid-1950s and early 1960s through the association of the abnormal hemoglobins (Hb H and Hb Bart's) with hypochromic microcytic anemia in the absence of iron deficiency (for a review see [63]). Identification of β-like globin chain tetramers in patients with these syndromes provided evidence that these conditions result from genetic defects in the α-like globin chains [72].

In 1949, Linus Pauling and his group surmised that a defect of hemoglobin was likely to be the cause of SCD, basing this on evidence indicating that the process of sickling might be intimately associated with the state and the nature of the hemoglobin within the erythrocyte [131]. Therefore, the authors compared the hemoglobins of SCD patients and carriers, and of normal individuals. Using zone electrophoresis, Pauling et al. observed a significant difference between the electrophoretic mobilities of hemoglobin derived from erythrocytes of normal individuals and from those with SCD [131]. In hemoglobin from subjects with the sickle cell trait about 25–40% of the hemoglobin turned out to be identical with that found in SCD, whereas the remainder was indistinguishable from normal. This result was compatible with the genetic data showing that SCD represents the homozygous state of a gene variant for which SCD carriers are heterozygous. Pauling and his co-workers therefore concluded that SCD was indeed the first example of a molecular disorder.

In 1956, Ingram discovered what precisely distinguishes normal from sickle hemoglobin [71]. Using a trypsin-based fingerprinting method, he showed that sickle cell hemoglobin was identical with the normal molecule in all peptides except one. Further analysis showed that sickle cell hemoglobin differed from normal hemoglobin in only one amino acid: glutamic acid was replaced by valine at position 6 of the globin chain. Glutamic acid has two COOH groups and one NH_2 group, whereas valine has only one COOH group, resulting in a charge difference, which explained the electrophoretic differences between normal and sickle hemoglobin.

In the early 1960s, further steps of great importance in hemoglobin research were the establishment and elucidation of the full amino acid sequence of hemoglobin chains and the elucidation of the three-dimensional structure of human hemoglobin [102, 132], which led to the identification of an increasing number of other hemoglobin variants (see also below). Subsequent advances have led to our understanding of structure-function relationships and to detection of various types of mutations, such as deletions and frameshifts. Isolation of the hemoglobin mRNA led to new insights into gene structure and function and opened new paths to the understanding of gene action [138].

Molecular work on the hemoglobins has proceeded at a rapid pace since then. The full DNA sequences of the various hemoglobin genes and their flanking sequences were elucidated in the early 1980s, and the hemoglobin genes and their regulation are probably better understood than any other mammalian gene. Mutations affecting the hemoglobins, particularly the thalassemias [174], have been elucidated, and models for the understanding of gene action at the molecular level have been proposed. In 1982, Orkin et al. demonstrated that a number of sequence variations were linked to specific β-globin gene mutations [111]. These groups of restriction fragment length polymorphisms (RFLPs), termed "haplotypes" (both intergenic and intragenic), provided a first screening approach making it possible to determine which *HBB* gene is mutated [4, 111]. RFLP analysis also made it possible to track a mutant allele for pre- and postnatal diagnosis [14]. At first, the identification of the disease-causing mutation was only possible through the construction of a genomic DNA library from the affected individual, in order to first clone the mutated allele and then determine its nucleotide sequence. Again, many human globin gene mutations have been identified through such approaches [6, 17, 112, 114, 115, 167]. At the same time, in order to provide a shortcut to DNA sequencing, a number of exploratory methods for identifying mutations in patients' DNA were developed. The first methods involved mismatch detection in DNA/DNA or RNA/DNA heteroduplexes [103]. Using these laborious and time-consuming approaches, a number of sequence variations were identified, which made the design of short synthetic oligonucleotides as allele-specific probes for genomic Southern blots possible. This experimental design was quickly implemented for the detection of *HBB* gene mutations [113, 135]. With the advent of the polymerase chain reaction (PCR), characterization of the β-globin gene mutation leading to SCD was possible from a considerably smaller amount of genomic DNA and was also significantly expedited [146, 147]. In addition, the battery of diagnostic tools for globin gene mutation screening was significantly enriched. As with many other genetic disorders, DNA amplification was coupled to a rich repertoire of methodologies, either for the detection of known mutations or for screening for unknown sequence alterations inside the human globin loci [130].

The major discoveries and milestones in hemoglobin research are summarized in Table 11.1.

Table 11.1 Landmark studies and history of human hemoglobin research

Discovery	References
First observation and investigation of sickle cell disease	[62]
First report on thalassemia	[25]
Characterization of sickle cell disease as a molecular disorder	[131]
Identification of the mutation leading to sickle cell disease at the protein level	[72]
Elucidation of hemoglobin's protein sequence and three-dimensional structure	[102, 132]
Elucidation of the full globin genes DNA sequences	[10, 86, 154, 156]
Determination of human β-globin gene cluster haplotypes	[4]
Prenatal diagnosis for sickle cell disease or β-thalassemia using DNA polymorphisms	[14, 75]
Molecular diagnosis of β-thalassemia using allele specific oligonucleotide probes	[112, 135]
Molecular diagnosis of sickle cell disease using restriction site analysis	[146]
Study of developmental regulation of globin gene switching in transgenic mice	[41, 80]
Discovery of the human β-globin Locus Control Region	[56]
Molecular diagnosis of sickle cell disease using PCR-RFLP analysis	[147]
Establishment of the globin gene competition model	[176]
Successful restoration of β-thalassemia phenotype in mice using lentiviral *HBB* gene transfer	[97]
Study of the human β-globin Active Chromatin Hub	[119, 129]

11.3 Genetics of Hemoglobins

11.3.1 Hemoglobin Molecules

Human hemoglobin consists of four globin chains. The general designation of a hemoglobin (Hb) molecule is $\alpha_2\beta_2$ (Hb A), signifying that the four globin chains comprise two pairs of identical polypeptides. Each globin chain carries a heme group, a nonprotein molecule attached at a specific site of the globin molecule. The four globin chains with their respective heme groups constitute the functional hemoglobin molecule that carries oxygen from the lungs to the tissues. A globin chain is either α- or β-*like*, in which case it consists of a string of 141 (α-*like*; ζ-, and α-globin), or 146 amino acids (β-*like*; ε-, γ-, δ-, and β-globin). Most normal human hemoglobins, which are expressed at different developmental stages, have identical α-globin chains, while the non-α (or β-*like*) globin chains differ from each other (see below). The primary DNA and protein sequence of all globin chains is well defined.

The principal hemoglobin of children and adults is Hb A or adult hemoglobin ($\alpha_2\beta_2$). The characteristic subunit of Hb A is the β-globin chain. Additionally, all adults carry a small amount (<3%) of Hb A_2 ($\alpha_2\delta_2$). The δ-globin chains differ in only ten amino acid positions from the β-globin chain. A small amount (<2%) of fetal hemoglobin (Hb F; $\alpha_2\gamma_2$) is also present postnatally in all individuals. The γ-globin chain differs considerably from both the α- and β-globin chains, whereas the α-globin chains of Hb A, Hb A_2, and Hb F are identical.

Several hemoglobins that are characteristic of embryonal and fetal development exist. Hemoglobin molecules of the embryonic erythropoietic stage consist of α-, ζ-, ε-, and γ-globin chains. The ζ- and ε-globin chains disappear after 8–10 weeks of embryonal life (Fig. 11.1a) [178]. The principal hemoglobin of fetal development is Hb F. There are two types of γ-globin chains with very similar properties: those with alanine at position 136 ($^{A}\gamma$) and those with glycine at the same amino acid position ($^{G}\gamma$). There is a third type of γ-globin chain, with threonine instead of isoleucine at position 75 in the γ-globin chain; this occurs with a frequency ranging up to 40% and is polymorphic in human populations [148]. Adult hemoglobin can be demonstrated in fetuses as early as at the 6–8-week stage [178].

The embryonic erythropoietic site in humans is the yolk sac, where the α-, ζ-, and ε-globin chains are produced. The transition from primitive to definitive erythropoiesis coincides with a switch in erythropoietic sites from the yolk sac to the fetal liver, where α- and γ-globin chain synthesis largely occurs. Conversely, α-, β-, and δ-globin chains in childhood and later in adulthood are produced in the bone marrow and spleen (Fig. 11.1b).

11.3.2 Hemoglobin Genes

The amino acid sequence of each of the globin chains is specified by a unique globin gene. The human globin genes are arranged in multigene clusters, namely, the α-*like* and β-*like* globin gene cluster, a frequent type of organization of mammalian genes.

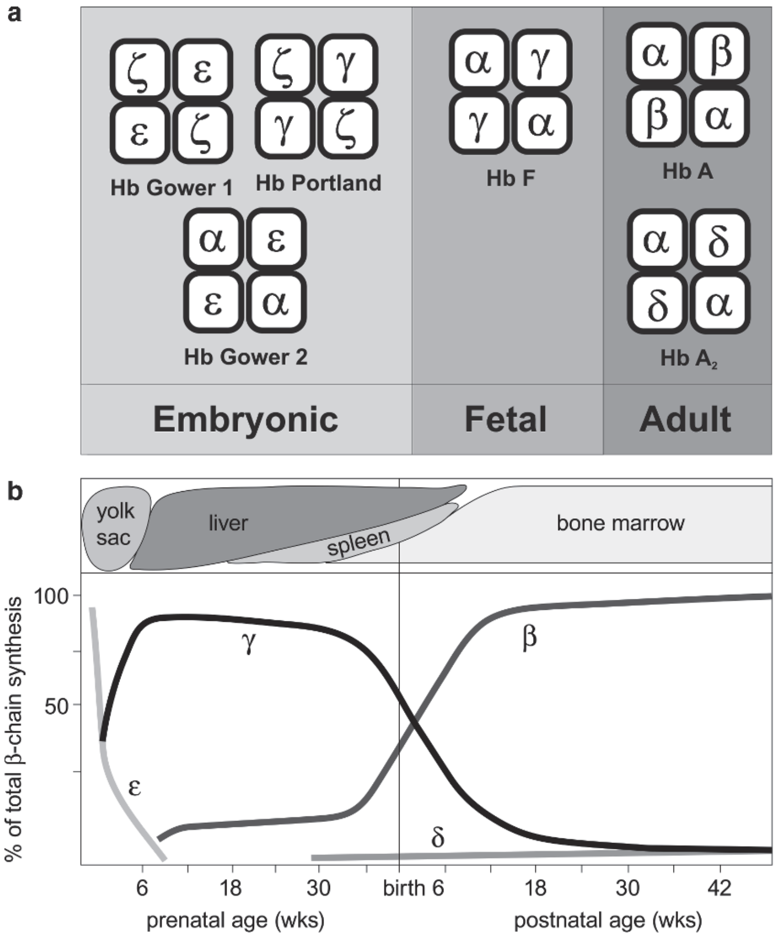


Fig. 11.1 (**a**) Hemoglobin tetramers during the embryonic, fetal and adult developmental stages. (**b**) Globin chain synthesis during development. The various tissues which contribute to hematopoiesis at the various developmental stages are shown above (see also text for details)

The α-globin gene exists in two copies, namely, α2 (*HBA2*)- and α1 (*HBA1*)-globin genes, yielding an identical protein product. The latter, together with the ζ-globin gene (*HBZ*) are resident on chromosome 16. The structure of the α-*like* globin gene cluster [from 5′ (upstream) to 3′ (downstream)] include: the embryonic *HBZ* gene, a *HBZP* pseudogene, two α-globin pseudogenes, namely, *HBAP2* and *HBAP1*, two identical functional α-globin genes, namely, *HBA2* and *HBA1*, and the θ-globin gene of unknown function, (*HBQ*1; Fig. 11.2a) [96]. Similarly, the location of the various genes on the β-globin gene cluster are: the embryonic ε-globin gene (*HBE*), two fetal (γ)-globin genes, namely, ^{G}γ-(*HBG2*) and ^{A}γ-(*HBG1*), the ψβ-globin pseudogene (*HBBP*), a δ-globin gene (*HBD*), and a β-globin gene (*HBB*; Fig. 11.2a). The 5′ to 3′ arrangement of the genes in both clusters is in the order of their ontogenetic expression during development. Extensive DNA sequence analysis has been carried out on all hemoglobin genes, and their primary sequence has been fully characterized [10, 24, 86, 154, 156].

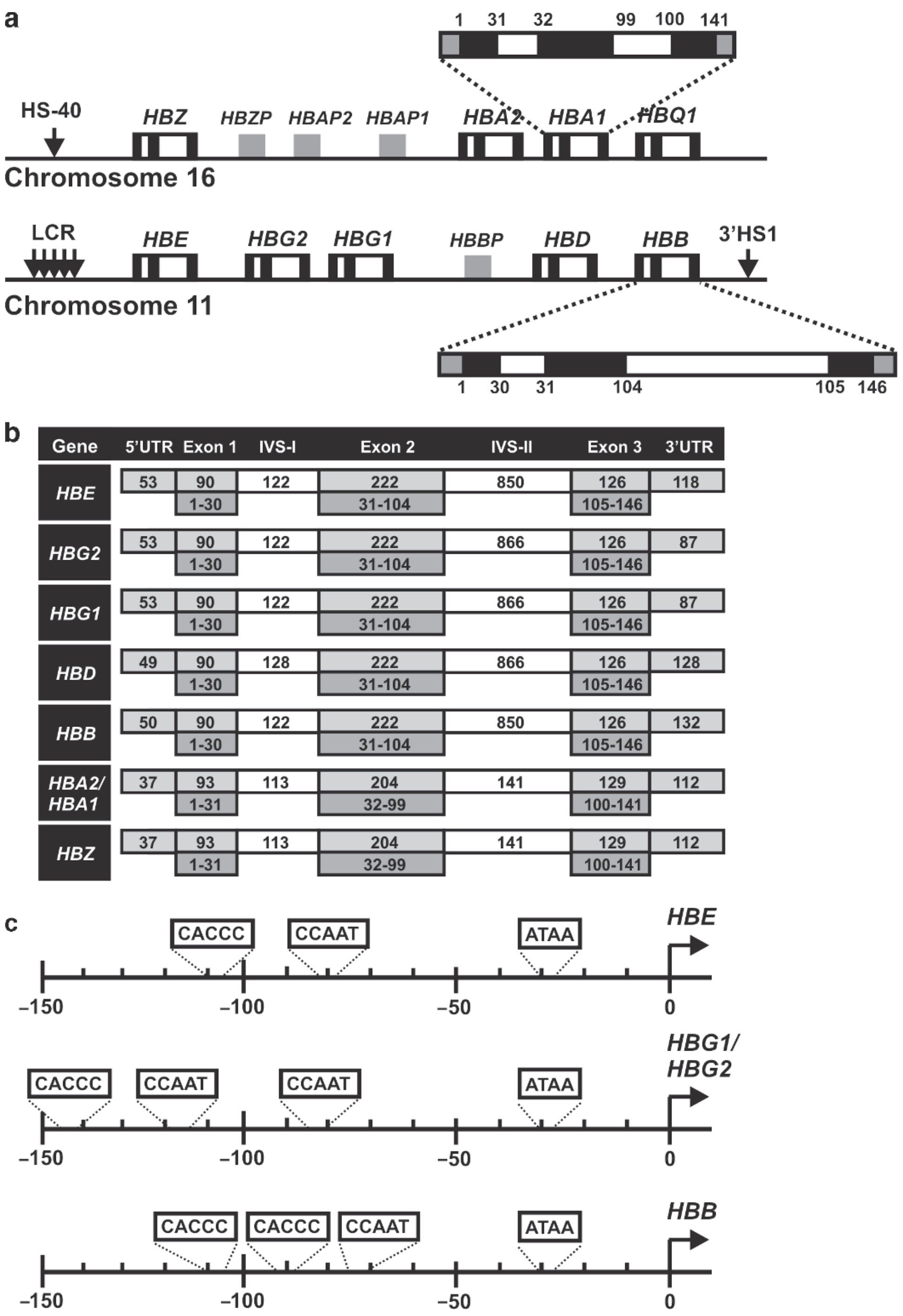
All the globin genes have many functional similarities in organization. Three exons or coding sequences code for the unique amino acid sequence of each globin chain. Between exons 1 and 2 and between exons 2 and 3 there are intervening sequences (IVS) or introns known as IVS-I and IVS-II, respectively (Fig. 11.2b).

11.3.3 Regulatory Elements

Transcriptional regulation of the human globin clusters is achieved by proximal and distal *cis*-regulatory elements. Promoter, enhancer, and silencer elements govern the proper developmental expression of individual globin genes. Each proximal regulatory element, whether promoter, enhancer, or silencer, harbors binding sites for erythroid-specific and ubiquitously expressed transcription factors, which interact with other co-factor(s) to regulate gene expression. Each globin gene has a characteristic pattern of transcription factor-binding sites, consisting of a “TATA box,” accompanied by two other globin-specific motifs, namely, the “CCAAT box” and the “CACCC box.” In addition, major regulatory elements, located at the 5′ end of each cluster, such as the hypersensitive site (HS)-40 in the α-globin gene cluster and the locus control region (LCR) in the β-globin gene cluster, contribute to high-level, tissue-specific, and developmentally controlled expression of the globin genes. The proximal regulatory elements are thought to interact with the LCR in achieving control of individual gene expression at the various developmental stages in a well-defined nuclear territory, termed the “active chromatin hub” (ACH; see also below).

The HBE Gene. The *HBE* promoter has canonical CACCC, CCAAT, and TATA boxes in a 5′>3′ orientation (Fig. 11.2c). Erythroid-specific transcription factor binding, such as GATA-1 and most likely NF-E2, is vital for proper tissue-specific expression of the *HBE* gene [54]. Studies in transgenic mice have shown that the *HBE* gene displays autonomous developmental control, namely, activation and silencing, at the fetal hematopoietic stage. The latter is achieved by a 275-bp silencer element, located 467 bp upstream of *HBE* Cap site. This silencer contains GATA-1- and YY1-binding sites, and when deleted in the context of transgenic mice the *HBE* gene loses its developmental specificity and remains expressed during definitive hematopoiesis [142].

The HBG2 and HBG1 Genes. Like their coding sequences, the sequences of the γ-globin gene promoters are almost identical owing to a recent gene conversion event (see below) and contain (in a 5′>3′ orientation) a CACCC box, two CCAAT boxes within a duplicated 27-bp segment, and the TATA box (Fig. 11.2c). In contrast to the *HBE* promoter and coding regions, in which no mutations have been found [120], both γ-globin gene promoters harbor many mutations, mostly in regulatory motifs, which result in persistent high fetal hemoglobin levels in adults, an inherited condition known as hereditary persistence of fetal hemoglobin (HPFH). These mutants have contributed to the enhancement of our understanding of the molecular mechanisms governing the down-regulation of the γ-globin genes in the adult. Although the distal CCAAT box harbors several mutations, leading to HPFH, the proximal CCAAT box is virtually mutation free, probably suggesting that mutations in the proximal CCAAT box are incompatible with fetal life or that these mutations are very mild. Although experimental evidence from transgenic mice suggests that the γ-globin, like the *HBE*, genes silence autonomously during adult life [32], the γ-globin gene silencer region(s) remains to be discovered. Stamatoyannopoulos



Gene	5'UTR	Exon 1	IVS-I	Exon 2	IVS-II	Exon 3	3'UTR
HBE	53	90 1-30	122	222 31-104	850	126 105-146	118
HBG2	53	90 1-30	122	222 31-104	866	126 105-146	87
HBG1	53	90 1-30	122	222 31-104	866	126 105-146	87
HBD	49	90 1-30	128	222 31-104	866	126 105-146	128
HBB	50	90 1-30	122	222 31-104	850	126 105-146	132
HBA2/ HBA1	37	93 1-31	113	204 32-99	141	129 100-141	112
HBZ	37	93 1-31	113	204 32-99	141	129 100-141	112

Fig. 11.2 (**a**) Schematic drawing of the human α-*like* and β-*like* globin gene clusters. Exons and intervening sequences of functional genes are depicted by *black* and *white boxes*, respectively, and pseudogenes as *light gray boxes*. *Dark gray boxes* represent the 5′ and 3′ untranslated regions. *Numbering* corresponds to the first and last amino acid of each exon. (**b**) Comparison of the sizes of the 5′ and 3′ untranslated regions, exons and intervening sequences of the human globin genes. *Numbers* in the *upper part*, i.e., *light gray and white boxes*, depict the length of each fragment (in bp), while the numbers in the *lower part* (*dark gray boxes*) indicate the amino acid range of each exon. (**c**) Position of the *cis*-regulatory elements of the human β-*like* globin gene promoters, relative to their Cap site (indicated with an *arrow*)

et al. have demonstrated that a 354-bp region, located -732 bp upstream of the γ-globin genes Cap site, may act as a silencing element and, when deleted, result in the continuing expression of γ-globin genes in adult hematopoiesis [159].

A 750-bp region downstream of the *HBG1* gene has been shown to display enhancer activity, based on cellular expression studies [13]. This fragment harbors eight footprints, three of which are GATA-1-binding motifs [139]. Subsequently, the same fragment has been shown to silence the expression of a reporter gene [91]. Altogether, these data suggest that the function of this "Aγ-enhancer" element remains enigmatic.

The HBB and HBD Genes. The *HBB* gene promoter contains (in a 5′>3′ orientation) two CACCC boxes, a CCAAT box, and a TATA box (Fig. 11.2c). Of the two CACCC boxes, the proximal one appears to play the more important functional role, since naturally occurring mutations in this box, as well as in the TATA box, have been shown to cause mild β-thalassemia. GATA-1 and KLF-1 [or erythroid Krüppel-*like* factor (ELKF)] are the most important erythroid-specific transcription factors that bind to the *HBB* promoter. In particular, targeted deletion of the murine *KLF-1* gene results in a lethal phenotype in the early definitive hematopoietic stage, owing to severe dyserythropoiesis [107]. The human *HBB* gene remains silent during fetal and adult erythropoiesis in *KLF-1*-targeted mice carrying a human β-globin locus transgene and presents with a nonpermissive chromatin configuration [177].

Also, two enhancers, within and downstream of the *HBB* gene, are likely to contol *HBB* gene expression [9]. The intragenic enhancer is located between IVS-II and exon 3, while the downstream enhancer is located just after the *HBB* polyadenylation site. Both enhancer elements contain at least four GATA-1-binding sites, which most likely account for their enhancer properties. Although the *HBB* and the *HBD* genes differ only in ten amino acids in their coding regions, they vary substantially in their promoter regions. *HBD* gene transcription is far less efficient than *HBB* gene transcription [162], which is reflected in the HbA_2 and HbA concentrations, respectively.

The HBA2/HBA1 and HBZ Genes. Located in a different chromosome than the β-*like* globin genes, *HBA2/HBA1* and *HBZ* genes have several unique features (Table 11.2). Like the fetal globin genes, *HBA2/HBA1* promoters and coding regions are virtually identical, containing TATA and CCAAT boxes but lacking a CACCC box, despite being GC rich. On the other hand, the *HBZ* gene has the typical globin gene promoter arrangement, with CACCC, CCAAT and TATA boxes in a 5′>3′ orientation. In all cases, GATA-1-binding sites have been identified, along with the ubiquitous transcription factor Sp1 motifs [172].

Table 11.2 Comparison of the general features of the α- and β-globin gene clusters, suggesting that the chromosomal regions on which the α- and β-globin gene clusters reside are structurally different, reflecting in the differences in transcription, repair, recombination and replication

	Gene cluster	
Features	**α-Globin**	**β-Globin**
Location		
Chromosomal location	Telomeric (16p13.3)	Interstitial (11p15.5)
GC content	54%	39.5%
CpG islands	Common	None
Gene density	High	Low
Alu family repeats	25%	~5%
LINE repeats	Rare	Present
Chromatin structure and transcription		
Chromatin	Open	Closed → Open
Matrix attachment sites	None detected	Common
Major regulatory element	Enhancer (HS-40)	Locus Control Region
Effect of deletion of major regulatory element on long -range chromatin structure	No	Yes
Expression in transient expression assays	Enhancer independent	Enhancer dependent
Expression in cell hybrids	Early	Late
Other features		
Replication timing	Early	Late → Early
Predominant mutations	Deletions	Point mutations
Evolution of intergenic regions	Rapid	Slow

The Human β-Globin Locus Control Region. The human β-globin locus control region (or LCR) is the major regulatory element governing proper transcriptional regulation of the human β-*like* globin genes. It is located at the 5′ end of the β-globin gene cluster, approximately 20 kb upstream of the *HBE* gene, and consists of five erythroid-specific DNaseI-hypersensitive sites (HS1-5). The first indication of its importance was obtained when Kioussis et al. [78], identified a deletion mutant in which the *HBB* gene was silent, but structurally normal [79]. Subsequently, two other deletion mutants have been identified, one of which was removing only four of the LCR HSs (HS2-5), leaving the entire β-*like* globin genes, though structurally intact, transcriptionally silent (Fig. 11.3a). Studies in transgenic mice have shown that linkage of the LCR to the *HBB* gene confers high-level copy number-dependent and position-of-integration-independent expression of this gene [56]. This latter feature is what distinguishes the LCR from a classic enhancer element. It is noteworthy that prior to the discovery of the LCR, *HBB* transgenes without LCRs were only expressed in 50% of the transgenic mouse lines and at about 0.1–3% of their expected transcription rate [80].

Each HS contains a 250- to 500-bp core region, which is largely responsible for the HS activities. The core regions contain multiple erythroid-specific and ubiquitous transcription factor-binding sites. From a plethora of experiments it has been shown that HS2-4 display enhancer activities, with HS2 being the strongest enhancer among them, most likely because of the presence of an NF-E2/AP1 transcription-binding site [106]. HS5 functions as a chromatin insulator [44], where a binding site for the protein CCCTC-binding factor (CTCF), which acts as a chromatin insulator [108], has been found. The function of HS1 remains to be defined.

Another property of the LCR is its ability to confer copy number-dependent and position-of-integration-independent expression on a linked gene [56]. Copy number-dependent expression is widely considered to be indicative of permissive chromatin structure, i.e., DNA that is accessible to transcription factors. Involvement of the β-globin LCR in creating open chromatin was first suggested from analysis of β-thalassemia deletional mutants, where part of the LCR was deleted but the β-*like* globin genes were structurally intact [46, 79] (Fig. 11.3a]. In keeping with this, only the

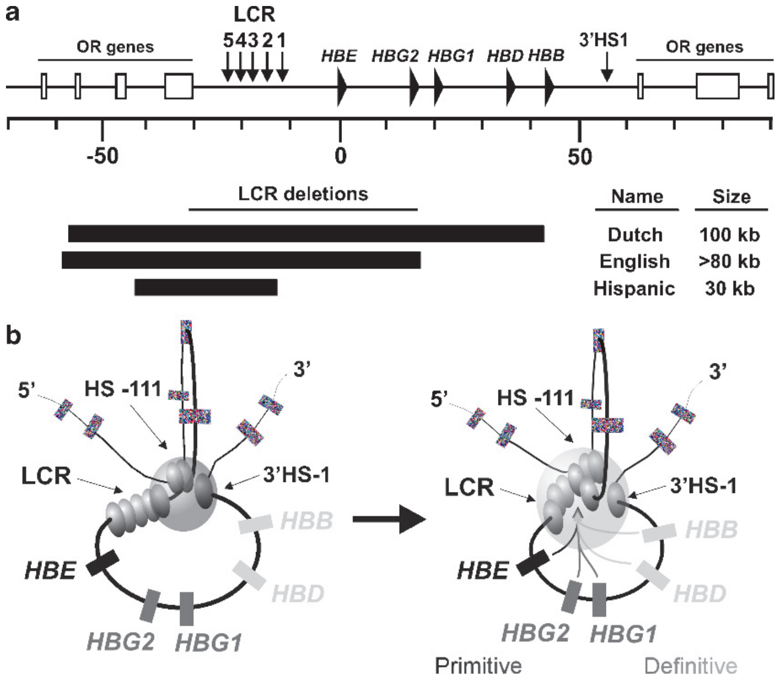


Fig. 11.3 (**a**) Representation of the large deletions identified to date, removing part or the entire LCR, leading to β-thalassemia. *Black arrows* represent the functional globin genes. (**b**) Model of the three-dimensional structure of the human β-like globin gene cluster. The chromatin hub (CH), indicated as a *dark gray circle*, is formed from the distant HSs, namely, HS-111 and 3′HS1 together with HS5 from the LCR. The genes are colored depending on their developmental order of expression, namely, embryonic (*black*), fetal (*dark gray*), and adult (*light gray*). Olfactory receptor genes are shown as *hatched gray boxes*. The active chromatin hub (ACH; shown as *light gray circle*) is formed by the remaining HSs of the LCR and the gene proximal regulatory elements, i.e., promoter, at the respective developmental stage, in which the globin gene(s) are active

full LCR (HS1-5) can provide position-independent chromatin-opening activity in transgenic mice carrying the entire β-globin locus [99]. In transgenic mice carrying single copies of gene constructs, including one of the LCR's HSs and a globin gene, only those carrying HS3 were able to confer copy number-dependent gene expression [40], suggesting that HS3 possesses the dominant chromatin-opening activity of the β-globin LCR.

Finally, the β-globin LCR can direct replication timing in a developmentally specific manner in vivo [153], resulting in the human β-globin locus being replicated late in most cell types, but early in erythroid cells [31].

The Human α-Globin Locus Control Region. The α-*like* globin genes also display tissue specificity in their developmental expression. The *HBZ* gene is expressed during the first trimester of embryonic life, whereas the *HBA2*/*HBA1* genes are expressed during the last two trimesters of gestation and later on in adult erythroid cells. Although tissue specificity and developmental expression pattern are identical to those of β-*like* globin genes, the regulatory mechanisms governing human α-*like* globin gene cluster expression display some distinct features (Table 11.2). The existence of an α-*like* LCR has been established by naturally occurring deletion mutants leading to α-thalassemia, in which the α-globin genes are silenced, albeit structurally intact [60, 144]. In addition, the *HBA2*/*HBA1* genes alone are expressed in low levels either in cultured erythroid cells or in transgenic mice, but when linked to the β-*like* LCR they are then expressed in high levels and with erythroid specificity [145]. A systematic search for LCR activity revealed an HS 40 kb upstream of the human α-globin gene cluster (termed therefore "HS-40"), displaying properties of a strong erythroid-specific enhancer [64, 151]. The HS-40 appears to function in a very similar way to HS2 of the human β-globin LCR [73].

The Human β-Globin Active Chromatin Hub. The spatial organization of the murine β-globin gene cluster in expressing and nonexpressing cells has recently been studied using the chromosome conformation capture (3C) technology [29] and the RNA TRAP method [18]. It has been shown that in erythroid cells the expressed adult mouse β-globin genes (β_{maj} and β_{min}) spatially interact with the HS of the LCR, located 60 kb away. The inactive embryonic β-*like* globin genes (εy and βh1), positioned in between the LCR and adult genes, do not participate in this interaction and loop out [18, 166]. Two sets of HS at either side of the murine β-*like* globin gene cluster, 130 kb apart from each other, are also present in the spatial cluster, namely, 3′HS1 and two additional 5′HS (called HS-60.7 and HS-62.5), with previously unknown function [44]. These HSs form a pre-ACH complex, termed the "chromatin hub" or CH, and are likely to be involved in globin gene regulation in erythroid cells. Most importantly, the transcriptionally silent olfactory receptor (OR) genes located in between the 5′HSs and the LCR, like the inactive embryonic globin genes, do not participate in clustering, and also loop out. The spatial clustering of *cis*-regulatory elements and active genes is termed an "active chromatin hub" or ACH (Fig. 11.3b).

Similar results have been obtained using transgenic mice carrying the human β-globin gene cluster [119], where it was also shown that the formation of the ACH is mediated by multiple regulatory elements [129]. Similar observations in *KLF-1*-targeted mice, in which the functional murine β-globin ACH is lost [37], suggest that KLF-1 is a key player in stabilization of the β-globin ACH in definitive erythroid cells.

It is postulated that the active organization of the human β-*like* globin gene cluster would use similar principles (but on a larger scale) to those used in the folding of an enzyme to create an active site or "pocket" [129]. Initial folding of the locus would be nonspecific in terms of exact position or site, but would provide the template to allow the proper and precise folding of the LCR (which would be analogous to creating an active site of an enzyme) and allow interactions with the globin genes (which, to continue, the analogy, is the substrate). In other words, the function of the CH lies in the fact that it would cause a spatial restriction by forming a loop containing the genes and LCR and hence stimulate ACH formation.

11.3.4 Molecular Control of Globin Gene Switching

Analysis of developmental expression of human globin genes has provided useful insights into the regulation of gene expression. The human β-*like* globin gene cluster had always had a central role in these experiments, since understanding of the molecular mechanisms governing the expression of the β-*like* globin genes would open new perspectives leading toward novel therapeutic strategies for β-thalassemia patients (see also below). The human β-*like* globin gene cluster displays two developmental switches, one from the embryonic to the fetal stage, coinciding from the transition from primitive to definitive hematopoiesis, and a second switch from the fetal to the adult stage during the perinatal period [158]. From different types of experiments it has been established that the regulation of the human β-*like* globin gene is controlled by a dual regulatory mechanism involving (a) *gene competition* for activation from the LCR and (b) *gene silencing* of those genes that are active during the previous developmental stage (Fig. 11.4).

Gene Silencing. Autonomous gene silencing of the globin gene, which is active at the previous developmental stage, has been demonstrated to be one of the two molecular mechanisms involved in human globin gene regulation. Deletion of the putative *HBE*-silencing elements resulted in the abrogation of *HBE* silencing, whose expression persisted during adult erythropoiesis [142]. Similarly, autonomous silencing has also been demonstrated for the *HBZ* gene [155]. However, silencing of the human fetal globin genes has been more difficult to demonstrate. When a 3.3-kb fragment containing the *HBG1* gene was linked to the LCR, it displayed proper developmental control, e.g., was expressed in the embryonic and fetal erythroid stage and not in the adult stage [32]. However, Enver et al. concluded that similar LCR-*HBG1* constructs displayed γ-globin gene expression throughout development [41]. Dissecting the human γ-globin gene promoter suggests that there are two negative regulatory elements [159]. The first is located downstream of position -141, where many mutations leading to non-deletional hereditary persistence of fetal hemoglobin (HPFH) were found (see also below). The second is localized further upstream, between positions -378 and -732, and appears to function as an adult-specific enhancer. In favor of this hypothesis is a mutation leading to increased Hb F levels in the adult [21, 92].

Gene Competition. In erythroid cell cultures or transgenic mice, the *HBB* gene is properly regulated in DNA constructs containing the LCR and the *HBG1* and *HBB* genes in the normal configuration. However, when the *HBG1* gene is removed, *HBB* loses its developmental specificity and remains active throughout development [41]. The model suggests that the LCR

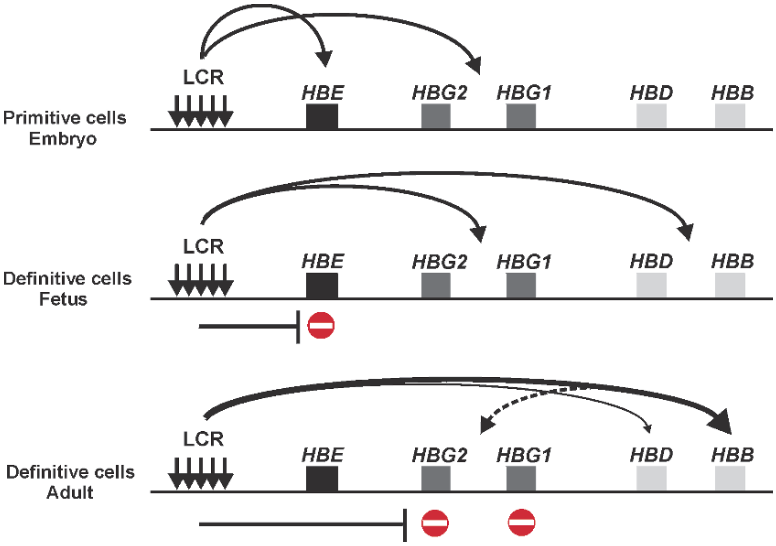


Fig. 11.4 Schematic drawing of the dual regulatory mechanism, which governs human globin gene transcription. The embryonic *HBE* gene is expressed during primitive hematopoiesis. Upon switch from primitive to early definitive hematopoiesis, the *HBE* gene is silenced autonomously and the *HBG1* and *HBG2* genes are activated. Finally, silencing of the human fetal globin genes occurs upon transition of hematopoiesis from early to late definitive erythroid tissues, where the *HBD* and *HBB* genes are transcribed at high levels. At this stage, *HBG1* and *HBG2* genes are expressed at very low levels (indicated with a *dashed line*)

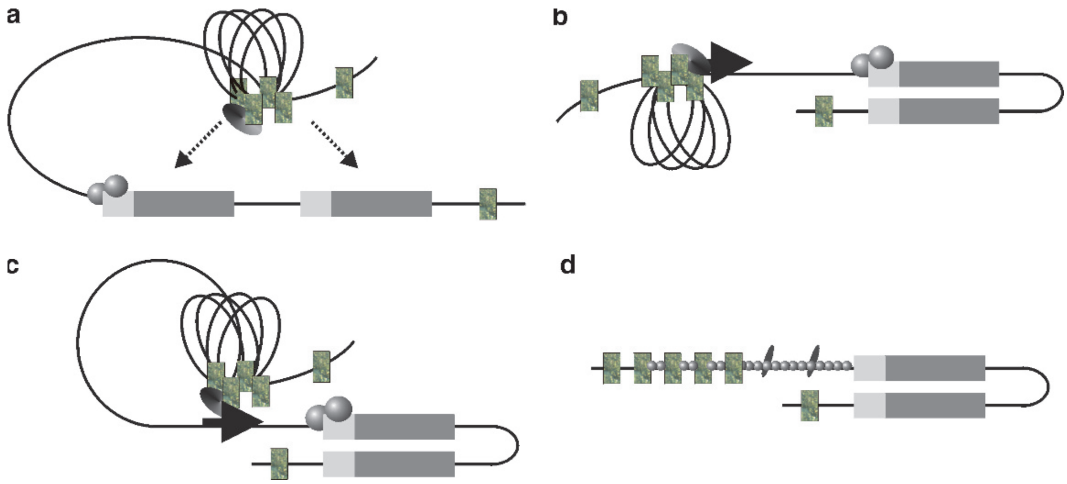


Fig. 11.5 (**a–d**) The four models put forward to explain globin gene competition, namely, looping (**a**), scanning (**b**), facilitated scanning (**c**) and linking (**d**). The outline of each model is provided in the text. *Dark gray boxes* indicate the structural genes, *light gray boxes*, the gene proximal regulatory elements, *large* and *small circles* correspond to the various transcription factors and histone modification proteins, respectively, and *hatched boxes*, to the HSs of the LCR

directly interacts with the gene proximal regulatory elements, e.g., promoters, and this interaction is primarily determined by the transcription factor environment. The gene that successfully interacts with the LCR is expressed, and the unsuccessful one is not. The model has been expanded to explain both switching processes in the human β-*like* globin gene cluster. Four different models have been proposed to explain LCR function in relation to globin gene competition: looping, tracking (or scanning), facilitated tracking, and linking (Fig. 11.5). Available data neither strongly support nor preclude any of them.

The *looping model* suggests that the HSs of the β-globin LCR form a "holocomplex," with the HS core elements creating an active site that binds transcription factors and the core-flanking sequences constraining the holocomplex in the proper conformation (Fig. 11.5a). This structure is looping, so that the LCR comes in close physically proximity to the appropriate gene promoter. Close association with gene-proximal promoter and enhancer elements allows the delivery of LCR-bound transcription proteins and other co-activators that interact with the basal transcription machinery, which is already bound at the promoter to form a stable transcription complex, thus enhanci ng globin gene expression [99, 176]. Further evidence supporting the notion of a holocomplex suggests that the LCR interacts with only one globin gene promoter at any given time and that these interactions may alternate between two or more promoters (also known as "flip-flop"), depending on the developmental stage [176]. The gene order and distance between the LCR and its target gene is a critical parameter in the flip-flop assumption, which has been shown to affect the probability that these two elements, namely, the LCR and the gene promoter, will interact [33]. This probability is constant for a gene at a specific stage of development, determined at each stage from the respective transcription factor environment. A variation of this model suggests that the LCR initially serves as a multiple element receptor that acts as a docking site for transcription factors to initiate chromatin remodeling [55]. Once chromatin-remodeling activity has been completed, the LCR directly interacts with downstream genes to facilitate their expression.

The *tracking (*or *scanning) model* suggests that erythroid-specific and ubiquitous transcription factors and cofactors bind to recognition sequences in the LCR and form an activation complex that migrates, or tracks, linearly along the β-*like* globin gene cluster (Fig. 11.5b) [168]. When this transcription complex encounters the basal transcription machinery located at the correct gene promoter, according to the developmental stage, the complete transcriptional apparatus is assembled and transcription of that gene is initiated.

The *facilitated-tracking model* incorporates aspects of both the looping and the tracking models (Fig. 11.5c) [12]. This model suggests that an LCR co-activator complex loops to contact downstream DNA in promoter-distal regulatory regions, where the transcription factor complex is released. This complex then tracks in small steps along the chromatin until it encounters the appropriate promoter with its associated bound proteins. A stable loop structure is then established and gene expression proceeds.

Finally, the *linking model* proposes that chromatin facilitator proteins bound throughout the β-*like* globin gene cluster define the domain to be transcribed and mediate the sequential stage-specific binding of transcription factors (Fig. 11.5d) [16]. Non-DNA-binding facilitator proteins form a continuous protein chain from the LCR to the globin gene to be transcribed, linking proteins bound at a transcriptionally primed gene to one another.

11.3.5 DNA Polymorphisms at the Globin Genes

Gene mapping by restriction enzyme analysis of the β-*like* globin gene cluster led to the identification of numerous single nucleotide polymorphisms (SNPs) between different individuals. Haplotypes, i.e., the pattern of SNPs on a given chromosome, are valuable diagnostic tools, and only a few of them can be found in different ethnicities. Initially, Kan and Dozy reported a DNA polymorphic sequence adjacent to the human *HBB* gene in relation to the sickle cell mutation [78]. Subsequently, at least 17 single nucleotide substitutions have been identified in the β-*like* globin gene cluster (intergenic) and in the *HBB* gene itself (intragenic) [4, 8, 75, 77, 112]; these are symbolized as either present (+) or absent (–) in different individuals. Most of the polymorphic sites are of ancient origin, since they are found in all ethnic groups, but their frequencies vary significantly among different ethnicities and populations (Table 11.3).

Table 11.3 Frequency of DNA polymorphic sites in the β-globin gene cluster in different populations (adapted from [8])

Polymorphic sites	Ethnic groups Greeks	 African–Americans	 Southeast-Asians
Taq I	1.00	0.88	1.00
Hinc II	0.46	0.10	0.72
Hind III	0.52	0.41	0.27
Hind III	0.30	0.16	0.04
Pvu II	0.27	N.D.	N.D.
Hinc II	0.17	0.15	0.19
Hinc II	0.48	0.76	0.27
Rsa I	0.37	0.50	N.D.
Taq I	0.68	0.53	N.D.
Hinf I	0.97	0.70	0.98
Hgi A	0.80	0.96	0.44
Ava II	0.80	0.96	0.44
Hpa I	1.00	0.93	N.D.
Hind III	0.72	0.63	N.D.
Bam HI	0.70	0.90	N.D.
Rsa I	0.37	0.10	N.D.

N.D. Not determined

A remarkable feature of the β-*like* globin gene cluster SNPs is their linkage disequilibrium. The most reasonable interpretation postulates a recombinational hotspot, leading to high recombination rates, which separates the flanking SNP clusters. If free recombination were involved among these SNPs, one would expect random associations of any two polymorphic sites over many generations, which would have resulted in a very large number of haplotypes [19]. Recent data suggest that SNPs within the β-globin gene cluster comprise two distinct linkage disequilibrium blocks, one extending from the LCR to the *HBD* gene and the other containing the *HBB* gene [95].

Apart from the main haplotype frame, a handful of SNPs, either within a haplotype context or independently, have been subsequently identified in different regulatory regions within the β-*like* globin gene cluster [34, 84, 110, 121, 122, 127, 137]. These polymorphisms have been correlated with different *HBD* and *HBB* gene mutations, and occasionally with phenotypes of varying severity in β-thalassemia and SCD patients.

11.4 Molecular Evolution of the Human Globin Genes

All of the normal human hemoglobins and their genes have an identical three-dimensional structure and a closely related primary DNA sequence, respectively, suggesting a common evolutionary origin. The closer the resemblance between two globin chains, the more recent the common ancestral sequence. The main evolutionary event in generating a gene family is gene duplication, an essential first step toward the creation of duplicates from a single ancestral gene and expansion of gene clusters. The duplicated copies can subsequently evolve independently to acquire new biological functions. Homologous recombination between repeated elements is the most frequent event leading to gene duplication and was first described by Shen et al. [152] in the human fetal globin genes.

Gene duplication is usually followed by additional recombination events. Unequal crossover can subsequently increase or decrease the number of genes in the family. In the human α-*like* or the β-*like* globin gene clusters, unequal crossover has not only been involved in their evolution but has been also implicated in pathogenic gain or loss of gene copies [67], which leads to phenotypes of various severity, with Hb Lepore [116] and Hb Kenya [68] as the most characteristic examples (see also below).

Gene sequence similarities strongly indicate that the human α-*like* and β-*like* globin genes originate from a single ancestral globin gene. Following gene duplication, the two genes were separated in different chromosomes, approximately 450–500 million years ago (Mya), and have evolved independently ever since (Fig. 11.6). The ancestral α-*like* globin gene has undergone several duplication events since then, leading to the *HBZ* gene (300 Mya), the *HBQ1* gene (260 Mya) and ultimately the *HBA2* and *HBA1* genes (52–72 Mya). Similarly, the ancestral β-*like* globin gene was duplicated some 150–200 Mya, yielding two gene copies of embryonic and adult developmental specificity. Duplication of the ancestral embryonic gene 100–140 Mya resulted in the embryonic *HBE* gene and a fetal-*like* globin gene, whose duplication 47 Mya yielded the *HBG2* and *HBG1* genes. Finally, the ancestral adult gene was duplicated 85–100 Mya, yielding the *HBB* and *HBD* genes.

Following the initial gene duplication, the gene copies accumulate nucleotide substitutions, small or large deletions, and insertions independently, as part of the evolutionary process. Consequently, the two gene copies diverge from each other, and finally each copy has the possibility of gaining an improved function or, on the other hand, of being silenced and becoming a pseudogene. Gene conversion, the nonreciprocal transfer of genetic information between two highly homologous genes , first observed in yeast [141], has been described in the *HBG2*/*HBG1* genes [152, 154], indicating that such events also occur frequently in mammalian cells. Sequence analysis has revealed that a 1.5-kb DNA fragment of the *HBG2* gene, including the promoter up to most of the IVS-II region, is virtually identical to the respective DNA fragment of the *HBG1* gene. This observation indicates that a gene conversion event occurred of such a kind that sequences from the *HBG2* gene converted those of the *HBG1* gene no later than 1 Mya [123].

Similarly, in 1983, Michelson and Orkin showed that, following the α-globin gene duplication event, the duplicated loci underwent a gene conversion event that homogenized their sequences from the 5′ regulatory to the IVS II region [98]. Most interestingly, in both gene copies gene conversion events are restricted to the upstream sequences, leading to homogenization of the 5′ regulatory and coding sequences. On the other hand, the absence of gene correction events at downstream sequences, such as the 3′ UTR, excludes this region from the concerted evolutionary process, leading to the accumulation of specific nucleotide variations. The latter may reflect the differences in the transcriptional and translational efficiency of the *HBA2* and *HBA1* genes [88].

Also, it is interesting that the conversion polarity is defined from the expression level of the participating genes. The gene that is expressed at a higher level ("master" gene) converts the sequence of one expressed

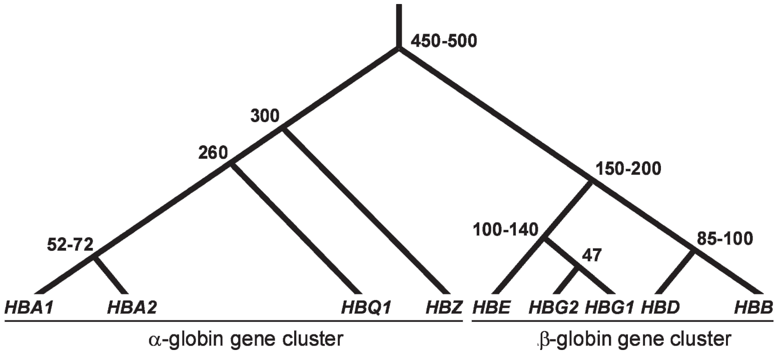


Fig. 11.6 Outline of the human α- and β-globin gene clusters' evolution. *Numbers* indicate the approximate date (in million years) when the respective gene duplication event took place

at a lower level ("slave" gene). This master/slave gene rule, which seems to be involved in the definition of the polarity of the conversion, can be clearly interpreted as an attempt by natural selection to prevent the possible inactivation of the slave gene when two functional gene copies are required [20].

On the other hand gene conversions are less frequent between the *HBD* and *HBB* genes, which has led to their marked sequence divergence, also reflected in their transcriptional efficiency. Nevertheless, interallelic gene conversion seems to have contributed to the spread of β-thalassemic mutations into different chromosomal backgrounds, defined from the respective β-*like* globin haplotypes [123]. Recent data from at least 14 different α-globin chain variants, due to an identical mutation in both the *HBA2* and *HBA1* genes, suggest that such mechanism may be also active in the human α-globin genes [100].

Gene conversion has been shown to have an important role in propagating pathogenic mutations in both globin gene clusters. The most characteristic example is Hb Parchman [1], which is most likely the result of *HBD* gene conversion by the *HBB* gene. Finally, the Cretan type of HPFH, which resulted from a likely gene conversion event identical to the one that originally homogenized the fetal globin gene sequences (Fig. 11.7), [126], further demonstrates the dynamic nature of this mechanism in molecular evolution.

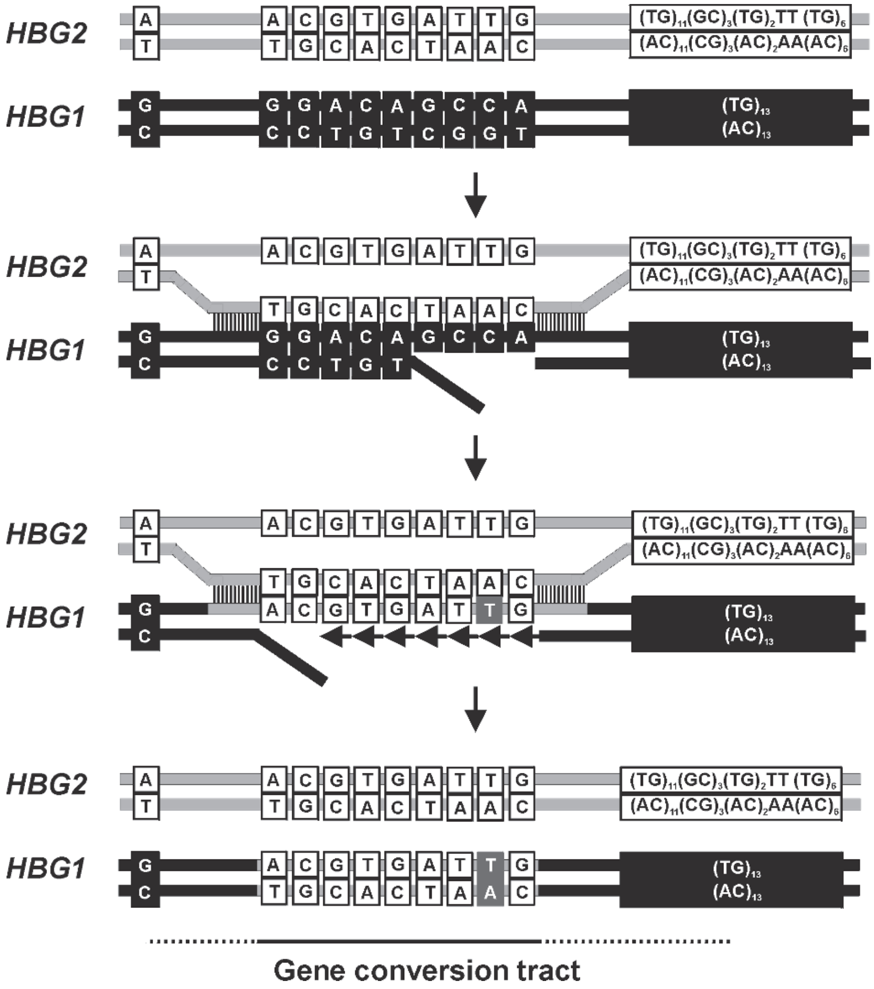


Fig. 11.7 Schematic drawing depicting the convergent evolution of the human fetal globin genes. A gene conversion event resulted in the homogenization of a 1.5-kb fragment of the *HBG1* gene from the *HBG2* paralog. The gene conversion tract length can be deduced from the flanking DNA base differences and spans from 423 to 1,550-bp (indicated as a *dashed line*). An identical de novo gene conversion event has recently been identified, leading to the Cretan type of nondeletional HPFH (shown as a *gray box*), indicative of the dynamic nature of this phenomenon [126]

11.5 Molecular Etiology of Hemoglobinopathies

Hemoglobinopathies refer to a wide range of inherited disorders, including the thalassemias and SCD. The vast majority of hemoglobinopathies result from genetic defects in the human α-*like* or β-*like* globin genes. In other words, the pathogenic mutations lie in *cis* to the globin gene cluster. However, there are a few examples where the mutation resulting in α- or β-thalassemia is located in *trans* of the globin gene clusters. In particular, few mutations in genes enconding for the erythroid-specific transcription factor GATA-1 on chromosome X [180] or the XPD subunit of the general transcription factor TFIIH on chromosome 19 [170] have been shown to cause β-thalassemia. In addition, mutations in the *ATRX* gene have been shown to result in both inherited and acquired forms of α-thalassemia. Interestingly, transcription of antisense RNA or mutations in unrelated genomic regions have also been shown to result in α-thalassemia.

11.5.1 Thalassemias and Related Conditions

A variety of conditions are characterized by genetically determined diminished or absent synthesis of a globin chain. These diseases are known as the thalassemias [25]. This term is derived from the Greek word "*θαλασσα*" (*thalassa*=sea*),* referring to the Mediterranean Sea, and was originally used to describe the Mediterranean origin of many gene carriers of these conditions [175].

The thalassemias are among the most common monogenic disorders in the world. The World Health Organization has estimated that as many as 270 million carriers of a globin gene genetic defect exist worldwide, 80 million of whom are carriers for β-thalassemia, and around 300–400,000 severely affected infants are born every year. Because of a selective advantage of heterozygotes against malaria, the frequencies of thalassemia are particularly high in the malarial tropical and subtropical regions of Asia, the Mediterranean, and the Middle East [45]. The carrier frequency of α-thalassemia varies from 1% (e.g., in southern Spain) to 90% (e.g., in tribal populations of India), while the carrier frequency of β-thalassemia varies from 1% (e.g., in northern Italy) to up to 70% (e.g., in some regions of Southeast Asia). Considerable genotypic variation occurs even within a single country. The epidemiology of the disease, however, is changing. In the developing countries, the numbers of affected children are on the increase owing to falling childhood mortality resulting from improved nutrition and better infection control, while in the more developed countries, epidemiology of the disease has been affected by a fall in total birth rate and preventive programs. One of the best-documented control programs involving education, counseling, and prenatal diagnosis that have succeeded in limiting the numbers of new births of affected individuals has been in Cyprus, where thalassemia was first recognized in the 1940s. Implementation of the control program resulted in fewer than 600 severely affected patients with thalassemia in Cyprus in total, with only two or three new cases each year (Fig. 11.8). Furthermore, because of recent population migrations, thalassemia has become an important part of clinical practice in Northern Europe, including the United Kingdom, and in the USA and Australasia [174].

The molecular etiology of the thalassemias is highly heterogeneous. It is noteworthy that advances in understanding the thalassemias at the molecular level have led to better comprehension of the nature and variety of human mutations in general. Depending upon which globin chain is absent or reduced, thalassemias can be classified as α-, β-, γ-, δ- and δβ-thalassemias (the last including exclusively deletion mutants and being further subdivided into ^{A}γδβ-, γδβ- and εγδβ-thalassemia). The commonest and clinically most important are the α- and β-thalassemias (Fig. 11.9), while δ-thalassemia is a clinically silent condition. Elucidation of globin gene regulation has been aided significantly by investigation of various thalassemia mutant alleles, which can be classified in the categories detailed below.

11.5.2 β-Thalassemia

The recessively inherited β-thalassemias are widespread throughout the tropical and subtropical areas of the world and likely owe their frequency to a selective advantage vis-à-vis *Plasmodium falciparum* malaria [173]. The apparent protective effect of thalassemia

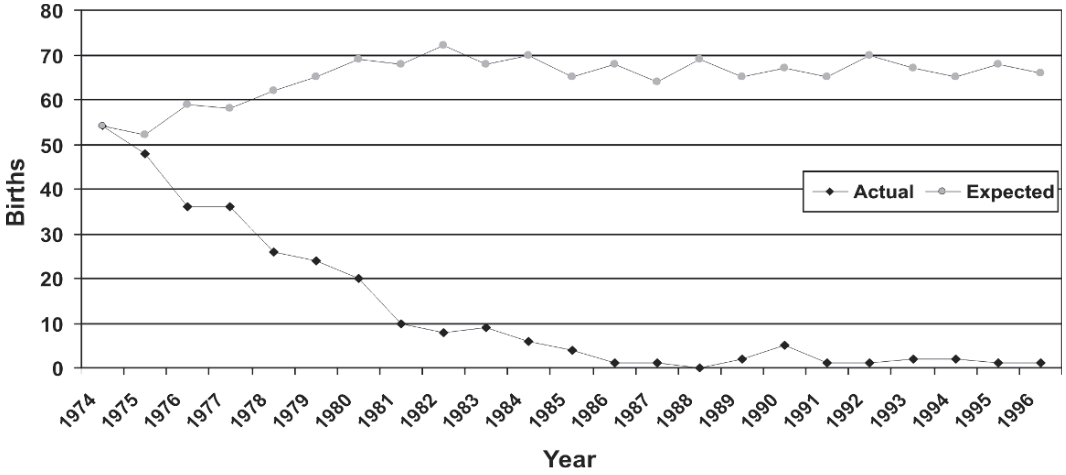
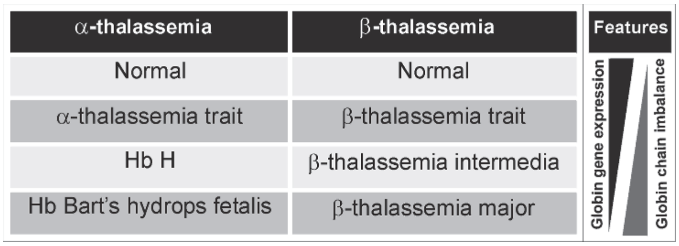


Fig. 11.8 Graph depicting the decline of births of homozygous β-thalassemia children in Cyprus compared with the expected number of homozygous β-thalassemia births. The graph is indicative of the success of the β-thalassemia prevention program in Cyprus

Fig. 11.9 Classification of α- and β-thalassemia depending on the degree of symptoms' severity, in relation to globin chain imbalance and gene expression. (From [130])



α-thalassemia	β-thalassemia
Normal	Normal
α-thalassemia trait	β-thalassemia trait
Hb H	β-thalassemia intermedia
Hb Bart's hydrops fetalis	β-thalassemia major

Features
Globin gene expression
Globin chain imbalance

against malaria may be related to enhanced immune recognition, and hence clearance, of parasitized erythrocytes [93].

Carriers (heterozygotes) of β-thalassemia are clinically normal with mild anemia and are largely unaware of their carrier status. Hb A_2 is slightly elevated, while the red cells are smaller and less filled with hemoglobin, leading to a decrease in the mean cellular hemoglobin (MCH) and mean cellular volume (MCV) values. Heterozygotes usually do not require medical attention or treatment. Severely affected β-thalassemia homozygotes, however, have marked anemia requiring blood transfusions. Hb A is completely absent in β^0 thalassemia homozygotes, and much decreased in β^+ thalassemia homozygotes. The disease is associated with growth failure and often leads to death in adolescence or earlier. Homozygosity for β^0 thalassemia alleles and compound heterozygosity for β^+/β^0 thalassemia alleles are severe hemoglobinopathies and are a serious public health problem in countries where these alleles are common. The simultaneous presence of α-thalassemia ameliorates the clinical severity of homozygous β-thalassemia. Hb S/β^+ thalassemia is common in black populations. Hb E/β-thalassemia is common in Southeast Asia and results in severe anemia, as seen in homozygous β^0 thalassemia. This severity is at least partially explained by the fact that the Hb E mutation itself also causes mild thalassemia (see below). Less severe β-thalassemic alleles include β^{++} and β^{Silent} reflecting the minimal deficit in β-globin chain synthesis.

The remarkable heterogeneity of *HBB* gene mutations explains the frequent finding of compound heterozygotes for β-thalassemia, i.e., affected patients who have inherited a different *HBB* gene mutation from each parent. The frequency of such compound heterozygotes is somewhat lower in population isolates, where a single thalassemia mutant may account for the majority of thalassemia alleles. For example, while the p.Q39X (*HBB*:c.118C>T) nonsense mutant comprises about 27% of all β-thalassemia mutant alleles in general Mediterranean populations (http://www.findbase.org), it accounts for most of the β-thalassemia mutations in Sardinia. Since homozygosity for a given thalassemia mutant may range from only mild reduction to complete absence of globin synthesis, and compound heterozygosity is frequent, a wide spectrum of thalassemias with different clinical severity will be encountered.

A complete list of all pathogenic mutations reported for the human *HBB* gene can be found in the HbVar database for hemoglobin variants and thalassemia mutations (http://globin.bx.psu.edu/hbvar). The commonest mutations leading to β-thalassemia are point mutations or short insertions or deletions (indels) and can be classified in the following categories:

1. Promoter Mutations. Thalassemia mutations that affect the noncoding 5′ upstream regions of the *HBB* gene are regulatory mutations, which affect gene transcription. These mutations have been found at the distal CACCC and TATA boxes, which diminish hemoglobin synthesis and manifest themselves as relatively mild thalassemias [6, 114]. No mutations have yet been found at the *HBB* CAAT box.
2. RNA Cleavage Mutations. These mutations affect the AATAAA polyadenylation signal in the 3′ untranslated region of the *HBB* gene [115].
3. Nonsense Mutations. These mutations result in a new stop codon and hence lead to premature termination of translation, resulting in a shortened and, therefore, nonfunctional globin chain. These mutations therefore lead to β^0 thalassemia. One of the commonest mutations of this category is p.Q39X (*HBB*:c.118C>T), which is mostly found in patients and carriers of Mediterranean origin.
4. Frameshift Mutations. Short indels produce frameshifts with garbled coding, causing effective termination of functional globin synthesis again yielding a shorter globin chain.
5. RNA-Processing Mutations. These mutations affect RNA processing (splicing), mostly altering the GT or AG dinucleotide consensus sequence at the donor or acceptor splicing sites of splice junctions, respectively. These mutations cause β^0-thalassemia. Mutations in the splicing consensus sequence beyond the invariant GT-AG sequence usually cause milder β^+-thalassemia. Often, cryptic splice sites, namely, those that are not used during normal splicing, are sometimes activated by mutations in intervening sequences and cause interference with normal splicing process. Such cryptic splicing sites can also be activated within exons, Hb E and Hb Knossos being the most characteristic examples.

11.5.3 Dominantly Inherited β-Thalassemia

Dominantly inherited β-thalassemias are heterogeneous at the molecular level and are due to mutations at or near the *HBB* gene. Many of these involve mutations of exon 3 of the *HBB* gene. The resulting β-globin chain variants are very unstable, and in many cases the products of the dominantly inherited β-thalassemias are not detectable and the mutant alleles only are deducted from the DNA sequence. The predicted synthesis is supported by the presence of substantial amounts of abnormal β-globin mRNA in reticulocytes, comparable to the amount produced by the normal *HBB* allele. Of the 33 known dominantly inherited β-thalassemia mutations, 22 are located in exon 3 of the *HBB* gene, producing unstable globin and thalassemia intermedia (a term used to characterize a wide clinical spectrum ranging from mild thalassemia conditions to asymptomatic forms that are only slightly more severe than the β-thalassemia trait; see http://globin.bx.psu.edu/hbvar). The mutations leading to dominant β-thalassemia are missense mutations, frameshifts, premature chain termination (nonsense) mutations, and complex rearrangements, often resulting in an early stop codon (6 alleles) or elongated globin chains (7 alleles). Nonsense or frameshift mutations that would produce truncated β-globin chains up to 72 residues in length are usually associated with a mild phenotype in heterozygotes. It is believed that the mRNAs associated with these mutations are not transported to the cytoplasm and hence, no mutant protein is synthesized. On the other hand, mRNAs with mutations in exon 3 are transported and translated normally. They produce long and highly unstable globin gene products that are capable of binding heme, but not combining with α-globin chains to produce any kind of stable Hb tetramer. Hence, these large truncated products tend to precipitate in the red cell precursors, together with excess α-globin chains, to produce large inclusion bodies [165].

11.5.4 δβ-Thalassemias and Hereditary Persistence of Fetal Hemoglobin

Various rare deletions in the human β-globin gene cluster have been described. These conditions are known as δβ-, ^{A}γδβ-, γδβ-, and εγδβ-thalassemias, depending on which genes have been removed by the deletion. Such deletions have been important for the identification of the human β-globin LCR (see above). None of these deletions can be recognized cytogenetically, since they are too small for microscopic detection.

Hereditary persistence of fetal hemoglobin (HPFH) is an inherited condition leading to increased Hb F production in adult life. HPFH is caused by deletions in the human β-globin gene cluster (deletion HPFH), removing the *HBD* and *HBB* genes, and is clinically dinstict from δβ-thalassemia. In particular, HPFH is characterized by higher HbF levels (30%), in contrast to δβ-thalassemia (24%), and has pancellular as against heterocellular distribution of Hb F. HPFH can also result from 18 point mutations and 1 short (13-bp) deletion (nondeletion HPFH), residing at the upstream promoter region of either the *HBG1* (10 mutations and 1 deletion) or *HBG2* (8 mutations) genes. These mutations are located in the distal CCAAT box and the upstream GC-rich region (200 bp upstream of *HBG1* and *HBG2* Cap sites) and up-regulate Hb F production, most likely by altering erythroid-specific transcription factor binding, which results in the continuous expression of the (otherwise silenced) fetal globin genes. Studies directed at understanding the regulation of the Hb F switch have far-reaching implications for treatment of thalassemia and SCD, since increased Hb F production in these disorders would be of marked therapeutic benefit.

11.5.5 α-Thalassemia

11.5.5.1 Deletion α-Thalassemia

Most α-thalassemias are caused by gene deletions. Sequence similarity in the 5′ and 3′ regions of the α-globin genes allows incorrect chromosomal alignment, followed by recombination with subsequent deletions and duplications. The crossover chromosomes bearing either a single α-globin gene (-α) or three α-globin genes (ααα) have both been observed. While malarial selection has amplified the frequency of the (-α) alleles among tropical and subtropical populations, the triple (ααα) allele seems to confer neither an advantage nor deleterious effects on its carriers and, hence, is much rarer [65].

Various phenotypes caused by deletions of one, two, three, or four α-globin genes have been documented. Absence of a single Hb α gene (-α/αα) produces little hematological impairment, since three genes remain active. Two principal types of deletion events cause the mild α-thalassemia (-α/αα). The so-called leftward crossover creates a single *HBA2* gene and deletes a 4.2-kb fragment. The so-called rightward crossover derives from misalignment of the *HBA2* and *HBA1* genes, with crossover producing a fusion gene and a 3.7-kb deletion (Fig. 11.10). The rightward single α-globin gene is the most common type of α-thalassemia in Africa and in the Mediterranean countries, while in Asia both leftward and rightward crossovers have been reported. Several deletions that removed the upstream regulatory region (HS-40) of the human α-globin gene cluster were found to silence α-globin gene expression [144].

Deletion of two α-globin genes (-α/-α or --/αα) produces mild anemia, while deletion of three α-globin genes (-α/--) causes a more severe anemia characterized by production of Hb H, a β_4 tetramer, owing to α-globin chain deficiency. Co-existing α-thalassemia in patients with SCD is associated with less severe anemia and improved survival. Finally, deletion of all four α-globin genes (--/--) is fatal perinatally and is known as hydrops fetalis, referring to the extensive edema of the stillborn infant (Fig. 11.9). Most of the hemoglobin molecules of such infants consist of a γ_4 tetramer (Hb Bart's). Survival of the fetus into late pregnancy is likely to be caused by the presence of functional Hb Portland ($\zeta_2\gamma_2$). The virtual absence of hydrops fetalis from African infants is related to the nonexistence of the chromosome bearing the two α-globin gene deletions in that population.

11.5.5.2 Nondeletion α-Thalassemia

Nondeletion mutations similar to those resulting in β-thalassemia would be expected. A variety of such mutations have in fact been found, most of which involve the *HBA2* gene. No regulatory mutations in the

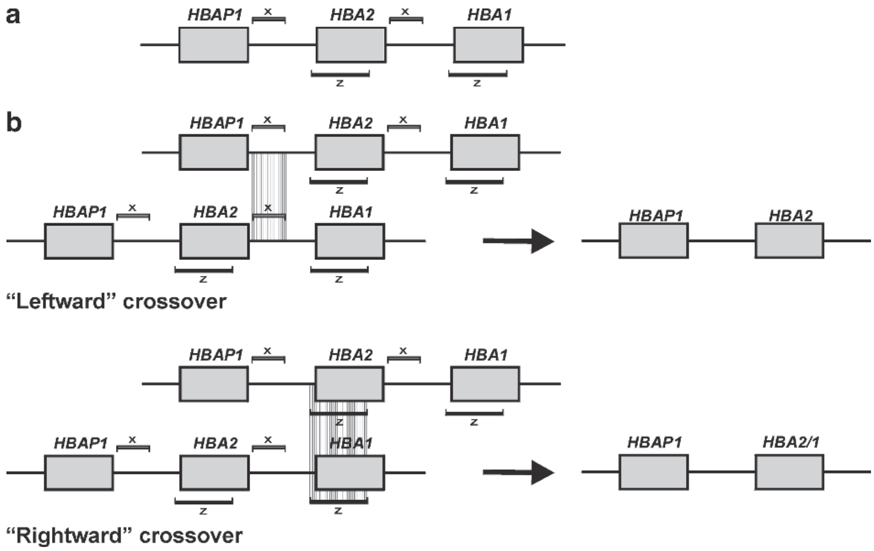


Fig. 11.10 Schematic drawing indicating the mechanism that resulted in the commonest deletion mutants leading to α-thalassemia. (**a**) Regions of homology are shown as "*x*" and "*z*." (**b**) Crossover between the x homology boxes yields a 4.2-kb deletion, termed "leftward," as the homology boxes are on the left side of the gene. Similarly, pairing of the "*z*" homology boxes in the rightward crossover yields a smaller, i.e., 3.7-kb, deletion; both result in a single α-globin gene

upstream region of both α-globin genes besides an LCR deletion (see above) have been detected. Only one splicing mutation, consisting of a 5-bp deletion that abolishes an acceptor site in IVS-I, has so far been found. Few point mutations have altered the human α-globin gene's termination codon. As a result, the α-globin chain is extended by 31 additional amino acids. These variants are unstable, and only small amounts (5%) of them can be detected in the blood. Hb Constant Spring is the most common of these mutants.

11.5.6 Other Mmutation Types Leading to Hemoglobinopathies

11.5.6.1 Fusion Genes

Deletions are frequent events of mispairing between homologous sequences of nucleotides during either meiotic or mitotic divisions in germ cell development. Examination of nucleotide sequences around the areas of deletions for various such mutants shows expected sequence homologies that facilitate mispairing. Recombination or crossover events following mispairing may lead to fusion genes, as the result of mispairing between similar but not identical genes. Therefore, nonhomologous crossover can eventually lead to fusion genes that encode the N-terminal portion of one globin and the C-terminal portion of another. Hb Lepore is the protein product of a *HBD-HBB* fusion gene (Fig. 11.11), with several types of Hb Lepore (http://globin.bx.psu.edu/hbvar), depending upon the site of crossover. Hb Kenya is another example of a protein that results from a fusion gene, owing to the misalignment between *HBG1* and *HBB* genes [109].

11.5.6.2 Duplications

Duplications may affect whole genes, such as the duplications during evolution that led to the various globin genes (see also Sect. 11.4). The commonest examples of globin gene duplication usually involve highly homologous genes, such as the human α- and γ-globin genes. In the former case, α-globin gene triplications are usually found, while in the latter, γ-globin gene triplications, quadruplications, and a quintiplication have been reported [67]. Duplication gene products would also be expected from crossover events as the counterpart of fusion genes. A chromosome with a *HBD*,

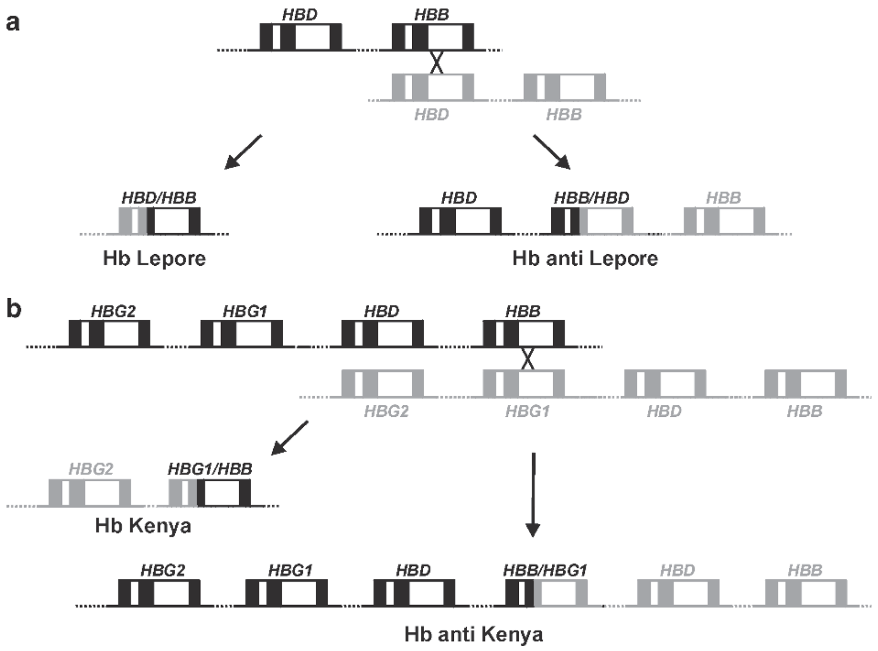


Fig. 11.11 Formation of hemoglobin fusion genes. Mispairing of the *HBD* and *HBB* genes yields a hybrid *HBD*/*HBB* gene (Hb Lepore) and an alternate product, termed Hb anti-Lepore. Such Hb anti-Lepore molecules have been identified, namely, Hb Miyada, Hb P, Hb Congo). Similarly, mispairing between the *HBG1* and *HBB* genes result in another fusion gene, namely, *HBG1*/*HBB* (Hb Kenya). The postulated Hb anti-Kenya molecule has never been found

HBB/*HBD*, *HBB* gene structure or Hb anti-Lepore have in fact been identified several times, while the expected Hb anti-Kenya chromosome (*HBG2*, *HBG1*, *HBD*, *HBB*/*HBG1*, *HBD*, *HBB*) has not yet been found.

11.5.7 Hemoglobin Variants

Hemoglobin variants are caused by a variety of mutational events affecting a given hemoglobin gene. The most common hemoglobin variants result from single amino acid substitutions of a globin chain, such as the *HBB*:p.E6V mutation, leading to SCD. If the electric charge of the mutant amino acid is different from the normal residue, the variant hemoglobin can be recognized by its altered behavior on electrophoresis or cation exchange chromatography. Mutations that do not change electrophoretic properties are usually detected if they exert a deleterious effect on hemoglobin function and cause disease. The majority of hemoglobin qualitative variants, regardless of charge differences, has a minor effect or none at all on hemoglobin function and is compatible with normal health. In general, amino acid substitutions of the exterior of the hemoglobin chains cause fewer perturbations of function than those replacing amino acids in the chain interior or close to the insertion of the heme group. Substitutions affecting normal helical turns of the chain often cause hemoglobin instability. Amino acid replacements affecting subunit contacts are often associated with abnormalities in oxygen affinity [157]. Most hemoglobin variants are rare, although a few, such as Hb S, Hb C, and Hb E, have reached higher frequencies in certain populations, presumably by positive natural selection.

The results of compromised hemoglobin function caused by hemoglobin variants can produce four different types of disease: (a) SCD due to distortion of the red cell membrane, (b) hemolytic anemia due to unstable hemoglobins, (c) methemoglobinemia attributable to more rapid hemoglobin oxidation and (d) erythrocytosis attributable to abnormal oxygen affinity causing hypoxia with resulting erythropoietin production. Synonymous polymorphisms, i.e., nucleotide variants in codons with no effect on protein's primary sequence also exist.

11.5.7.1 Sickle Cell Disease

Hb S is a recessive disease caused by the substitution of valine for glutamic acid in the sixth codon of the β-globin chain. This particular mutation affects the solubility and crystalization of hemoglobin under conditions of hypoxia. Patients with SCD produce Hb S and lack Hb A. With a relatively low degree of hypoxia, the Hb S of such patients polymerizes into filaments of high molecular weight that associate to form bundles of fibers. These abnormal hemoglobin crystals distort the red cell membrane to its characteristic sickling shape. Some of these cells remain irreversibly sickled and are destroyed prematurely. Sickled cells increase blood viscosity and impede normal circulation in small blood vessels. The resultant hypoxia leads to more sickling, with a vicious cycle of more stagnation and characteristic episodic sickle crises with abdominal and musculoskeletal pain. After several years, necrosis of poorly perfused tissues, such as the spleen, occurs, and this organ atrophies [150].

Carriers of the mutation leading to SCD have only 25–40% Hb S and are clinically normal. Their red cells contain both Hb A and Hb S, and have a normal red cell life span. In vivo sickling occurs only under conditions of severe hypoxia, such as atmospheric conditions at altituds of over 3,000 m [149].

Hb F, when present together with Hb S in a red cell, decreases the extent of sickling [39]. Hb F reduces the crystalization of Hb S so that patients with SCD and large amounts of Hb F have few or no symptoms of SCD. In a few of these instances Hb F is contributed by a HPFH mutation [124]. In general, there is an inverse correlation between the amount of Hb F and the severity of symptoms in SCD. Any manipulation that would increase fetal hemoglobin production would therefore cause clinical improvement in SCD. Co-existing α-thalassemia in patients with SCD is associated with less anemia and improved survival.

Interestingly, there are several genetic factors that modulate the phenotypic outcome of SCD [161]. Hb F plays a key role as a genetic modifier of SCD. The sickle cell mutation arose independently on different chromosomes, as determined by the underlying haplotypes [7, 118]. The latter has been shown to significantly impact on the observed Hb F levels and, hence, on the observed phenotype. In particular, SCD patients bearing the Bantu, Benin, and Cameroon haplotypes present with a severe phenotype with painful occlusive crises, while SCD patients bearing the Senegal and Arab/Indian haplotype have milder symptoms attributable to the increased Hb F levels [83]. Moreover, there are more genetic modifiers that have been found in different chromosomes, influencing the overall Hb F production in SCD patients, namely, on chromosomes Xp [36], 6p [26], and 8q [47], also known as quantitative trait loci (QTL). Similarly, α-thalassemia reduces the concentration of Hb S and, therefore, Hb S polymerization [62].

Hb E/Hb C. Hb E (*HBB*:p.E26K) is the second most common hemoglobin variant, which reaches high frequencies in Southeast Asia, particularly in regions of Laos and Thailand 1102]. The β^E mutation is also only a mildly pathogenic mutation, to the extent that Hb E homozygotes have a clinical picture resembling classic β-thalassemia trait [43]. The β^E mutation affects *HBB* gene expression by improving the efficiency of a normally inactive donor site for RNA splicing between codons 25 and 27 of the *HBB* gene [112]. Through this mechanism, the mutation leads to a mild deficiency in normal β-globin mRNA and to production of small amounts of structurally abnormal mRNA. Hb E homozygotes have microcytic red cells but no significant anemia or other clinical problems. However, co-existence of β-thalassemia with Hb E gives rise to a more severe β-thalassemia-*like* phenotype.

Hb C (*HBB*:p.E6K) is the third most commonly encountered structural hemoglobin variant worldwide. Hb C is frequently found in West Africa [104] and also among American Blacks, though at much lower frequencies. As with Hb S, the *HBB*:p.E6K mutation results in reduced solubility of the Hb C oxy- and deoxygenated forms and the formation of crystals. Crystalization of Hb C can be inhibited in the presence of Hb F [66]. Hb C homozygotes have mild anemia, markedly dehydrated red cells, and moderate spleen enlargement, while Hb C heterozygotes are mostly asymptomatic. Co-existence of Hb S with Hb C result in the same, but less frequent, painful vaso-occlusive crises as are seen in Hb S homozygous patients [171].

11.5.7.2 Unstable Hemoglobins

Over 130 unstable hemoglobins have been described, the majority of which are in the β-globin chain. Many unstable hemoglobins have amino acid substitutions or deletions affecting the heme pocket of the globin chain.

Clinical manifestations vary from mild instability that is not clinically apparent, to severe instability, which causes increased red blood cell destruction. The instability of these hemoglobins is often caused by premature dissociation of the heme from the globin chain. Such heme-depleted globin is precipitated as intracellular material known as Heinz bodies and interferes with cell membrane function. The diagnosis of unstable hemoglobins, if not associated with electrophoretic mobility alterations, is difficult and may require isolation of the precipitated globin chains for further analysis in specialized laboratories. Unstable hemoglobins have been found to result from recurrent mutations, yielding identical hemoglobin molecules (i.e., Hb Köln, Hb Hammersmith).

Methemoglobinemia Attributable to Hb M. Hb M was the very first globin abnormality to be discovered [174]. Methemoglobinemia is caused by the more rapid oxidation of divalent to trivalent iron. Nine different mutations can produce Hb M, six of them producing it by tyrosine replacement of the histidine residues that anchor the heme group in its characteristic pocket of the globin molecule and stabilize the heme iron. Mild hemolysis is common in patients with Hb M. Patients with Hb M mutations of the α-globin chain are cyanotic from birth. Those with Hb M mutations of the β-globin chain do not develop severe cyanosis until 6 months of age, when the γ-globin chains are replaced by β-globin chains. However, Hb M mutations of the γ-globin chain are manifested with cyanosis at birth, which disappears in a few months after β-globin chains have replaced γ-globin chains.

Erythrocytosis due to Hemoglobins with Abnormal Oxygen Affinity. Upon oxygenation, the area of contact between the α/β globin subunits shifts. Deoxyhemoglobin is normally stabilized by a hydrogen bond between α42Y and β99D, while oxyhemoglobin is stabilized by a bond hetween α94D and β102N [101]. Stabilization of the oxy conformation or destabilization of the deoxy conformation by a mutation may result in increased oxygen affinity. More than 90 hemoglobins with increased oxygen affinity are known to exist. Most hemoglobins with high O_2 affinity have substitutions of the C-terminal of the β-globin chain or at binding sites of diphosphoglycerate (DPG), which are normally involved in maintenance of stability of the deoxy conformation [11]. The increased oxygen affinity reduces oxygen delivery to the tissues, with resultant hypoxia. Hypoxia leads to release of the hormone erythropoietin, which stimulates red cell production with resultant erythrocytosis. Only a few hemoglobins with reduced oxygen affinity have been detected [11]. In these cases, increased oxygen delivery to the tissues caused by the reduced affinity for hemoglobin results in reduced production of erythropoietin and mild anemia.

11.6 X-Linked Inherited and Acquired α-Thalassemia

In contrast to the usual deletion and nondeletion forms of α-thalassemia resulting from *cis*-acting genetic defects, there are two other forms of α-thalassemia, one associated with a variety of developmental abnormalities in a rare, severe form of X-linked mental retardation (ATR-X syndrome) [52] and another acquired abnormality in association with a multilineage myelodysplasia, also known as α-thalassemia myelodysplasia syndrome (ATMDS) [160]. ATR-X syndrome is a rare condition that, to date, has been identified in approximately 100 families from many regions of the world. Patients with ATR-X syndrome carry mutations in the *ATRX* gene [53] residing on the X-chromosome (Xq13.1-q21.1). ATR-X patients present with a strikingly uniform phenotype, comprising severe mental retardation, characteristic dysmorphic facies, genital abnormalities, and an unusual, mild form of Hb H disease, accompanied by reduced *HBA1* and *HBA2* mRNA levels.

Also, although abnormal patterns of hemoglobin synthesis are nearly always inherited, occasionally persons with previously normal hematologic function develop aberrant hemoglobin synthesis as an acquired abnormality, usually within the context of hematologic malignancy. This situation is known as ATMDS. It has been demonstrated that both inherited and acquired mutations in *ATRX* gene cause α-thalassemia (ATR-X syndrome and ATMDS, respectively), suggesting that *ATRX* has a central role in the regulation of α-globin gene expression [52]. The mechanism by which *ATRX* mutations down-regulate α-globin expression is still unknown. The *ATRX* gene encodes a chromatin-remodeling factor, a member of the SWI2/SNF2 family of proteins. Although ATMDS and ATR-X syndrome are rare conditions, they have provided important insights into the general principles underlying more common, clinically important, diseases such as α-thalassemia.

11.6.1 Regulatory SNPs and Antisense RNA Transcription Resulting in α-Thalassemia

A common situation in human molecular genetics is a well-defined clinical phenotype for which the mutation causing the disease cannot be found in the associated gene/s or regulatory sequences. In contrast to the majority of thalassemia patients, in whom the underlying genetic defect is well characterized and located within the structural globin genes or their regulatory elements, there is a fraction of patients whose mutation could not be found in the latter genomic regions.

α-Thalassemia can result from transcription of antisense RNA, leading to an α-globin genes silencing, Tuffareli et al. showed that in an α-thalassemia, transcription of antisense RNA from the juxtaposed, to the *HBA2*, truncated *LUC7L* gene, owing to an 18.3-kb deletion, mediates silencing and methylation of the associated CpG island [169]. In other words, the antisense RNA from the truncated *LUC7L* gene runs through the *HBA2* gene and interferes with normal transcription in the sense direction in *cis*. Hence, although the *HBA2* gene retains all of its local and remote *cis*-regulatory elements, its expression is silenced and its CpG island becomes completely methylated early in development.

Finally, a rare form of α-thalassemia has been shown to occur via a gain-of-function regulatory SNP (rSNP) [28] in a nongenic region between the α-globin genes and their upstream regulatory elements. In particular, this rSNP creates a GATA-1 binding site, nucleates the binding of a pentameric erythroid complex including the transcription factors SCL, E2A, LMO2, and Ldb-1, which are frequently found with GATA-1 at erythroid regulatory elements, and binds RNA polymerase II. The rSNP, therefore, creates a new promoter-*like* element that interferes with normal activation of all downstream α-*like* globin genes. These findings not only demonstrate an additional mechanism causing human genetic disease, but also illustrate that SNPs of functional significance can also be identified in genomic regions, such as multispecies conserved noncoding (CNC) regions, harboring no *cis*-regulatory elements.

11.6.2 β-Thalassemia Attributable to Transcription Factor Mutations

Apart from the plethora of *HBB* mutations leading to β-thalassemia, a few β-thalassemia patients with their phenotype resulting from mutations in genes encoding for general and erythroid-specific transcription factors have been described. Viprakasit et al. have demonstrated that *HBB* gene expression is affected by a mutation in the XPD subunit of the general transcription factor TFIIH [170]. In particular, it has been shown that β-globin chain synthesis is only affected in trichothiodystrophy (TTD), and not in xeroderma pigmentosum (XP), patients. Using standard hematological assays, TTD patients have been identified with substantially elevated Hb A_2 levels, one of the hallmarks of β-thalassemia, reduced mean red cell volume and mean cell hemoglobin values, and no *HBB* genetic defect. In addition, α-globin chain levels were unaffected [170]. These data provide insights into the mechanism that enables a defect in a general transcription factor to affect the expression of specific genes, the *HBB* gene being the first example to confirm this hypothesis. Also, the fact that *XPD* mutations affect *HBB* but not *HBA2/HBA1* gene expression also suggests that *HBB* and *HBA/HBA1* gene promoters may differ in their requirement for TFIIH and, by implication, initiation of transcription from the human *HBB* and *HBA/HBA1* gene promoters may occur via different mechanisms. This would be consistent with many previously described structural and functional differences between the *HBB* and *HBA/HBA1* genes (Table 11.2).

Finally, although mutations in the erythroid-specific transcription factor GATA-1 have been reported to also result in β-thalassemia [180], there has been no mutation found in the erythroid-specific transcription factor KLF-1 in atypical β-thalassemia patients [42].

11.7 Population Genetics of Hemoglobin Genes

The presence of a relatively large number of DNA polymorphisms at the human β-globin gene locus has enabled the elucidation of the geographic origin and spread of several mutations leading to hemoglobinopathies. Initial evidence suggested that the mutation leading

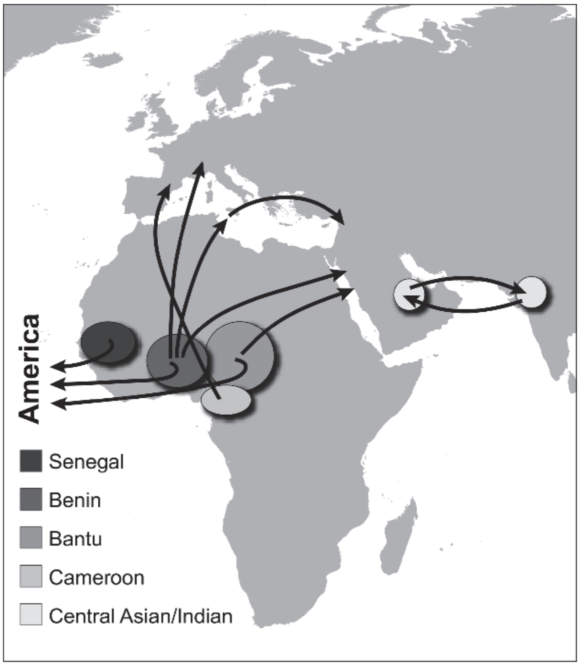


Fig. 11.12 Multicentric origin of the sickle cell mutation in Africa and Asia. *Arrows* indicate the migration routes of each β^S chromosome in various European and Asian populations and the Americas

to SCD (β^S, p.E6V) occurred at least four times in different geographic areas in Africa (Senegal, Benin, Central African Republic and the Bantu-speaking area) [85, 118] and once in Asia (Arab/Indian) [81] and then spread in various regions because of the selective advantage of Hb S carriers vis-à-vis malaria. In total, the β^S mutation has been observed in at least 16 different chromosomal backgrounds [7]. Interestingly, European populations are extremely homogeneous in terms of the β^S mutation origin. In particular, the Benin type of the β^S mutation is predominantly found in Mediterranean populations, namely, Sardinian, Greek, Turkish, and Cypriot, while both the Benin and Senegalese types of the β^S mutation are spread out over the west and northwestern parts of Africa (Fig. 11.12). However, the β^S mutation found in the African-American populations is largely heterogeneous owing to population migrations to the American continent (Fig. 11.2).

As with the β^S, the β^E mutation has been found on three different chromosomal backgrounds in Southeast Asia [5] and on two in European population [76].

While the haplotype data suggest that several mutational events were the origin of Hb S and Hb E chromosomes, it is likely that recurrent mutations are but one of three possible mechanisms, the other two being recombination and gene conversion events (see also Sect. 11.4). The latter events are likely mechanisms to explain the spread of several thalassemia mutations in different chromosomal backgrounds within a single population or ethnic group or closely related ones. In other words, the various common β-thalassemia mutations usually occurred in a unique haplotype with subsequent expansion of such chromosomes because of malarial selection. The p.Q39X nonsense mutation (*HBB*: c.118C>T), having been found in all nine haplotypes in the Sardinian population, stands out as the most characteristic example [136]. Other examples include: (1) *HBB:*c.79G>A [β^E mutation [5], (2) *HBB*:c.20delA [77], (3) *HBB*:c.124_127delTTCT [61] and (4) *HBB*:c.92+5G>T and *HBB*:c.316-197C>T [181].

Interestingly, the chromosomal background of a certain *HBB* mutation leading to hemoglobinopathies is linked to the symptoms' severity. To this end, SCD patients bearing the Senegalese or Asian/Indian haplotypes present with milder symptoms than do patients bearing the Bantu, Benin and CAI haplotypes [83, 118], owing to the higher Hb F levels in the former patient group. Similar data have been recently provided for Hb E/β^{o}-thalassemia compound heterozygotes in Southeast Asian populations [95].

11.8 Diagnosis of Hemoglobinopathies

Although symptom-free, individuals with thalassemia trait are characterized by a specific hematological profile, which can be indicative for their genotype. A number of comprehensive guidelines for laboratory diagnosis of hemoglobinopathies have been published; all this lies outside the scope of this chapter and will not be discussed in detail.

11.8.1 *Carrier Screening*

The aim of carrier screening is to identify carriers of hemoglobinopathies in order to assess the risk of a couple having a severely affected child and to provide information on the options available to avoid such an eventuality. Ideally, screening is performed before pregnancy. There are several possible strategies for screening, depending on factors such as disease frequency, heterogeneity of the genetic defects, resources available, and social, cultural and religious factors. Knowing the frequency and heterogeneity of the hemoglobinopathies in a population is critical for the planning of an adequate strategy of carrier identification and for selection of the most suitable laboratory methods.

There are two types of screening: mass screening, provided to the general population before and during childbearing age, and targeted screening, which is restricted to a particular population group, such as couples preparing to marry, before conception or in early pregnancy. Screening may be "retrospective," when couples already have an affected child, or "prospective," when carriers are identified before having an affected child. Screening can be also targeted at different age groups, such as newborn, currently only recommended for sickle cell disease, and adolescent screening [3]. Premarital testing is carried out in several Mediterranean countries (Greece, Italy, and Cyprus). In Cyprus, couples are required to produce a certificate of carrier testing before they can be married. This, together with prenatal diagnosis and genetic counseling, has contributed to the marked decrease in the number of observed homozygous births since the beginning of the program in mid-1970s (Fig. 11.8).

Carrier identification strategies should ensure that no carrier eludes detection. There are two possible methodological approaches for β-thalassemia carrier identification: (a) a primary screen to determine red cell indices, followed by a secondary screen involving hemoglobin analysis in subjects with reduced MCV and/or MCH, recommended in countries with low frequency and limited thalassemia heterogeneity; and (b) complete screening based on determining red cell indices, hemoglobin pattern analysis, and Hb A_2 measurement, recommended in populations where both α- and β-thalassemias are common and where interaction of α- and β-thalassemias could lead to misdiagnoses attributable to the normalization of red cell indices. A flowchart illustrating the strategy used to identify carriers in high-risk populations is shown in Fig. 11.13.

In brief, considering that the iron status (metabolism) is normal, reduced red blood cell (RBC) indices, i.e., mean corpuscular hemoglobin (MCH<28 pg) and mean corpuscular volume (MCV<81fl) levels, are suggestive of thalassemia heterozygosity. If accompanied by elevated Hb A_2 levels, a diagnosis of β-thalassemia trait, co-existing or not with α-thalassemia, is made. Reduced RBC indices accompanied by normal Hb A_2 levels are indicative of α-thalassemia trait, co-existence of α- and δ-thalassemia traits, or co-inheritance of δ- and β-thalassemia alleles. In the latter case (also known as normal Hb A_2-β-thalassemia), caution should be exercised, as the β-thalassemia condition can be overlooked, leading to misdiagnosis.

Reduced MCH and MCV values accompanied with elevated Hb F levels are strong indicators of δβ-thalassemia or compound heterozygosity of β-thalassemia

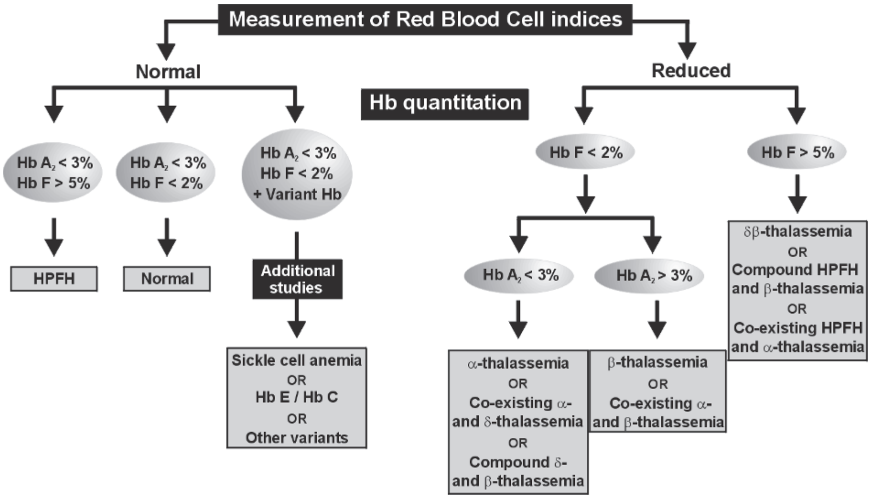

Measurement of Red Blood Cell indices
Normal
Reduced
Hb quantitation
Hb A2 < 3% Hb F > 5%
Hb A2 < 3% Hb F < 2%
Hb A2 < 3% Hb F < 2% + Variant Hb
Hb F < 2%
Hb F > 5%
HPFH
Normal
Additional studies
Sickle cell anemia OR Hb E / Hb C OR Other variants
Hb A2 < 3%
Hb A2 > 3%
δβ-thalassemia OR Compound HPFH and β-thalassemia OR Co-existing HPFH and α-thalassemia
α-thalassemia OR Co-existing α- and δ-thalassemia OR Compound δ- and β-thalassemia
β-thalassemia OR Co-existing α- and β-thalassemia

Fig. 11.13 Carrier screening and differential diagnosis of hemoglobinopathies. (From [130])

and HPFH, or co-existence of α-thalassemia with HPFH. In cases where the MCH and MCV values are normal, qualitative, and quantitative hemoglobin analysis is required in order to distinguish between a normal individual, an HPFH hetero- or homozygote (Hb F>5%), and a carrier of Hb S or any other structural hemoglobin variant (Hb C, Hb E, or other).

These cut-off indices are the most widely used; however, appropriate reference values should be independently defined for each population, as there may be slight differences according to the types of thalassemia alleles present. In addition, there should be regular quality control programs in order to monitor the accuracy of laboratory results.

11.8.2 Hematological and Biochemical Methods

With the exception of newborn screening, hematological and biochemical investigations of an individual for a suspected hemoglobinopathy provide useful insights into the hematological phenotype. Routine biochemical investigations mainly consist of electrophoretic and/or chromatographic analysis of an individual's hemoglobin fractions and globin chains.

Hematological Indices. The individual's hematological profile consists of the measurement of the RBC indices and includes hemoglobin concentration, hematocrit, RBC number, MCH, MCV, and red cell distribution (RDW, an indicator of RBC size variation). Routinely, a blood film accompanies the RBC indices. Depending on the severity of the thalassemia condition, minimal or major decrements in most of the RBC indices are observed. Also, quantitation of the hemoglobin fractions, using a variety of electrophoretic and chromatographic techniques, is an essential part of the hematological profile (see below). Iron deficiency alters RBC indices. If necessary, additional investigations can be performed, such as hemoglobin heat stability and sickling tests, oxygen dissociation studies, and in vitro globin chain synthesis, in order to detect and/or better characterize a structural hemoglobin variant.

Biochemical Methods. Electrophoresis has been the method of choice in a traditional hematological laboratory for qualitative and quantitative analysis of the various hemoglobin fractions. Cellulose acetate electrophoresis at alkaline pH (8.2–8.6) and citrate agar or agarose gel electrophoresis at acid pH (6.0–6.2) allows separation of the major hemoglobins, i.e., Hb A, Hb F, Hb S/D, Hb C/E/O-Arab, and a number of less common hemoglobin variants. Because of its simplicity, cellulose acetate electrophoresis remains among the most popular methods for hemoglobin screening. However, apart from being laborious, these electrophoretic techniques have the disadvantages of poor precision and accuracy of hemoglobin quantitation. Also, urea-triton gel electrophoresis provides rapid analysis of very small amounts of hemoglobin from hemolysates and permits examination of globin chain composition as well as globin synthetic ratios [2]. In some cases, mutant globin chains could also be separated with this electrophoretic technique. Finally, isoelectric focusing (IEF) on agarose gels can be used to separate different hemoglobin fractions and globin chains.

Chromatographic methods are also widely used for hemoglobin quantitation and initial screening of hemoglobin variants. Cation exchange-high performance liquid chromatography (CE-HPLC) has become the method of choice to quantify the various normal and abnormal hemoglobin fractions [143]. This method tends to replace electrophoretic techniques for primary screening of hemoglobin of clinical significance and to be at least an additional tool for identification of hemoglobin variants [74]. In recent years, reversed-phase HPLC (RP-HPLC) of globin chains has become an important tool in the study of hemoglobin abnormalities [87]. It has mostly been used for measuring the γ-globin chain ratios in various hemoglobin disorders, but owing to its high sensitivity, the proposed method may be also useful in the diagnosis of hemoglobinopathies and in the detection and study of hemoglobin variants, even those that are indistinguishable through the battery of electrophoretic tests.

11.8.3 Molecular Diagnostics of Hemoglobinopathies

Although a plethora of molecular diagnostic techniques are available for genetic testing of hemoglobin disorders, DNA sequencing is the ultimate method for the definitive identification of unknown sequence alterations, which should always be coupled to additional mutation scanning methods. In the vast majority of analyzes for molecular diagnosis of thalassemias, DNA is extracted from peripheral blood leukocytes, while chorionic villus and amniotic fluid cells are used as DNA source for prenatal diagnosis, fetal cells, and fetal DNA isolated from the maternal plasma and serum [22]. The noninvasive sampling methods are still under development and hence not widely offered for globin genes mutation screening. In preimplantation genetic diagnosis (PGD) polar bodies or blastomeres, either at the cleavage or at the blastocyst stage, are isolated from preimplantation embryos and these cells are used for DNA extraction to perform genetic diagnosis.

The available mutation detection methodology for human globin genes include screening for known mutations, using restriction endonuclease analysis and allele-specific mutation detection or amplification, and whole-gene scanning methods where the mutation in question in not known, using denaturing gradient gel electrophoresis (DGGE) or single-stranded conformation polymorphism (SSCP) analysis, both of which are characterized by their high discriminatory potential [130]. In recent years, denaturing HPLC (DHPLC) has been gradually taken into use by several diagnostic laboratories, as it provides a semiautomated, fast and reliable alternative to DGGE.

Regarding routine detection of large deletion mutants leading to α-thalassemia and a few other β-type hemoglobinopathies, gap-PCR provides an alternative to Southern blot analysis for routine detection of such mutants [23], as it is a rapid, cost-effective approach and simple to use. Today, gap-PCR is the ultimate method for reliable detection of the common deletional α-thalassemia alleles, Hb Lepore, or several deletion mutants in the human β-globin gene cluster, leading to δβ-thalassemia or deletional HPFH. The rationale behind gap-PCR is the generation of wild-type and deletion-specific amplification products from one each, respectively, of two oligonucleotide primer pairs, from which one primer is common. Alternatively, a three-color multiplex ligation-dependent probe amplification (MPLA) approach has been described [59]. This approach utilizes two sets of probes, one of which is α- and the other, β-globin cluster specific, and aims to detect rearrangements in these clusters. This approach is a rapid and sensitive method for high-resolution analysis of the globin gene clusters and an attractive alternative to conventional techniques and gap-PCR. Ultimately, oligonucleotide tiling microarrays could also be used to detect all of these abnormalities.

High-Throughput Globin Gene Mutation Screening. Recent developments in automation and miniaturization technologies have created new standards and changed the practice of molecular diagnostics in the postgenomic era. DNA microarrays have allowed high-throughput mutation detection and large-scale DNA sequencing. There are several microarray-based human globin gene mutation screening methods [27], based on single-base extension, arrayed primer extension, or a microelectronic allele-specific oligonucleotide (ASO)-*like* and multiple ligation probe amplification (MLPA)-*like* approaches. Finally, pyrosequencing is also gaining momentum for resequencing of globin genomic regions.

11.8.4 Prenatal and Preimplantation Genetic Diagnosis of Hemoglobinopathies

Prenatal and preimplantation genetic diagnoses of hemoglobinopathies both aim at reducing the number of affected individuals in at-risk populations.

Prenatal diagnosis became possible in the early 1970s, and in most European countries prenatal diagnosis is available for couples at risk for hemoglobin disorders, with the option of selective termination of pregnancy after genetic counseling [134]. However, it is widely discussed that the latter cannot be considered an optimal or easy solution. Chorionic villus sampling under ultrasound guidance in the first trimester of pregnancy (10–12 weeks of gestation) usually provides sufficient amounts of DNA for further analysis, with a relatively low risk of procedure-related pregnancy loss (0.6%).

Virtually all the DNA diagnostic methods reported for thalassemia diagnosis are suitable for prenatal diagnosis. It is highly recommended, however, that fetal DNA analysis be performed by two independent DNA diagnostic techniques, both yielding unambiguous results before a diagnosis can be made. Maternal contamination of the fetal tissue is the most common problem, which can lead to false-positive or false-negative results. The inheritance of polymorphic markers could always be used to “diagnose” contamination.

PGD is an early form of prenatal diagnosis, in which oocytes or preimplantation embryos are genetically analyzed, so that only those that are judged to be free of the genetic defect under consideration are transferred back to the mother. Detection of mutations in the *HBB* and *HBA2*/*HBA1* genes, leading to SCA and β- and α-thalassemia, has received considerable attention, because of their high frequency in certain populations. A handful of protocols have been reported for PDG of hemoglobinopathies [130].

11.8.5 Genetic Counseling

Genetic counseling is very important in view of the phenotypic diversity of thalassemia. Generally, the inheritance of two pathogenic *HBB* mutations results in a blood transfusion-dependent thalassemia. However, there are mild β-thalassemia mutations resulting in thalassemia intermedia. For instance, when both parents carry the mild *HBB*:c.-138C>T mutation, the homozygous state generally results in a very mild clinical phenotype. This type of mild thalassemia poses an ethical dilemma, both to parents and to health professionals, as far as prenatal diagnosis and possible termination of pregnancy are concerned. Nevertheless, there are situations in which the couple can be reassured that the phenotype of their affected child will be mild, such as (a) when the parents are silent carriers of β-globin gene mutations, i.e., the *HBB*:c.-151C>T mutation, resulting in very mild clinical phenotype in the homozygous state, (b) when mild and severe β-thalassemia mutations are likely to be interacting in the compound heterozygous state, and (c) when β-thalassemia is co-inherited with α-globin gene triplication (ααα) or an HPFH condition. Prenatal diagnosis is not offered for these cases [133].

Counseling couples who are at risk for SCD is often perceived as relatively simple, but in fact it can be significantly more complex, because of the variation in severity of SCD [150]. In contrast, counseling couples at risk for α-thalassemia is more straightforward, because of the usually severe phenotype for an affected fetus and the likelihood of life-threatening obstetric risks for the mother [133].

11.9 HbVar Database for Hemoglobin Variants and Thalassemia Mutations

In the late 1990s, Titus Huisman published two books that recorded information on hemoglobin variants and thalassemias, entitled *A Syllabus of Human Hemoglobin Variants* (2nd edn) [70] and *A Syllabus of Thalassemia Mutations* [69], which were a rich source of information not only about the mutations, but also about the methods used in detection and analysis, their biochemical properties, associated clinical effects, ethnic distribution, and other data. The sheer amount of information tabulated, the continuous accumulation of new mutation data, and their complexity dictated the need for construction of a globin-specific database as an up-to-date and accessible repository of this information, which has occurred in three discrete stages. HbVar is a comprehensive locus-specific database for the globin genes, realized from a multicenter academic initiative to provide up-to-date information on the various genetic defects leading to hemoglobinopathies [58]. This database, accessible at http://globin.bx.psu.edu/hbvar,), includes detailed information on pathology, hematology, clinical presentation, and laboratory findings (range of hemoglobin levels, hematocrit, etc.) on most of the published and unpublished hemoglobin variants and thalassemia mutations, while considerable biochemical data on the variants is also recorded, including techniques used to identify, isolate, and determine their structure, stability, function, and qualitative distribution in ethnic groups and geographic locations [128]. These data can be easily accessed through summary listings or user-generated queries, which can be highly specific.

Also, HbVar has been linked with the *GALA* database [49], since for several studies the information in HbVar needs to be combined with the wealth of information about features of genomic DNA, such as gene structures, interspecies sequence conservation, and many others, while an online repository for experimental protocols for detecting the globin gene variation has been also developed, as part of HbVar associated resources [50]. Finally, HbVar was one of the locus-specific databases implemented for the PhenCode project, in an effort to combine phenotypic, genotype and clinical data [51].

HbVar is useful not only for the research community, for geneticists and physicians as an aid in diagnosis, but also to other interested individuals, such as patients and their parents, people involved in the provision of genetic services and counseling and pharmaceutical industries. Online Mendelian Inheritance in Man (OMIM; http://www.ncbi.nlm.nih.gov/omim) and the Human Gene Mutation Database (HGMD; http://www.hgmd.cf.ac.uk) also contain information on globin gene mutations. However, OMIM lacks the important querying functionality, and being core (general) databases, these are less complete than HbVar.

11.10 Therapeutic Approaches for the Thalassemias

The gradual elucidation of the pathophysiology of thalassemia has led to the development of new strategies in attempts to cure or mitigate the disease. These potential therapeutic approaches can be divided into three categories, aiming to: (1) address the reduced or absent globin chain synthesis directly; (2) compensate for the reduced or absent globin chain synthesis; and (3) treat thalassemia complications, e.g., by decreasing iron overload and reducing oxidative stress. Apart from these approaches, there are also others, such as RNA interference (RNAi) to abrogate expression of mutant *HBB* alleles, that have only been experimentally used [163]. Although these strategies have not yet been used in practice, they hold promise for their future therapeutic potential [140].

11.10.1 Hematopoietic Stem Cell Transplantation

Hematopoietic stem cell transplantation is conceptually the simplest, and so far the only, approach that may lead to a definitive cure for β-thalassemia. Patients who are likely to benefit most from such treatment are those with early but severe transfusion-dependent disease [48]. However, even in the ideal case scenarios of leukocyte antigen (HLA)-identical family donors, this treatment is associated with 5% mortality and the receipients patients still have a high risk of developing tissue damage with time, such as impaired growth, gonadal failure, and chronic graft-vs-host disease (GVHD) [82]. These disadvantages, namely, donor shortage and GVHD (in the case of allogeneic stem cell transplantation), can be overcome by gene therapy of autologous hematopoietic stem cells (see below).

11.10.2 Pharmacological Reactivation of Fetal Hemoglobin

An alternative therapeutic approach involves pharmacological up-regulation of the fetal globin genes, which are otherwise silent in the adult hematopoietic stage, to compensate for the reduced or absent β-globin chain synthesis in patients with β-type hemoglobinopathies. There are three classes of pharmacological agents that have been shown to be capable of inducing Hb F to therapeutic levels: (1) recombinant human erythropoietin (rhEPO) preparations, acting on human erythropid progenitors cells during erythroid differentiation, (2) short-chain fatty acid derivatives, exerting their effect by promoting cell differentiation and enhancing gene expression, and (3) chemotherapeutic cytotoxic agents, which terminate actively cycling progenitors and, hence, trigger rapid erythroid regeneration and formation of mature red blood cells that contain Hb F. This approach has a number of disadvantages. First of all, the reactivation of human fetal globin genes expression is transient. Secondly, the mechanisms underlying the pharmacological induction of Hb F synthesis remain unclear, complex, and likely to involve multiple pathways, including alteration of the chromatin structure, e.g., by inhibition of histone deacetylase (HDAC) activity, hypomethylation of the human fetal globin promoters, and acceleration of erythroid cell differentiation.

Hydroxycarbamide (hydroxyurea; HU) is the only Federal Drug Authority (FDA)-approved agent used to stimulate Hb F synthesis with proven efficacy in SCD. Despite its use, the mechanism of action by which HU induces Hb F still remains uncertain. HU is cytotoxic; it inhibits ribonucleotide reductase, which interferes with DNA synthesis in the dividing late erythroid progenitors. This leads to a transient arrest of hematopoiesis, enhancing the premature commitment of earlier progenitor cells that still possess the primitive Hb F program [117]. HU has a relatively good safety profile with a low risk of carcinogenicity. Although HU has been shown to be highly effective in reducing the frequency of painful crises in SCD, with increases of Hb F levels, its efficacy in β-thalassemia patients has been less convincing. There have been few reports of its clinical efficacy in the severe transfusion-dependent β-thalassemia patients [15]. The future of HU looks more promising in the thalassemia intermedia syndromes in which sustained modest increases in Hb F can be maintained.

Short-chain fatty acids, such as butyrate and its analogs, are thought to act as HDAC inhibitors. One of the major caveats with the use of butyrates therapeutically is that they have short half-lives; they require continuous intravenous administration for demonstrable efficacy, and they may also inhibit erythroid cell growth. 5-Azacytidine and decitabine are DNA methyltransferase inhibitors, thus acting as hypomethylating agents. These drugs have been shown to have an action that is beneficial to β-thalassemia patients [30, 89].

11.10.3 Pharmacogenomics and Therapeutics of Hemoglobinopathies

The concept that genetic variation contributes to variability in drug responses is widely accepted and has been validated in many research settings. The application of pharmacogenetic testing and pharmacogenomics in hemoglobinopathy therapeutics is particularly attractive, because of the limited therapeutic possibilities presently available and the narrow therapeutic drug index, namely, iron chelation and fetal hemoglobin (Hb F)-inducing agents.

Hb F response of β-hemoglobinopathies patients to HU treatment is variable, particularly in β-thalassemia, with approximately 25% of the patients being poor or nonresponders [117]. Therefore, a capacity to predict a patient's Hb F response to HU and/or other drugs used for the same purpose would aid the selection of patients for treatment and reduce toxicity from unhelpful dose escalation. Polymorphisms in genes regulating Hb F expression, HU metabolism, and erythroid progenitor proliferation might modulate the patient response to Hb F-inducing pharmacological agents.

Correlation of SNPs linked to the human β-globin locus with HbF induction upon HU treatment is a controversial issue. Two association studies investigated β-globin locus-related SNPs, indicating that the most significant modulating factor involved in good and moderate response to HU was positively correlated with the *HBG1*-158 T allele [179], while in another study this correlation was not confirmed [35, 90]. On the other hand, association studies in genomic regions not linked to the human β-globin locus revealed putatively useful pharmacogenetic markers for HU treatment [94].

Data supporting the use of pharmacogenetic testing for HU treatment of hemoglobinopathies are currently very limited [125], and similar studies should also be conducted with more pharmacological agents and different treatment modalities. However, candidate gene studies attempting to associate SNPs with phenotypes of varying severity related to hemoglobinopathies should be interpreted with caution, as a candidate gene approach is necessarily limiting and unlikely to examine all genes affecting a phenotype. Whole-genome association pharmacogenetic studies are only just beginning, facilitated by the most technological advances in microarray-based transcription profiling and genotyping (G.P. Patrinos and F. Grosveld, unpublished work). Such studies may identify different types of genes: (a) genes encoding for putative stage-specific transcription factors that down-regulate the γ-globin genes or sustain high Hb F levels, (b) novel erythroid genes that participate in regulatory pathways involved in erythroid cell differentiation and which can potentially alter the stringent developmental program governing globin gene expression and/or (c) genes involved in the Hb F-inducing drug metabolism. Results from such studies may also better orientate pharmacogenetic marker identification only in those genes that are differentially expressed in good and poor responders to Hb F-inducing therapy. This will in turn facilitate the design of customized high-throughput pharmacogenetic tests for hemoglobinopathies based on the arsenal of the currently available genetic testing methods for globin gene mutation screening.

11.10.4 Gene Therapy

Gene therapy, i.e., the insertion of normal DNA directly into cells to correct a genetic defect of β-thalassemia aims to achieve therapeutic levels of functional *HBB* gene expression leading to more than 20% of total hemoglobin production. Efficient gene therapy requires erythroid lineage specificity and should be carried out by safe and efficient viral or nonviral transfection of autologous hematopoietic stem cells. Early attempts included conventional oncoretroviral systems carrying the *HBB* gene and β-globin LCR "core" elements of the β-LCR and adeno-associated virus (AAV) vectors, but both were unsuccessful because of vector instability, low viral titers, and variable, low β-globin gene expression [38] and position-variegated expression. In 2000, May et al. reported that a lentivirus vector carrying a large human *HBB* gene fragment under the control of the β-globin LCR could yield stable therapeutic levels of *HBB* gene expression [97]. Unlike retrovirus, lentiviral vectors are highly suitable for gene therapy of hemoglobinopathies, as they can transduce nondividing cells, are characterized by relative genomic stability and larger packaging capacity, e.g., can accommodate larger DNA fragments, and are much safer, owing to their self-inactivating design that include the removal of the viral long terminal repeats (LTRs) upon integration. Therefore, lentiviral vectors can stably include larger DNA inserts without rearrangements, allowing the incorporation of larger LCR fragments and chromatin insulator elements to overcome the problem of positional effects with the ability to sustain therapeutic levels of expression. The pioneering study of the Sadelain group [97] paved the way in the field, and preclinical studies, using third-generation lentiviral vectors, are currently under way; provided that the risk of insertional oncogenesis is adequately addressed [57], they hold great promise for clinical trials on gene therapy for hemoglobinopathies.

Acknowledgments We regret that, owing to space limitations, much of the relevant work could not be cited.

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Human Genetics of Infectious Diseases

12

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Abstract A lingering question in the field of infectious diseases is that of the considerable clinical variability between individuals in the course of infection, raising fundamental questions about the actual pathogenesis of infectious diseases. There is increasing epidemiological and experimental evidence to suggest that human genetics plays a major role in susceptibility/resistance to infectious diseases. There seems to be a continuous spectrum of human predisposition to infectious diseases, from monogenic to polygenic inheritance. Many monogenic primary immunodeficiencies have been clinically described and genetically deciphered, and most predispose affected individuals to multiple infections. Other monogenic traits conferring pathogen-specific susceptibility in otherwise healthy individuals are increasingly being described. Examples of Mendelian specific resistance to infectious agents are also being discovered. At the population level, major genes are being identified in a small, but growing number of common infectious diseases. Truly polygenic predisposition to a human infectious disease remains to be definitively demonstrated experimentally, despite the unquestionable identification of individual (but not necessarily interacting) susceptibility genes. Studies of the human genetics of infectious diseases have considerable clinical implications, as improvements in our understanding of the pathogenesis of infectious disease pave the way to both genetic diagnosis and immunological interventions. The genetic investigation of infectious diseases, seen as 'experiments of Nature', also provides a unique approach to definition of the function of host defense *genes in natura* — i.e. in the setting of a natural, as opposed to experimental, ecosystem governed by natural selection.

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Contents

12.1 Introduction

The determinism of human infectious diseases is still widely misunderstood, with these diseases commonly thought to be purely infectious. As exposure to a microbial agent is obviously required for infection and disease to occur, infectious diseases are often regarded as textbook proof-of-principle examples of purely environmental diseases. Conversely, some inherited metabolic disorders, such as phenylketonuria, are commonly seen as perfect examples of purely genetic traits. However, in the case of phenylketonuria, mental retardation is entirely dependent on the patient's exposure to the amino acid phenylalanine (85). If phenylalanine was absent from our diet, phenylalanine hydroxylase deficiency would be considered merely a neutral polymorphism in human populations. *Conditional* genetic susceptibility to disease, whether Mendelian or more complex, is probably not a trivial exception but a general rule that also applies to infectious diseases. Nonetheless, for most scientists and physicians, the more overt the disease-causing environmental factor, the less other factors, such as human genetics, are taken into account. It is precisely for this reason that infectious diseases are often erroneously considered to be determined solely by the microbial environment. However, even Pasteur's microbial theory of disease states that the microbe is necessary but not sufficient for the development of infectious diseases [66] (Fig. 12.1 and Fig. 12.2).

Once these diseases had been shown to be infectious, the next most important question concerned the astounding level of interindividual clinical variability in populations infected with the same microbe. Speculations about and investigations of natural variability in the development of infectious diseases were boosted in the early twentieth century by Charles Nicolle's discovery of the coexistence of symptomatic and asymptomatic infections in naive human populations [60]. In addition to microbial variation, three theories have been proposed to account for this heterogeneity. Nonmicrobial environmental factors may be involved, with air temperature or humidity, and the availability of an animal vector particularly crucial (the ecological theory of infection) [28]. Nongenetic host factors, such as age or, since the last century, personal vaccination history may have a key role (the

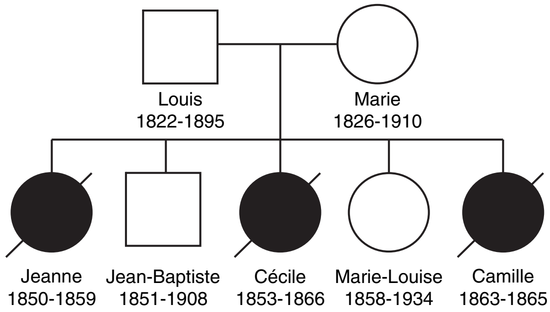

Fig. 12.1 Pasteur's pedigree and the genetic theory of infectious diseases. Louis Pasteur, the founder of the microbial theory of disease, lost three young daughters to "fever" between 1859 and 1866. A few years later, in 1870, he discovered that microbes caused disease in silk worms [65], paving the way for a general microbial theory of disease. Retrospectively, it is clear that his daughters died of infectious diseases. This illustrious family is representative of most families worldwide and throughout history until recent improvements in hygiene and the advent of vaccines and antibiotics, which resulted from the microbial theory. It was not uncommon for at least half the siblings in a family to die of infection. The microbial theory of disease identified the microbial cause of disease, but did not resolve the question of intrafamilial clinical heterogeneity in families exposed to the same microbial environment. As illustrated in Pasteur's own pedigree, one son and one daughter survived into adulthood, despite probable exposure to at least one of the microbes that killed their other siblings. We show here Pasteur's pedigree as it would have been drawn at around 1866 if the genetic theory of infectious diseases had emerged at that time. It is possible that the three children who died carried a Mendelian trait predisposing them to infectious diseases, or at least some form of genetic predisposition

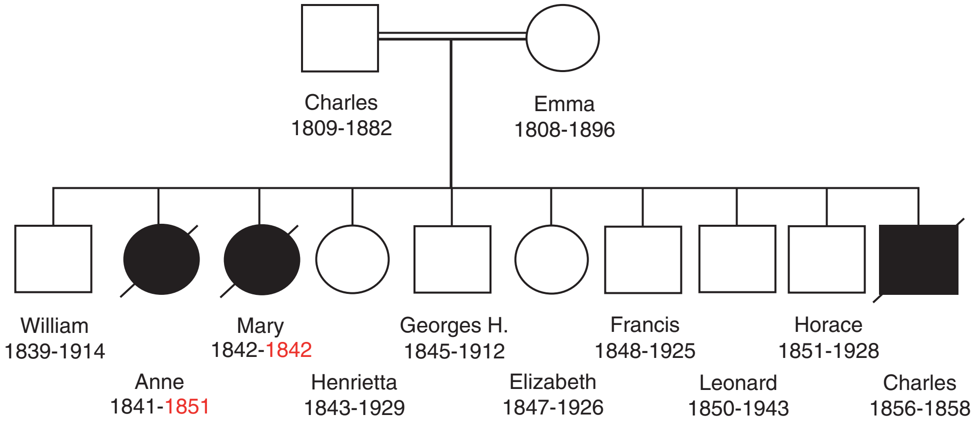

Fig. 12.2 Darwin's pedigree and the genetic theory of infectious diseases. Charles Darwin, the founder of the theory of natural selection, also lost three children to febrile infectious diseases between 1842 and 1858 (Fig. 12.1). In 1859, one year after the last of these three deaths, he published 'On the origin of species by means of natural selection', proposing the theory of natural selection of species. We show here Darwin's pedigree as it would have been drawn in 1858 if the genetic theory of infectious diseases had already emerged. Darwin married a first cousin and it is therefore possible that the three children who died of infection carried a recessive genetic predisposition to infectious diseases

immunological theory of infection) [66]. Finally, epidemiological evidence has accumulated since the 1930s, that shows a particularly important role of human genetic factors in immunodeficiency and susceptibility to infectious diseases (the genetic theory of infection) [20, 46]. The first evidence supporting the genetic theory of infectious diseases came from observations of the ethnic or familial aggregation of both rare and common infections, which even followed a Mendelian (monogenic) pattern of inheritance in some kindreds [4]. Follow-up studies of adoptive children also showed that predisposition to infectious diseases was largely inherited, paradoxically more so than in diseases associated with less well-known environmental risk factors, such as cancer [82]. Finally, the concordance rate of infectious diseases was higher in monozygotic than in dizygotic twins, implicating host genetic background in susceptibility to disease [4, 46].

The field of human genetics of infectious diseases entered the molecular and cellular era in the early 1950s, with the discovery of X-linked agammaglobulinemia by Bruton [16] and that of the protective role of the sickle cell trait for severe forms of *falciparum* malaria by Allison [7]. According to the dominant paradigm following these two landmark discoveries, predisposition to infectious disease segregates in a Mendelian or polygenic pattern of inheritance. An ever-growing number of rare Mendelian syndromes conferring susceptibility to multiple infectious agents is being reported. These syndromes include, in particular, conventional primary immunodeficiencies (PIDs) associated with multiple infections, [63]. For more common infectious phenotypes (such as malaria), genetic predisposition is thought to involve many genes, each of which has a modest marginal effect. However, the distinction between Mendelian predisposition in individuals with rare infections (one gene, multiple infections) and complex predisposition in populations with common infections (one infection, multiple genes) has become somewhat blurred in recent years [19, 22]. First, nonconventional PIDs conferring predisposition to a single type of infection in otherwise healthy individuals are increasingly being recognized [23, 67]. Second, Mendelian resistance to more virulent pathogens has also been described (e.g. [11, 55]). Third, a so-called polygenic susceptibility may primarily reflect the impact of a predominant gene, often referred to as a major gene [1]. Finally, the commonly diffused concept of polygenic inheritance remains to be demonstrated, at least at the individual level. We provide below an overview of the various genetic susceptibilities underlying human infectious diseases (Fig. 12.3), illustrated by key examples.

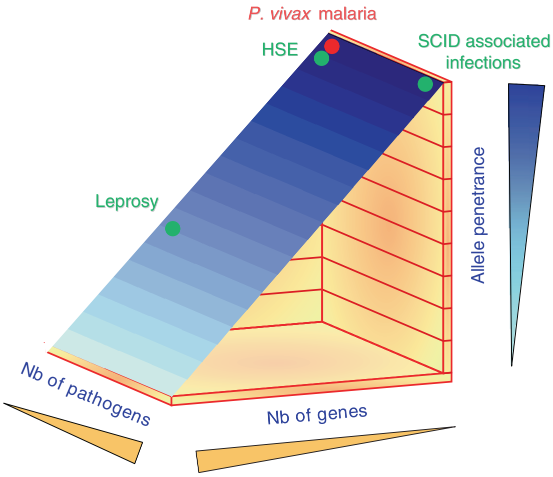

Fig. 12.3 Schematic representation of the continuous genetic models underlying human infectious diseases. This figure summarizes the spectrum of genetic susceptibilities predisposing to infectious diseases at the individual level. Different situations can be distinguished according to the number of genes with an additive impact on genetic susceptibility (in *green*) or resistance (in *red*), the marginal effect of each of these genes and the number of pathogens to which the individual is susceptible. Four textbook examples are shown on the graph: severe combined immune deficiency (SCID)-associated infections (a unique gene with complete penetrance predisposes individuals to a broad spectrum of infectious agents), herpes virus encephalitis (HSE; a single gene with high but incomplete penetrance conferring predisposition to a single infectious agent), *P. vivax* malaria (a single gene with high penetrance conferring resistance to a single infectious agent,) and leprosy (a small number of genes with intermediate penetrance conferring predisposition to a single infectious agent)

12.2 Mendelian Predisposition to Multiple Infections

Perhaps the most compelling evidence that human genetics does indeed determine the development of infectious diseases arises from the group of Mendelian disorders known as PIDs, which were first described as such in 1952 [16, 17]. More than 200 PIDs have been clinically described. Each is individually rare, but the genetic etiology of over 100 of these diseases has been elucidated [61, 63]. A comprehensive review of them is beyond the scope of this chapter, and interested readers are referred to previous reviews and textbooks [61, 63]. Typically, these disorders are monogenic (Mendelian) and confer predisposition to multiple infectious diseases (one gene, multiple infections) – the number and nature of which vary from case to case. They affect immune responses in various ways, in hematopoietic cells, nonhematopoietic cells, or both. The most severe known PID is reticular dysgenesia (RD), which is characterized by a complete lack of leukocytes of both lymphoid and myeloid origin [14]. Severe combined immunodeficiency (SCID) is more common and almost as severe, and is defined as an intrinsic (i.e., hematopoietic) lack of development of autologous T cells [34]. The Di George and Nude syndromes are immunological phenocopies of SCID of extrinsic (i.e., nonhematopoietic) origin. Children with SCID can be further classified according to the underlying genetic etiology, which may or may not affect other lymphoid lineages, such as B and NK lymphocytes. X-linked SCID (typically B+ NK–) is caused by mutations in the cytokine receptor common γ–chain. Other patients with SCID carry deleterious mutations in genes involved in the machinery generating antigen-specific receptors on T and B cells. Children with SCID present with multiple infectious diseases caused by viruses, bacteria, fungi, and parasites in the 1st year of life, typically starting by the age of 2–3 months. A broad range of weakly virulent microorganisms can kill such patients. SCID and RD neatly illustrate the life-threatening impact of Mendelian genetics in terms of infectious diseases.

SCID patients have also proved interesting in terms of the information they provide about the efficacy to save life and the risks of death associated with hematopoietic stem cell transplantation and gene therapy. SCID is invariably fatal when it follows its natural course, even in tertiary hospitals, typically in the 1st year of life. SCID patients progressively succumb to the succession of infections, even if treated with anti-infectious agents. The first successful hematopoietic stem cell transplantation (HSCT) in humans was actually performed in 1968, in a child with SCID [38, 40]. Despite the rarity of graft rejection, it was soon realized that children undergoing HSCT could die of graft-versus-host disease or severe infections. More than 30 years later, a series of ten children with X-linked SCID were treated with gene therapy [25]. A retroviral vector encoding a wild-type copy of the common cytokine γ–chain was introduced into CD34-positive bone marrow cells, which were thought to include committed hematopoietic progenitors and self-renewing stem cells. Remarkably, nine of the ten children with SCID developed normal numbers of functional T cells, which were maintained for up to 7 years in the first child treated [34]. This was the first clinical success of human gene therapy. However, four of the nine children eventually developed acute T-cell leukemia, 3–5 years after gene therapy [34, 42]. Molecular oncogenesis in the first two patients was found to be related to insertion of the retroviral vector into the *LMO2* gene, which encodes a transcription factor normally produced in T-cell progenitors, and an oncogene driving the proliferation of certain T-cell leukemias [42]. The molecular mechanism underlying the T-cell leukemia in the remaining two patients has yet to be reported. However, insertional mutagenesis and aberrant transcription of an oncogene were probably involved in leukemogenesis in all four patients. Sadly, one of these four patients with leukemia died. The other three were treated with chemotherapy, and one also underwent HSCT. The other five patients from the first trial are still doing well. The gene therapy trial was suspended when it was found that at least four of the nine patients had developed T-cell leukemia. One of the patients treated with a similar protocol in the UK has also since developed leukemia [37, 43]. Gene therapy for SCID, by heterologous recombination, has provided the long-awaited proof-of-principle that human gene therapy can be clinically beneficial. It has also revealed that despite the initial success, gene therapy as performed in this trial could eventually be detrimental.

12.3 Mendelian Predisposition to Single Infections

Interestingly, not all PIDs confer predisposition to multiple infections. An increasing number of disorders (summarized in Table 12.1) are known to confer Mendelian predisposition to a single type of infection [23, 67]. Epidermodysplasia verruciformis (EV) was described clinically as early as 1922. The genetic origin of this syndrome was proposed in 1933, and its viral etiology was documented in 1946 [64]. It was therefore probably the first PID ever described – preceding even Bruton's agammaglobulinemia – in that it corresponds to a monogenic predisposition to infection. The lack of an overt immunological phenotype and the extremely narrow spectrum of infections, limited to those caused by certain oncogenic papillomaviruses, probably precluded the use of the term "PID" at this time. The first two EV-causing genes, *EVER1* and *EVER2*, were described in 2002 [72]. An X-linked form of predisposition to lethal infection by Epstein-Barr virus was reported in 1975 [70], and two causal genes have been identified to date [26, 73]. Mendelian predispositions to bacterial infections have also been described. Patients with properdin deficiency or defects in the terminal components of complement (C5–C9, forming the membrane attack complex) display

Table 12.1 Mendelian holes in immunity to infection

Infectious agent	Clinical phenotype	Immunological phenotype	Gene	References
Neisseria	Invasive disease	MAC deficiency	*C5, C6, C7, C8A, C8B, C8G, C9*	Reviewed in [67]
	Invasive disease	Properdin deficiency	*PFC*	
Mycobacteria	MSMDDisseminated tuberculosis	IL-12/23-IFN-γ deficiency	*IFNGR1, IFNGR2, STAT1, NEMO, IL12B, IL12RB1*	Reviewed in [33]
Streptococcus pneumoniae	Invasive disease	IRAK-4 deficiency	*IRAK4, MYD88*	[13, 68]
Epstein-Barr virus	X-linked lymphoproliferative disease	SAP deficiency	*SH2D1A, BIRC4*	[26, 73]
Human papillomavirus	Epidermodysplasia verruciformis	EVER1/EVER2 deficiency	*EVER1, EVER2*	[72]
Herpes simplex virus 1	Encephalitis	Impaired production of type I IFN	*UNC93B1, TLR3*	[24, 89]
Trypanosoma evansi	Febrile episodes	No trypanolytic activity	*APOL1*	[86]
Plasmodium vivax	Natural resistance	Lack of receptor for pathogen	*DARC*	[55]
Human Immunodeficiency virus-1	Natural resistance	Lack of receptor for pathogen	*CCR5*	[6, 51, 78]
Norovirus	Natural resistance	Lack of receptor for pathogen	*FUT2*	[49, 50]

a selective predisposition to invasive meningococcal disease [67]. A predisposition to invasive pneumococcal disease also led to the discovery of IRAK-4 and Myd88 deficiency [13, 68]. The most thoroughly characterized of these syndromes is probably Mendelian predisposition to mycobacterial diseases (MSMD). Despite its clinical description in the 1950s, it was not until 1996 that the first genetic etiology of this syndrome – IFN-γR1 deficiency – was identified [44, 59]. In the last 10 years, up to 13 genetic defects affecting six genes have been reported, including autosomal recessive, autosomal dominant, and X-linked recessive traits, partial and complete defects, and complete defects with a lack of protein or nonfunctional expressed proteins [18, 33]. The six genes are physiologically related, as all are involved in IL-12/23-dependent, IFN-γ-mediated immunity [18, 32, 33]. Finally, Mendelian predisposition to a parasitic infection was recently documented in a patient with APOL1 deficiency who developed trypanosomiasis [86].

These disorders paved the way for the study of herpes simplex virus encephalitis (HSE), the most common form of sporadic viral encephalitis in Western countries. Since its discovery in 1941, it has remained unclear why only a small fraction of otherwise healthy individuals exposed to herpes simplex virus 1 (HSV-1) develop HSE. Moreover, none of the known PIDs, including RD and SCID, increase the risk of HSE. In a genetic epidemiological survey, about 10% of children with HSE were found to have been born to unrelated parents from consanguineous families, strongly suggesting Mendelian inheritance of predisposition to HSE, in at least some patients. Two genetic etiologies of HSE have recently been discovered: autosomal recessive UNC-93B deficiency [24] and autosomal dominant TLR3 deficiency [89]. These disorders lead to impaired recognition of dsRNA intermediates of HSV-1 in the central nervous system, resulting in impaired interferon production, itself resulting in enhanced viral replication and cell death. The virus does not spread to other organs because other cell types seem to be capable of controlling HSV-1 by TLR3-independent processes. Similarly, patients seem to use other means to control other viruses. The incomplete clinical penetrance probably reflects the influence of other factors, such as age at HSV-1 infection or modifier genes. In any event, it probably accounts for HSE being sporadic in the vast majority of kindreds. The identification of a Mendelian basis for HSE provides the first demonstration that a sporadic, life-threatening infectious disease may result from a group of monogenic disorders. It also suggests that other severe infectious diseases, particularly in children, may have a monogenic basis. The field of Mendelian predisposition to infectious diseases thus covers an immense spectrum, ranging from RD, in which patients have no leukocytes and are vulnerable to most (but not all) microbes, to HSE, in which leukocytes are not involved in the immunodeficiency and otherwise healthy patients are vulnerable to primary HSV-1 infection of the central nervous system.

12.4 Mendelian Resistance

Genetically determined resistance to an infectious agent is the obligate mirror of the susceptibility phenotype. Four Mendelian traits have been found to confer resistance to specific infections, as they result in a lack of the receptors used by the invading microbes (Table 12.1). Consequently, individuals carrying the common wild-type alleles are intrinsically susceptible to these particular pathogens, whereas individuals carrying the rare mutant alleles display almost complete and apparently specific protection against these pathogens. Protection against *Plasmodium vivax*, an agent of malaria, is conferred by a lack of erythrocyte expression of the Duffy antigen receptor for chemokines (DARC), a key receptor for the parasite [55]. The resistance trait is recessive, and the single nucleotide mutation affects the GATA-1-binding site in the promoter of the *DARC* gene, thereby selectively preventing gene transcription in erythroid cells [85]. Recessive resistance to human immunodeficiency virus-1 (HIV-1) infection has been found to be conferred by mutations affecting the extracellular domain of another chemokine receptor, CCR5. CCR5 functions with CD4 as a coreceptor for HIV-1 on CD4+ T cells [9]. Subjects homozygous for the most common *CCR5* deleterious mutation, a 32 bp deletion (Δ32), display strong protection against infection by CCR5-tropic HIV-1 [27, 51, 78]. The erythrocyte P antigen is the cellular receptor for parvovirus B19, and the rare people with the p-phenotype, whose erythrocytes do not have this receptor, are resistant to B19 infection, which causes erythema infectiosum [15]. Finally, resistance to norovirus (i.e., Norwalk-like viruses), a leading cause of gastroenteritis, was recently shown to be associated with alleles of the *FUT2* gene [49] encoding an α[1, 2]-fucosyltransferase that regulates the expression of ABH histo-blood group antigens on the surface of epithelial cells and in mucosal secretions [53]. Several inactivating *FUT2* mutations are responsible for the nonsecretor phenotype (Se−), with a lack of expression of ABH antigens on epithelial cells and complete resistance against symptomatic norovirus infection in experimental and natural conditions [50, 84].

Alleles conferring Mendelian resistance to virulent pathogens would be expected to be under strong positive selection pressure. This is clearly the case for the *DARC* mutation, which is not found in Europe but has a frequency of up to 80% in African populations, in which *P. vivax* is endemic [55]. Similarly, variants of erythrocyte disease-causing genes conferring a "major" (but not Mendelian) resistance, as defined in the next section, against *P. falciparum* malaria are much more common in endemic countries. The best example is provided by the worldwide spread of the deleterious hemoglobin S (HbS) allele [47]. Homozygosity for this allele causes life-threatening sickle cell disease (drepanocytosis), but heterozygosity protects against severe *P. falciparum* malaria [7], resulting in heterosis, in which heterozygotes have a selective advantage over both types of homozygote [87]. In contrast, the HbC allele confers recessive, but not dominant, resistance against *P. falciparum* malaria, possibly accounting for the limited spread of this allele in one geographical region (West Africa) [58]. The first population genetics studies of *CCR5* found that the main resistance allele, Δ32, originated from a single ancestor of European origin [83]. The relatively recent estimated date of the mutation event (about 2,000–3,000 years ago), the frequency of the allele in the European population (10% in Western and Central Europe), and the long-range linkage disequilibrium pattern at the *CCR5* locus are highly suggestive of positive selection [62]. However, the intensity [77] and nature [9] of the selective pressure remain to be determined. The situation may be even more complicated, as CCR5-Δ32 homozygosity was recently reported to be associated with symptomatic West Nile virus (WNV) infection [39], indicating that selective pressure may also be negative, depending on the microbial environment. Finally, a single nonsense *FUT2* mutation, G428A, is the most common mutation (>95%) responsible for the Se- phenotype in populations of European and African descent [84]. It remains unclear whether the recurrence of this mutation is due to a hotspot and/or a founder effect under positive selective pressure. Mendelian resistance genes have provided the best overall illustrations of natural selection on the human genome. There are probably many other similar human mutations that have been or are being selected because they confer Mendelian resistance to virulent pathogens.

12.5 Major Genes

The "major gene/locus" concept was developed in the 1960s, following the introduction of the polygenic model by Fisher [35], when clinical geneticists needed a framework that could explicitly specify the effect of single genes in the expression of common diseases [30, 48]. A major gene differs from a Mendelian effect in displaying incomplete penetrance, and its phenotypic expression may be influenced by both environment and other genes in the individual. This concept was first formalized and developed in the context of complex segregation analysis, a statistical method based in its more general expression on a model of inheritance in which a given phenotype may result from the joint effects of a major locus, a polygenic component, and environmental factors [45, 48]. Several major genes identified by segregation analyzes have been reported since the 1970s in a number of complex traits, including infectious disease-related phenotypes in leprosy, malaria, schistosomiasis, and some viral infections [19]. The development of highly polymorphic genetic markers has recently led to the use of the major gene concept for loci identified in the context of genome-wide screening by means of linkage studies. Indeed, the loci detected in genome-wide scans, particularly in those based on the commonly used affected sib-pairs design, should have a substantial influence on the phenotype under study that could be qualified as a major effect [75, 76]. Using this definition, several major loci have been mapped in infectious diseases [19], the first one to chromosome 5q31–q33 and controlling levels of infection with the parasite *Schistosoma mansoni* [55], and the most recent to chromosome 8q12 and conferring a predisposition to pulmonary tuberculosis [10]. However, the major gene concept may evolve further, particularly in the context of technological advances in genomics. Furthermore, major genes may be specific to a given population (depending on ethnic origin, history, age of onset, etc.), and, for an infectious disease (e.g., leprosy), specific for a given phenotype (e.g., paucibacillary leprosy).

The HbS trait may be considered the first major gene identified in a common infectious disease [7], based on both its frequency in some African populations and its estimated effect on severe malaria (relative risk ~10) [47]. However, no genome-wide linkage screen has been conducted for severe malaria, probably because of the rarity of this phenotype and the even greater rarity of families with multiple cases. Consequently, leprosy is the only infectious disease for which a complete successful positional cloning approach, including genome-wide linkage screening followed by refined linkage disequilibrium mapping, has led to the identification of major susceptibility variants. Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* that continues to affect more than 300,000 new subjects per year [88]. Both the development of leprosy per se upon exposure to *M. leprae* and its clinical features (ranging from paucibacillary to multibacillary forms) depend on human genes [6, 18]. The first evidence for this was provided by twin studies carried out in the 1960s, followed by several segregation studies, most detecting the presence of a major gene [2, 3]. Two major genes were only recently mapped by genome-wide linkage studies. The first of these studies focused on paucibacillary leprosy in India and detected a major locus on chromosome 10p13 [80] that has yet to be precisely identified. The second was carried out in Vietnam and mapped a major gene for susceptibility to leprosy per se to chromosome 6q25 [57]. Further linkage disequilibrium studies identified variants of the regulatory region shared by *PARK2*, a gene encoding an E3-ubiquitin ligase called Parkin, and *PACRG* (Parkin coregulated gene) as genetic risk factors for leprosy [56]. In addition to identifying a novel pathway of immunity to *M. leprae* [79], this study was the first to report successful positional cloning of a major locus in a common infectious disease.

12.6 Multigenic Predisposition

It is common to distinguish two patterns of multigenic inheritance, oligogenic and polygenic, according to the number and marginal size effect of the genes influencing the disease. Oligogenicity implies that the phenotype is dependent on two or more major genes, in addition to other factors. Polygenicity implies that the phenotype results from the effect of a large number of genetic loci, each having a small effect. An implicit but fundamental idea underlying multigenic inheritance is that, mechanistically, these definitions apply at the *individual* level. As this idea of a *cumulative* effect of the genes at the individual level is implicit, this point is often overlooked, and it is common practice to describe multigenic predisposition at the *population* level. However, multigenic predisposition at the population level does not necessarily reflect multigenic inheritance at the individual level, as genetic or even phenotypic heterogeneity may be involved, with certain genes acting as major genes in certain individuals or groups of individuals in the population considered. As discussed above, there is no unambiguous definition of a “major gene,” and it is even possible to use a mixture of several definitions. Indeed, it would be legitimate to consider tuberculosis to be an example of an oligogenic disease (one major gene on 8q12 derived from genome-wide linkage analysis [10] and another, *NRAMP1,* from candidate gene analysis [12, 41, 52]) if both genes were found to affect the *same* phenotype in the *same* sample. We pointed out above that two major genes conferring a predisposition to leprosy have been identified on chromosomes 10p13 and 6q25. However, it would not be accurate to cite this as an example of oligogenicity, because the first of these two genes seems to affect only the paucibacillary form of the disease, whereas the second affects leprosy per se. Conversely, in the Vietnamese sample used to identify *PARK2/PACRG* [56], *LTA,* encoding lymphotoxin alpha, was recently identified as a second major susceptibility gene for leprosy per se [5]. The study of genetic predisposition to leprosy has therefore led not only to the first successful positional cloning of a major locus in a common infectious disease, but also to the first demonstration of an oligogenic predisposition to a common infectious disease.

The identification of a polygenic predisposition requires a large number of individuals, both because of the small expected effect attributable to each “polygene” and because of the additive nature of these polygenic effects. It is therefore not surprising that the proof-of-principle of such genetic mechanisms at the individual level has been provided by studies of susceptibility to infectious diseases in animal models of experimental infections. As a textbook example, it has been shown in a murine model of malaria caused by infection with *Plasmodium chabaudi* AS (which mimics several pathophysiological aspects of the blood-stage infection in humans, including host response and genetic control of parasitemia and ultimate outcome of infection) that at least five loci control parasitemia and nine control survival [36]. Several genes have often been reported to have a potential influence on the onset of a given infectious disease, but there is currently no proof of polygenic predisposition per se in individual human beings. For example, a number of genes (e.g., *HLA-DR, NRAMP1, IL12RB1*) have been reported to have a role in tuberculosis, but it has never been determined whether these genes act independently and additively on the same phenotype in the same sample. Thus, references to the polygenic nature of predisposition to a human infectious disease have generally been made at population level and should be considered to reflect ignorance of events at the level of individuals. However, with the advent of new chip-based technologies for massive genotyping, it should soon become possible to perform genome-wide scans for association, by capturing a large proportion of the genetic variation through the genotyping of millions of SNPs, in thousands of cases and controls. This need for a large sample for the detection of polygenes is entirely consistent with these genes playing a role in individuals. Indeed, the small size of their effects makes it more likely that the genes detected in such a sample play a role in the vast majority of the individuals, indicating true polygenic susceptibility. This comprehensive approach has started the dissection of the polygenic contribution to several complex diseases in humans such as type I diabetes [81] or Crohn's disease [29, 74]. In infectious diseases, the first whole-genome association was recently published. This study of the genetic control of HIV-1 viral load identified several polymorphisms that explained nearly 15% of the phenotypic variation among asymptomatic individuals [31].

12.7 Concluding Remarks

Infectious diseases are therefore largely genetically determined, probably more so than many other human diseases. The increase in life expectancy observed in the twentieth century occurred despite the retention of poor immunity to particular infectious agents in the genomes of most individuals [21]. There has been no sudden natural selection of high-quality immune system genes worldwide; this persistent immunodeficiency has simply been masked by medical progress. The genetic theory of infectious diseases constitutes a paradigm shift in medicine but does not conflict with the microbial theory of diseases, and Pasteur himself stated in his seminal survey of diseases affecting silk worms, that in the course of "flacherie," which he designated as "hereditary," "[It] is not the microbe that is transmitted from the parents to the offspring, but the predisposition to disease." However, although the genetic theory of infectious diseases has benefited from recent exchanges between different disciplines [21], only a very small fraction of human infectious diseases are understood at the genetic level.

Conventional PIDs have been studied in most detail, over the last 50 years. Association studies have so far identified only a handful of convincing susceptibility alleles (e.g., HbS). Linkage studies have recently identified a set of major genes (leprosy), and novel PIDs have provided a Mendelian basis for predisposition to certain infectious diseases (HSE), bridging the gap between the two fields dealing with genetic predisposition to infection. Much progress is expected in these two fields in the near future. We can also expect more Mendelian resistance genes to be discovered. There is therefore considerable determinism in the human genetics of infectious diseases, and the field as a whole is in its infancy. In addition to the identification of new genes, one key question concerns definition of the proportion of Mendelian and more complex predispositions or resistances in individuals and populations [8, 69]. There is clearly no such thing as a strict Mendelian segregation of phenotypes, because no single-gene organisms exist. All phenotypes are therefore multigenic in essence. It is therefore important to define the hierarchy of the genes involved in predisposition to infection at both the individual and population levels.

This field has considerable clinical implications, as illustrated in this chapter, in terms not only of diagnosis, but also of the development of new therapeutic interventions, such as the life-saving effects of exogenous IFN-γ in patients with mycobacterial disease resulting from insufficient endogenous IFN-γ production, which are of a similar magnitude to those of insulin in diabetic patients. The biological implications have been equally considerable, not only in terms of understanding the pathogenesis of infections but also in terms of immunology, as the human genetics of infectious diseases provides an ideal way to define the function of immune system genes *in natura*, in the setting of a natural ecosystem [71].

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Gene Action: Developmental Genetics

13

Stefan Mundlos

Abstract Developmental genetics studies the mechanisms how genes initiate and control the process by which a single cell can give rise to a mature organism. This includes mechanisms of early patterning, as well as later events that result in the formation and maturation of organ systems. Developmentally active genes exert their effects through many pathways and mechanisms including diffusing morphogens, cell migration, proliferation, and border formation. Transient structures such as the somites, the branchial arches and the apical ectodermal ridge serve as scaffold and signaling centers during embryogenesis. Gene defects frequently result in abnormal development with specific phenotypes that reflect the gene's essential functions during embryogenesis. In many instances this results in a combination of malformations that are characteristic for a specific syndrome.

Contents

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13.1 Genetics of Embryonal Development

The study of embryonal development focuses on the process by which a single cell can give rise to a mature organism. This involves the early steps of development that set the pattern for the overall bauplan of the body and the development of individual organs studied in model systems, such as the insect eye, the vertebrate limb, or the nervous system. The study of developmental biology, however, goes beyond the study of embryos. It includes the regeneration of lost organs, such as the newt limb, or the lizard tail, and the control of postembryonic growth, a process that includes metamorphosis and aging. Development is less an adaptation and reaction to certain stimuli and more a programmed set of events in which the transient stages are more

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important than the permanent ones. The information for this process is contained within the genome. The genome holds the code that tells the embryo to develop and controls differentiation of cells, thus directing the entire process. But this is not a one-way process, since the embryo, on the other hand, is able to control its genome, mediating between genotype and phenotype, between the inherited genes, its environment, and the adult organism. Since gene regulation is obviously the key event in the organization of this process, many mechanisms have been proposed and experimentally verified. But how this intricate control system is able to induce and maintain differences between cells, enabling them to differentiate, remains largely unclear. The answer will probably not come from considering merely DNA and its interactions with RNA and proteins. The feedback between the cells, their genome, and the environment will have to be considered. The organism, the regulative pathway including a network of cooperating partners, and the interacting environment have to be studied as a whole for true understanding of this process to be possible. The detection of a mutation in a patient with a heart defect links the function of this gene with heart development, but how this gene contributes to a critical process during development has to be investigated in detail using functional analysis. Mutations in humans can lead to new insights into the factors that contribute to certain developmental processes, but only the study of the entire regulatory network will yield a thorough understanding that will ultimately enable us to comprehend complex human traits and their interaction with environmental stimuli.

Because human experimentation is subject to obvious limitations, appropriate model systems are needed to study the molecular basis of developmental processes. Over the past century developmental biologists have established a wide variety of model systems that have greatly contributed to our understanding of the basic mechanisms of animal and human development. These include the fruit fly *Drosophila melanogaster*, the nematode *Caenorhabditis elegans*, the sea urchin *Lytechinus variegatus*, the zebra fish *Danio rerio*, the frog *Xenopus laevis*, the chick, and the mouse, to mention the most important ones. *Drosophila* was the first animal model to be studied in great detail, because it is genetically amenable and therefore suitable for large-scale mutation screening, genetic analysis, and manipulation. Many of the mechanisms underpinning early embryonic development were established in this species and, surprisingly at the time, were found to be conserved throughout the animal kingdom. The sea urchin has been most instrumental in the study of early development, in particular gastrulation, whereas *C. elegans* is a more recent model system appreciated for its stereotyped developmental program, an almost invariant cell lineage, and easy manipulation. *Xenopus* and the chick have been used as vertebrate model organ systems because both species produce robust embryos that develop outside of the mother, allowing easy manipulation. Both systems, however, have the disadvantage that they cannot be genetically manipulated. In this sense the mouse is the preferred organism, because of its suitability for genetic manipulation (in particular gene targeting) and its closeness to humans. The zebra fish combines many of the above-mentioned advantages owing to its accessibility and genetic amenability.

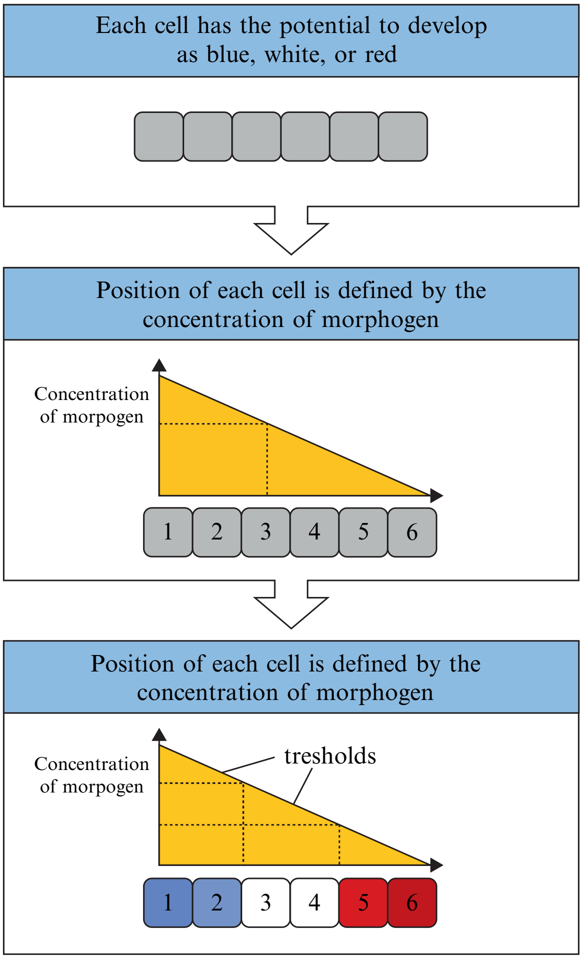
13.1.1 Basic Mechanisms of Development

The problem of how the embryo determines a pattern is one of the most central questions in biology. How is the cell fate orchestrated in a three-dimensional space by a set of instructions, the genes, that are the same in every cell? This question has captivated biologists and scientists from many other disciplines who have infused the field with their viewpoints. Clearly, the embryo uses a variety of ways to determine its own gestalt and function. At the molecular level several different processes are incorporated that affect the behavior of cells. Cell *proliferation* leads to an increase in cell number and thus expansion and growth of the embryo. New structures can be generated by differentially increasing the rate of proliferation. *Growth* may also be accomplished by the synthesis of macromolecules or an increase in cell size. Programmed cell death or *apoptosis* is an important mechanism to remove transient structures. For example, our fingers and toes are created by the death of interdigital cells in the hand and foot plates. Cell *migration* is the movement of an individual cells or groups of cells with respect to other cells in the embryo, as observed, for example, in neural crest and germ cells that undergo extensive migration and consequently populate parts of the embryo that are very distant from their original locations. During further development, *differentiation* takes place, a process by which cells become structurally and functionally specialized.

Cells become organized in tissues by sticking together, a process called condensation. This can take place through the expression of complementary adhesion molecules on their surfaces, and/or they may form associations with their extracellular matrix. Adhesion molecules function in the same way as a receptor-ligand interaction, except that in this case the adjacent cells carry either the receptor or the ligand. By this mechanism borders can be induced and maintained, ensuring that the cells on either side of the border develop according to a different scheme. The delta/notch pathway represents such a system, where one cell expresses notch whereas the other carries the delta receptor on its surface, each inducing its own set of gene expression. Posttranslational modification of proteins is an important way to modify their interaction with other proteins [13]. For example, fringe, a known modifier of notch signaling, has been shown to function as a fucose-specific *N*-acetylglucosaminyltransferase, demonstrating that notch signaling can be regulated by protein modification. More recently, microRNAs, small RNA molecules that inhibit the translation of specific mRNAs, were shown to be involved in gene regulation in development. All of these mechanisms are instrumental in inducing differentiation, but how a body plan and thus a three-dimensional structure is established cannot be explained by these mechanisms alone. One emerging concept that is likely to be central to the problem is the presence of so-called morphogens, signaling molecules that determine cell fate.

13.1.2 Mechanisms of Morphogenesis

In the oldest sense of the word, a morphogen is a substance that is produced by cells and organizes a pattern by spreading to other cells [1]. Because morphogens are produced at one location, usually referred to as the signaling center, their concentration is thought to decline as a function of distance from the source. Thus, cells that are close to the signaling center will receive a high concentration of morphogen, while those further away receive lower doses. The hypothesis of smoothly declining gradients, originally proposed by Wolpert, assigns positional values to cells that are ultimately translated into cell fate determination (Fig. 13.1). The diffusing morphogen produces a gradient, which is superposed by other morphogen gradients. This results in cross-threshold values at which genes are turned on or off. This hypothesis has been supported by the identification of substances such as bicoid and decapentaplegic (Dpp) in *Drosophila* and, subsequently, polypeptides of the fibroblast growth factor (FGF), Wnt, Hedgehog, and transforming growth factor (TGF) families in vertebrates that act as intracellular or extracellular morphogens. During early *Drosophila* development morphogens can diffuse freely because the zygote nucleus undergoes a series of divisions in a common cytoplasm, the syncytial blastoderm. But how do morphogens move in the intercellular space? It is still not clear how gradients of


Each cell has the potential to develop as blue, white, or red
Position of each cell is defined by the concentration of morphogen
Concentration of morpogen
1
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Position of each cell is defined by the concentration of morphogen
Concentration of morpogen
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Fig. 13.1 Morphogens determine cell fate. Each cell has the potential to develop as red, white, or blue. A gradient of morphogen produced by a signaling center results in a different concentration at each cell, depending on the distance from the source. A threshold level determines cell fate and identity. A high concentration results in blue cells, medium concentration, in white, and low concentration, in red cells. (From [35], p. 24, Fig. 1.25, by permission of Oxford University Press)

morphogens specify positional information, in particular the relative roles of morphogen diffusion and cell–cell interaction. Several models exist that try to explain how cell identity can be specified over long and short distances. Simple diffusion refers to randomly moving molecules that encounter little impediment from the tortuous intercellular spaces if concentration difference is the driving force of their movement. To direct the effect of a morphogen, evolution has developed intricate ways by modifying the spreading of freely moving molecules. Altering diffusivity allows the morphogen to accumulate to much higher levels near its source, paradoxically resulting in an increased range of action. Extracellular heparan sulfate proteoglycans, for example, promote the transport of *Drosophila* hedgehog protein and are essential for FGF signaling. Consequently, enzymes that change the local content of extracellular matrix heparan sulfate can have a great influence on hedgehog as well as FGF signaling. Other ways to modify diffusivity are lipid modifications; these occur in morphogens of the hedgehog and Wnt families. Morphogens may also be transported through cells by endocytotic vesicles, passing the morphogen on from one cell to the next. Receptor-mediated endocytosis and subsequent rapid degradation may serve to produce a sharp decline in protein concentration. Availability of morphogens may be altered by producing nonactive depot forms, as it is in the case of the transforming growth factors, which are activated upon proteolytic cleavage. Inhibitors may bind to morphogens, thus preventing their diffusion and/or binding to their cognate receptors. The BMP inhibitor Noggin, for example, completely inhibits BMP signaling, but is expressed at distinct sites that only partially overlap with BMP expression, thus directing and diversifying the signal (Fig. 13.2).

A wide variety of different signaling centers have been described that are essential for many developmental processes. In the *Drosophila* egg two "organizing centers" initiate anterior and posterior gradients, each forming its own structures at the poles and interacting with the other gradient to form the central portion of the embryo. Nüsslein–Volhard identified bicoid and hunchback as the proteins crucial for the anterior gradient and thus head and thorax formation, whereas nanos and caudal were found to form the posterior gradient and thus the abdominal segments [6, 7]. The anterior proteins inhibit the translations of the posterior proteins and vice versa, and as a result of this interaction a four-protein gradient is produced in the early embryo, which governs the first steps of *Drosophila* embryogenesis (Fig. 13.3). Signaling centers in vertebrates are, for example, the notochord and the floor plate, both producing the morphogen sonic hedgehog (Shh). As discussed below, different neurons develop in the neural tube depending on their distance from the signaling center and thus the concentration of the morphogen. A similar situation is seen in the limb, where Shh is expressed exclusively in the so-called zone of polarizing activity, a distinct region in the posterior part of the limb bud which controls the asymmetry of our limbs from the thumb to the little finger (Fig. 13.4) [30]. Depending on the distance from the center a pattern is built that determines which cells will finally develop into the individual digits. Duplications of this center to the anterior side result in mirror image duplications producing another set of digits. Many other signaling centers are known, and some of them are discussed below.

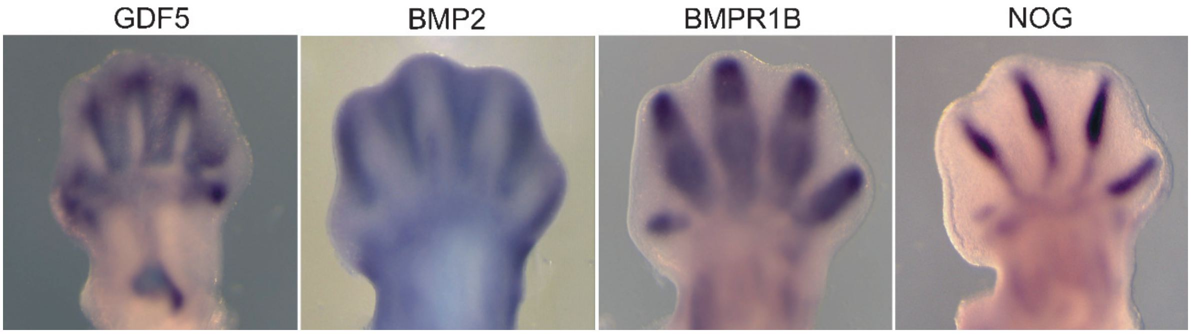

Fig. 13.2 Diverse expression patterns of morphogen, receptor, and inhibitor. Expression pattern of GDF5, BMP2, the receptor BMPR1B, and the inhibitor Noggin during development of the mouse digits. (Courtesy of P. Seemann)

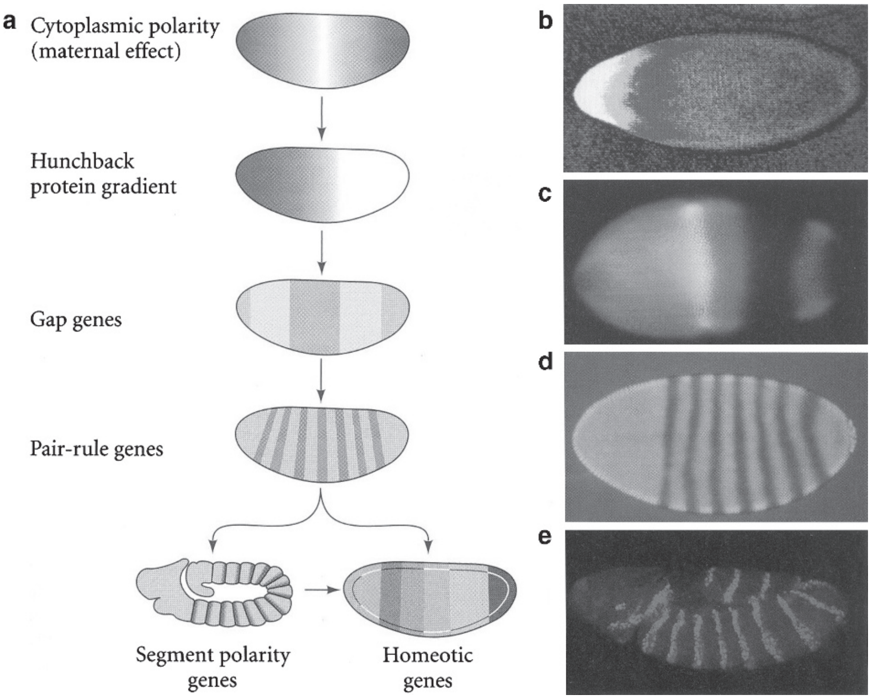

Fig. 13.3 (**a–e**) A generalized model of *Drosophila* pattern formation. (**a**) Anterior-posterior pattern is first generated by maternal effect genes that are located as sequestered mRNAs near the anterior tip (bicoid) and the posterior tip (nanos) of the unfertilized egg. After fertilization, the mRNAs are translated into proteins that can diffuse in the syncytial blastoderm, forming gradients that in turn activate Hunchback protein, which differentially activates gap genes, that define broad regions of the embryo. Gap genes activate pair-rule genes, giving the first indication of segmentation in the fly embryo. Pair-rule genes are expressed in a "zebra-stripe" pattern with an alternating pattern of vertical bands of cells expressing and not expressing a pair-rule gene. Together, these genes control the expression domains of the homeotic genes that define the identity of each segment. (**b**) Maternal effect genes. Bicoid protein concentration is highest at anterior tip (*bright yellow*) and diminishes towards the middle of the embryo (*red*). (**c**) Gap gene expression. Distribution of hunchback (*orange*) and Krüppel (*green*) overlap in the middle part (*yellow*). (**d**) Pair-rule genes. Pair-rule gene fushi tarazu forms seven stripes across the embryo. (**e**) Segment polarity genes. Expression ofengrailed dividing the embryo into a repeated series of segmental primordia along the anterior-posterior axis. (From [10], Fig. 9.17a–e)

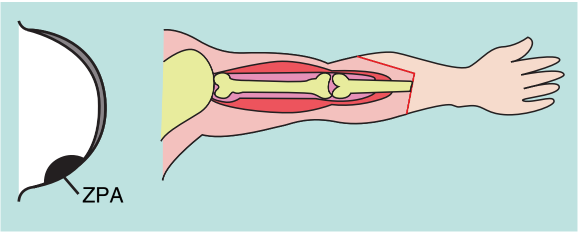

Fig. 13.4 Morphogen controls asymmetry of the limbs. Sonic hedgehog (*Shh*) is expressed in the posterior margin of the developing limb in a region called the zone of polarizing activity (ZPA). Digital identity from thumb to little finger is determined by the proximity to the signaling center and thus the concentration of Shh protein. (From [33], Fig. 2)

Morphogens need effectors, molecules that govern gene expression and by this means determine cell fate. In general this is not accomplished by single molecules but by an entire set of so-called transcription factors that bind to specific DNA sequences in the regulatory regions of target genes, thus resulting in an orchestration of gene expression. One example are the homeotic or *Hox* genes, originally identified in *Drosophila* for their ability to deliver positional identity to cells, meaning the information that tells each cell where it is in the embryo and how it has to behave to generate a regionally appropriate structure. Mutations in *Hox* genes produced a number of flies in which one body

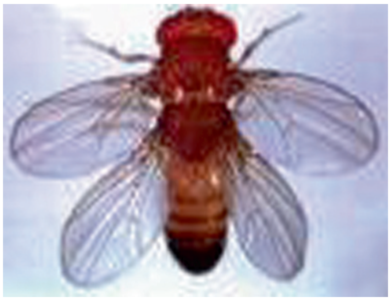

Fig. 13.5 Ultrabithorax mutant. A mutation in the bithorax complex of the *Drosophila Hox* gene cluster results in the homeotic transformation of the posterior halteres (a balancing organ) into wings, resulting in a fly with two pairs of wings instead of one. (From [10], p. 284)

part developed in the likeness of another (called a homeotic transformation). In the mutant *Antennapedia* legs instead of antennae grow out of the head. In the mutant bithorax a second set of wings develops instead of the haltere, a small structure normally used by the fly to keep balance (Fig. 13.5). Molecular analysis has revealed that eight homeobox genes exist in *Drosophila* and that they are arranged in a cluster in the fly genome. Furthermore, the genes are expressed in an overlapping pattern along the head-to-tail axis, dividing the body into discrete zones (Fig. 13.6). The particular combination of genes expressed in each zone appeared to be essential for this the cell's positional information, since manipulating the genes either by mutating them or by overexpressing single *Hox* genes resulted in body part transformation [19, 21]. Very similar clusters of *Hox* genes are found in mammals, but here four clusters are present that are also expressed in an overlapping fashion, with most 3′ genes of a cluster being expressed most anterior and most 5′ in the dorsal region of the embryo (Fig. 13.6) [19]. Mutations in HOX genes in humans do not result in transformation of body parts, but instead lead to a variety of malformations involving the limbs and genitals (Fig. 13.7).

13.2 The Stages of Development

The development of animals actually begins before fertilization of the egg with the production by the female of substances that nourish and control the development of the zygote into a multicellular organism. After fertilization the zygote divides mitotically to produce the cells of the body, usually without much growth in overall size. In most invertebrates the resulting ball of cells is called a blastula, but in vertebrates the term morula is used. Even at this stage some cells have been determined to form specific tissues in the body. This was shown by experiments in which a piece of blastula is surgically excised and transplanted to a different position or onto another organism. In some cases the cells survive and continue their original path of development irrespective of their new environment. Thus, even before there is any visible distinction, cells are assigned to a specific fate. Cleavage divisions produce a hollow sphere of cells, the fluid-filled blastocele, which is called a blastocyst in mammals.

After the first rapid series of cell divisions, the blastula/morula undergoes a massive reorganization called gastrulation. This process converts an essentially nondescript sphere of cells into an organism with distinct cell layers, often called the primary germ layers. As originally studied in the sea urchin, gastrulation starts with an invagination of a subset of cells located at one side of the blastula into the blastocoel, the central cavity of the blastula. Through extensive cell movements the primary germ layers are formed. As a result, the embryo consists of the outer layer, the ectoderm, which produces the cells of the epidermis and the nervous system, the inner layer, the endoderm, which produces the lining of the digestive tube and its associated organs, and the middle layer, the mesoderm, which gives rise to several organs, including heart, kidney, gonads, and the skeleton. Although similar in principle, gastrulation follows different mechanisms in different species (Fig. 13.8). The major characteristic of avian and mammalian gastrulation is the primitive streak. This structure is visible as a thickening of the outer cell layer at the posterior region of the embryo caused by the ingression of mesodermal cells into the blastocoel and by migration of lateral cells towards the center. This streak marks the anterior–posterior axis of the embryo. A depression (primitive groove) forms within the streak, through which cells migrate into the blastocoel. Other migrating cells move between the two layers and form the mesoderm. At the anterior end of the primitive streak a regional thickening forms, called the primitive knot or Hensen's node. This structure is equivalent to the amphibian's blastopore, where migrating cells from the outside turn inward and travel along the inner surface of the outer cell sheets

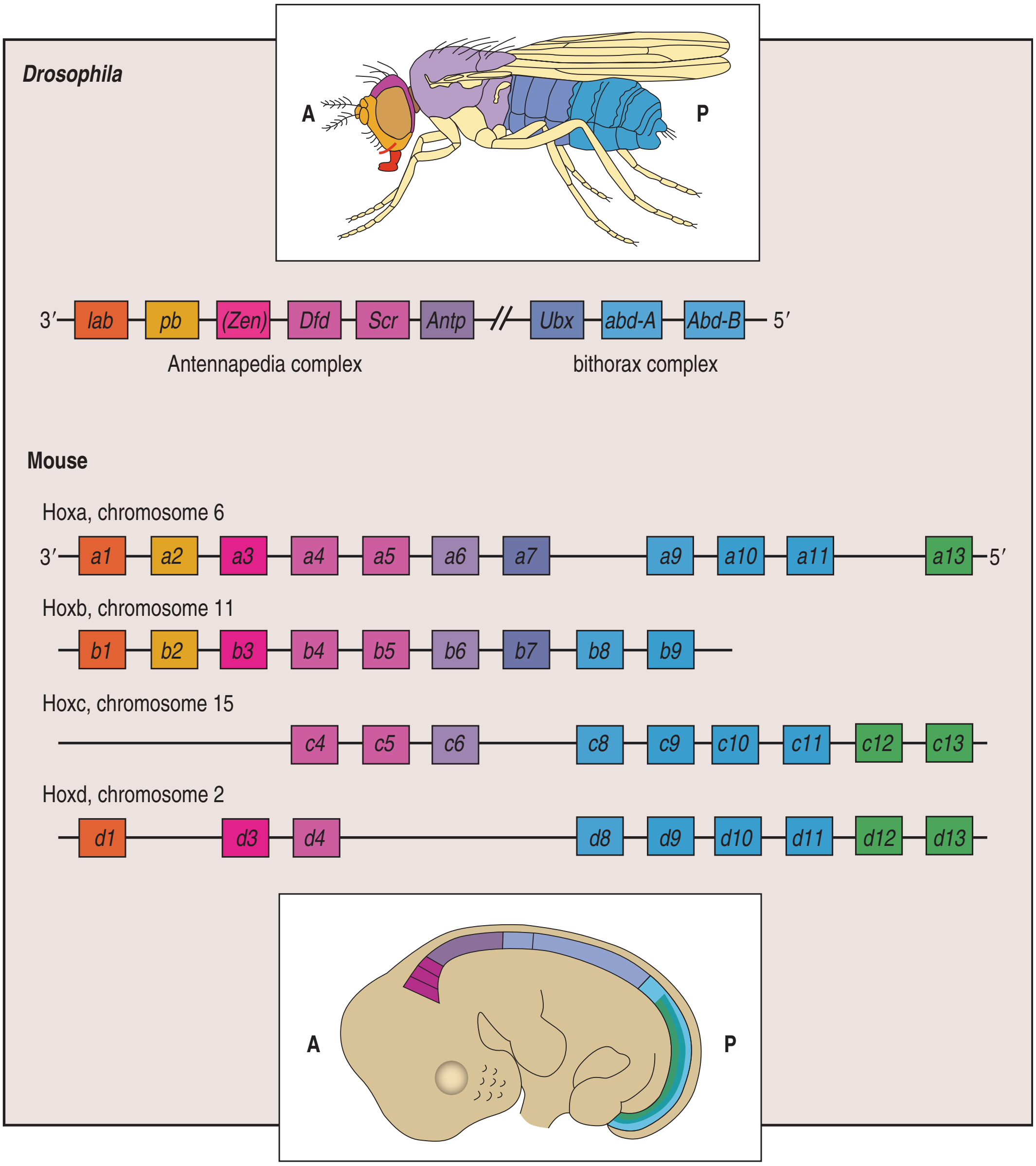

Fig. 13.6 *Hox* genes in *Drosophila* and mammals. *Hox* genes belong to a group of regulatory proteins (transcription factors) that control gene expression. In *Drosophila* there is one *Hox* gene cluster known as HOM-C, which consists of two distinct composites, the Antennapedia and the bithorax complex. In vertebrates *Hox* genes are arranged in four *Hox* clusters called A, B, C, and D, which are likely to have developed from genomic duplications of ancestral *Hox* clusters. In each cluster the order of the genes from 3′ to 5′ corresponds to the sequence in which they are expressed along the anterior-posterior axis of the embryo. Thus, the most 3′ located gene (*red*) is expressed first and furthest in the anterior direction, whereas the most 5′ (*blue*/*green*) is expressed last and furthest in the posterior direction (a phenomenon called spatial and temporal colinearity, respectively). Vertebrates have four *Hox* gene clusters (*Hox*A, -B, -C, -D), which originate from an ancestral cluster, possibly related to the single *Hox* cluster in the lancelet, a simple chordate. Genes that have arisen by duplication and divergence are referred to as paralogs, and the corresponding genes in each cluster (e.g., *Hoxa9*, *Hoxb9*, *Hoxc9*, *Hoxd9*) are known as a paralogous subgroups. Genes of a paralogous subgroup are more similar than genes within a cluster. (From [35], p. 156, Box 4A, by permission of Oxford University Press)

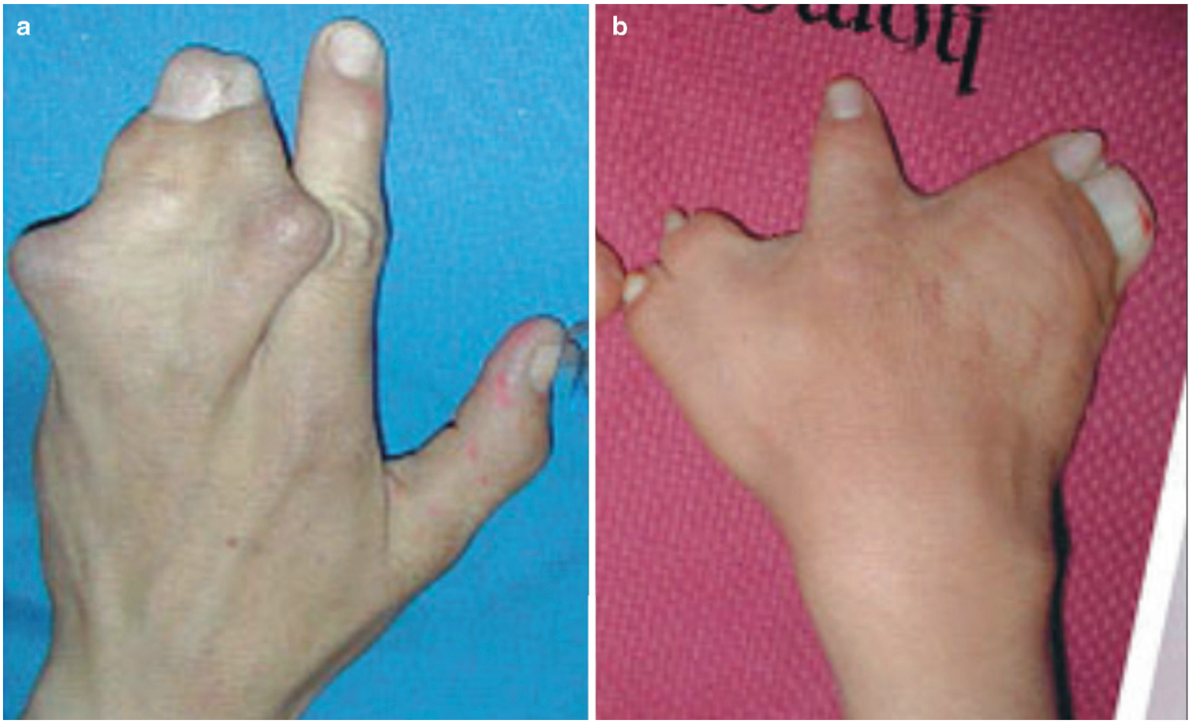

Fig. 13.7 (**a**, **b**) Synpolydactyly caused by a polyalanine expansion mutation in HOXD13. (**a**) Heterozygous mutations result in an additional finger between the third and fourth fingers and a syndactylous web between them. (**b**) Homozygous individual showing severe shortening of fingers, syndactyly, and polydactyly

(Fig. 13.9). Cells migrating through the primitive knot contribute to the foregut, the head mesoderm, and the notochord, whereas those that migrate through the lateral portions of the primive streak give rise to the other endodermal and mesodermal tissues. During this time a relatively small inner cell mass has become a bilaminar disk of ectoderm and endoderm, each with its own fluid-filled cavity, the amniotic sac, and the yolk sac.

In mammals early development follows the same principle, but not all cells contribute to the embryo, since a major proportion of cells is concerned with establishing tissues that are needed as a life support, namely the extraembryonic membranes and the placenta. Five to six days after conception the human blastocyst arrives in the uterus and attaches to the uterine wall (Fig. 13.10). The blastocyst now consists of an outer cell layer, the trophoblast and an inner layer, the embryoblast. The latter cells congregate at one end of the blastocele to form the inner cell mass. The trophoblast proliferates rapidly and differentiates into an inner layer of cytotrophoblast and an outer multinucleated layer, the syncytiotrophoblast, which starts to invade the uterine wall. During the second and third weeks of human development the invaded tissue becomes vacuolated and rapidly fills with blood. Chorionic villi grow into the vacuoles, bringing the maternal and embryonic blood supply into close contact and allowing the exchange of nutrients and waste products. The fully developed organ consiting of trophoblast tissue and the blood vessels is called the chorion. The chorion fuses with the uterine wall to create the placenta. Besides its role in the exchange of nutrients, the chorion has other important functions as an endocrine organ producing chorionic gonadotropin.

One of the early mesodermal derivatives is the circulatory system, which develops during the third week by vascular channels that arise in the splanchnic mesoderm lining the yolk sac; later a primitive heart that begins pumping by the end of the third week. The narrow connecting stalk that links the embryo to the trophoblast eventually forms the vessels of the umbilical cord. The embryo is now connected to its supply for

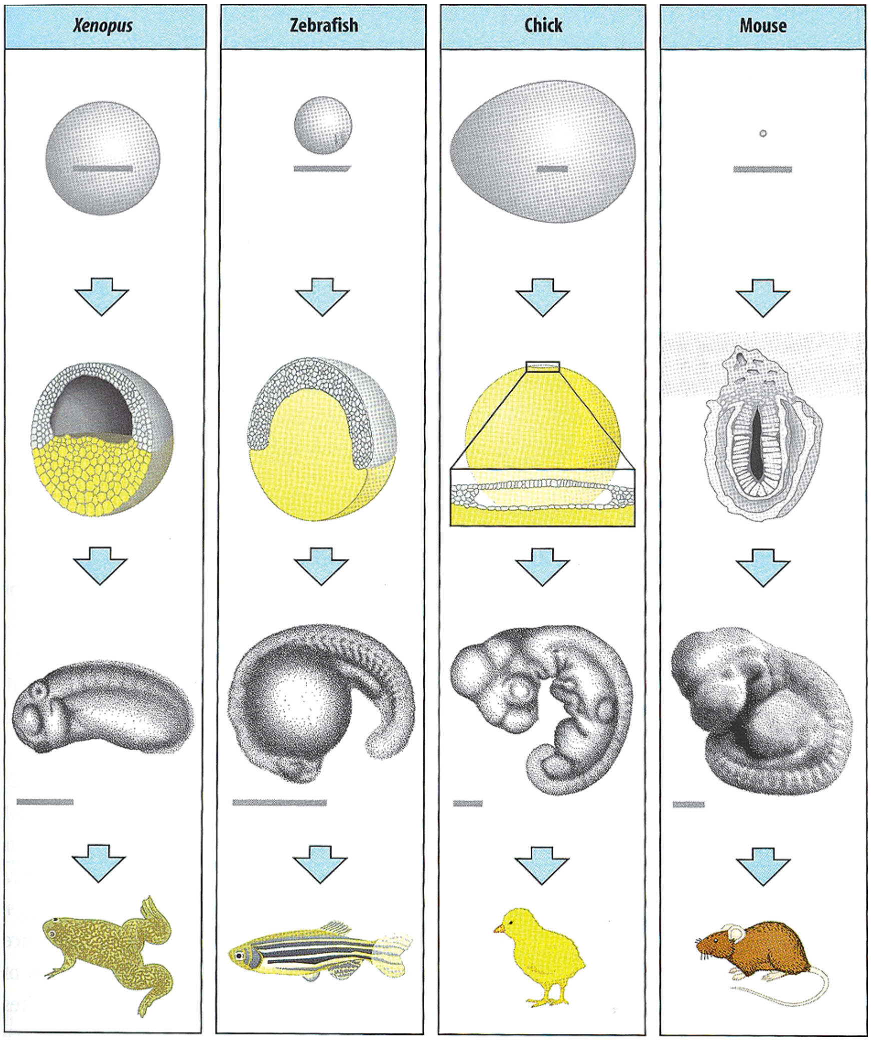

Fig. 13.8 Differences and similarities in development. Vertebrates show considerable differences at the start of their development. The size of the egg is very different (*scale bars* 1 mm except for the chicken egg: 10 mm). The *second row* shows a cross section through the blastula, with the egg yolk shown in *yellow*. At this time the mouse embryo has implanted into the uterine wall and has developed extraembryonic tissues. The embryo itself is the U-shaped structure in the *middle*. Thereafter, gastrulation commences and the embryos develop into polarized bodies consisting of ectoderm, mesoderm, and endoderm. In the following stage (*third row*) all embryos show a certain degree of similarity. The head has formed, and the neural tube, somites, and notochord are present. After this stage their development diverges again, giving rise to such diverse structures as wings, fins, and legs. (From [35], p. 91, Fig. 3.2, by permission of Oxford University Press)

nutrition, hormones and other essential substances, and the stage is set for the period of further patterning and major organogenesis. Early patterning events and the subsequent organ development are extremely complex processes that have been studied in a multitude of model systems. In this overview a few of these systems will be presented in an exemplary way without aiming at a full description of vertebrate organ development.

13.3 Formation of the Central Nervous System

Neurulation is the process by which the embryo forms a neural tube, the rudiment of the central nervous system. The formation of the neural tube is directly related to gastrulation, one of the most important processes in early development. The

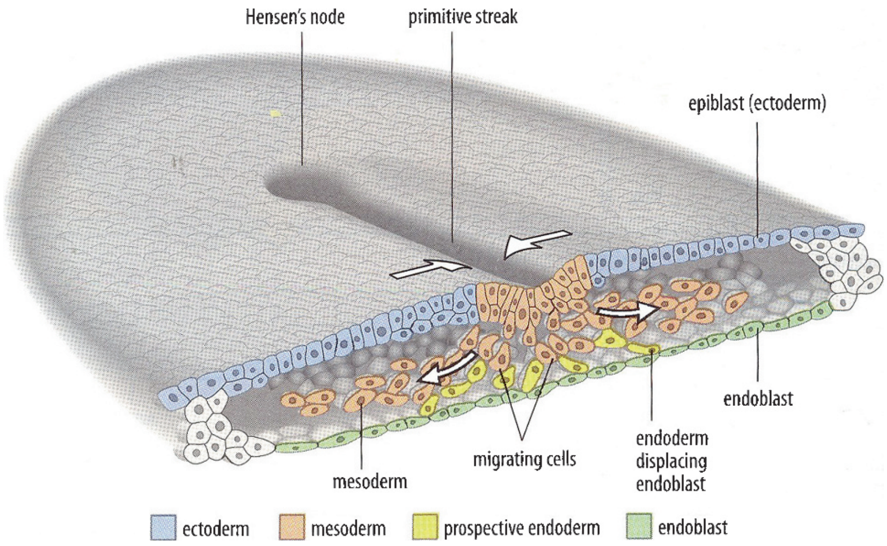

Fig. 13.9 Gastrulation in the chick embryo and Hensen's node. Cells migrate through the primitive streak into the interior of the blastoderm, where they give rise to the endoderm and the mesoderm. At the anterior end of the primitive streak an aggregation of cells known as Hensen's node can be seen. As the streak regresses, the node moves to the posterior end leaving behind the notochord and the first somites. (From [35], p. 102 Fig. 3.15, by permission of Oxford University Press)

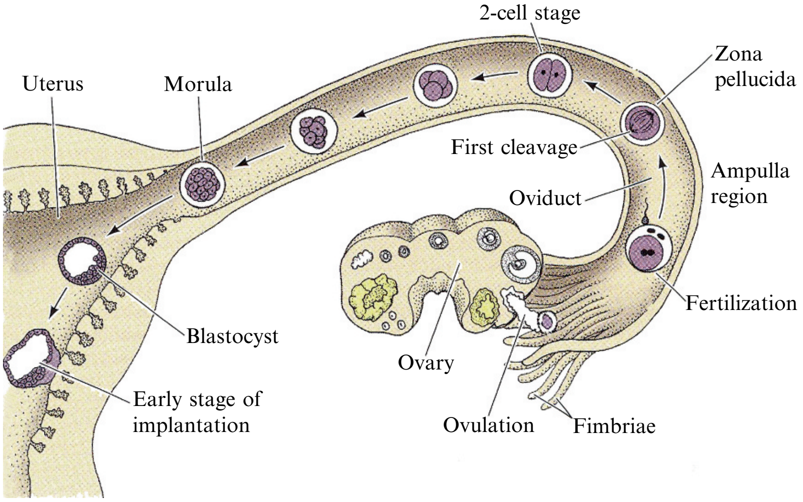

Fig. 13.10 Development of the human embryo from fertilization to implantation. Fertilization of the human oocyte takes place in the ampulla region of the oviduct, and the first cleavage occurs about a day later. The embryo keeps dividing at a slow rate as the cilia of the oviduct push the embryo towards the uterus. In contrast to most other embryos, mammalian blastomeres do not all divide at the same time, and they thus frequently contain odd numbers of cells. The zona pellucida surrounds the embryo and prevents it from attaching to the oviduct. Upon entry into the uterus the blastocyst escapes from the zona pellucida. In the mouse this is accomplished by digesting a small hole in it, through which the blastocyst "hatches." The uterine epithelium (endometrium) secretes a matrix that allows attachment of the blastocyst to the uterine wall. A cocktail of proteases secreted by the trophoblast enables the blastocyst to bury itself within the uterine wall. (From [10], p. 348, Fig. 11.27)

interaction between the dorsal mesoderm and its overlying ectoderm results in the formation of a hollow tube, which will differentiate into the brain and the spinal cord. The ectoderm folds at the most dorsal point, forming an outer epidermis and an inner neural tube. The two layers are connected by a specialized subset of cells, the neural crest. Folding ultimately results in the formation of the spinal cord from the inner layer and closure of the epidermal layer, with the neural crest being situated in between. The neural tube closes as the paired neural folds are brought together at the dorsal midline. This process does not happen at one time point, but is an ongoing process that can best be observed in the chick embryo. While major regions of the neural tube may already be formed in the cephalic (head) region of an embryo, the caudal (tail) region may still be undergoing gastrulation, i.e., the migration of cells from the outer layer inside. Thus, tube formation progresses from head to tail in a zipper like format. In the cephalic region the wall of the tube is broad and thick and a series of swellings and constrictions define the future brain compartments. In those parts of the embryo that form the spinal cord, the neural tube remains a simple tube (Fig. 13.11).

In humans this process follows the same principle. However, the timing and the site of neural tube closure is different. In contrast to the process in the chick, human closure starts in the middle of the embryo and both, the anterior and the posterior neuropores, are open. Closure of the tube progresses from this initial site in both directions, toward the caudal and the cephalic part of the embryo. The cephalic part closes first, followed by the caudal part. Human malformations involving this process are common (Fig. 13.12). They present as spina bifida and anencephaly. In the latter condition there is failure of the neural plate fusion in the cephalic region while spina bifida is observed if the caudal part does not fuse. The reasons for this are complex, caused by genetic as well as environmental factors. Dietary factors such as cholesterol and folic acid appear to be important for normal development. It has been estimated that around 50% of neural tube defects can be prevented when pregnant women take supplemental folic acid. Cholesterol, on the other hand, appears to be necessary for the function of the Sonic hedgehog (Shh) signaling molecule, a morphogen that is essential for neural tube and brain development.

Neural crest cells start a long migration throughout the embryo, contributing to a vast number of tissues, including the bones of the skull, the teeth, the neuronal cells of the gut, and the heart. In humans several syndromes are known that are due to defects in neural crest migration and/or differentiation. For example,

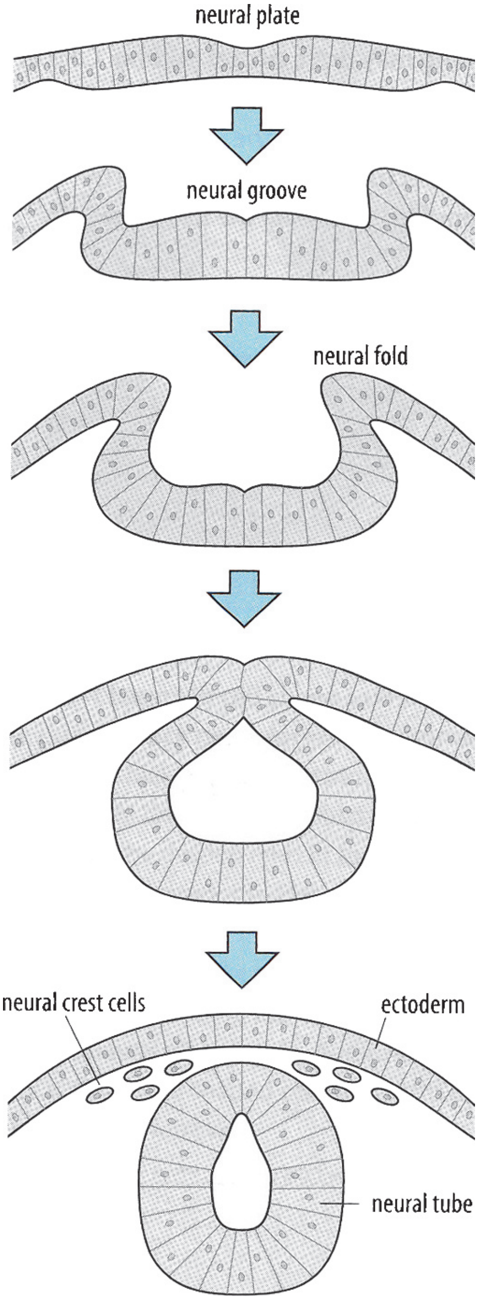

Fig 13.11 Development of the neural tube. Schematic cross sections through the early embryo are shown, representing different stages of neurulation from anterior (head, *top*) to posterior (tail, *bottom*). During neurulation the neural plate bends inwards, creating the neural groove with the neural folds at either side. With further development, the neural folds rise up, extend to the lateral side, and finally form a tube when they meet in the midline. This tube then detaches from the ectoderm, which becomes the epidermis. Neural crest cells which originate from the tip of the folds migrate away towards their distinct destinations. (From [35], p 283, Fig. 7.34, by permission of Oxford University Press)

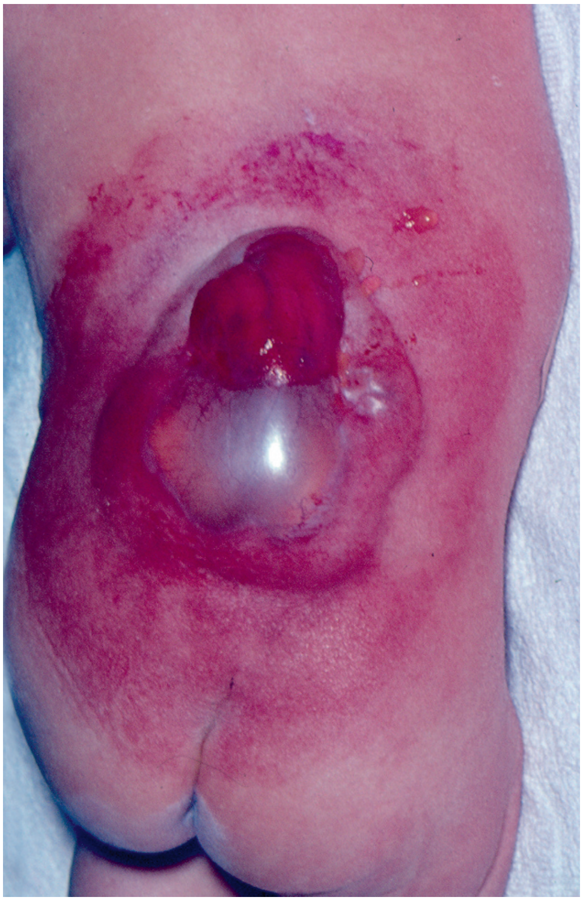

Fig. 13.12 Neural tube defect

mutations in PAX3 result in Waardenburg syndrome, a condition characterized by a specific facial appearance and unpigmented scalp hair. This pigmentation defect is believed to be due to missing neural crest cells, since these cells contribute to the pigment epithelium in the skin and hair.

While the posterior part of the tube is still being formed, the anterior or cephalic part undergoes drastic changes. In humans it subdivides into three primary vesicles, the forebrain (prosencephalon), the midbrain (mesencephalon), and the hindbrain (rhombencephalon). The anterior part of the prosencephalon bulges out laterally, building the two parts of the telencephalon, whereas the more caudal part of the prosencepahlon becomes the diencephalon. Furthermore, secondary bulges, the optic vesicles, extend laterally from each side of the prosencephalon. The mesencephalon does not become subdivided. Its lumen will eventually become the aqueduct connecting the ventricles. The rhomencephalon elongates and becomes subdivided into the metencephalon and the myelencephalon which eventually give rise to the cerebellum and the medulla oblongata, respectively (Fig. 13.13). During this process the early brain increases dramatically in size; however, this increase is primarily due to an increase in cavity size, and not to tissue growth. It has been speculated that increased fluid pressure inside the vesicle is the driving force for

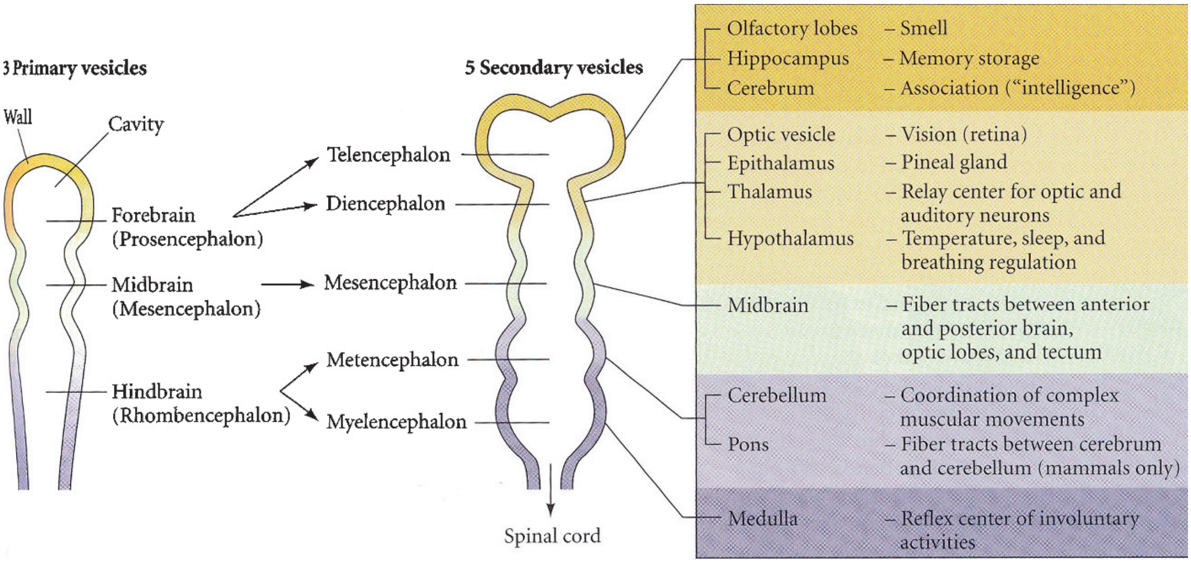
3 Primary vesicles
Wall
Cavity
Forebrain (Prosencephalon)
Midbrain (Mesencephalon)
Hindbrain (Rhombencephalon)
5 Secondary vesicles
Telencephalon
Diencephalon
Mesencephalon
Metencephalon
Myelencephalon
Spinal cord
Olfactory lobes – Smell
Hippocampus – Memory storage
Cerebrum – Association ("intelligence")
Optic vesicle – Vision (retina)
Epithalamus – Pineal gland
Thalamus – Relay center for optic and auditory neurons
Hypothalamus – Temperature, sleep, and breathing regulation
Midbrain – Fiber tracts between anterior and posterior brain, optic lobes, and tectum
Cerebellum – Coordination of complex muscular movements
Pons – Fiber tracts between cerebrum and cerebellum (mammals only)
Medulla – Reflex center of involuntary activities

Fig. 13.13 Early human brain development. The three primitive brain vesicles, forebrain, midbrain, and hindbrain, are subdivided as shown on the *right*. These give rise to the adult brain derivatives listed in the *box*. (From [10], p. 381, Fig. 12.9)

this rapid expansion, since reduction of the pressure in chick embryos results in enlargement at a slower rate and the formation of fewer cells.

One important molecule that controls these early patterning events is Sonic hedgehog (Shh)[14]. Hedgehog was first discovered during a screen for mutants in *Drosophila melanogaster*, in which mutations in the single *Hh* gene that presents in this species give rise to an embryo that is covered in spiky cuticular processes called denticles, inspiring the "hedgehog" name. Shh, the vertebrate homologue of Hh, is a secreted molecule that undergoes extensive posttranslational modification including lipid modification, which influences the movement of Hh molecules between cells and its autocatalytic processing in an active and an inactive part. Through its receptor-patched (ptc) and the activation of the transcription factors Gli (cubitus interruptus (Ci) in *Drosophila*) Shh/Hh specifies neuronal identity over short and long distances. Shh's ability to specify identities as a function of its concentration is especially well illustrated by the vertrebrate neural tube, where it has a pivotal role in the generation of the diverse types of neurons that are required for the assembly of the spinal cord, the forebrain, and the retina. Shh patterns the neural tube from its two expression sites, the notochord and the floorplate, a triangular wedge of cells located at the ventral midline of the neural tube. The decreasing concentration of Shh from ventral to dorsal establishes distinct progenitor domains which prefigure and predict defined classes of neurons (Fig. 13.14). Without Shh this specification does not takes place and the neural tube consists mainly of dorsal type neurons and, for example, completely misses motorneurons.

How can one signal result in such diverse outcome? Expression of additional factors that modulate Shh signaling may result in a completely different effect. For example, regional differences to Shh signaling have a crucial role in the generation of complexity in the CNS of vertebrates. Shh is expressed in the developing brain in a narrow strip of cells in the vertebrate diencephalon, which is known as the zona limitans intrathalamica. Cells that lie posterior to this structure form the thalamus, whereas those located anterior give rise to the prethalamus. The target genes of *Shh*, *Ptch1* and *Nkx2.2* are expressed on both sides, but other important transcription factors (*Dlx2* on the anterior side and *Gbx2* on the posterior side) are expressed asymmetrically in response to *Shh*. Thus, the response to *Shh* may vary over time and space, further diversifying the response of cells to this important signal.

In humans, heterozygous mutations in SHH have been associated with variable forms of midline facial and brain dysmorphism, in particular holoprosencephaly (HPE) [27]. According to its clinical severity, three types of HPE have been delineated: alobar HPE, the

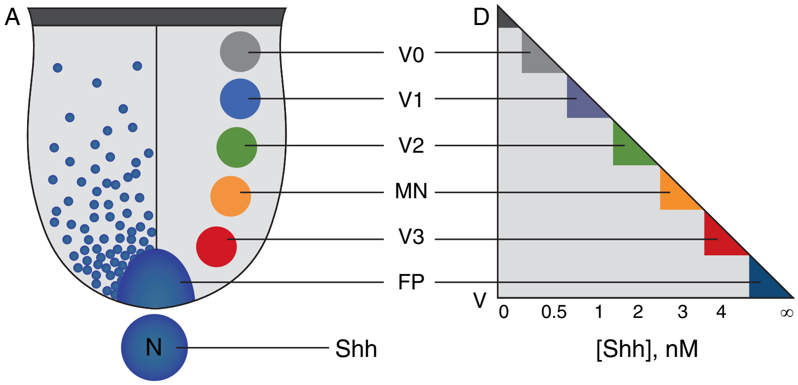

Fig 13.14 *Shh* and the control of spinal cord patterning. *Shh* expressed in the notochord and the floorplate defines the fate of ventral neural progenitors of the spinal cord. The progressively decreasing concentration of Shh protein regulates the expression domains of a series of transcription factors (as indicated by *color code*). *Shh* either represses or induces target genes at different concentration thresholds resulting in a complex pattern of expression combinations. The combinatorial expression of transcription factors in distinct domains determines the type of neuron that arises from each domain. (Reprinted from [15], by permission from Macmillan Publishers Ltd, *EMBO Reports*, copyright 2003)

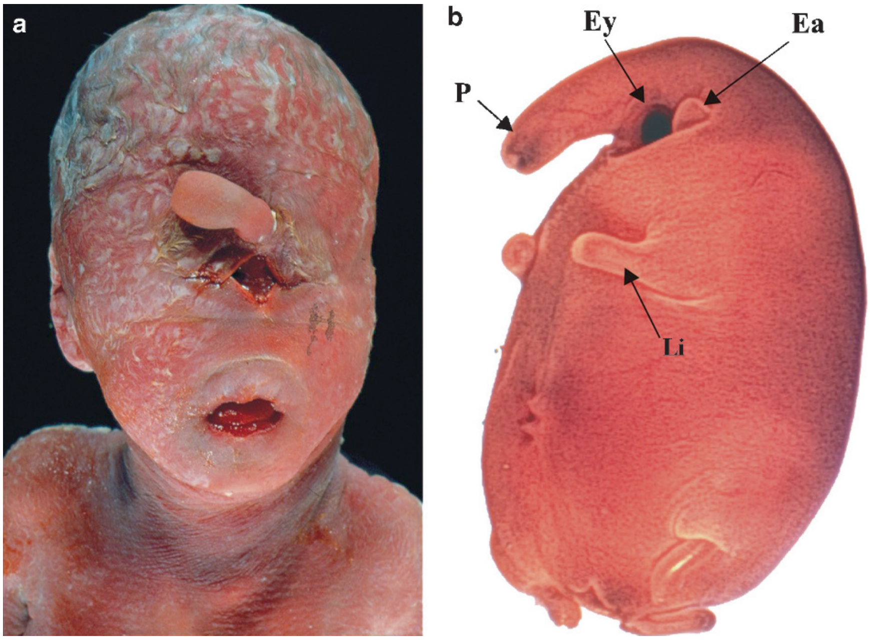

Fig. 13.15 (**a**, b) *Shh* regulates many aspects of development. (**a**) Patient with holoprosencephaly. Note nose-like structure in the middle with single eye underneath. (Courtesy of J. Kunze) (b) Mouse with inactivated *Shh*. Note single midline eye (cyclops) and nose-like structure above the eye (proboscis) (*P* Proboscis, *Ey* Eye, *Ea* Ear, *Li* Limb)

most severe form, is characterized by complete failure of the forebrain to divide into left and right hemispheres (Fig. 13.15). This form is associated with a single ventricle, and severe anomalies of the face, such as cyclopia, midline cleft, and single nostril are common. In semilobar HPE the interhemispheric fissure is partially present, whereas in lobar HPE the two hemispheres are separated but there is usually microcephaly. All three types, including completely asymtomatic carriers, can be observed within one family. This extreme variability raises the question of whether environmental factors can influence the outcome of this condition. Studies conducted in mammals and birds show that the severity of HPE defects correlates with the stage in which interruption in *Shh* signaling occurs [4]. Furthermore, levels of cholesterol may influence Shh processing and thus its activity. Inactivation of *Shh* in the mouse results in HPE and cyclopia, thus supporting the strong conservation of the pathway.

The initial patterning phase of the human cerebral cortex is paralleled and followed by three overlapping stages that form the structure of the brain. From weeks 5/6 to 6/20, stem cells deep in the forebrain proliferate and differentiate into young neurons or glial cells. During the second phase, between weeks 6/7 and 20/24, neurons migrate away from their place of origin in a radial fashion towards the pial surface, where each successive generation passes the previous one and settles in an inside-out pattern within the cortical plate. When migration is complete, the cortex consists of six layers of neurons that form discrete connections between the neurons and different parts of the CNS. Finally, from week 16 until well into postnatal life organization of the cortical layers takes place, a process associated with synaptogenesis and apoptosis. In this dynamic process more than one stage can occur simultaneously.

Developmental disorders accompanied by brain malformations are important causes of developmental delay, mental retardation, and epilepsy [12]. One of the best-described forms of human brain malformation is lissencephaly, a condition characterized by absent (agyria) or decreased (pachygyria) convolutions, producing a smooth cerebral surface attributable to loss of the folds of the brain, an abnormally thick cortex, and the loss of cortical lamination (Fig. 13.16). Subcortical band heterotopia is a related disorder that can be observed in different regions of the same brain, defining a spectrum of disorders with variable severity, all related to poor cortical architecture in the brain's development. Mutations in the *LIS1* gene cause lissencephaly [26].

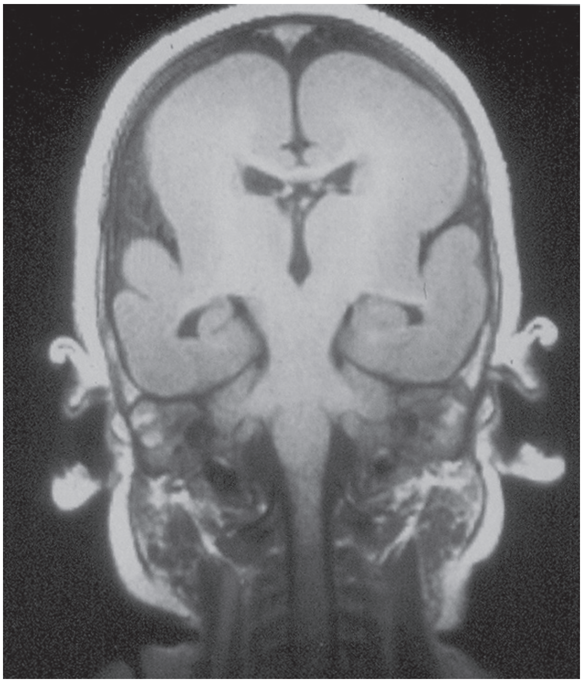

Fig. 13.16 MRI of patient with lissencephaly. Note "smooth" surface of brain owing to absence of gyri

Lis1 has been shown to influence neural progenitor proliferation and migration, possibly by interacting with microtubulin-binding proteins such as dynein and dynamitin. Loss of *Lis1* results in disruption of microtubule function, which interferes with forward translocation of the cell soma during migration and spindle orientation during mitosis. A second gene, DCX, causing an X-linked from of lissencephaly is likely to exert its effect on neuronal migration through its polymerization with microtubules.

Microcephaly vera means a reduction in brain size without marked brain malformation. Affected individuals have very small heads and a variable degree of mental retardation. Mutations in four genes have been shown to cause microcephaly vera; all are associated with the spindle poles of mitotic cells, and the mutations in them are consistent with a centrosomal mechanism in the control of cell division. Loss of microcephalin, for example, leads to premature chromosome condensation in G2 phase and delayed decondensation postmitosis through the condensin proteins. Interestingly, microcephalin has been implicated in human evolution including human brain size based on a strong positive selection in the human evolutionary lineage [8].

13.4 The Somites

Somites are a transient organizational structure of the developing embryo located on both sides of the neural tube consisting of epithelial cells with a periodic structure that originate from the paraxial mesoderm [11]. The formation of new somites and their detachment from the paraxial mesoderm has to occur in a highly ordered fashion simultaneously on both sides of the neural tube in the cranio-caudal direction. A time code for the formation and the budding of new somites is given by oscillations of cycling genes that lead to waves of notch-signaling sweeping up the paraxial mesoderm from the posterior to the anterior pole (Fig. 13.17). Beside this molecular clock, a stable gradient of *Fgf8* expression from the posterior to the anterior pole of the embryo allows a spatial coordination of somite border formation. Dll proteins are notch ligands that reside at the cell surface. Their differential expression determines both the size and the polarity of the somites. Any disturbance in this polarity results in abnormally spaced somites and in fusion of adjacent somites, as exemplified in the mouse mutant pudgy, which carries a mutation in *Dll3*. The phenotype observed in these mice resembles human vertebral malformations.

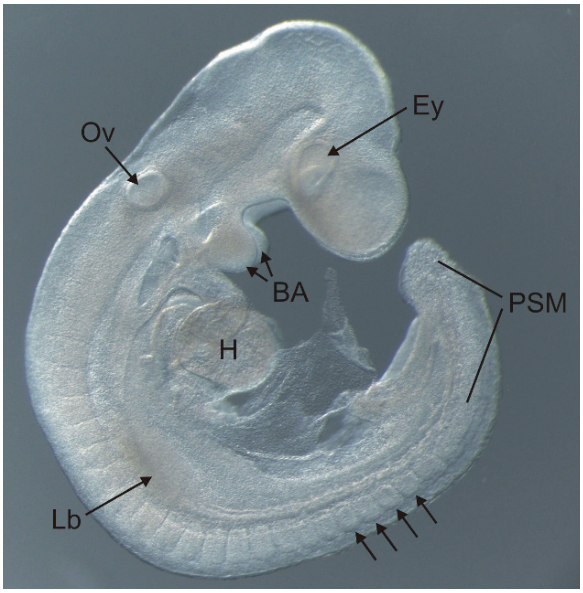

Fig. 13.17 Nine-day mouse embryo. Individual somites (*arrows*) can be seen, as can the paraxial mesoderm (PSM) (*Ey* Eye, *Ov* Otic vesicle, *Lb* Limb bud, *H* Heart, *BA* Brachial arches)

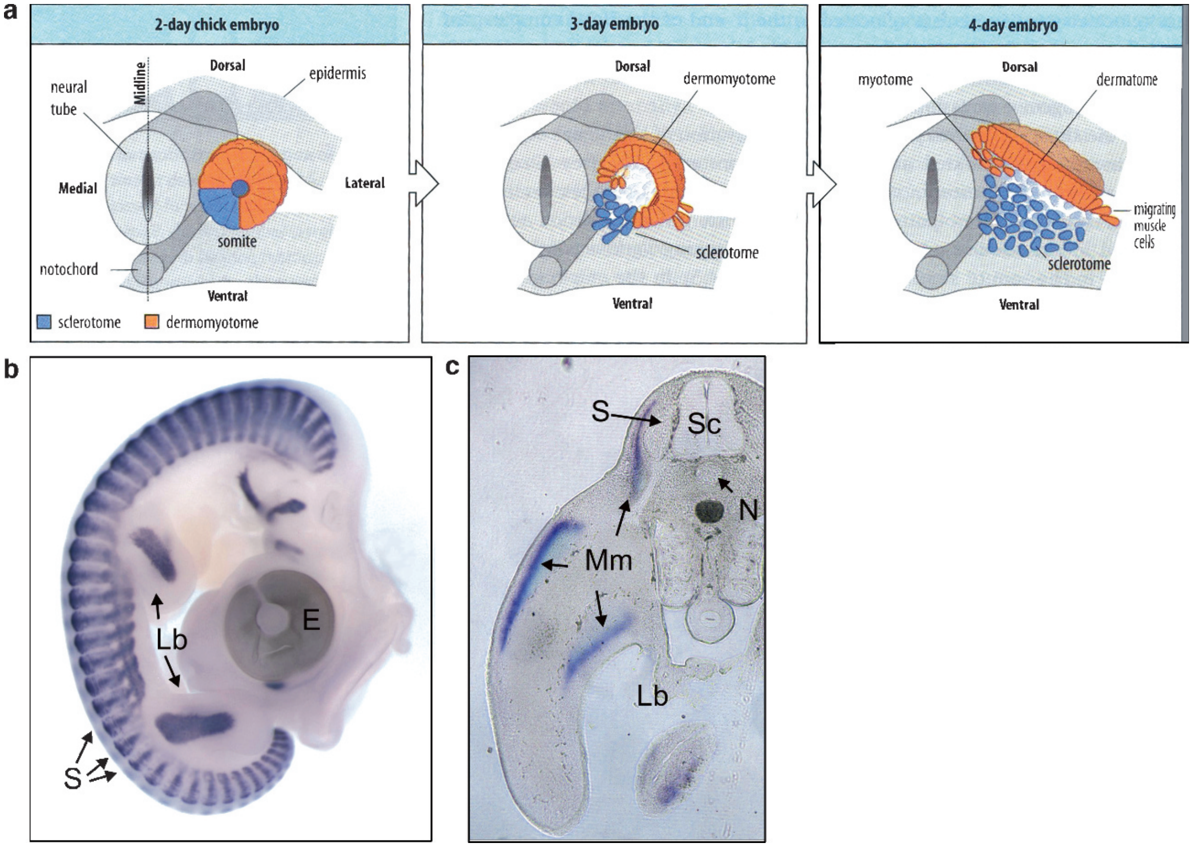

Fig. 13.18 (**a–c**) Development and fate of the somite. (**a**) Somites are transient structures that are formed periodically at each side of the neural tube from presomitic mesoderm. The medial quadrant of each somite gives rise to the sclerotome, whose cells will eventually form the vertebrae and the posterior part of the ribs. The rest of the somite forms the dermatome, which gives rise to the dorsal dermis and the myotome, the origin of all trunk and limb muscles. (**b**) Whole-mount in situ hybridization of a chick embryo showing expression of *MyoD*, a gene expressed during early muscle differentiation. Note periodic expression in somites and in the limb buds. (**c**) Cross section of embryo shown in **B** at anterior limb level. Staining shows migrating muscle cells (*Mm*) lateral to the somite (*S*) and in the limb bud (*Lb*) (*N* Notochord, *Sc* Spinal chord, *E* Eye. (From [35], p 164, Fig. 4.15, by permission of Oxford University Press)

As the somite moves rostrally, it matures and differentiates into the dermatome, the myotome, and the sclerotome (Fig. 13.18). A range of different tissues is generated from these structures. The dermatome originates from the central region of the dorsal layer of the somite (at this stage called the dermatomyotome). It generates the mesenchymal connective tissue of the back skin, the dermis. The myotome originates from the two most lateral portions of the somite. Its cells produce a layer of muscle precursor cells, the myoblasts, which form the epaxial muscles that will give rise to the intercostal and back musculature, and the hypaxial muscles of the body wall, limbs, and tongue. The epaxial part of the myotome is induced by signals from the neural tube (*Wnt1* and *Wnt3a*) and the floorplate (Shh), whereas the hypaxial region is induced by Wnt proteins from the epidermis and bone morphogenetic protein (BMP4) from the lateral plate mesoderm. The latter signal is likely to cause the migration of myoblasts away from the dorsal region into the body wall and the limbs. Further muscle development is established by a cascade of gene activation directed by a set of transcription factors, referred to as the myogenic regulatory factors, including *MyoD* and *Myf5*.

The sclerotome is the most medially located part of the somite and the primary origin of the axial skeleton. *Shh* is the major signal from the notochord that initiates and controls sclerotome formation. Transplantation of parts of the notochord (or *Shh*-producing cells) to other regions of the somite will result in the induction of sclerotome cells at these sites. Sclerotome cells migrate towards the notochord and eventually surround it completely where they condense to form the anlage of the vertebrae. On each side of the neural tube the anterior part of a somite contributes to the caudal part of the vertebral body and the neural arch, whereas the posterior part of the next rostrally located somite is responsible for the rostral part of the vertebral body and the neural arch. It is evident that any disturbance of this process will result in abnormal anlagen of the vertebrae and thus in vertebral malformations. The notochord degenerates by apoptosis but sections of it remain and will eventually form parts of the intervertebral disk, the nucleus pulposus. In addition to the three major compartments of the somite, the sclerotome, the dermatome, and the myotome, two additional regions have been described. The syndetome is another layer of specified cells located between the myotome and the sclerotome. These cells are the precursors of cells that eventually form the tendons, which connect muscle to bone. The fifth compartment is present only in the trunk somites and contains cells that will form the vascular wall of the aorta and the intervertebral blood vessels.

Abnormalities of the ribs and/or vertebrae are a relatively common finding in human malformation syndromes. Those that primarily affect the axial skeleton are summarized under the term spondylocostal dysostoses (SCD) (Fig. 13.19). While the majority of these genetically heterogenous conditions remain unexplained, some types of SCD have been shown to be caused by mutations in members of the notch pathway such as DLL3 and NOTCH2, or in lunatic fringe (LFNG), a secreted protein necessary to maintain oscillation of Notch in the paraxial mesoderm. Affected individuals show a wide variety of vertebral malformations, including fusions and half-vertebrae.

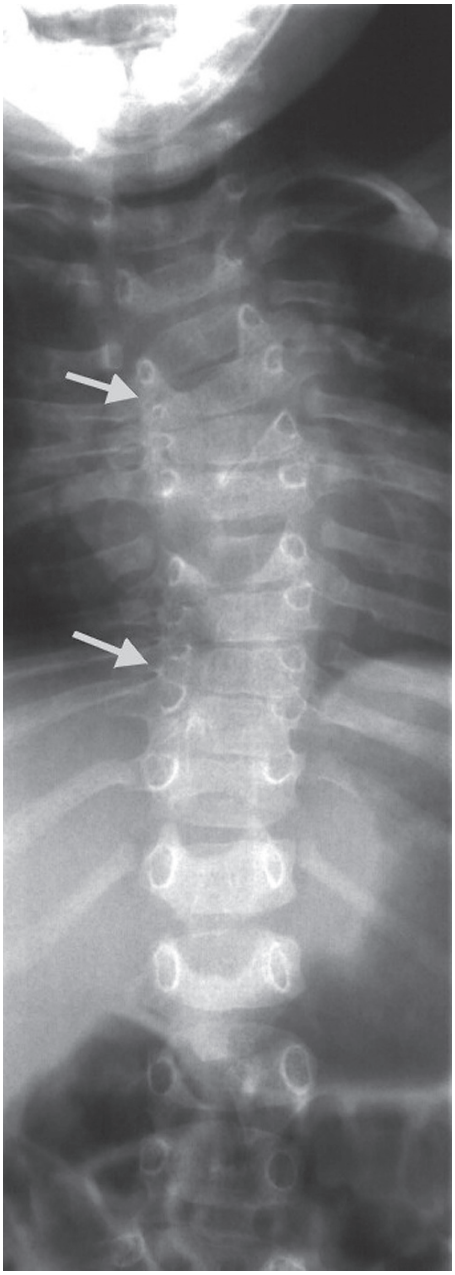

Fig. 13.19 Radiograph of a patient with spondylocostal dysostosis and mutation in DLL3. Organization of the vertebral column is chaotic and many vertebrae are fused or present only as halves

13.5 The Brachial Arches

The brachial or pharyngeal arches are another transient structure in the developing embryo that disappears along with the development of the neck and facial structures. They appear at about 3–4 weeks of human development as a series of bulges found on the lateral surface of the embryo. Four arches are found on each side, separated by clefts on the outside and so-called pharyngeal pouches on the inside (Fig. 13.17). Each pharyngeal arch is covered externally by ectoderm and internally by endoderm. The core of each arch contains neural crest cells, which surround a central group of mesodermal cells. Each cell type gives rise to a distinct set of tissues. The ectoderm generates the epidermal covering of the arches, whereas the endoderm forms the endothelial lining of the pharynx, the thyroid, the parathyroid, and the thymus. The mesoderm forms both the musculature and the endothelial cells of the arch arteries. The neural crest cells generate the connective tissue of the neck and the skeletal tissues of each arch including Meckel's cartilage and the middle ear (stapes, incus, malleus). Although the arches represent repeated series of similar structures, they have been shown to give rise to distinct parts of the body. The most anterior arch forms the jaw, the malleus, and the incus of the middle ear, the second forms the stapes and the styloid ligament, and the third and fourth arches form the hyoid bone and the thyroid and the cricoid cartilage (Fig. 13.20).

During development, each arch must be patterned to receive its own identity and to be positioned within the embryo along the three axes. A number of mechanisms are known to achieve this, but it is clear that the endoderm plays a prominent role. It is the site where most of the signaling molecules are expressed and the endoderm has been shown to be responsible for the induction of particular arch components including cartilage formation and the precursors of the cranial sensory ganglia. Neural crest cells migrate from the neural tube into the arches. Transplantation studies in both amphibian and avian embryos have demonstrated that crest cells acquire positional information when they are within the neural tube and transfer this information into patterning cues when they have reached the arches. Crest cells migrate in three streams separated by two regions, rhombomeres 3 and 5, which are basically depleted of crest cells. BMP4 have been shown to induce cell death of crest cells in these rhombomeres, while the flanking cells are protected by expression of the BMP inhibitor Noggin. However, other studies point to roles of the mesoderm and the surface ectoderm in patterning the arches, and there is evidence that *Hox* genes play the major part in this process. Inactivation of *Hoxa2*, for example, results in homeotic transformation of elements derived from the second arch into first arch derivatives, including partial duplications of Meckel's cartilage and the ossification centers of the middle ear bones.

The combination of hearing loss (sensorineural and/or conductive), preauricular pits, branchial fistulas, and renal dysplasia characterizes the branchio-oto-renal syndrome. With the exception of the renal problem, these anomalies are considered defects of the brachial arches, and EYA1, the gene mutated in this condition, is expressed in the neural cells of the first arch and in the second, third, and fourth pharyngeal pouches and clefts. Defects of the brachial arches result in

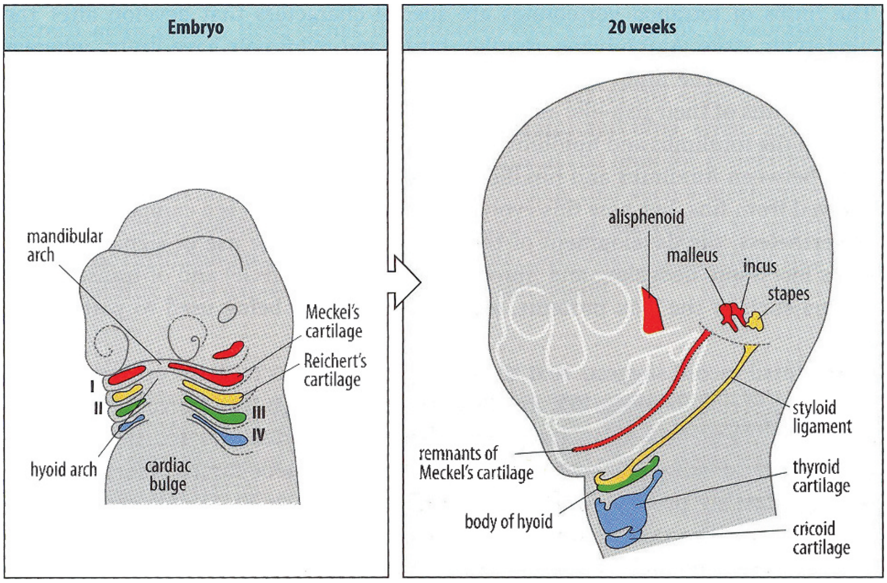

Fig. 13.20 Fate of brachial arch cartilage in humans. Cells from the brachial arches give rise to the three auditory vesicles, the hyoid, and the pharyngeal skeleton. The fate of the various elements is shown by the *color coding* (From [35], p. 502, Fig. 14.6, by permission of Oxford University Press)

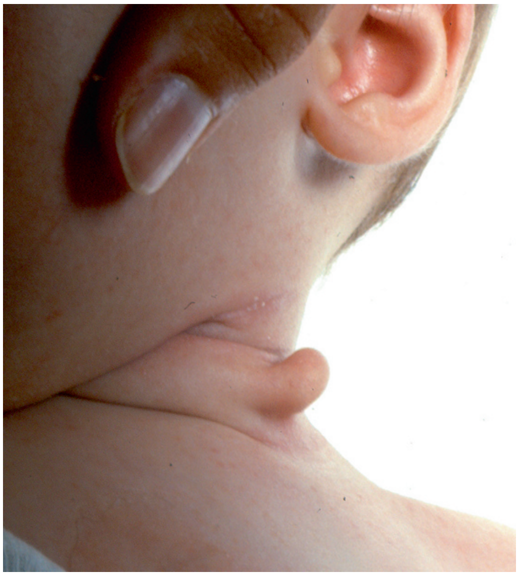

Fig. 13.21 Patient with brachial cyst. (Courtesy of J. Kunze [34], p. 8, Table 4.15)

malformations of the neck, such as brachial fistulas or cysts (Fig. 13.21).

13.6 Development of the Limbs

The limb skeleton originates from the lateral plate mesoderm. All other limb structures, including muscles, nerves, and vasculature, originate from the somitic mesoderm. Outgrowth of the limb bud is the result of a series of interactions between the mesoderm and the overlying ectoderm. As the limb bud grows out, mesenchymal cells begin to differentiate to form the various tissues of the limb in a proximo-distal sequence with structures being laid down progressively from a region of undifferentiated mesenchymal cells at the tip of the limb bud, known as the progress zone. The positional identity, and thus differentiation, of each cell is controlled by a three-dimensional coordinate system consisting of the dorso-ventral, the proximo-distal and the antero-posterior axes. Each axis in controlled by a particular set of signaling molecules/pathways produced by a defined population of cells (signaling centers). The combination of these signals informs the undifferentiated cells in the mesenchyme about their position and their fate in order to form the appropriate structures. Three signaling regions that convey this information have been identified: the apical ectodermal ridge (AER), mediating limb bud outgrowth (proximo-distal axis), ectoderm covering the dorsal sides of the bud governing dorso-ventral pattern, and the zone of polarizing activity (ZPA) controlling antero-posterior pattern (Fig. 13.22). Many of the signaling molecules that are produced by these signaling centers have been identified and characterized and intracellular signaling transduction pathways are being unraveled [32]. Furthermore, mutations in human limb malformations are being identified that help to understand the normal and abnormal mechanisms of limb development [28].

The AER is an anatomical structure consisting of densely packed ectodermal cells located at the very tip of the limb bud. Several different fibroblast growth factors (FGFs) are expressed and secreted by the AER and have been shown to be both, essential and sufficient to initiate and control outgrowth of the limb. FGF signaling is conveyed through the FGF receptors, which are expressed in the underlying mesenchyme. Experimental removal of the AER results in an arrest of limb outgrowth and thus truncations of the limb, depending on the time point of the intervention (Fig. 13.23). Mutations that result in AER loss have a similar outcome, leading to various degrees of limb defects. In humans this mechanism is associated with the ectrodactyly phenotype. This severe limb malformation is characterized by variable degrees of central defects of the digits resulting in cleft hands/feet, or, in the most severe cases, adactyly/monodactyly (Fig. 13.24). Ectrodacyly is genetically heterogeneous, but mutations in TP63L appear to be the most common cause.

The ZPA is a region of mesenchyme located at the posterior limb bud margin. Sonic hedgehog (*Shh*) is expressed in this region and has been shown to be the main mediator of antero-posterior patterning. Implantation of *Shh*-expressing cells can rescue surgical ZPA removal, and expression from the anterior margin of the limb bud results in the formation an anterior ZPA with subsequent mirror duplication of the entire autopod (Fig. 13.25). The restricted expression of *Shh* in the anterior part of the limb bud appears to be regulated by a conserved region approx. 1 Mb away from the *Shh* promotor known as the ZRS. Mutations in ZRS result in polydactyly in humans, mice, and cats by dysregulating *Shh* expression in the limb bud, thus creating an additional anterior *Shh* expression domain

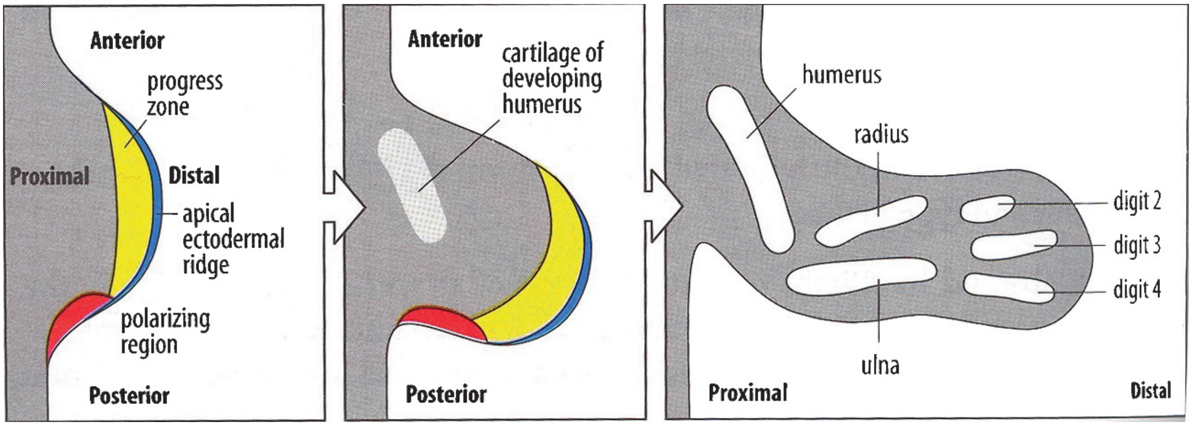

Fig. 13.22 Development of the chick wing with its centers of differentiation and signaling. Cells in the limb bud receive signals from the zone of polarizing activity (ZPA) localized at the posterior margin of the limb, and the apical epidermal ridge (AER), a specialized anatomical structure at the very tip of the bud. The ZPA specifies position along the anterior-posterior axis, whereas the AER controls the proximo-distal axis. Signals from the AER keep cells in the so-called progress zone in an undifferentiated and proliferative state. Once they leave this zone they start to differentiate, giving rise to the mesodermal derivatives of the limb, e.g., the skeleton. Thus, the individual skeletal elements are laid down in a sequential order from proximal to distal. (From [35], p. 142, Fig. 9.6, by permission of Oxford University Press)

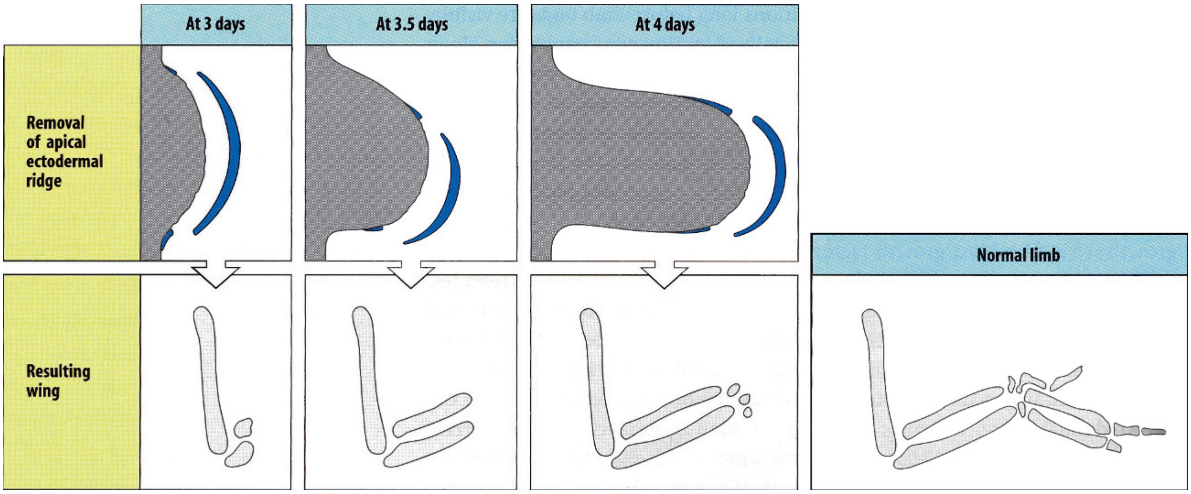

Fig. 13.23 Removal of the AER results in arrest of limb outgrowth and truncation. Surgical removal of the AER results in limb truncations at different levels, depending on the time point of removal. (From [35], p. 341, Fig. 9.7, by permission of Oxford University Press)

(Fig. 13.25b–d). Thus, this regulatory mutation has the same effect as the ZPA transplantation, i.e., activation of ectopic anterior *Shh* expression and subsequent mirror image duplications.

Under the influence of *Shh* the zinc finger transcription factor *Gli3* is converted into the activating form *Gli3A*, whereas otherwise the repressor form *Gli3R* predominates, which in a negative feedback downregulates *Shh*. By this mechanism *Gli3* expression is much stronger in the anterior than in the posterior limb bud, where *Shh* levels are high, thus creating an antero-posterior gradient that has been shown to be important for limb patterning. Mutations in GLI3 result in the Greig and Pallister–Hall syndromes, two conditions characterized by various degrees of polydactyly associated with midline malformations and benign tumors (hamartomas) (Fig. 13.26). In mice it has been shown that *Gli3* deficiency leads to ectopic expression of

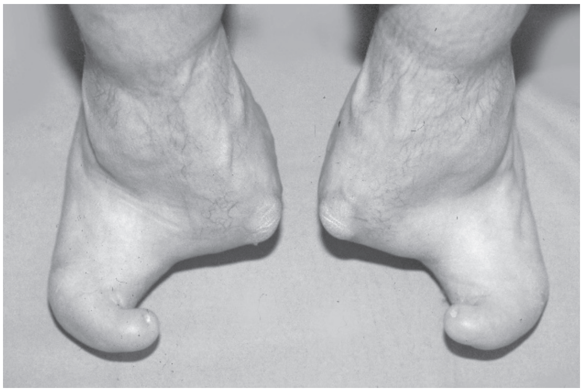

Fig. 13.24 Ectrodactyly associated with mutation in TP63L

Shh in the anterior margin of the limb bud in addition to the normal expression of *Shh* in the ZPA. This results in a double dose of *Shh* and a second ZPA, thus explaining the polydactyly.

A member of the Wnt family of growth factors, Wnt7a, has been shown to be important in dorso-ventral patterning. Expression of Wnt7a in the dorsal ectoderm of the limb bud up-regulates Lmx1b, which belongs to the family of LIM homeodomain transcription factors and forms a dorso-ventral gradient. This close functional relationship explains why Wnt7a-deficient and Lmx1b-deficient mice both develop autopods with a double ventral phenotype. Mutations in

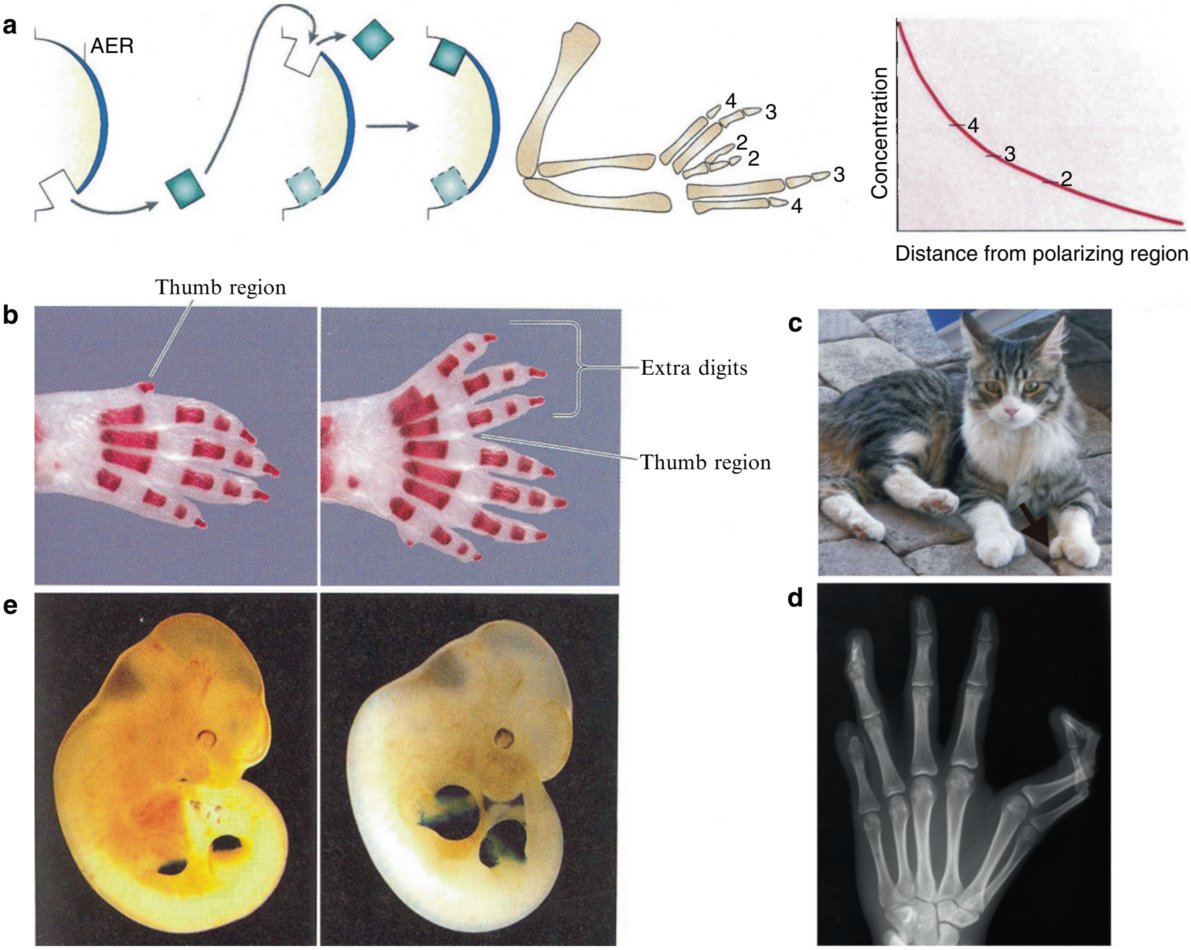

Fig. 13.25 (**a–e**) Digit duplications by AER duplication. (**a**) If an AER is grafted from a donor limb to the anterior region of a recipient a mirror image duplication of the digits is observed. This is due to a double dose of Shh morphogen from both the anterior and the posterior side. Since the distance from the source specifies digit identity, two posterior sides are created. (Reprinted from [32], by permission from Macmillan Publishers Ltd, *Nature Reviews Molecular Cell Biology*, copyright 2005) (**b–d**) Mutations in the regulatory region of the Shh gene in mouse (**b**), cats (**c**), and humans (**d**) result in polydactyly due to ectopic *Shh* expression at the anterior margin of the limb bud. (**e**) Transgenic mouse embryos stage E 12.5 expressing *LacZ* (*blue staining*) under the control of wt (*left*) or mutant (*right*) *Shh* limb regulatory region. Note expression restricted to the posterior limb in embryos expressing the wt construct and expanded expression domain in the mutant. ((**b**, **e**) from [10], p. 517, Fig. 16.17a–d)

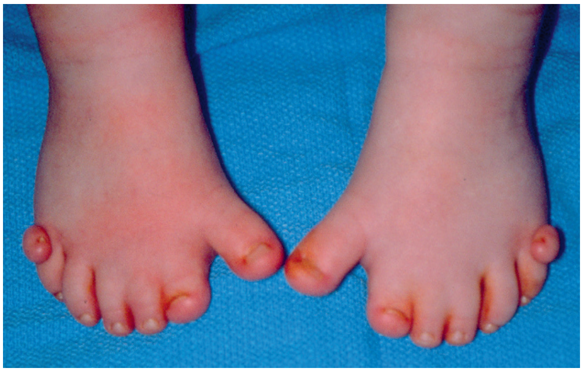

Fig. 13.26 Polydactyly in Pallister–Hall syndrome caused by mutation in GLI3

LMX1B in humans result in nail-patella syndrome, a condition characterized by absent/hypoplastic patellae and dysplastic nails. Both structures represent the dorsal part of the limb, and thus nail-patella syndrome represents a "ventralizing" phenotype. Mutations in WNT7A cause a range of phenotypes, again associated with loss of dorsal structures (nails, patellae), but in most severe cases these mutations are associated with phocomelia, probably because of a complete breakdown of all three signaling centers.

Hox genes from the 5′ region of the A- and D-clusters show characteristic stage-dependent expression patterns that determine the shape and identity of the limb skeletal elements. They are expressed in overlapping patterns, with most 5′ genes having the smallest, the most posterior, and the most distal expression domain. During later stages of development these domains change in a very dynamic way, resulting in an expression of the most 5′ *Hoxd* gene, *Hoxd13* over the entire hand/foot plate. Gene inactivation experiments in the mouse have shown that these domains also correspond to later anatomical regions (Fig. 13.27) in that loss of *Hoxd11* and *Hoxa11* results in absence of radius/ulna and tibia/fibula, whereas loss of *Hoxd11*, *-12*, *-13*, and *Hoxa13* results in a severe reduction of hands/feet. This pattern of expression is probably achieved by a repression mechanism that is gradually released during development located in a chromosomal region upstream of *Hoxd13*. Mutations in *Hox* genes result in limb malformations as illustrated by synpolydactyly, a condition characterized by fusion of fingers (syndactyly) together with an additional finger in the syndactylous web (polydactyly) (see also Fig. 13.7).

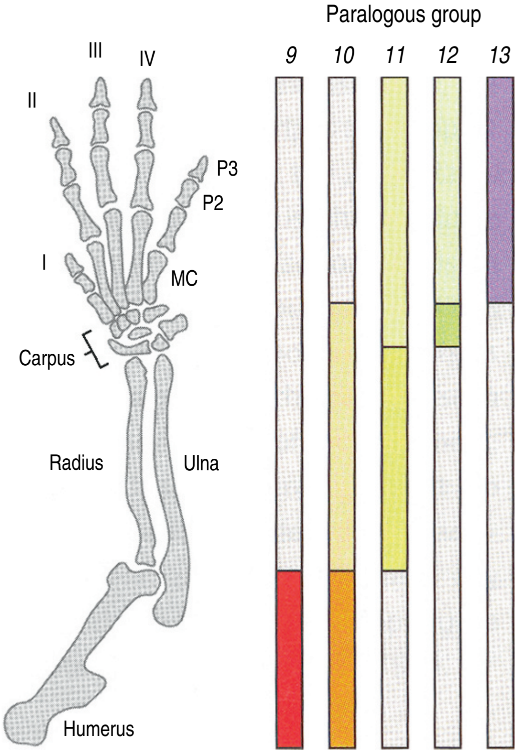

Fig. 13.27 (**a, b**) *Hox* gene patterning of the forelimb. (**a**) *Hox* genes of one paralogous group are expressed in distinct domains of the developing limb that correspond to adult skeletal elements. Whereas *Hoxd13* is exclusively influences patterning of the hand (autopod), *Hoxd11* together with *Hoxd10* are important for pattering of radius and ulna (zeugopod). *Hoxd9* and *Hoxd10* function together to pattern the humerus (stylopod). (From [35], p. 353, Fig. 9.18). (**b**) *Hoxd13* expression in the limb buds and the genital ridge of a mouse embryos stage E12.5. By permission of Oxford University Press

13.7 Development of the Circulatory System

Presumptive heart cells originate in the early primitive streak and form two groups of cells lateral to Hensen's node. At the same time, angiogenic clusters form that contain the first blood cells. These are soon surrounded by a double-walled tube consisting of the inner endocardium and the outer epimyocardium, which will form the endocardium, i.e., the inner lining of the heart and the heart muscles, respectively. As the foregut is closed, the two tubes are brought together and fuse to a single pumping chamber. In humans this occurs at 3 weeks of gestation. By 5 weeks the heart has developed into a two-chambered tube with one atrium and one chamber. The partitioning into the two chambers is accomplished by cells that migrate into a hyaluronate-rich structure, the endocardial cushion, that is later located between the ventricles and the atria. Meanwhile the atrium is partitioned by the development of two septa that grow ventrally towards the endocardial cushion. These septa stay open, thus providing blood flow from the right to the left atrium. In the 7th week of human development the ventricles begin to be separated by the growth of the ventricular septum towards the endocardial cushion [31]. Figure 13.28 shows a schematic of human heart development.

For a proper orientation of the pulmonary (right) and systemic (left) ventricles and for the alignment of the heart chambers with the vasculature the linear heart tube undergoes rightward looping [25]. The direction of cardiac looping is determined by an asymmetric axial signaling system that also affects the position of the lungs, liver, spleen and gut. Before organ formation begins, this signaling cascade directs the asymmetrical expression of Sonic hedgehog (*Shh*) and Nodal, a member of the TGFβ family. In humans mirror image reversal of right–left asymmetry (situs inversus) is often associated with normal organogenesis, but discordance of cardiac and visceral asymmetry (situs ambiguus, also called heterotaxy) is associated with malformations of the heart and other organs. In the latter condition asymmetry in structure and placement of organs still develops, but, owing to the lack of definitive positional information, this happens on a stochastic basis. Cardiac defects typically occurring with situs ambiguus include, but are not limited to, atrial septal defects, ventricular septal defects, transposition of the great arteries, double-outlet right ventricle, anomalous venous return, and aortic arch anomalies. But what determines right–left asymmetry in the first place? Evidence that ciliary function might be involved came from studies of Kartagener syndrome, a condition characterized by situs inversus together with recurrent pulmonary infections attributable to defects in cilia function. Elegant experiments performed with video microscopy have shown that motile cilia are present in the center of the mouse node that propagate directional fluid flow. In addition, it was shown that this flow is abnormal in several mouse mutants with laterality

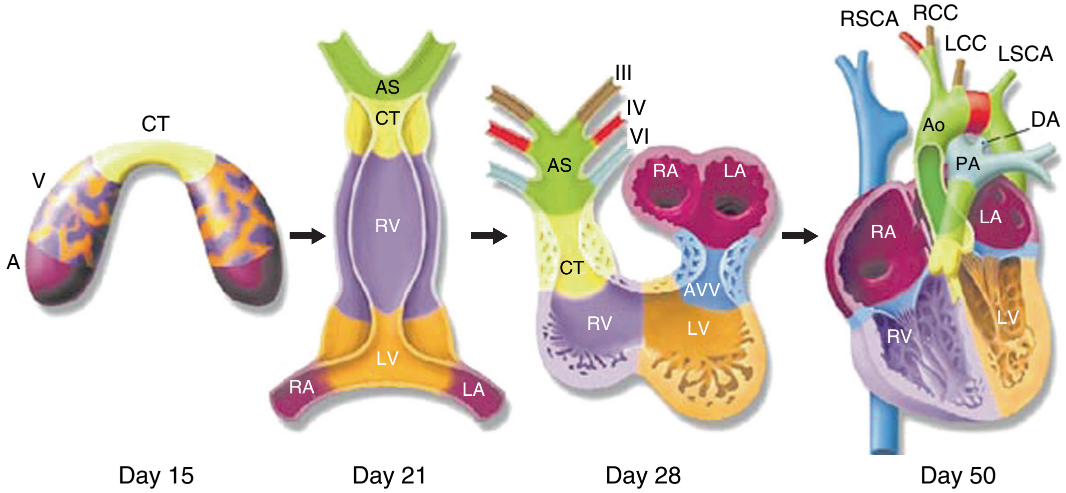

Fig. 13.28 Human heart development. Cardiac development is depicted in four consecutive steps. The heart consists of an inner endocardium, which is the endothelial sheet, and the outer myocardium, which is contractile. The first sign of the developing heart is the formation of a crescent (*left-most panel*), which fuses along the midline to give rise to a tube; this is patterned along the anterior-posterior axis to form the various chambers of the heart. This two-chambered heart is the basic adult form in fish, but in higher vertebrates the tube undergoes looping, a process controlled by the left-right asymmetry of the antero-posterior axis, and further partitioning to form a four-chambered heart. Cells from neural crest origin contribute to the outflow tracts populate the bilaterally symmetrical aortic arch arteries (III, IV, and VI) and aortic sac (AS) that together contribute to specific segments of the mature aortic arch. Mesenchymal cells form the cardiac valves from the conotruncal (CT) and atrioventricular valve (AVV) segments. Corresponding days of human embryonic development are indicated (*A* Atrium, *Ao* Aorta, *DA* Ductus arteriosus, *LA* Left atrium, *LCC* Left common carotid, *LSCA* Left subclavian artery, *LV* Left ventricle, *PA* Pulmonary artery, *RA* Right atrium, *RCC* Right common carotid, *RSCA* Right subclavian artery, *RV* Right ventricle, *V* Ventricle) (Reprinted from [31], by permission from Macmillan Publishers Ltd, *Nature*, copyright 2000)

defects. In the current model, this cilia-directed flow creates an asymmetric accumulation of growth factors such as Nodal and/or Shh that subsequently governs asymmetry. In Kartagener syndrome this is presumably disturbed because the mutations in dynein result in nonfunctional cilia.

The individual segments of the heart can only function if the cardiac valves are properly placed to ensure the unidirectional flow of blood through the heart. Development of the valves starts with the formation of regional swellings, known as cardiac cushions, that form the anlage of the valves. To form the valves a transformation of endocardial cells into mesenchymal cells has to take place, mediated by members of the TGFβ family. Inactivation of Smad6, a transcription factor activated upon TGFβ signaling leads to abnormally thick-ended, gelatinous valves. Transformed cells migrate into the cushions and differentiate into the fibrous tissue of the valves. Atrioventricular canal or endocardial cushion defect represents a developmental abnormality of this process, a congenital heart defect frequently observed in trisomy 21. However, which gene(s) cause this defect is unknown.

Many other genes have been implicated with congenital heart defects. Mutations in TBX5 cause Holt–Oram syndrome, a condition characterized by heart defects, frequently associated with arrhythmia, and limb malformations. TBX5 was shown to be an essential factor for the development of the right ventricle and the outflow tract [3]. In addition, Tbx5 appears to function in the left ventricle and atria by influencing the expression of other transcription factors and proteins that are required for cardiac function. Tbx5 associates with Nkx2.5, another transcription factor shown to be essential for normal heart development. Mutations in NKX2.5 have been identified in families with atrial septal defects and cardiac conduction abnormalities [2]. Sporadic mutations have also been found in individuals with outflow tract alignments defects such as tetralogy of Fallot, or tricuspid valve anomalies. An evolving general theme of congenital heart defects appears to be a considerable variability in the type and severity of cardiac malformations among individuals with mutations in the same gene or even with the same mutation. Mutations in one gene are not predictive for a certain heart defect but rather appear to be responsible for a range of abnormalities, with certain types of defects occurring more often than others.

13.8 Development of the Kidney

The urogenital system, i.e., the kidneys, the gonads, and their respective duct systems, develop from the intermediate mesoderm, the region located between the lateral plate mesoderm (origin of the limbs) and the paraxial mesoderm (origin of the somites). Development of the permanent kidney, the metanephros, is preceded by the formation of a transient structure, the pronephros. The pronephric duct is the first visible sign, a bilateral structure consisting of a single-cell-thick epithelium that extends caudally until it reaches the cloaca. Connected to this tube a linear array of tubules derived from mesenchymal cells adjacent to the primary nephric duct forms. The more caudal tubules contain glomeruli and convoluted proximal tubule-like structures that serve as transient filtration units that degenerate as the development of the permanent kidney takes place [5]. Figure 13.29 shows a summary of kidney development.

The adult kidney or metanephros begins to develop when an outgrowth of the primary nephric duct, termed the ureteric bud, extends into the surrounding metanephric mesenchyme. The ureteric bud branches and first mesenchymal cells begin to aggregate near the tips of the bud. This interaction of mesenchyme with epithelium drives the entire following developmental process. The aggregates first form a polarized renal vesicle, which is still in contact with the bud. Subsequently the so-called comma and S-shaped bodies form and become polarized along a proximo-distal axis as they undergo mesenchyme-to-epithelial conversion. Subsequently the distal end fuses with the ureteric bud to form a single, continuous epithelial tube, which in its distal part will give rise to the proximal tubule, Henle's loop, and the distal tubule. Endothelial cells invade the cleft of the S-shaped body forming the glomerular filtration unit. Interactions between endothelial cells and the mesenchyma-derived glomerular epithelial cells lead to the formation of podocytes and thus the glomerula basement membrane that serves as a filtration barrier. This process of branching, aggregation, mesenchyme-to-epithelial conversion, fusion, and endothelial invasion continues from the inside to the outside in such a way that the oldest nephrons are located closer to the medulla and the youngest ones more peripherally in the nephrogenic zone. In humans this process continues during the

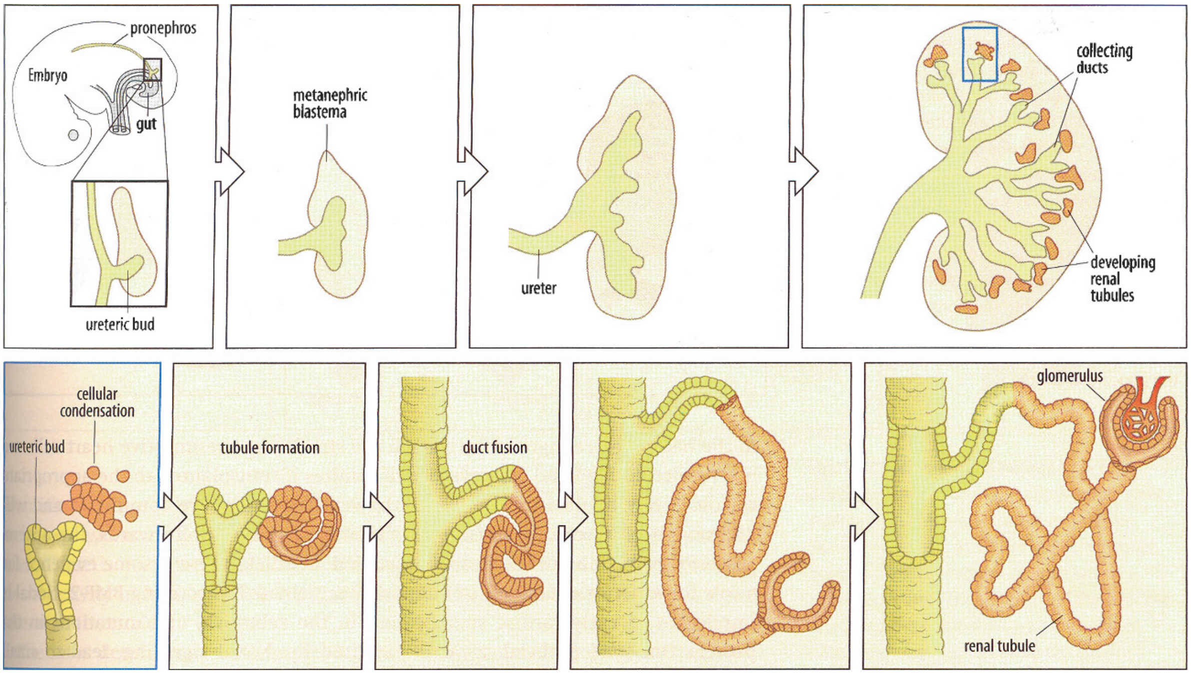

Fig. 13.29 Kidney development. The permanent kidney develops from a loose mass of mesenchyme, the metanephric blastema, which is induced by cells from the ureteric bud. The ureteric bud itself is induced by the mesenchyme to grow and branch. The metanephric blastema eventually develops into the glomerulus and the renal tubule, whereas the ureteric bud will form the collecting ducts that connect the kidney to the ureter. The formation of glomeruli involves several distinct steps, including comma- and S-shaped bodies. The human kidney continues to grow from metanephric blastema until birth. (From [35], p. 377, Fig. 9.46)

entire fetal period until the final size of the kidney is reached.

This complex process in governed by a large number of signaling pathways, including the *Pax/Eya/Six* genes as well as *Lim1* and *Odd1*. Ureteric outgrowth and branching morphogenesis are controlled by the Ret/Gdnf pathway. In the mouse *Wnt9b* and *Wnt4* genes are critical for aggregation and transformation of metanephrogenic mesenchyme into tubular epithelium. If separated from the utereteric bud mesenchymal cells die. FGF2 and BMP7 are two factors secreted by the ureteric bud that prevent apoptosis and promote aggregation. The competence to respond to uteric bud inducers is thought to be regulated, among other genes, by Wt1, a transcription factor originally found to be mutated in a heritable form of childhood kidney tumor, the Wilms tumor. If this factor is missing, the uninduced cells of the metanephrogenic mesenchyme die and no kidney is formed. Later in development Wt1 is found expressed in podocytes of the glomerular basement membrane. Besides leading to tumors, specific mutations in WT1 can result in Denys–Drash syndrome, a condition characterized by Wilms tumors, nephrotic syndrome (severe proteinuria) leading to end-stage renal failure before the age of 3 years, and pseudohermaphroditism with children having either ambiguous external genitalia or a normal female phenotype with an XY karyotype. The latter phenotype points to an important role of Wt1 in genital development. It is similar to the renal phenotype in that Wt1-deficient mice develop no gonads.

The caudal part of the ureteric bud becomes the ureter, which inserts into the bladder and is a part of the urogenital system that, if nonfunctional, will result in hydronephrosis. The bladder develops out of the cloaca, which is divided by a septum into the rectum und the urogenital sinus. The latter will also give rise to the urethra. *Hox* genes appear to be important for this process. Mutations in HOXA13 result in hand-foot-genital syndrome, a condition characterized by short thumbs/toes and abnormalities of the cloaca, the male and female reproductive tracts and the urethra.

13.9 Skeletal Development

The skeleton arises from three distinct sites: the axial skeleton consisting of the vertebrae and ribs originates from the somites, the appendicular skeleton has its origin in mesenchymal cells located in the lateral plate mesoderm, and most of the craniofacial bones are of neural crest origin. Patterning genes determine the number, size, and shape of the future skeleton and guide cells to migrate to the sites of future skeletogenesis, where they aggregate to form the skeletal anlage. The next step is the overt differentiation of these cells into cartilage-forming chondrocytes in endochondral skeletal elements, or into bone forming osteoblasts in areas of membranous bone formation [16, 17]. Figure 13.30 schematically summarizes skeletal development.

In areas of endochondral bone formation, the condensed cells differentiate into chondrocytes that produce cartilage. The transcription factor Sox9 has been shown to be essential for this process. Sox9 is expressed in the early cartilaginous condensations and, at later stages, in growth plate chondrocytes. One of its regulatory targets is *Col2a1*, the gene encoding the major collagen in cartilage. Sox9 binds specifically to sequences within the first intron of this gene. Mutations in SOX9 cause campomelic dysplasia, a lethal chondrodysplasia characterized by bowed long bones (femur and tibia in particular), small scapula, small rib cage, and sex reversal in XY males. Inactivation of Sox9 in mice causes early lethality, but conditional inactivation in the limbs shows that Sox9 is essential for the differentiation of precursor cells into chondrocytes.

The TGFβ/BMP/GDF5 pathway plays an important role in the regulation of condensation and differentiation of precursor cells into chondrocytes. Signaling of the Tgfβ superfamily members requires the binding of the ligand to cell surface receptors consisting of two types of transmembrane serine/threonine kinase receptors classified as type I and type II. The type II receptor transphosphorylates and thus activates the type I receptor. The intracellular substrates of the activated type I receptors are the Smads. Smads 1, 5, and 8 are phosphorylated and then translocated to the nucleus, where

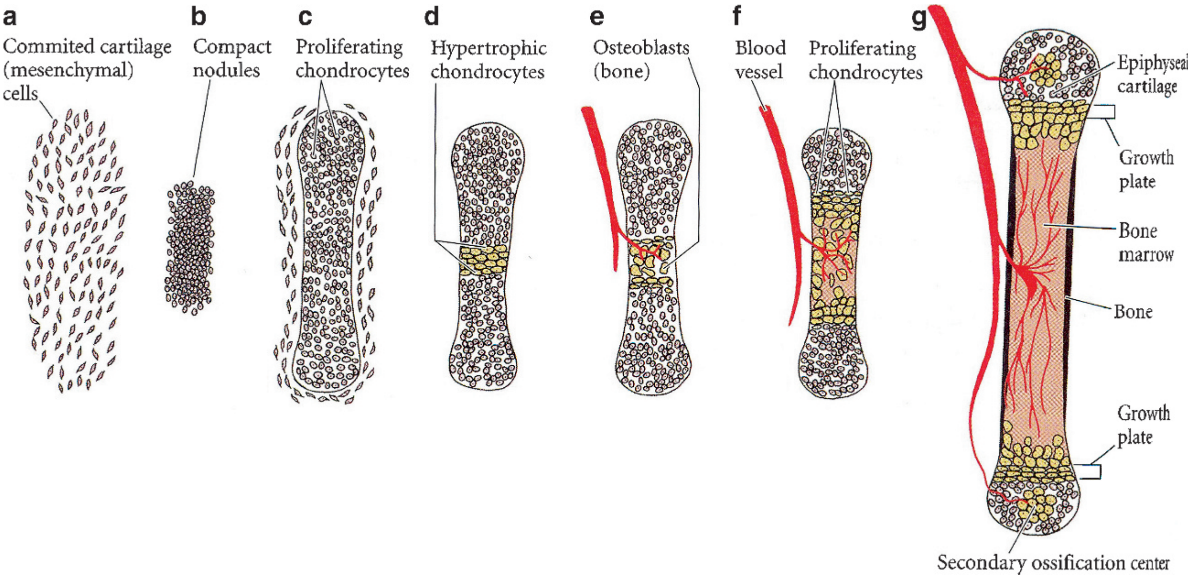
a
Commited cartilage (mesenchymal) cells
b
Compact nodules
c
Proliferating chondrocytes
d
Hypertrophic chondrocytes
e
Osteoblasts (bone)
f
Blood vessel
Proliferating chondrocytes
g
Epiphyseal cartilage
Growth plate
Bone marrow
Bone
Growth plate
Secondary ossification center

Fig. 13.30 Skeletal development**.** The first sign of skeletogenesis is the condensation of precursor cells at the site of future bone formation. The condensed cells differentiate into cartilage to form the cartilaginous anlage of the future bone or directly into bone at sites of desmal ossification. Chondrocytes in the center of the cartilaginous anlage begin to hypertrophy, and their matrix begins to mineralize. At this stage the first cortical bone begins to form as a thin layer of osteoid around the shaft of the anlage. In addition, vessels invade the cartilage, introducing monocytic progenitor cells that differentiate into osteoclasts that remove cartilage and bone. A growth plate forms at each end of the bone, in which most of the growth is generated until adulthood. Secondary ossification centers develop in the cartilage heads of the bone. (From [10], p. 456, Fig. 14.14)

they participate in the transcriptional regulation of the expression of genes involved in cartilage and bone formation [22]. BMP signaling is controlled by the binding of BMPs to inhibitors in the extracellular space. One potent inhibitor is Noggin, a gene originally identified for its role to induce head formation in *Xenopus*. Activation of BMP signaling either by overexpression, activation of the receptor, or inhibition of the inhibitor results in grossly enlarged cartilaginous anlagen. The likely mechanism for this effect is the recruitment of mesenchymal precursor cells to the cartilage condensations and to the perichondria, which contribute cells to the anlage by appositional growth. Mutations in GDF5, another member of the BMP/TGFβ family or its receptor BMPR1B result in various limb phenotypes, mainly characterized by shortening of the digits (brachydactyly). Mutations in the inhibitor NOGGIN or activating mutations in GDF5 cause joint fusions (symphalangism, synostosis syndrome), indicating that the tight regulation of BMP signaling is also essential for joint formation [29].

In areas of membranous bone formation, the condensed cells differentiate into osteoblasts which produce bone matrix. Genetic experiments in mice have demonstrated that Cbfa1/Runx2 is essential for this process. Runx2 is a member of a small family of transcription factors that are homologous to the *Drosophila* runt gene. In Runx2 null mice no endochondral or membranous bone is formed owing to an arrest in the early steps of osteoblast differentiation (Fig. 13.31). In contrast, the cartilaginous template is relatively normal. Mutations in RUNX2 cause cleidocranial dysplasia, a skeletal dysplasia characterized by patent fontanelles, aplastic/hypoplastic clavicles, supernumerary teeth, and short stature [24].

In contrast to the bones of the skull, which are formed by a direct transformation of mesenchymal cells into osteoblasts, the major part of the skeleton is formed by endochondral ossification in which a cartilaginous template is formed first, which is subsequently replaced by bone. Central to this process is the formation of a growth plate, a highly organized structure that generates the entire longitudinal growth. Growth plate chondrocytes are invariably arranged in three layers: (1) reserve chondrocytes, (2) proliferating chondrocytes, and (3) hypertrophic chondrocytes. A very complex interplay of different signaling pathways regulates the rate of proliferation and the conversion of proliferating chondrocytes into hypertrophic chondrocytes.

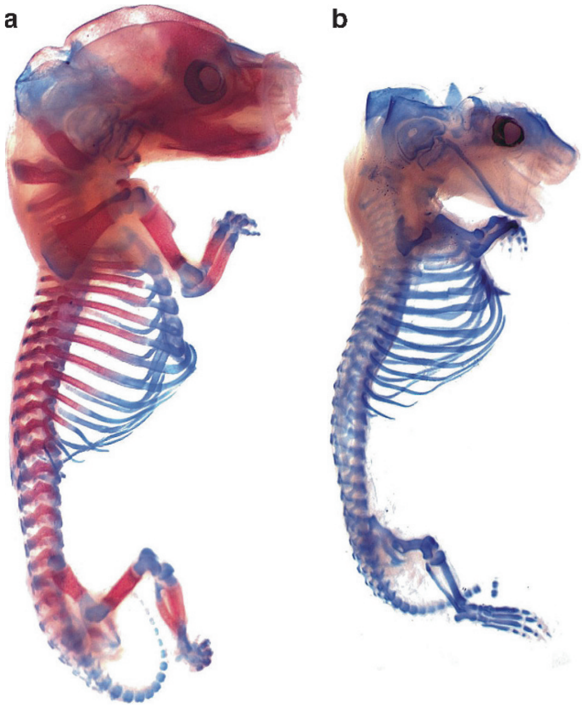

Fig. 13.31 (**a, b**) Role of Runx2 in bone development. Inactivation of Runx2 transcription factor results in complete loss of bone. Mouse skeleton with cartilage stained blue and bone stained red. Note *red* and *blue* staining in control (**a**) but no red staining in Runx2−/− mice (**b**)

During the process of chondrocyte differentiation, the matrix changes dramatically through the production of other components such as collagen type 10, the expression of metalloproteinases, and the calcification of matrix. At the same time, blood vessels begin to penetrate the calcified cartilage, bringing in osteoclasts that remove cartilage and osteoblasts, which build new bone. With further growth, the central and primary centers of ossification expand towards the ends of the bones and secondary centers of ossification form within the cartilage remnants. The growth plate, now localized between the epiphysis (secondary center of ossification) and the metaphysis (distal end of former primary ossification center) generates all longitudinal growth until the end of puberty when primary and secondary ossification centers fuse. At this point, the cartilage of the joints is the only cartilage that remains of the former anlage (Fig. 13.32).

Two major signaling pathways that control proliferation and differentiation of chondrocytes have been identified, the Indian hedgehog (*Ihh*)/parathyroid

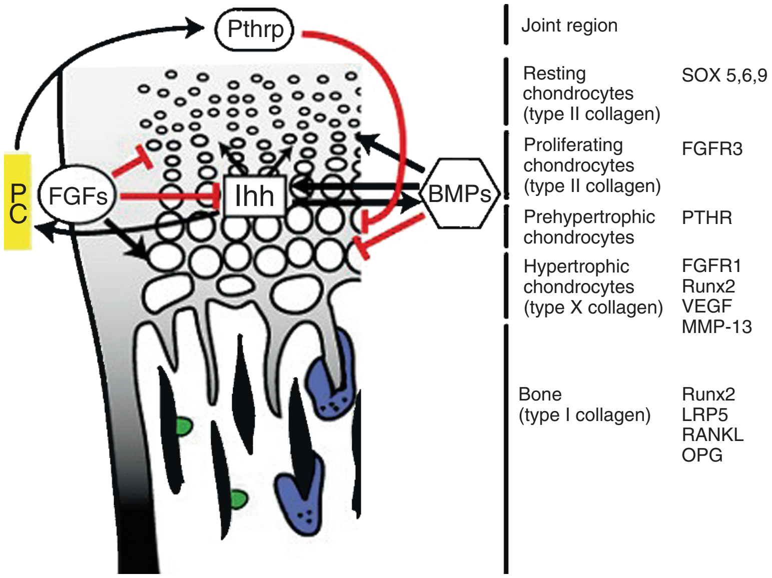
Pthrp
P
C
FGFs
Ihh
BMPs
Joint region
Resting chondrocytes (type II collagen)
SOX 5,6,9
Proliferating chondrocytes (type II collagen)
FGFR3
Prehypertrophic chondrocytes
PTHR
Hypertrophic chondrocytes (type X collagen)
FGFR1
Runx2
VEGF
MMP-13
Bone (type I collagen)
Runx2
LRP5
RANKL
OPG

Fig. 13.32 Schematic of growth plate and its regulation**.** Beginning from the joint region, chondrocytes are arranged in four layers, representing their stages of differentiation: resting, proliferating, prehypertrophic, and hypertrophic chondrocytes. The later are replaced by bone by the joint action of osteoblasts (*green*) and multinuclear osteoclasts (*blue*). Genes that are characteristically expressed within each layer are given on the *right* side. A complex signaling network regulates proliferation and differentiation of chondrocytes. Prehypertrophic chondrocytes express *Ihh*, which regulates PTHrp, which is produced by the joint region, via the perichondrium. PTHrp, in turn, inhibits differentiation of proliferating chondrocytes. FGFs inhibit *Ihh* and chondrocyte proliferation and stimulate differentiation of chondrocytes. BMPs act antagonistically to the FGFs. *Gray shading* symbolizes the degree of calcification of the extracellular matrix

hormone related peptide (PTHrP) pathway and the FGF pathway. Indian hedgehog (*Ihh*), the second mammalian hedgehog ortholog, plays a central role in the regulation of chondrocyte proliferation and hypertrophy. Through a yet unknown cascade of events *Ihh* indirectly regulates the expression of PTHrP. *Ihh*, and PTHrP form a feedback loop whereby *Ihh* up-regulates the synthesis of PTHrP, thereby indirectly slowing down the process of chondrocyte hypertrophy. Mutations in the PTHrP pathway cause pseudohypoparathyroidism (PHP) and pseudopseudoparathyroidism (PPHP). In PHP abnormalities in calcium and phosphate metabolism (hypocalcemia, elevated PTH levels) are accompanied by obesity, mental retardation, short stature, subcutaneous calcifications, and short digits (Albright hereditary osteodystrophy), whereas PPHP patients have osteodystrophy with normal calcium and PTH levels.

FGF signaling plays a major role in the regulation of chondrocyte proliferation and differentiation. More than 20 different FGFs are currently known, and four distinct FGF receptors (FGFR) have been described that bind and are activated by members of the FGF family. FGFs are potent mitogens for chondrocytes as well as for osteoblasts and stimulate bone formation in vitro and in vivo. Inactivation of Fgfr3 in the mouse results in overgrowth of the long bones, whereas expression of an activating mutation results in dwarfism, indicating that Fgfr3 functions as a negative regulator of chondrocyte proliferation and/or hypertrophy. Mutations in FGFR3 cause the most common form of skeletal dysplasia, achondroplasia. Affected individuals are characterized by disproportionate short stature (130 cm adult height). Further activating mutations in FGFR3 cause hypochondroplasia, a less severe variant, and thanatophoric dysplasia, the most severe and lethal form of this dysplasia group.

Cartilage is characterized by a unique extracellular matrix that accounts for around 90% of the tissue volume. The main component is fibrous collagen, which confers tensile strength. The growth plate cartilage contains type II, IX, X, and XI collagen. In the resting and proliferating cartilage type II collagen predominates, which can form fibers together with type IX and type XI. Type X collagen, in contrast, is specific for hypertrophic cartilage. Proteoglycans, and especially aggrecan, are highly abundant. They are giant molecules, which have a gel-like consistency when dissolved in water. Consisting of a core protein to which different kinds of glycosaminoglycans (GAGs) are attached, the proteoglycans are highly sulfated and thus negatively charged. This allows them to bind large amounts of cations and water molecules and to be mutually repellent, which is thought to contribute to the elasticity of the cartilage. Glycoproteins are a third group of matrix components comprising for example perlecan, fibronectin, tenascin, and cartilage oligomeric matrix protein (COMP). Mutations in any of these cartilaginous matrix components result in skeletal dysplasias with growth deficiencies.

In bone, type 1 collagen is the most abundant protein. In contrast to cartilage, bone matrix contains few proteoglycans, but instead consists largely (two thirds) of hydroxyapatite, to ensure that it is rigid. Mutations in either of the two genes that encode for the two chains that contribute to collagen type I (COL1A1 and COL1A2) result in osteogenesis imperfecta, a group of diseases primarily characterized by brittleness of bones and recurrent fractures (Fig. 13.33).

Homeostasis of bone mass and remodeling during skeletal growth are accomplished by an antagonistic action of bone producing osteoblasts and bone resorbing osteoclasts. Osteoclasts originate from mononuclear hematopoietic precursor. When osteoclasts attach to bone they create the so-called ruffled membrane, which contains high levels of V-type H^+-ATPases that pump protons into the tightly sealed resorption lacuna between osteoclast and bone surface. Strong extracellular acidification is crucial for bone resorption, as low pH is needed to dissolve the hydroxyapatite of the bone tissue and to degrade the organic components of the bone matrix, above all type I collagen, by acidic proteases such as cathepsin k. Dysfunction of osteoclasts results in an abnormal accumulation of bone also called osteopetrosis. The recessive forms are life-threatening conditions caused by loss of the bone marrow cavity. They can be caused by defects in the osteoclast acidification machinery, either affecting the osteoclast proton pump TIGR, the chloride transporter CLC-7 [18], or carboanhydrase type II, a protein responsible for synthesizing the protons transported by the H^+-ATPase. Mutations in cathepsin k, which is indispensable for bone resorption since it is the only protease able to cleave the intact collagen triple-helix, result in the sclerosing disorder pycnodysostosis.

13.10 Abnormal Development: Definitions and Mechanisms

The complexities of embryonal development are reflected by the vast number of phenotypes produced by abnormal development. Anomalies that are of prenatal

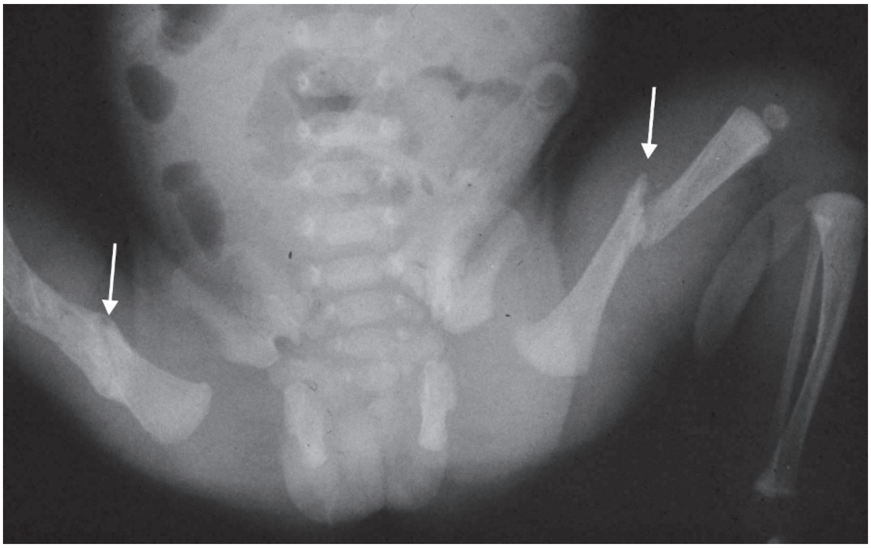

Fig. 13.33 Newborn with type II osteogenesis imperfecta**.** Note fresh fracture of left femur and old fracture of right femur with callus formation (*arrows*)

onset are generally subsumed under the heading congenital. Such defects are either visible at birth or can be diagnosed at a later time point when they manifest as disease (e.g., deafness, anomalies of teeth). 'Congenital' does not necessarily mean genetic or inherited, because congenital defects can also be caused by maternal disease (e.g., diabetes), intraterine infections (e.g., rubella), or mechanical forces (e.g., uterine constraint caused by oligohydramnion).

Congenital anomalies contribute to a great extent to neonatal morbidity and mortality. It has been estimated that, depending on the methodology of assessment, 4–8% of all newborns are born with a medically relevant anomaly. Thus, approx. 1 in 20 newborns has a recognizable and medically relevant anomaly. This proportion is higher among children who die during the neonatal phase (20%) and is much higher in miscarried babies. In Western countries congenital anomalies are the most frequent cause of neonatal mortality.

Abnormalities of development will have a different outcome depending on the cause, time point, and magnitude of the insult. For example, defects that affect the embryo during the preimplantation phase usually result in either complete restoration or loss of the embryo, whereas deficiencies that occur later can result in either death of the embryo or in birth defects. For the clinician a careful clinical analysis may reveal important information that is of high relevance not only for understanding the condition, but also for successful counseling. As illustrated in Fig. 13.34, all developmental anomalies can be categorized into four subgroups.

13.11 Malformations

Primary malformations are caused by an intrinsic defect of the embryo, i.e., they are genetic or due to an interaction of the embryo's genome with its environment. They can be inherited or may develop as a result of a de novo mutation that has occurred in the oocyte or the sperm, or during early embryogenesis. Malformations are due to inactivation or dysregulation of important developmental genes during the postimplantation phase. These genes regulate early pattering processes and/or organogenesis. Because regulation of early development is their most important role, the developmental defect happens very early and the

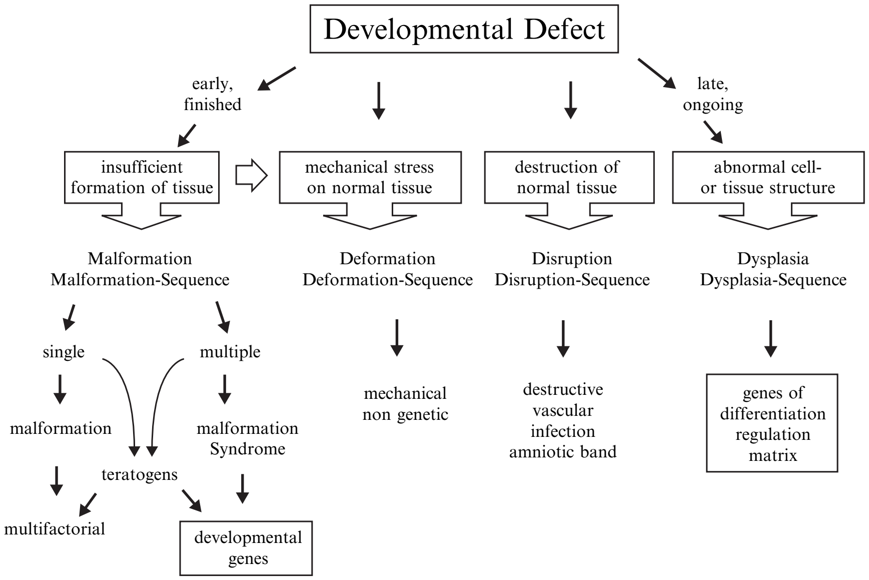

Fig. 13.34 Types of structural defects. Congenital defects can be subdivided in four major groups, malformations, deformations, disruptions, and dysplasias. As shown, their etiology can be genetic, multifactorial, and non-genetic. Each type may induce secondary changes, also called sequence. (From [20], p. 282, Fig. 31.2)

pathogenesis of the condition is thus completed at the time point when it is observed.

The majority of malformations are likely to be caused by a combination of environmental and polygenic factors. Abnormalities in the maternal metabolism, such as diabetes mellitus, are such an environmental factor which, together with yet unknown susceptibility genes, may result in severe malformations, such as caudal regression. A smaller number of birth defects is due to chromosomal aberrations or is caused by single gene defects.

Defects caused by polygenic factors are most commonly "single site defects," i.e., they affect one site or organ of the body while leaving others intact. They occur at relatively high frequency and are either present or absent, i.e., they show little overlap with minor defects. Such malformations include cleft lip and palate, isolated cleft palate, neural tube defects, club foot, congenital heart defects, and pyloric stenosis to mention the most frequent ones. They tend to cluster in families but do not conform to Mendel's laws of gene transmission. Risk calculation has determined that: (1) first-degree relatives have a risk approximating the square root of the population risk; (2) second-degree relatives have a sharply lower risk than first-degree relatives; (3) the higher the number of affected individuals in one family, the higher the risk; (4) consanguinity increases the risk; and (5) the more severe the malformation, the higher the risk. For some defects the influence of environmental factors is larger than for others. For example, neural tube defects can be prevented to a large extent if folic acid is supplemented during early pregnancy. In fact, the folate-neural-tube defect relation represents the only instance in which a congenital malformation can be prevented by changing the environment. In contrast, the frequency of concordance and discordance in monozygotic and dizygotic twins argues against both a single-gene etiology and a major environmental influence in most other conditions.

Cleft lip with or without cleft palate is one of the most common birth defects in the world, with an average prevalence of 1/700. Transforming growth factor (TGFA) a is a growth factor expressed during palatogenesis in the mouse, but mice with inactivation of TGFA have abnormal skin, hair, and eyes, but no clefts. TGFA was found to be associated with human clefts in a first study in 1989 and has been confirmed in several studies since. Taken together, these studies show that TGFA is probably a modifier of clefting in humans, which is consistent with an oligogenic model for clefting in humans. Several causative genes for inherited syndromic forms of cleft lip/cleft palate have been identified and some studies show that they may also contribute to the occurrence of isolated forms.

In contrast to the above-mentioned polygenic conditions, chromosomal imbalances and single-gene disorders are usually associated with a combination of anomalies, hence their name "syndrome" (Greek, meaning "convergence"). For the clinician this means that the search for other associated anomalies is pivotal, since it helps to distinguish single-gene defects from polygenic conditions. As only the combination of symptoms defines a syndrome, a clinical diagnosis cannot be made on the basis of a single defect. In that respect the detection of minor defects may be as helpful as that of major anomalies. The pattern of anomalies defines the condition and, in addition, the functional role of the gene affected. Chromosomal aberrations, including microdeletions, show their "own" pattern, which is usually less defined and broader (e.g., a combination of specific patterning defects with mental retardation) than that of single gene defects because multiple genes are involved that may act in a additive or even epistatic way.

Malformations affecting single sites may result in consecutive changes of other sites, giving rise to a malformation sequence. For example, innervation problems of the tongue resulting in a reduced pressure on the lower jaw result in reduced growth of the mandible, also called micrognathia. The latter is a consequence of the initial defect and thus secondary. Arthrogryposis is a congenital contracture of the fingers with partial joint fusions. In most cases this is not due to a primary defect in joint formation, but to a secondary effect caused by reduced fetal movement either because of reduced muscle mass or reduced muscle innervation. Both examples illustrate that the most obvious abnormality is not necessarily the most informative. Most primary defects lead to secondary effects, and sometimes it is difficult to separate cause from effect.

13.12 Disruptions

Disruptions define destructive problems acting on a normal fetus. The fetus has the ability to develop normally but fails to do so. Such disruptions may be of vascular, infectious, or teratogenic origin. They usually affect several different tissues in a particular anatomic region without adhering to regions defined by developmental processes. For example, an amniotic band can cut through a certain part of an embryo, destroying muscle, skin, bone, and other tissues that are not embryologically related. Probably secondary to amnion rupture, small strands of amnion can encircle developing structures (most frequently the limbs), leading to annular constrictions, edema, disruptive necrosis, amputation, or syndactyly. At birth aberrant strands can be noted and/or remnants of the rolled-up amnion are present at the placental base. In the milder cases this leads to asymmetric amputation-like phenotypes of the digits. In the most severe cases the entire limb may be lost and secondary deformational defects consequent on decreased fetal movement may occur. Other disruptions can be caused by intrauterine infections: rubella, for example, leads to the destruction of certain organ systems (in this case the inner ear) that would have developed in a regular way if the insult had not occurred. Teratogenic substances such as thalidomide or valproate may cause disruptions because they interfere with normal signaling and/or cell proliferation

13.13 Deformations

In contrast to malformations, which are caused by a primary and intrinsic problem in morphogenesis, and disruptions, which represent the breakdown of a previously normal tissue, deformations are abnormalities caused by mechanical forces. They usually occur during the late phase of pregnancy, but can nevertheless have a pronounced influence on fetal development. Deformations may be intrinsic, i.e., due to abnormal development of the fetus itself, or extrinsic, produced by constraint in utero of an otherwise normal fetus. Such extrinsic forces may produce a single localized deformation, such as a deformed foot, or they may cause a deformation sequence. The potter sequence is an example for the complex effects of mechanical forces. In cases of serious oligohydramnion caused by amniotic fluid leakage or due to reduced production of fluid (aplasia of kidneys, urethral valves, etc.) thoracic growth is restrained and the full growth and maturation of the lungs is thwarted, making them incapable of aerobic expansion and oxygen exchange. The nose is flattened and the limbs are in an aberrant position, often with stiff joints owing to insufficient movement. Breech presentation is another important cause of deformation. Owing to the abnormal position in utero, the head is elongated, approaching a scaphocephalic form, and there are redundant skin folds in the posterior neck, presumably due to the constant retroflexion of the head. The legs are usually hyperflexed in front of the fetus, and any gradation of hip dislocation may occur. Most of the deformations have an excellent prognosis once the fetus is released from the constraining environment. However, some need treatment to release contractures, while others are difficult to treat if the deformation interferes with normal organ development.

13.14 Dysplasias

Dysplasias are caused by gene defects that affect the formation and growth of tissues. In contrast to malformations that are due to abnormal events during early embryonic patterning, dysplasias affect the embryo at a later time point, when the patterning phase is completed. Accordingly, the gene defects that cause dysplasias frequently continue to be active after birth. This offers potential treatment options that are hard to envision for malformations. On the other hand, owing to the ongoing gene activity, some dysplasias carry the potential risk for malignant transformation. Mutations in the cartilage-specific COL2A1 gene cause different forms of skeletal dysplasias ranging from lethal types (achondrogenesis), severe dwarfism (SED congenita), to normal stature with premature osteoarthrosis and myopia. Even the most severe cases have normal skeletal patterning, i.e., they have a normal number of fingers, toes, vertebrae, etc., but all bones are very short owing to a defect in tissue (cartilage) formation. Likewise, mutations in one of the collagen type 1 chains (COL1A1, COL1A2) cause brittle bone disease or osteogenesis imperfecta. Here the affected tissue is not cartilage but bone, because COL1A1 and COL1A2 are predominantly expressed in bone and are essential for the mineralization of bone and thus for its strength.

13.15 Terminology of Congenital Defects

Congenital defects come in a bewildering diversity. Further complicating the situation is the extreme variability in some conditions combined with reduced penetrance. Over the past clinical geneticists set out to order these conditions by giving names to diseases that looked similar. Fine discrimination of the phenotype is necessary to distinguish similar entities. For example, achondroplasia was frequently misdiagnosed among individuals who have small stature and chondrodysplasia that only superficially resemble true achondroplasia. Only the clear delineation of signs and symptoms made definite diagnosis on clinical grounds possible. This phenotypic approach proved to be very successful, since most clinically defined conditions were subsequently identified as having similar or identical gene defects. Certain defects that occurred together were defined as a "syndrome," implying that all patients with this condition are likely to have the same gene defect. The identification of mutations in these patients has shown that the theory holds true, at least in principle. However, similar phenotypes may also be caused by different gene defects, a phenomenon known as genetic heterogeneity. It turns out that gene defects that result in similar phenotypes are likely to be linked within a common molecular pathway. The disruption of such a pathway results in a certain phenotype regardless of where it takes place. Noonan syndrome, for example, is a relatively common condition characterized by small stature, characteristic facies, and heart defects. Mutations in KRAS, a proto-oncogene well known from its mutations in tumors, have been shown to be responsible for Noonan syndrome. Mutations in other components of the Ras pathway including PTPN11, SOS1, and HRAS were shown to cause Noonan or Noonan-like phenotypes illustrating how defects in one pathway may lead to overlapping phenotypes [9]. Conditions that are caused by gene mutations within one molecular pathway and that are thus associated with similar phenotypes have also been called "molecular disease families" [23]. The ongoing identification of gene defects associated with specific phenotypes will eventually enable us to link phenotype and genotype and to better understand differences and similarities in human birth defects.

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Cancer Genetics

14

Ian Tomlinson

Abstract This chapter outlines the contribution of inherited genetic variation to cancer susceptibility and of acquired somatic mutations to cancer growth. The chapter deals with all forms of cancer susceptibility from rare, high-penetrance Mendelian syndromes to common alleles with small effects with importance for population risk. The section on somatic cancer genetics deals with underlying processes, such as genomic instability, and the types of functional change that result in the growth of both benign and malignant tumors. Genetic and epigenetic changes in cancers, from chromosomal scale to small mutations, are discussed. Overall, rather than performing an exhaustive survey in this large field, the chapter outlines the principles of cancer genetics using examples from both common and rare tumor types.

Contents

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14.1 Inherited Risk

14.1.1 Introduction

The fact that cancer can aggregate in some families has been known for many years. In some of these families, the phenotype has been striking in terms of early onset, multiple tumors of particular combinations of lesions, including some nontumor features. These observations have allowed the cancers in such families to be distinguished from so-called sporadic lesions. Examples of such kindreds include those with von Hippel-Lindau (VHL) syndrome, in which affected individuals may develop the otherwise very rare tumors cerebellar hemangioblastoma or pheochromocytoma, but may also develop clear-cell renal carcinoma, which is suffered by 0.5–1% of the general population [23]. By contrast, in neurofibromatosis (NF), patients may develop hundreds of benign nerve cell tumors, but malignancy is not common [32]. In familial adenomatous polyposis (FAP), the characteristic lesion is the humble colorectal adenoma, but patients usually develop hundreds or thousands of these tumors, combined with upper-gastrointestinal tumors, desmoid tumors, and congenital hypertrophy of the retinal pigment epithelium [15].

The realization that many of the common cancers might have some genetic basis has been gradual. Occasionally, these cancers present in large families and inheritance appears to be Mendelian. Breast and ovarian cancers caused by *BRCA1* or *BRCA2* mutations and colorectal cancers caused by mismatch repair gene mutations are two examples. Such families tend to present with a few distinguishing features, such as low age at presentation, multi-generational inheritance, and characteristic tumor histology, but these kindreds cannot always be distinguished from those with familial disease resulting primarily from chance. There is, however, evidence from genetic epidemiology to suggest that the relatives of patients with otherwise unremarkable cancers are often at increased risk of the disease themselves. The magnitude of this increased risk and of the genetic contribution to it remain controversial, as will be discussed below.

14.1.2 Identification of Mendelian Cancer Susceptibility Genes

Following naturally from the observation of cancer-prone families, linkage analysis with subsequent positional cloning was for several years the chief method used to identify dominant Mendelian cancer predisposition genes. Here, by following anonymous, polymorphic DNA markers through families and testing for co-segregation with disease, the locations of the cancer genes could be detected. These locations were subsequently refined through further linkage searches to detect critical recombinants, often based on a finer scale map of polymorphic markers, and by allied techniques, such as the identification of large-scale germline changes including microdeletions. Ultimately, a large panel of genes within a particular genomic region usually had to be screened for germline mutations before the culprit was identified. Some of the cancer predisposition genes identified in this fashion are shown in Table 14.1. In many cases, particularly when the phenotype was not highly unusual, a single, relatively large, family provided the crucial localization data.

A relatively recent example of tumor predisposition gene identification in the Mendelian dominant setting is that of succinate dehyrogenase subunit D (SDHD): *SDHD* mutations cause early-onset or multiple paragangliomas and pheochromocytomas (HPGL) [4]. Ascertainment and careful curation of data on families segregating paragangliomas (particularly carotid body tumors) and pheochromocytomas had shown that this condition was likely to be inherited as a Mendelian dominant trait with incomplete, variable penetrance. A genome-wide linkage screen with highly polymorphic markers mapped the gene to chromosome 11q23.1, this location being refined by the use of 16 novel simple tandem repeat polymorphisms generated by the researchers themselves. Genes within the region were identified using available physical maps and additional mapping data, e.g., from the construction of bacterial and yeast artificial chromosome contigs [5]. Screening of candidate genes revealed that affected individuals harbored protein-inactivating mutations in *SDHD*. That linkage searches can be successful despite reduced penetrance has more recently been shown by the successful

Table 14.1 Mendelian tumor susceptibility genes

Disease[a]	Gene symbol[b]	Tumor spectrum[c]	Classification	Notes
Ataxia telangiectasia		Leukemia, generally increased risk of malignancy	Tumor suppressor; caretaker	Predisposed to DNA double strand breaks; heterozygotes have increased breast carcinoma risk
Bloom syndrome		Generally increased risk of malignancy, especially hematological	Tumor suppressor; caretaker	Increased mitotic recombination
Hereditary pituitary adenoma	AIP	Pituitary adenoma	Tumor suppressor; gatekeeper	Possible somatotropinoma excess
Familial adenomatous polyposis	APC	Intestinal adenomas and carcinomas, desmoids, thyroid carcinomas, hepatoblastomas, adrenal tumors, brain tumors	Tumor suppressor; gatekeeper	Also attenuated disease variant
Hereditary breast/ovarian carcinoma	BRCA1	Breast carcinoma, ovarian carcinoma, pancreatic cancer, prostate cancer	Tumor suppressor; ?both	
	BRCA2			
Hereditary diffuse gastric carcinoma	CDH1	Diffuse gastric carcinoma; ?lobular breast carcinoma	Tumor suppressor; gatekeeper	
Familial melanoma	CDKN2A/TP16	Cutaneous melanoma; pancreatic carcinoma	Tumor suppressor; gatekeeper	Also known as familial atypical multiple mole melanoma-pancreatic carcinoma (FAMMPC) syndrome
Hereditary mixed polyposis	CRAC1	Colorectal hyperplastic polyps, adenomas, and carcinomas	Not known	Gene not yet cloned
Cylindromatosis; Brooke-Spiegler syndrome	CYLD1	Turban tumors/trichoepitheliomas	Tumor suppressor; gatekeeper	
Fanconi anemia	FANCA	Leukemia, generally increased risk of malignancy	Tumor suppressor; caretaker	Predisposed to DNA double strand breaks
	FANCB			
	FANCC			
	BRCA2			
	FANCD2			
	FANCE			
	FANCF			
	FANCG			
	BRIP1			
	FANCL/PHF9			
	FANCM			

(continued)

Table 14.1 (continued)

Disease[a]	Gene symbol[b]	Tumor spectrum[c]	Classification	Notes
Hereditary leiomyomatosis and renal cell carcinoma	FH	Uterine and cutaneous leiomyomas, type II papillary renal cell carcinoma, uterine leiomyosarcoma; Leydig cell testicular tumor	Tumor suppressor; gatekeeper	
Birt-Hogg-Dube syndrome	FLCN	Fibrofolliculomas, trichodiscomas, acrochordons, renal carcinoma (mostly chromophobe and oncocytoma)	Tumor suppressor; gatekeeper	
Peutz-Jeghers syndrome	LKB1/STK11	Intestinal hamartomas, increased risk of colorectal and several other carcinomas	?	Possible tumor suppressor
Multiple endocrine neoplasia type I	MENIN/MEN1	Pancreatic endocrine tumors, parathyroid adenoma, pituitary adenoma, adrenocortical adenoma	Tumor suppressor; gatekeeper	
Hereditary papillary renal cell carcinoma	MET/HGFR	Papillary renal carcinomas	Oncogene; gatekeeper	
Hereditary non-polyposis colon carcinoma	MSH2	Carcinomas of the colorectum, endometrium, stomach, urothelium and skin (sebaceous), and possibly other sites; brain tumors	Tumor suppressor; caretaker	MLH3 and PMS1 also previously suggested; carcinomas show microsatellite instability
	MLH1			
	MSH6			
	PMS2			
MYH-associated polyposis	MYH/MUTYH	Intestinal adenomas and carcinomas	Tumor suppressor; caretaker	
Neurofibromatosis type I	NF1	Multiple neurofibromas, neuromas of other sites, meningioma, hypothalamic tumor, neurofibrosarcoma, rhabdomyosarcoma, parathyroid adenoma, pheochromocytoma	Tumor suppressor; gatekeeper	
Neurofibromatosis type II	NF2	VIII cranial nerve tumors, meningiomas, schwannomas of spinal cord, sometimes other neuromas or neurofibromas	Tumor suppressor; gatekeeper	
Nijmegen breakage syndrome	NIBRIN	Probable generally increased risk of malignancy, especially hematological	Tumor suppressor; caretaker	
Carney complex	PRKAR1A	Atrial myxoma, endocrine tumors, psammomatous melanotic schwannomas	Tumor suppressor; gatekeeper	MYH8 mutations in variant disease
	MYH8			
Gorlin syndrome	PTCH1	Basal cell nevi and carcinomas	Tumor suppressor; gatekeeper	

Disease[a]	Gene symbol[b]	Tumor spectrum[c]	Classification	Notes
Cowden syndrome; Lhermitte Duclos disease; Bannayan-Ruvalcaba-Riley syndrome	PTEN	Multiple hamartomas, including epithelial trichilemmomas, papules, keratoses and verrucous lesions, benign and malignant breast tumors, multiple lipomas, thyroid carcinoma, endometrial carcinoma, cerebellar dysplastic gangliocytoma, intestinal hamartomas	Tumor suppressor; gatekeeper	Considerable, largely unexplained phenotypic heterogeneity
Hereditary retinoblastoma	RB1	Retinoblastoma, osteosarcoma	Tumor suppressor; gatekeeper	
Werner syndrome	RECQL2	Generally increased risk of malignancy, especially sarcoma	Tumor suppressor; caretaker	
Rothmund-Thomson syndrome	RECQL4	Osteosarcoma; possibly generally increased risk of malignancy	Tumor suppressor; caretaker	
Multiple endocrine neoplasia type II	RET	Pheochromocytoma, medullary thyroid carcinoma, parathyroid adenoma, carcinoid, ganglioneuroma	Oncogene; gatekeeper	
Inherited AML	RUNX1/CBFA2	Acure myeloid leukemia	Oncogene?; caretaker	
Hereditary	SDHB	Paraganglioma (including carotid body tumor),	Tumor suppressor;	May also be risk of clear-cell
pheochromocytomas	SDHC	pheochromocytomas	gatekeeper	renal cell carcinoma
and paragangliomas	SDHD			
Juvenile polyposis	SMAD4/MADH4/DPC4	Intestinal polyps, colorectal carcinoma, upper-gastrointestinal carcinoma	Tumor suppressor; gatekeeper	Landscaper suggested for SMAD4
	BMPR1A/ALK3			
Tylosis	TOC	Carcinoma of esophagus	Not known	Gene not yet cloned
Li–Fraumeni syndrome	TP53	Rhabdomyosarcoma, soft tissue sarcomas, osteosarcoma, breast carcinoma, brain tumor, adrenocortical carcinoma	Tumor suppressor; ?both	CHEK2 also a low-penetrance susceptibility gene for breast and other cancers
	CHEK2			
Mosaic variegated aneuploidy	TP53	Rhabdomyosarcoma, Wilms tumor, leukemia	Tumour suppressor; caretaer	
	CHEK2			
Tuberous sclerosis	TSC1	Multiple renal angiomyolipoma, cardiac rhabdomyoma, ependymoma, renal carcinoma, astrocytoma	Tumor suppressor; gatekeeper	
	TSC2			
Von Hippel-Lindau syndrome	VHL	Multiple angiomas, cerebellar hemangioblastoma, pheochromocytoma, clear-cell renal cell carcinoma, pancreatic carcinoma	Tumor suppressor; gatekeeper	

(continued)

Table 14.1 (continued)

Disease[a]	Gene symbol[b]	Tumor spectrum[c]	Classification	Notes
Wilms tumor	WT1	Nephroblastoma	Tumor suppressor; gatekeeper	Associated with Wilms tumor–aniridia–genitourinary anomalies–mental retardation syndrome and Beckwith–Wiedemann syndrome
Xeroderma pigmentosum	XPA ERCC3 XPC ERCC2 DDB2 ERCC4 ERCC5 POLH	All types of skin carcinoma	Tumor suppressor; caretaker	Nucleotide excision repair deficiencies

[a] Most commonly used name

[b] "Official" and some unofficial names in common use

[c] Most commonly reported neoplastic lesions; other characteristic lesions are not shown

identification of germline aryl hydrocarbon receptor interacting protein (*AIP*) mutations in pituitary tumor families [55]. Here, a single, very large family was used to map a susceptibility locus based on 16 individuals affected by somatotropinona or mixed adenoma, who were separated by tens of meioses and by many relatives who were obligate gene carriers but showed no evidence of tumor.

Increasingly, linkage analysis and positional cloning have been supplemented by other techniques. Where a Mendelian disease is genetically heterogeneous, screening of sequence or functional homologs has provided an alternative method or a short cut to the gene (see Table 14.1 for details). After *SDHD* was identified as the cause of HPGL, for example, the other components of the SDH heterotetramer were screened for in families with similar phenotypes, and mutations were found in *SDHB* and *SDHC* [3, 36]. The identification of *LKB1/STK11* as the gene for Peutz-Jeghers syndrome relied on linkage analysis that was focused on the short arm of chromosome 19 by the discovery of deletions of this region in polyps from Peutz-Jeghers patients [19].

In addition to the above examples, several other Mendelian tumor syndromes are caused by mutations in more than one gene, each of which encodes a functionally related protein. In many of these cases, the connection between the genes has only become apparent after positional cloning identified the susceptibility loci. In tuberous sclerosis, for example, the proteins hamartin and tuberin were found to have decreased expression after linkage analysis demonstrated that there were two genes implicated, one at chromosome 9q34 and one at chromosome 16p13.3. The genes turned out to be *TSC1* and *TSC2*, respectively, and a mutation in either gene results in a similar phenotype [11]. Both proteins act as tumor suppressors and interact with each other to form a heterodimer that acts within the PI3K/AKT/mTOR signaling pathway. Other examples are provided by the condition of gastrointestinal juvenile polyposis, in which both susceptibility genes (*BMPR1A, SMAD4*) lie in the TGF-beta/BMP signaling pathway, and by neurofibromatosis, where type 2 is caused by mutations in the protein merlin, which is involved in regulation of the Ras/Rac signaling pathway and type 1 by mutations in neurofibromin, which contains a Ras GTPase-activating protein domain and negatively regulates Ras.

The fact that several bioscientific disciplines can contribute to disease gene identification is exemplified by the case of the MMR genes in Lynch syndrome (hereditary nonpolyposis colon cancer, HNPCC). Linkage analysis was intrinsically difficult in this disease, because of the problems in distinguishing the phenotype from sporadic colorectal cancer (see above). Gene identification was, however, clinched by a combination of evidence sources: linkage analysis in large colorectal cancer families; replication errors in familial and sporadic cancers that were reminiscent of similar changes in mismatch repair-deficient yeast; and the identification of germline mutations in MMR genes in HNPCC patients [31]. This approach was taken a step further by the discovery of *MYH/MUTYH* mutations as the cause of a recessively inherited form of colorectal polyposis. Here no linkage analysis or positional cloning was undertaken, and the original study was focused not on *MYH* but on the *APC* gene [1]. However, in examination of polyps from a family with multiple colorectal adenomas, the gene was identified on the basis of a strikingly unusual spectrum of somatic mutations in the *APC* gene. The preponderance of G>T changes within *APC* suggested that oxidative damage to DNA was not being efficiently repaired, perhaps as a result of an underlying defect in base excision repair (BER). This was subsequently confirmed by the finding of biallelic mutations in the gene encoding the MYH glycosylase, an enzyme that removes adenine residues which have mispaired with the oxidized base 8-oxo-7,8-dihydroxy-2’-deoxyguanosine.

14.1.3 Unidentified Mendelian Cancer Susceptibility Genes

Linkage analysis, positional cloning, and allied methods cannot succeed in gene identification for all Mendelian tumor syndromes, the chief problems being genetic heterogeneity and the presence of phenocopies within families. Candidate gene screening and searches for mutational signatures (such as in MAP and HNPCC) are not always successful. Therefore, while most Mendelian cancer genes have been identified, there almost certainly remains a small, but important, group of patients who have a single-gene disorder but whose underlying genetic problems have not been found. This contention remains true even if a generous allowance is made for the imperfections of mutation detection methods when screening known genes. In the absence of one or a few large kindreds that would on their own provide power for linkage analysis, these genes may remain unidentified until, by good fortune, a candidate gene search is successful or "something turns up," as it did for MYH.

14.1.4 Germline Epimutations

Some genes can be inactivated by promoter methylation rather than mutation in the soma, but it had generally been thought that stable methylation could not be transmitted through the germline. This reasoning did not, however, exclude acquisition of methylation early enough during development for it to affect sufficient cells to mimic a de novo mutation. This phenomenon, although apparently uncommon, has been reported for the DNA mismatch repair gene *MLH1*, which may acquire promoter methylation relatively easily [47]; the epimutation caused an HNPCC phenotype. There was also evidence that stable methylation might be transmitted through the germline in some unknown fashion. Recently, some cases of *MSH2* epimutation have been shown to result from germline deletions of an upstream gene, *TACSTD1*, which leads to transcript read-through and methylation of the *MSH2* promoter by some unknown mechanism [29]

14.1.5 A Heterozygote Phenotype in Mendelian Recessive Tumor Syndromes?

For recessive tumor predisposition genes, the combined population frequency of disease alleles may be >1%. At this level, the issue of disease risks in heterozygotes potentially becomes important. The mechanism for these effects might, for example, be mild haploinsufficiency or somatic loss of the wild type allele in tumors. The most long-standing example of such an effect comes from heterozygotes for ataxia telangiectasia (*ATM*) mutations. First-degree relatives of ataxia telangiectasia patients have been reported to be at increased risk of breast cancer [48], and recent molecular studies suggest that women with one mutant copy of ATM have an approximately two-fold increased risk of this cancer [39, 51]. Some other heterozygote effects continue to be more controversial. Carriers of single Bloom syndrome (*BLM*) mutations, especially the relatively common Ashkenazi change, a frameshift mutation in exon 10, have been found in some studies to be at increased risk of colorectal cancer [18], but others have found no such effect. There is, similarly, an ongoing debate about the colorectal cancer risk in carriers of single *MYH* mutations; very few studies have found significantly increased risks, but several have found small, nonsignificantly raised risks [10, 49]. Meta-analyses have not resolved this issue to date. Excluding a small risk when mutation carriers are uncommon is extremely difficult, and the case of *MYH* illustrates this problem well.

14.1.6 Some Cancer Genes are Involved in Both Dominant and Recessive Syndromes

For a number of Mendelian dominant cancer predisposition syndromes, there is now clear evidence of an additional, recessive phenotype. *BRCA2* mutant homozygotes or compound heterozygotes have Fanconi anemia [21]; and individuals with bi-allelic MMR mutations have neurofibromatosis, childhood lymphoid malignancies, and brain tumors [12, 16, 31]. Interestingly, even though somatic loss of the wild type allele underlies the dominant versions of these syndromes, the tumor spectrum is not identical to that of the recessive syndrome. For the MMR mutants, for example, perhaps the brain tumors or neurofibromas require multiple additional events that only rarely occur subsequent to allelic loss in mutant/wild type heterozygotes, but additional postulates are required to explain why the mutant homozygotes do not develop very-early-onset bowel tumors. In some cases, the homozygote syndrome is not associated with tumors at all: individuals with heterozygous fumarate hydratase (*FH*) mutations develop leiomyomas and renal cell cancer (HLRCC), but homozygotes die early in life from nonspecific global developmental delay, hypotonia, and acidosis [52].

14.1.7 Phenotypic Variation, Penetrance, and Rare Cancers in Mendelian Syndromes

The Mendelian cancer syndromes vary greatly in the number, aggressiveness, and tissue spectrum of tumors that develop (Table 14.1). In several syndromes, it is common for mutation carriers to reach old age without being affected, whereas others are affected exceptionally early in life. It is not easy to explain these variations, but animal models suggest that modifying genetic loci may sometimes have a role, as, of course, might environmental exposures. Genotype-phenotype associations also occur in Mendelian syndromes. The *APC* gene in FAP provides an excellent example of such an association. Some mutations, especially around codon 1309, cause thousands of colorectal adenomas, probably because these tumors can acquire their "second hits" by LOH, this phenomenon being related to the tumors needing the optimal level of Wnt signaling that is caused by the mutation combination [27]. Most other FAP patients have a few hundred adenomas, and their polyps have "second hits" caused by protein-truncating mutation, but a few so-called attenuated FAP patients have fewer than 100 adenomas, and some have none at all. These AFAP cases have germline mutations in the first four exons of APC, or the second half of the last exon, or in the alternatively spliced exon 9. It seems that many polyps from AFAP patients require "three hits" at APC, including loss or additional mutation of the germline mutant allele [45]. Again, it is likely that a requirement for optimal Wnt signaling is the reason.

Similar genotype-phenotype associations exist for other diseases, including conditions resulting from germline mutations in two or more different genes. In some cases, an explanation is at hand. There are, for example, suggestions that splice-site MMR mutations produce a "weak" phenotype, and it is plausibly argued that this is because they produce some functional protein and hence have less effect on the mutation rate. In other cases, however, the cause of the association is not fully understood. VHL disease (Table 14.1), for example, is often clinically divided into type 1 (without pheochromocytoma) and type 2 (with pheochromocytoma). It can be further subdivided into type 2A (with pheochromocytoma), type 2B (with pheochromocytoma and renal cell carcinoma), and type 2C (with isolated pheochromocytoma without hemangioblastoma or renal cell carcinoma). Protein-truncating mutations are more common in type 1 disease, whereas missense changes predominate in type 2, suggesting that the latter encode proteins that retain some function(s). Intriguingly, type 2C mutations, for example, may encode proteins that retain the ability to down-regulate hypoxia-inducible factor alpha subunit, thought to be critical in the pathogenesis of VHL tumors [26].

While such considerations can help to explain variation among individuals with the same disease, variation between diseases generally requires different explanations. Why are there so many polyps in FAP and neurofibromas in NF, yet so few identifiable lesions in, for example, carriers of E-cadherin mutations? Why do mutations in ubiquitously expressed genes only cause cancer in certain tissue types? Some possible causes for the variation between different tumor syndromes [44] are:

- Our failure to recognize or identify precursor lesions
- The stage of tumorigenesis at which mutation acts (earlier mutation causing more tumors)
- The cell turnover of target organ/tissue/cell
- The importance of the gene at different stages of development or for a particular specialized cell function
- Probabilistic effects, such as the likelihood of cell death unless the next mutation in the pathogenetic pathway happens within a particular time window
- The critical role of a gene, perhaps in relation to the environmental stresses acting on the target organ/tissue/cell

Perhaps even more difficult to explain is the similarity in some phenotypes, such as *MYH*-associated polyposis and FAP, which are caused by mutations in apparently unrelated proteins.

Another phenomenon that is increasingly described is the small absolute risks (although sometimes large relative risks) of certain cancer types in some of the Mendelian syndromes. Examples are shown in Table 14.1. They include modestly increased risks of prostate cancer in carriers of *BRCA2* mutations [54] and of solid malignancies in those affected by several of the syndromes caused by DNA breakage that had previously been strongly associated only with hematological malignancies.

14.1.8 Predisposition Alleles Specific to Ethnic Groups

Increasingly, cancer susceptibility mutations that are specific to particular ethnic groups have been identified. The Ashkenazi population is probably the best studied of these groups, and specific alleles have been identified in genes such as *BLM* (nt2281delATCTGA, insTAGATTC), *BRCA1* (185delAG, 5382insC), *BRCA2* (6174delT), *APC* (I1307K), and *MSH2* (A636P). For *MYH*, particular mutations are overrepresented in the Pakistani (Y90X), Indian (E466X), and Mediterranean (nt1395-7delGGA) populations [8]. Several other examples exist and must be borne in mind when undertaking mutation screening in clinical practice.

14.1.9 Germline Mutations can Determine Somatic Genetic Pathways

The identity of the germline susceptibility gene or allele can determine the somatic tumorigenic pathway that tumors follow. Breast cancers with germline *BRCA1/2* mutations, for example, generally have ductal morphology, high grade, loss of hormone receptors, absence of Her2/Neu amplification, and specific somatic molecular changes (the so-called basal phenotype [53]. HNPCC colorectal cancers are often found in the proximal colon and acquire changes, such as beta-catenin and K-ras mutations, which are very rarely found in their sporadic counterparts that have acquired somatic silencing of *MLH1*. Intriguingly, it has also been shown that genetic pathways of tumorigenesis may vary among ethnic groups, perhaps caused by their different genetic backgrounds [2].

14.1.10 Non-Mendelian Genetic Cancer Susceptibility

For most of the common tumors, there is evidence that first-degree relatives of cases are at a slightly (typically two-fold) increased risk of developing the same disease. These risks are higher for some cancers, such as those of the testis or thyroid. For some cancers, there is also sometimes a smaller increased familial risk of developing cancer in general. These risks are usually higher when the proband has early-onset disease or the family history is more extensive. Whilst some of this risk might be the result of shared environment, twin studies and segregation analyses have shown that genetic factors are highly likely to account for much of the increased risk [28].

The nature of the genetic risk of the "common cancers" remains unknown, although the relative risks associated with the known pathogenic variants in the Mendelian cancer genes are too high to explain the risks observed in the general population. Genes with moderate (say, fivefold) effects on risk may in theory exist, particularly for the more "genetic" cancers, such as those of the colorectum, breast, and prostate. However, efforts to identify such genes have largely been unsuccessful and most studies are currently focused on alleles that have relatively small effects on risk (or, in other words, have low penetrance). In all cases, these alleles may "interact" with other alleles (at the same or different loci), or with the environment, in such a way that risks are much higher (or lower) if an individual has several of them. There are essentially three major, nonexclusive models for the genetic contribution to the common cancers. In the first model – the "common variant-common disease" hypothesis – a relatively small number of frequent polymorphic alleles is supposed to increase disease risk. The second, "rare variant," model assumes that the predisposing alleles are generally uncommon (typically <2% frequency) and might even take the form of multiple different variants within the same gene. The third, "polygenic," model assumes that many, many variants contribute to disease, each conferring a very small risk; such variants may in some cases act via intermediate phenotypes or molecular traits, such as levels of gene expression.

It is arguable that the third model will be extremely difficult to show directly, except by exclusion of the other models, since the relative risks conferred by each allele (and even by a combination of several alleles) will be too low to detect other than by huge studies. Model three may also encompass what might be regarded as normal variants that are under genetic control and associated with cancer risk, such as body mass, skin pigmentation, breast density, and hormone levels. A two-stage search, based on finding the genes for these traits and then associating the traits with cancer, may be appropriate here.

Efforts to demonstrate model one are currently focused on case-control (association) studies involving thousands of patients. The disease-causing variation is generally assumed to take the form of single nucleotide polymorphisms (SNPs) or copy number variants. Platforms are now available to genotype thousands of coding SNPs or millions of noncoding SNPs genome wide. Even within the ranks of those who favor the "common variant-common disease" model, there is a divergence of opinion as to the best method of detecting causal variation, with one group favoring a screen based on known functional variation [50] and the other, a screen based on linkage disequilibrium (using so-called haplotype-tagged SNPs from efforts such as the HapMap study [9]. In the latter case especially, even when a disease-associated allele has been identified and verified by several studies it may prove particularly difficult to pin down the causal variant, given the linkage disequilibrium-based SNP selection involved.

The results of genome-wide SNP screens are at an early stage, but the validity of the common disease-common variant model has been confirmed for all the common cancers and for some rarer cancer types, including childhood solid tumors and hematological malignancies. More than ten SNPs have been found to be associated with each of prostate, breast, and colorectal cancer, although most of these are tagging variants rather than causal for disease. In general, the following conclusions can be drawn from the genome-wide association studies of cancer (Fig. 14.1) [13]:

- Most cancer predisposition SNPs are organ specific, an exception being rs6983267 on chromosome 8q, which is associated with increased risks of prostate, colorectal, and ovarian cancers.
- The relative risks conferred are somewhat lower than expected (typically 1.1–1.3 per allele).
- Power considerations suggest that more, undetected predisposition SNPs exist and that these probably have weak effects.
- SNPs in candidate genes and/or nonsynonymous SNPs are generally absent from those detected.
- Some clinicopathological associations have been found (e.g., with tumor site, stage, or grade).
- Risks at multiple SNPs are additive or log-additive, and no gene-gene interactions have been found to date.
- The most plausible models suggest that many variants have their effects through (long-range) changes in gene expression.
- Although the genes tagged by the SNPs appear to have various functions, there are hints of some pathways being consistently affected (e.g., the bone morphogenetic protein pathway in colorectal cancer).

Intriguingly, there is also evidence for model two, the rare variant' hypothesis [17]. Its proponents argue that variants with all but very small functional effects are unlikely to exist at allele frequencies higher than 1–5%, because natural selection will tend to eliminate them from the population as long as they cause some cancers to develop before reproductive age, or if they have deleterious effects unrelated to cancer. In general, surveys have shown that alleles predicted in silico to have deleterious effects do tend to be rarer, although it is difficult to apply this reasoning a priori to cancer. Rare variants might, moreover, confer higher relative risks than common variants, whilst not violating the

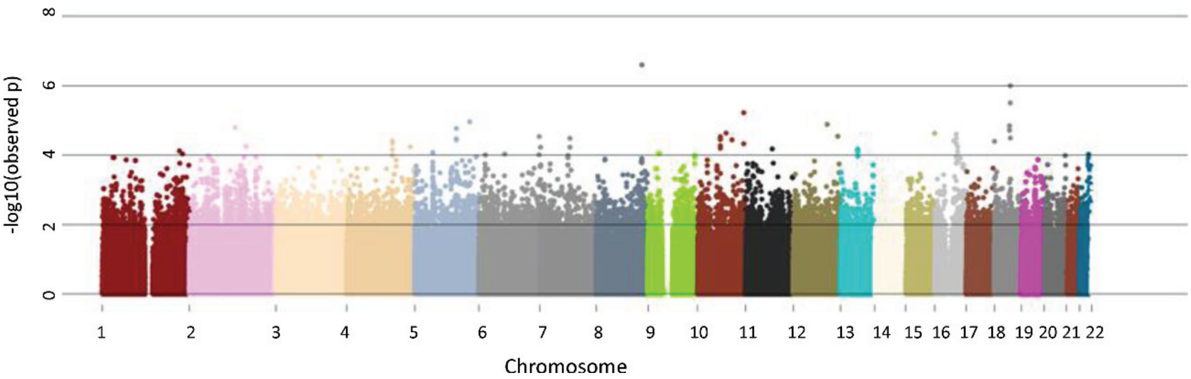

Fig. 14.1 "Manhattan" plot of GWA study data on colorectal cancer. Note the signals on chromosomes 8 and 18, both of which have subsequently been shown to result from true susceptibility loci

observed, population-based relative risk constraint. (Similar arguments can be applied to the effects of recessively acting SNP alleles.) The twin difficulties of identifying rare, disease-associated, variants are, however, discovering them and empowering analysis sufficiently to detect a genuine association (the latter is again also applicable to recessive alleles). Despite these great problems, there are a few potential solutions. Large numbers of rare variants now exist owing to HapMap and related SNP discovery programs, and selection of cases by age of onset and/or family history can greatly increase the power of association-based analyses. The *CHEK2* 1100delC allele is a case in point. This allele, which has a population frequency of ~1%, was originally proposed to be the cause of some Li–Fraumeni syndrome families (see Table 14.1). Although subsequently shown that this was unlikely to be the case, the role of CHEK2 – like BRCA1/2 – in the double strand break (DSB) repair pathway caused the variant to be tested in a set of familial breast cancer cases. It was estimated that carriers of the variant are at a two-fold increased risk of breast cancer [33]. Carriers of other rare *CHEK2* variants may also be at increased breast cancer risk, and perhaps at raised risk of other cancers too, although the latter remains controversial. It is not clear whether the examples of *CHEK2* can be applied generally, in the sense that candidate genes can be selected on the basis of current knowledge of molecular pathways in which susceptibility genes act. For breast cancer, it seems that this model is correct, in that not only are heterozygotes for pathogenic mutations in *ATM* at raised breast cancer risk (see above), but also carriers of rare variants in the related DSB repair genes *BRIP1* [42] and, possibly, *PALB2* [38] have a modestly raised risk of the disease. It is notable, however, that similar screens in the MMR or the Wnt pathways have not yet yielded similar associations between rare variants and colorectal cancer risk.

14.1.11 Concluding Remarks

Many cancers and benign tumors probably have an inherited component. Best characterized are the Mendelian (single-gene) disorders that are associated with a high lifetime tumor risk. There is great heterogeneity in these conditions in terms of the genes involved, tumor site, multiplicity of tumors, molecular pathways involved, and underlying mechanisms of tumorigenesis. Most Mendelian tumor susceptibility genes are dominantly inherited tumor suppressors that normally restrain cell proliferation in some way, or recessively inherited DNA repair or genome integrity alleles. Mendelian syndromes probably account for 1–2% of all human malignancies, and they are best classified on a molecular, rather than a clinical, basis.

The remaining genetic contribution to cancer is “complex,” but may explain up to one third of all cases. The alleles involved are likely to have low penetrance, but their successful identification depends on the size of their effects, on their frequencies, and on multiple replication studies. Eventually, however, it may be possible to offer genetic testing for multiple cancers to the general population, based on panels of polymorphisms and/or rarer variants. Some of these alleles may provide a general cancer predisposition, whereas others will be site specific. It will then be possible to offer preventive measures to those at higher genetic risk. In addition, it may be possible to identify germline determinants of tumor aggressiveness or response to treatment (including pharmacogenetics) and of prognosis.

The potential financial burden of population-level genetic testing will be great. It is therefore important to target this testing wherever possible, principally to alleles with relatively large effects on disease risk and to cancers for which effective, selective prophylaxis is possible. There is otherwise a danger that the existing and predicted gains of cancer genetics will not be applied equitably and efficiently.

14.2 Somatic Cancer Genetics

14.2.1 Mutations Cause Cancer

The mutational theory of carcinogenesis has had general acceptance for many years. In short, this theory suggests that the acquisition of changes in the DNA of a susceptible progenitor cell can confer the characteristics of cancer, namely unrestrained growth, invasion, and metastasis. Cancer is thus seen largely as a cell-autonomous condition, whereby tumorigenesis is initiated in a single cell, and as a result of selection, that cell's progeny increase in numbers over their normal counterparts. This process is known as clonal expansion. It is often stated, although rarely formally proven, that the initial mutations must occur within a stem cell in the normal tissue. More recently, it has been proposed that even within a cancer, there are stem and nonstem cell populations, and molecules such as CD133 have been proposed as cancer stem cell markers [34]. The stem cell model of carcinogenesis does not necessarily assume, however, that the cells outside the tumor clone have no part to play in tumor development, and there is indeed increasing evidence that the microenvironment is extremely important in determining tumor behavior.

The nature of the selective advantage that cancer-causing mutations must confer appears to vary among cancer types. For example, the *ras* oncogene family is activated by mutation in many different cancer types, and it causes activation of Pi3-kinase, Mek/Erk, and other signaling pathways. VHL mutations are found in renal and other cancers and cause activation of the hypoxic response pathway. E-Cadherin mutations are found in lobular breast and intestinal gastric cancers and lead to failure of cell adhesion. Retinoblastoma (*RB1*) mutations disrupt the balance between cell cycle arrest/senescence and proliferation.

In many cancer types, the acquisition of mutations tends to follow particular genetic pathways. In other words, while there seems to be no absolute requirement, certain mutations are usually co-selected. Thus, in colorectal cancer, mutations of K-ras and p53 often occur together, whereas mutations of BRAF and p53 are very rarely found together in the same tumor. Moreover, some mutations seem to be selected very early in tumorigenesis – an example being changes in adenomatous polyposis coli (APC) – and others, such as p53, are found in tumors that have already acquired one or more mutations in other genes. In general, the reasons for these findings are currently unknown, but presumably relate to factors such as the interplay between signaling pathways, interactions among tumor cells, and the relationships between tumor and nontumor cells; for example, a large invasive tumor will have very different requirements from a small benign tumor in terms of its many factors, including the availability of oxygen and nutrients, thus causing different mutations to be selected.

14.2.2 Chromosomal-Scale Mutations, Copy Number Changes, and Loss of Heterozygosity

Owing to the relatively long history of cytogenetics, including the examination of tumor karyotypes, chromosomal-scale changes were the first genetic changes to be discovered in cancers. Many, perhaps most, cancers were found to have gross karyotypic abnormalities, comprising a polyploid DNA content (often 3 N or 4 N), multiple aneusomies, structural changes (including large deletions, duplications, inversions, and translocations), and grossly abnormal chromosomes such as double minutes. Newer cytogenetic techniques, such as multicolor-fluorescence in situ hybridization (MFISH; Fig. 14.2), have confirmed and characterized this tendency of cancers to acquire a highly abnormal chromosome complement. Why many tumors are aneuploid and polyploid is, however, unclear, as is the mechanism (e.g., duplication of the whole chromosome complement versus sequential chromosomal gains).

In some tumor types, the chromosomal-scale changes tend to be highly specific. In hematological malignancies, and sarcomas in particular, recurrent translocations tend to place an oncogene under the control of another gene that is highly expressed in the relevant cell type (see http://www.ncbi.nlm.nih.gov/sites/entrez?db = cancer chromosomes). These translocations

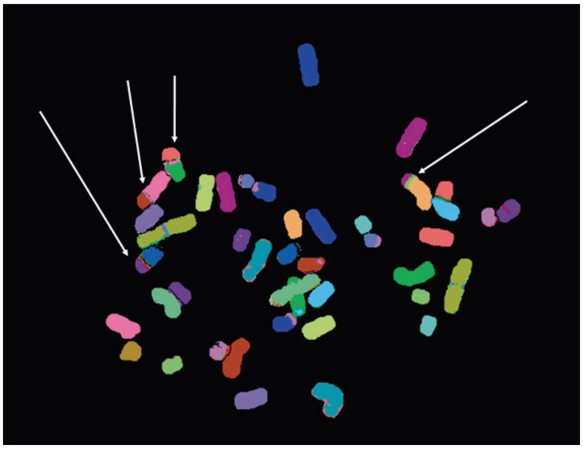

Fig. 14.2 M-FISH analysis of breast tumor cell line AP. The chromosome are pseudo-colored, showing several changes including translocations (examples *arrowed*) and numerical changes

may result in fusion genes or put the oncogene in an inappropriate molecular environment. Whilst each translocation must occur extremely rarely, they are found because they provide a strong selective advantage and, in some cases, may be essential for tumorigenesis. Examples include the classic Philadelphia chromosome t(9;22)(q34;q11) that fuses BCR and ABL in CML and the PAX3-FOXO1 fusion in rhabdomyosarcomas.

In carcinomas, recurrent translocations are relatively uncommon, for reasons that are unclear. Nevertheless, it is usual for a whole variety of chromosomal-scale mutations to be present in carcinomas. Most commonly, these result in copy number changes – gain or deletion of material relative to the overall DNA content of that tumor. Sometimes, however, the copy number changes target specific genes. For example, amplifications may target oncogenes such as EGFR or HER2/ERBB2 [41]; and deletions may target tumor suppressor genes, such as p16 or p53. However, in most cases, chromosomal-scale copy number changes in carcinomas have no obvious gene target. Whether these mutations represent background changes or subtle, selected effects on gene dosage, perhaps at multiple loci on a chromosome, is unclear. However, the fact that some changes appear to be recurrent and to differ in their frequencies among tumor types suggests that many copy number changes are not simply background events. Techniques such as high-density, microarray-based comparative genomic hybridization (CGH) are uncovering copy number changes that range in size from whole chromosomes down to a few kilobases.

A commonly used concept in cancer genetics is loss of heterozygosity (LOH) [7]. LOH can be defined as a loss of or decrease in the intensity of one allele at a polymorphic locus in a tumor, relative to paired normal tissue. The term LOH was introduced as a way of describing molecular observations associated with the inactivation of one copy of a tumor suppressor gene in a diploid cell. However, LOH has rarely been used in this correct context, and its extension to the analysis of cancers in which specimens are impure and nondiploid has created a great deal of confusion. Consider, for example, a perfectly triploid cancer. Every locus in that tumor will show LOH if a threshold of 50% decreased intensity of one allele is used. However, to ascribe functional significance to these observations would clearly be nonsensical. It may be time to accept that the term LOH should be restricted to a copy-neutral situation (i.e., when a chromosomal region becomes homozygous without deletion or gain of genetic material). This process commonly occurs by mitotic recombination (break-induced replication) and seems to be a favored way of inactivating tumor suppressors such as *APC* and *RB1*).

14.2.3 Activating Mutations and Oncogenes; Inactivating Mutations and Tumor Suppressor Genes

Conventionally, genes that undergo somatic mutations (defined so as to include all changes from those at whole-chromosome to single-base level) in cancer have been divided into those that cause gain-of-function (oncogenes) and those that cause loss-of-function (tumor suppressor genes). Perhaps unsurprisingly, this classification is too simple to describe the variety of mutations found in tumors, but it remains true that most mutated genes probably do fall into one of these two broad classes.

The original class of oncogenes was discovered as the normal cellular counterparts of proto-oncogenes found in transforming viruses. Examples include *MYC*, *SRC*, *KIT*, and *ABL*. Since that time, any dominantly acting mutation associated with gain of function has come to be classed as an oncogene. An example of an oncogene without a proto-oncogene equivalent is the Wnt signaling effector, beta-catenin which is mutated in several types of intestinal cancer [35]. In general, oncogenes can be activated by several different mutational mechanisms, including: point mutation or deletion of critical residues; translocation or rearrangement causing overexpression; and amplification (high-level copy number gain). Sometimes, an oncogene can be activated by more than one mechanism in different cancers, or even in the same cancer. For example, the hepatocyte growth factor receptor *MET* is activated by point mutation in some papillary renal cell cancers, but also frequently undergoes copy number gain of the mutant allele as a further "hit" [57].

Tumor suppressor genes conventionally require both copies to be inactivated by two mutational hits for there to be an effect on tumorigenesis. Small-scale mutations that target tumor suppressors are often, although by no means always, nonsense or frameshift changes that result in a truncated or absent protein. Missense changes that target critical residues may also be found if they inactivate protein function. Deletions or copy-neutral LOH are also common. A typical tumor suppressor is a gene such as *VHL*, which is mutated in the great majority of clear-cell renal cancers, mostly by protein-truncating mutation affecting one allele and deletion affecting the other. Loss of function of this ubiquitin ligase adaptor leads to failure to degrade HIF and other substrates, and hence to inappropriate activation of the hypoxia signaling pathway [23].

Although oncogenes can in principle be activated in a similar fashion in both diploid and aneuploid/polyploid cells, tumor suppressors classically require all copies to be inactivated. Thus, three "hits" would be required fully to inactivate a tumor suppressor in a trisomic cell, and four, in a tetrasomic cell. So far, there is little evidence that tumor suppressors harbor several mutations consistent with their inactivation in a polyploid cell, raising the intriguing possibility that tumor suppressor changes are usually relatively early events in tumorigenesis, albeit for largely unknown mutational or selective reasons.

The list of atypical oncogenes or tumor suppressors (or genes that cannot readily be placed into either category) has recently been lengthening. There is a relatively long-standing controversy as to whether p53, apparently one of the archetypal tumor suppressors, has some dominant negative effect in the heterozygous state associated with missense mutations in the protein's DNA-binding domain, despite there usually being two hits found in the gene in tumours [6]. *APC* mutations, found in the great majority of colorectal cancers, are constrained such that there is selection for retention of some protein function owing to a requirement for an optimal resulting level of Wnt signaling [27]. Mutations in the *CDC4/FBXW7* ubiquitin ligase adaptor are usually found in the heterozygous state with the wild type allele, suggesting a dominant negative effect, but may also be present in the conventional two-hit state in some tumors [25]. In addition, several genes mutated in cancer have been proposed to have their effects through haploinsufficiency, especially in mouse models, but further evidence is required before such a model, however plausible, can be accepted.

14.2.4 Epimutations

In the normal genome, CpG dinucleotides outside gene promoters are very often methylated at the cytosine residue. In cancers, there is global hypomethylation of unknown cause, but also a tendency for some promoter CpG islands to become hypermethylated and for expression of the gene concerned to be silenced. In many cases, just like mutations, these methylation changes are background events, but sometimes they target a gene the inactivation of which is selected. Indeed, for some tumor suppressor genes, mutation can affect one allele and hypermethylation the other. Examples of methylated genes with probable functional significance in cancer include the mismatch repair gene *MLH1* (see below), *CDH1*, *MGMT*, and *RUNX3*. In other cases of promoter methylation with proposed functional effects, the patterns and/or role of methylation are complex. At *APC*, for example, only the 1A promoter is methylated and, whilst this affects mRNA levels, it is far from clear that functional consequences result.

Hypermethylation in cancer does not have a known cause, and it is intrinsically different from mutation, in that multiple CpGs within a promoter must usually be methylated for there to be gene silencing. Little is known about whether this is a stochastic process or whether an initiating event triggers regional methylation. A further area of ignorance is why some genes tend to be inactivated by methylation rather than mutation: is this intrinsic to the gene concerned, perhaps reflecting some unknown aspect of normal gene sequence, structure or function?

14.2.5 How Many Mutations are Needed to Make a Cancer?

There is ongoing debate as to the number of mutations, probably acquired in a stepwise fashion, that are required for a malignant tumor to grow. Calculations based on cancer incidence rates have suggested that four to six mutations may be required, but these studies do not take full account of requirements for bi-allelic changes, the molecular processes that underlie complex mutations such as amplification, and the influences of epigenetic changes [20]. Moreover, these studies essentially measure rate-limiting steps rather than total mutation numbers. Recent efforts have enabled the DNA of a few colorectal and breast cancers to be sequenced on a genome-wide scale [56]. Remarkably, these efforts did not turn up large numbers of undiscovered mutations. In colorectal cancer, for example, all of the frequently mutated genes (largely *APC*, K-ras, p53, *SMAD4*, and *CDC4*) were already known, and no good evidence of any metastasis-specific genetic changes was found. Although other, low-frequency genetic changes occur, and may provide a small selective advantage, there is no good evidence that they drive tumorigenesis. Thus, it appears that relatively few (<5) small-scale mutations are required for a cancer to occur. However, this number takes no account of epigenetic changes, mutations in regulatory regions, changes to noncoding sequences such as microRNAs, copy number alterations, and polyploidy. Until the importance of such changes has been determined, the number of mutations required for carcinogenesis must remain uncertain, but it seems likely to be fewer than 20, and perhaps fewer than 10. This number is, of course, several orders of magnitude lower than the actual number of mutations found in any cancer. The processes of normal development and tissue turnover, added to the clonal expansion during tumorigenesis, mean that millions or billions of mutations are expected to occur in the typical cancer, simply as a result of normal rates of DNA damage, replication errors, and imperfect repair.

14.2.6 The Role of Genomic Instability and Molecular Phenotypes

An ongoing and unresolved controversy in cancer genetics is the role of genomic instability, meaning a specific underlying tendency to acquire mutations at a higher rate than normal cells. At one level, a relatively subtle form of genomic instability is probably caused by factors primarily selected for other reasons, such as oncogene activation or inactivation of tumor suppressors (e.g., *PTEN*). However, discussion of genomic instability rarely encompasses this subtle form. Rather, genomic instability is considered in terms of primary defects in processes such as DNA repair, chromosome segregation, cell cycle checkpoint failure, assembly of the mitotic spindle, or telomere structure.

Proponents of the central place of genomic instability in tumorigenesis argue that cancers require too many mutations for them not to require genomic instability [30]. This argument is relatively weak, since the process of clonal expansion can generate sufficient tumor cell progeny to allow five or six mutations to be acquired at normal mutation rates, let alone rates that may be modestly elevated as a result of, say, oncogene activation. More tellingly, it is argued that the observed numbers of mutations in cancers is probably many, many thousands, even though almost all of these are nonselected (or passenger) changes [46]. Others have responded by contending that normal mutation rates can explain even these large numbers of mutations without difficulty, and some authors have even suggested that genomic instability may not provide a selective advantage to tumors under all circumstances, particularly in the early stages of tumorigenesis [43].

What is incontrovertible is that a few cancers do have a specific tendency to genomic instability. Germline mutations in the following pathways, amongst others, cause genomic instability and a greatly increased risk of cancer: base excision repair (*MYH/MUTYH*); nucleotide excision repair (the xeroderma pigmentosum loci); spindle checkpoint (*BUBR1*); homologous recombination repair (*BLM*); mismatch repair (*MLH1, MSH2, MSH6, PMS2*); and double strand break repair (*ATM*). Most of these occur in the context of rare recessive conditions. By contrast, there are very few examples of the equivalent somatic changes in the common cancers. However, bi-allelic methylation of *MLH1* is found in an important minority of cancers, chiefly those of the colorectum and endometrium [24]. Loss of MLH1 expression leads to failure of repair of spontaneously mismatched bases, especially in simple repeat sequences such as microsatellites, and thus to a mutator phenotype termed microsatellite instability (MSI, MIN; Fig. 14.3).

The mutator phenotype in MLH1-deficient tumors raises an issue of tumor classification. MLH1-negative colorectal cancers tend to have a high frequency of mutations in genes such as *BRAF* and *TGFBR1*, and few changes in K-ras and *SMAD4;* MSI+cancers also strongly tend to have a near-diploid karyotype. Thus, it can be said that MSI+colorectal cancers tend to follow a particular genetic pathway. The molecular phenotype of MSI can thus conveniently classify a subset of cancers. By contrast, MSI- negative cancers are frequently aneuploid and polyploid and are said to be chromosomally

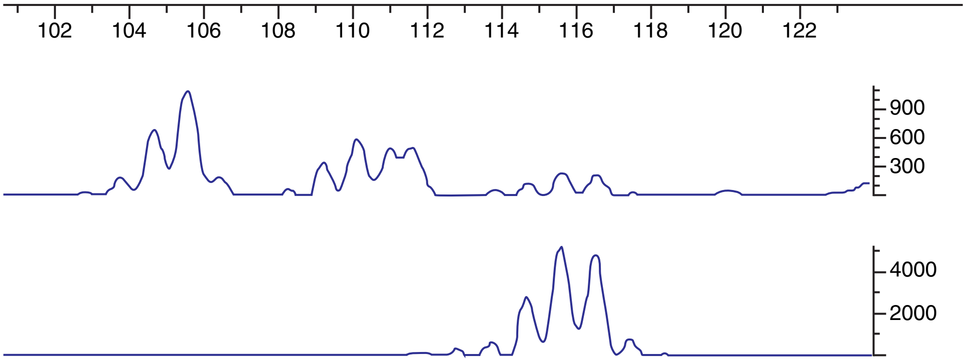

Fig. 14.3 Microsatellite instability in a colorectal tumor. The *lower trace* shows genotype at marker BAT26 in normal cells. The genotype is shifted to the left in the tumor (*above*), indicating deletion of adenine residues within the oligonucleotide tract at this site, and hence microsatellite instability

unstable (CIN+); this is an imprecise term and does not necessarily reflect an underlying tendency to genomic instability. MSI and CIN seem to be (almost) mutually exclusive. There may also be a group of MSI-CIN- cancers, although whether these have some other, undiscovered from of instability remains unknown. A third molecular phenotype, a global tendency to CpG island methylation, is generally, although not universally, accepted to exist; this so-called CIMP phenotype overlaps substantially with the MSI phenotype. Undoubtedly, these molecular phenotypes will become refined for most cancer types in the future.

14.2.7 The Conundrum of Tissue Specificity

Some genes, such as p53 and K-ras, are mutated or otherwise altered in many cancer types, whereas others, such as *APC*, have a very restricted occurrence in tumors of one or a few sites. In general, some tissue specificity of mutations is the norm. In some cases, this phenomenon can readily be explained. For example, changes in the PU.1 transcription factor are specific to hematopoietic cells and associated with the development of AML [40]. However, in most cases, it is not clear why some mutations are strongly associated with certain tumor types. DNA mismatch repair genes are ubiquitously expressed, yet *MLH1* is principally inactivated by promoter methylation in carcinomas of the large bowel and endometrium. There is increasing interest in a model of tumorigenesis in which highly specific levels of dysregulation are required for neoplastic growth. Particular types of cells have Achilles heels, but the specific molecular and general environments generally make these different among tissues. It follows that different genes tend to be altered in different tissue types.

14.2.8 Clinicopathological Associations, Response and Prognosis

Somatic mutations and epimutations are almost certainly the primary determinants of how cancers behave. Many associations between molecular changes (DNA, mRNA or protein) and clinicopathological associations, response and prognosis have been reported, although these are too numerous to list here. In colorectal cancer, for example, MSI+cancers tend to occur in older individuals in the proximal colon, to show mucinous histology and a high number of tumor-infiltrating lymphocytes. MSI is also associated with a generally good prognosis [37] and perhaps with a poorer response to 5-FU-based therapy [22]. The reasons for these associations are unclear, but may be related to the immune response elicited by MSI+cancers. With increasing use of molecularly targeted therapeutic agents, the associations between molecular changes and treatment response will become more important. This is already being seen for the use of anti-EGFR therapy in colorectal cancer, for which K-ras mutation is a strong negative predictive marker.

14.2.9 Posttherapy Changes

The use of chemotherapy and radiotherapy enforces strong extrinsic selection on tumors. While this will be strong enough to kill most or all of the tumor cells in many cases, where tumors do recur they often acquire specific resistance as a result of mutations in genes involved in the pathway targeted by the therapy. For example, *BRCA1/2*-mutant breast cancers that show resistance to poly(ADP-ribose) polymerase (PARP) inhibitors have been shown to have acquired reversion mutations that restore near-normal function to the mutant BRCA1 or -2 protein (yet, interestingly, do not cause cancer regression) [14]. Ovarian cancers treated with cisplatin have been reported to acquire MSI, owing to dependence of the effect of the drug on intact DNA mismatch repair and hence strong selection for resistance-conferring mismatch repair gene silencing.

14.2.10 Concluding Remarks

The cancer genome is increasingly becoming tractable to analysis. Methods such as high-density microarrays and massively parallel sequencing promise to deliver all of the important genetic and epigenetic changes in cancers. The challenge then becomes categorization of cancers, functional analysis of these changes, the acquisition of large sample series to test the roles of genetic changes as biomarkers and, ultimately, the design of new anticancer therapies.

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The Role of the Epigenome in Human Cancers

15

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Abstract Deregulation of the epigenome is an important mechanism involved in the development and progression of human diseases such as cancer. As opposed to the irreversible nature of genetic events, which introduce changes in the primary DNA sequence, epigenetic modifications are reversible. The conventional analysis of neoplasias, however, has preferentially focused on elucidating the genetic contribution to tumorigenesis, which has resulted in a biased and incomplete understanding of the mechanisms involved in tumor formation. Epigenetic alterations, such as aberrant DNA methylation and altered histone modifications, are not only sufficient to induce tumors, but can also modify tumor incidence and even determine the type of neoplasia that will arise in genetic models of cancer. There is clear evidence that the epigenetic landscape in humans undergoes modifications as the result of normal aging. Thus, it has been proposed that the higher incidence of certain disease in older individuals might be, in part, a consequence of an inherent change in the regulation of the epigenome. These observations raise important questions about the degree to which genetic and epigenetic mechanisms cooperate in human tumorigenesis, the identity of the specific cooperating genes, and how these genes interact functionally to determine the diverse biological paths to tumor initiation and progression. The answers to these questions will partially rely on sequencing relevant regions of the 3 billion nucleotide genome, and determining the methylation status of the 30 million CpG dinucleotide methylome at single nucleotide resolution in different types of neoplasias. Here, we also review the emergence and advancement of technologies to map ever larger proportions of the cancer methylome, and the unique discovery potential of integrating these technologies with cancer genomic data. We discuss the knowledge gained from these large-scale analyses in the context of gene discovery, therapeutic application, and building a more widely applicable mechanism-based model of human tumorigenesis.

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Contents

15.1 Introduction

Cancer is typically described in terms of genes that are mutated or deregulated. This gene-based model is derived, in large part, from whole-genome but low-resolution analytical methods, which certainly have biased the process of gene discovery. Higher resolution, high-throughput technical advances in DNA sequencing, genome scanning, and epigenetic analysis have produced an impressive cadre of new cancer gene candidates to fit to the model. A new challenge is thus to distinguish the gene alterations that are active drivers of cancer from those that are passengers, or more passively involved. However, significant portions of the cancer genome and epigenome remain uncharted, suggesting that even more cancer genes and potential targets for diagnosis and therapy remain to be discovered. This realization has stimulated national and international collaborative efforts to fully map various cancer genomes and epigenomes [59, 75, 90, 129], with notable successes in pilot phases. Here we discuss the technologies that have propelled these efforts, the resulting gene discoveries, and the fundamental principles of the pathogenic mechanisms of cancer, with particular emphasis on epigenetic studies of DNA methylation. Because epigenetic mechanisms can cause genetic changes and vice versa, we also review known epigenetic–genetic interactions in the context of an integrated mechanism-based model of tumorigenesis.

15.2 DNA Methylation in Development and Cellular Homeostasis

DNA methylation is essential for normal development, chromosome stability, maintenance of gene expression states, and proper telomere length [18, 23, 43, 64, 67, 95, 96, 109, 114, 123, 160, 163, 180]. DNA methylation involves the transfer of a methyl group to the 5-position of cytosine in the context of a CpG dinucleotide via DNA methyltransferases that create (DNMT3A, 3B) or maintain (DNMT1) methylation patterns. DNMT3A and DNMT3B share sequence similarity with DNMT3L, an enzymatically inactive regulatory factor that interacts with histone tails that are unmethylated at H3K4 and recruits DNMT3A2 to facilitate de novo methylation [86, 125]. Genetic knock-out of *Dnmt1*, *Dnmt3a* or *Dnmt3b* in the mouse embryo results in embryonic or perinatal lethality, underscoring the essential role of DNA methylation in normal developmental processes [109, 124].

In human and mouse, DNA methylation patterns are first established during gametogenesis. However, the genetic material contributed by each of the gametes undergoes profound changes after fertilization. A recent report indicates that the paternal genome is actively demethylated in mitotically active zygotes [72]. This active demethylation phase is followed by a passive and selective loss of DNA methylation that continues until the morula stage [141, 178]. DNA methylation patterns are then reestablished after implantation and maintained through somatic cell divisions [61]. Interestingly, amidst the sweeping genome-wide methylation changes during embryonic development, the methylation status of imprinted genes remains unchanged [164, 178].

The haploid human methylome consists of approximately 29,848,753 CpGs, nearly 70% of which are methylated in normal cells. Just 7% of all CpGs are within CpG islands [136], and most of these are unmethylated in normal tissues. Normally methylated sequences include those few CpG islands associated with the inactive X chromosome and some imprinted and tissue-specific genes, as well as pericentromeric DNA (e.g., Sat2 repeats on chr1 and chr16), intragenic regions and repetitive sequences. In fact, 45% of all CpGs in the genome are in repetitive elements, thus accounting for a large proportion of the total 5-methylcytosine [20, 31, 38, 39, 136, 156]. Normal DNA methylation patterns may vary among individuals [53, 140], potentially stemming from environmental exposure [177], stochastic methylation events [60], or trans-generational inheritance [119]. The importance of interindividual epigenomic variance has been postulated to influence the development of disease, and also the time of disease onset. An intriguing potential example of this phenomenon is illustrated by psychiatric diseases, such as bipolar disorder and schizophrenia in monozygotic twins. In some instances, only one member of the twin pair develops the pathology, while in others the time of disease onset between the twins may differ by several years or even decades. Importantly, molecular studies have failed to identify a genetic component that may account for this phenotypic discordance [16].

Several studies have focused on the influence of nutrition on DNA methylation. Of particular interest is the role played by a set of nutrients directly involved in regenerating or supplying methyl groups. Since methyl groups are labile, chronic deficiency in methyl-supplying nutrients can result in the direct or indirect alteration of SAM-to-*S*-adenosylhomocysteine (SAH) ratios, consequently reducing the cellular potential for methylation reactions, including DNA methylation [15]. Nutrients that regenerate or supply methyl groups fall into the category of lipotropes, and they include folate, choline, methionine, and vitamin B_{12}. Riboflavin and vitamin B_6 might also contribute to the modulation of DNA methylation processes, since both of these nutrients are integral parts in 1-carbon metabolism [183]. Studies in which rodents were subjected to diets deficient in different combinations of folate, choline, methionine, and vitamin B_{12} showed a reduction in the SAM-to-SAH ratio in those animals. Furthermore, DNA hypomethylation could be detected at the genomic level not only in specific tissues, but also at specific loci [127, 128, 147, 168]. High methyl–donor content in the diet of pregnant agouti mice can partially suppress the phenotypic manifestations of a genetic mutation (an IAP element insertion) in their offspring [171, 175]. Taken together, these results suggest that the mechanisms regulating the epigenome can be influenced by environmental factors and can interact with genetic elements to alter phenotype. Moreover, the modulation exerted by environmental factors on the epigenome can potentially contribute and/or trigger the development or onset of disease. In light of this evidence, high-resolution mapping of the methylome, ideally at single CpG dinucleotide resolution, may provide a new avenue for understanding the disease or susceptibility factors that could be used to detect at-risk individuals.

15.3 DNA Methylation is Disrupted in Human Primary Tumors

DNA methylation patterns are severely altered in primary human tumors. This includes aberrant hypermethylation of CpG islands in promoter regions, which is frequently associated with gene silencing [5, 23, 24, 45, 89], and genome-wide and locus-specific hypomethylation [39, 46–48, 58, 61]. Typically, aberrant CpG island methylation is assessed in genes already known to have a role in tumor development, especially in tumor samples that do not harbor genetic alterations of the gene. This candidate gene approach has identified aberrant methylation-mediated silencing of genes involved in most aspects of tumorigenesis, commonly altering the cell cycle [52, 66, 110, 116, 139, 158], blocking apoptosis [22, 94, 99, 161] and DNA repair [25, 26, 40, 41, 70, 74, 91]. In general, aberrant CpG island methylation tends to be focal, affecting single genes but not their neighbors [3, 185]. Two genomic loci however, are subjected to epigenetic silencing over an entire chromosomal domain of 150 kb in one case and 4 MB in the other [55, 121 154]. It is likely there will be more examples of this type of long-range epigenetic silencing yet to be uncovered.

These and other studies have established an important role for aberrant methylation in tumorigenesis and prognostication, but have focused on only a small number of the estimated 15,000 CpG island-associated promoters in the genome [4], and only on those genes first identified through genetic screens. Among those CpG islands analyzed, many have only been "sampled" for methylation at fewer than 5 of potentially 100 or more CpGs in a single island, and only on one DNA strand. Even more revealing of the early stage of cancer methylome analyses is the fact that, of the roughly 15,000 of non-CpG island-associated promoters that could also be influenced by aberrant methylation at specific CpGs, few have been studied in cancer [153]. Concurrent with promoter hypermethylation, many human tumors exhibit a global decrease in 5-methylcytosine, or genomic hypomethylation, relative to matching normal tissues [38, 47, 49, 57, 58]. In severe cases, hypomethylation can affect more than 10 million CpGs in a single tumor [12]. Three mechanisms by which hypomethylation contributes to malignancy have been proposed, including transcriptional activation of oncogenes, loss of imprinting (LOI), and promotion of genomic instability via unmasking of repetitive elements and by causation of mutations [18, 47, 49]. Most surprisingly, even though hypomethylation has been known about for more than two decades, the vast majority of genomic loci affected by cancer hypomethylation are unknown [46, 49, 83, 142, 143, 179], though presumably a significant proportion of DNA methylation loss occurs in repetitive sequences [76]. A resurgence of interest in hypomethylation, along with newer technologies for assessing hyper- and hypomethylation discussed herein, should address these sizable gaps in our knowledge of the cancer methylome.

15.4 Genome-Wide DNA Methylation Analyses

Analyzing the human genome for changes in DNA methylation is a challenging endeavor. A majority of the approximately 29 million CpG dinucleotides in the haploid genome are located in ubiquitous repetitive sequences common to all chromosomes, which hampers determination of the precise genomic location where many DNA methylation changes occur [101, 134]. In addition, gene-associated CpG islands encompass a minor fraction of all CpG sites, and their hypermethylation therefore has only a limited affect on global 5-methylcytosine levels in cancer cell DNA [35]. However, since changes in CpG island methylation can abrogate gene expression [88], identifying aberrant CpG island methylation often, but not always, identifies genes whose expression is affected during, or because of, the tumorigenic process.

Restriction Landmark Genomic Scanning (RLGS) was the first method to emerge as a genome-wide screen for CpG island methylation and was originally described in 1991 [69, 137]. In RLGS, genomic DNA is digested with the rare-cutting methylation-sensitive restriction enzymes such as *Not*I or *Asc*I. The recognition sequences for these enzymes occur preferentially in CpG islands [27, 111], effectively creating a bias towards the assessment of DNA methylation in gene promoters. Importantly, *Not*I and *Asc*I recognition sequences rarely occur within the same island, effectively doubling the number of CpG islands interrogated for DNA methylation in any given sample [29]. Following digestion, the DNA is radiolabeled and subjected to two-dimensional gel electrophoresis. DNA methylation is detected as the absence of a radiolabeled fragment, which stems from the enzymes' failure to digest a methylated DNA substrate. The main strengths of RLGS are that PCR and hybridization are not part of the protocol, allowing for quantitative representation of methylation levels and a notably low false-positive rate relative to most other global methods for detecting DNA methylation. Additionally, a priori knowledge of sequence is not required [151], making RLGS an excellent discovery tool [30, 105, 152, 184]. However, RLGS is limited to the number of *Not*I and *Asc*I sites in the human genome that fall within the well-resolved region of the profile. In practice, the combinatorial analysis of both enzymes can assess the methylation status of up to 4,100 landmarks [1264].

The success of the Human Genome Project [166] helped stimulate the development of newer methods for genome analysis, which were then adapted for DNA methylation analyses, ranging from single gene, intermediate range and high throughput (e.g., 100–1,000 loci/genes in 200 samples) [8, 37], to more complete methylome coverage (array-based methods, next-generation sequencing) [20, 82, 84, 98, 118, 145, 170, 172]. To allow for more in-depth discussion of these methods, we unfortunately had to exclude discussion of a number of other very effective PCR and array-based methods. Arrays originally designed for genome-wide analysis of DNA alterations have been adapted for methylation analysis. A main advantage of array platforms is their potential to increase the number of CpGs analyzed, and the technically advanced state of array analysis in general. Critical parameters for methylation arrays for analysis of human cancer include effective resolution, methylome coverage (total number of CpGs analyzed), reproducibility, ability to distinguish copy number and methylation events, and accurate validation through an independent method.

Differential methylation hybridization, the first array method developed to identify novel methylated targets in the cancer genome [81], has served as a basis for many newer generation array methods. In this assay, DNA is first digested with *Mse*I, an enzyme that cuts preferentially outside of CpG islands, and then ligated to linker primers. The ligated DNA is subsequently digested with up to 2 methylation sensitive restriction enzymes, such as *Bst*UI, *Hha*I or *Hpa*II. Since these enzymes are 4-base-pair restriction endonucleases, their recognition sequence is ubiquitous in GC-rich genomic regions, such as CpG islands. After the second round of enzymatic digestion, the DNA is amplified by PCR using the ligated linkers as primer binding sites. Detection of DNA methylation is accomplished by fluorescently labeling the PCR product from a test sample, such as tumor DNA and then co-hybridizing it with the PCR products derived from a control sample, such as normal tissue DNA. Aberrantly methylated fragments are refractory to the methylation-sensitive restriction endonuclease digestion, resulting in the generation of PCR products. On the other hand, an unmethylated fragment would be digested, preventing PCR amplification. Therefore, the comparison of signal intensities derived from the test and control samples following hybridization to CpG island arrays provides a profile of sequences that are methylated in one sample and not the other. One potential drawback of most methylation array methods is the need to use potentially unfaithful linker ligation and linker PCR amplification, which is prone to false positives. Nevertheless, massive improvements in oligonucleotide arrays, particularly for allelic methylation analysis, hold promise of even greater methylome coverage to methylation array-based methods in the future [20, 71, 98, 118, 145, 172].

Bacterial artificial chromosome (BAC) arrays have also been successfully introduced as a means of high-throughput DNA methylation analysis [20, 90], and complete tiling path arrays are available now [85]. In one application with BAC arrays, genomic DNA is digested with a rare cutting methylation-sensitive restriction enzyme, the digested sites are filled-in with biotin, and unmethylated fragments are selected on streptavidin beads and then co-hybridized to the BAC array with a second reference genome. In contrast to other array methods, ligation and PCR are not used in this protocol. The use of rare cutting restriction enzymes ensures that most BACs will contain only a single site or single cluster of sites, allowing single-CpG-effective resolution of the methylation analysis and accurate validation. Tiling path BAC arrays can be easily adapted for use with different restriction enzymes to significantly increase the number of analyzable CpGs. However, genome coverage using restriction enzymes is limited by the presence of their recognition sequence in the targets of interest.

The particular combination of array- and methylation-sensitive detection reagent is also critical for tumor methylome analysis. These reagents include methylation-sensitive restriction enzymes, 5-methylcytosine antibody, methylated DNA-binding protein columns, or bisulfite-based methylation detection. Bisulfite is a chemical that allows conversion of cytosine to uracil, but leaves 5-methylcytosine unconverted [56]. This method is a staple of single gene analysis and high-throughput analysis of small sets of genes [73, 107]. However, owing to the significantly reduced sequence complexity of DNA after bisulfite treatment, its use for array application has been more limited [2, 182]. DNA selected through methyl-binding protein columns or by 5-methylcytosine antibody immunoprecipitation has also been applied to microarrays [97, 121, 131, 172, 186, 187]. The effective resolution of methylation using either method is dependent in part on the average DNA fragment size after random shearing, generally 500 bp to 1 kb. It is not yet clear how many methylated CpG residues are needed for productive methylated DNA-antibody binding to occur, or whether the antibody has significant sequence bias. An advantage of this approach is that it is not as limited to specific sequences as restriction enzyme-based approaches. The 5-methylcytosine antibody approach has been used to successfully map the methylome of *Arabidopsis thaliana* [186, 187], with results largely confirmed by shot-gun bisulfite sequencing of the same genome [21, 114]. This approach has also been applied to human cancer cell lines [97, 121].

Methylation-sensitive restriction enzymes, whether rare or common cutters, can theoretically provide single-CpG-precision/effective resolution. In practice, however, common cutters, even when applied to oligonucleotide arrays, will not yield single-CpG resolution because up to ten oligonucleotides spanning multiple common cutter sites are averaged into one value. Additionally, because protocols using common cutters require ligation and PCR [82, 98, 145], the distance and sequence between sites precludes a large proportion of these sites from analysis, reducing genome coverage. The restriction enzyme *McrBc* has also been tested for methylation detection [112, 121], although the resolution of methylation events is undefined owing to the unusual recognition site of *McrBc* (two methylated CpGs separated by 40–3,000 bp of nonspecific sequence).

An innovative large-scale SAGE-like sequencing method has also been employed for methylation analysis of breast cancer and the surrounding stoma cells [80]. Gene expression arrays can also be used to identify methylation-related silencing of genes by focusing on silent genes that are reactivated in tumor cell lines exposed to a DNA demethylating agent [92, 93, 146, 159].

Reduced representation bisulfite sequencing, a large-scale genome-wide shotgun sequencing approach [115], has been successfully employed to investigate loss of DNA methylation in DNMT[1^{kd},$3a^{-/-}$,$3b^{-/-}$] ES cells. An advantage of this method is that it is amenable to gene discovery without preselecting targets, though sites exhibiting heterogeneous methylation might be confounding when represented by only a single sequence read. Substantially increasing the depth of sequencing may mitigate this limitation somewhat. Also, since clone libraries can be constructed, the system can be automated to maximize efficiency.

Human epigenome projects of normal human cells have taken a standard sequencing-based bisulfite strategy, which gives single-CpG resolution of methylation status [36, 85, 130]. While these projects are not primarily designed to determine the methylation status of 29 million CpGs, the efforts to date have been immense and impressive, including different cell type, and interindividual and interspecies comparisons. Combining either bisulfite, the 5-methyC antibody, methyl-binding protein columns, or restriction enzymes with next-generation sequencing also holds great promise. These and other studies are adding to whole new disciplines within epigenetic research, including population epigenetics and comparative epigenetics. In addition to the main goals of these projects, the data will also be of substantial value for comparison with cancer methylome data, whether from arrays or sequencing bisulfite-converted DNA.

15.5 Gene Silencing Vs. Gene Mutation

Tumor suppressor genes are typically discovered through study of familial cancers and through mapping allelic loss of heterozygosity (LOH) in sporadic human tumors [17]. Regions exhibiting recurrent, nonrandom deletion are selected for further identification of a candidate tumor suppressor gene by attempting to identify a second hit involving a point mutation or homozygous deletion [100]. Thus, until recently, surveys for point mutations have been confined largely to regions of recurrent LOH or genomic amplification. Current proposals for sequencing entire cancer genomes aim to identify genes that have escaped detection by lower resolution approaches, to provide new targets for therapy, and to further improve the experimental modeling of cancer. Pilot projects have proven the utility of this approach with great success [32, 33, 150]. A recent zenith in sequencing, including 13,023 genes in 22 tumor cell lines, yielded a wealth of new candidate cancer genes and potential therapeutic targets [150]. A current challenge is to distinguish mutations likely to contribute to the tumorigenic process from the many inconsequential mutations that riddle the cancer genome.

A related hypothesis is being addressed concurrently by taking an unbiased approach to mapping nonrandom and tumor type-specific epigenetic alterations that result in gene silencing [6, 27, 87, 138]. These studies address the hypothesis that there may be tumor suppressor genes that have escaped detection because they are seldom inactivated by genetic lesions, but often silenced by epigenetic mechanisms [185]. Using Restriction Landmark Genome Scanning (RLGS) [69], the first of many large-scale methylation

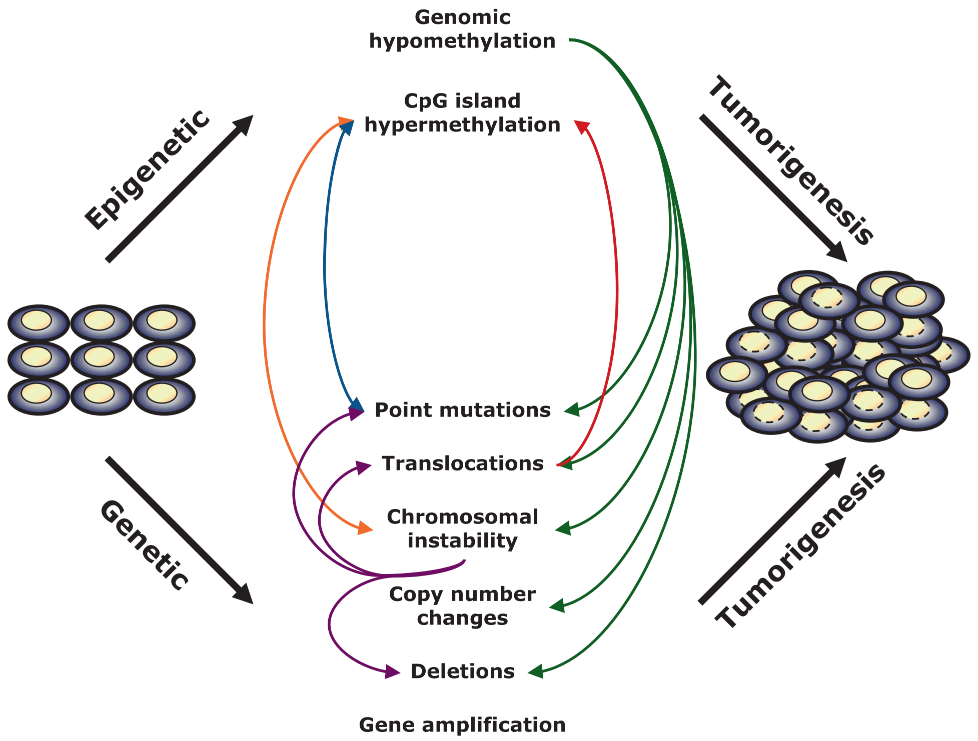

Fig. 15.1 A mechanism-based model of the pathogenesis of human cancer. Data from mouse models of cancer or hereditary human cancer indicate that genetic changes or epigenetic changes alone can initiate tumor formation. Sporadic cancers, which comprise 90–95% of all cancers, almost uniformly exhibit both genetic and epigenetic defects genome-wide, and these mechanisms show substantial interaction (*arrows*). That is, epigenetic events can cause genetic events, and vice versa. Determining the relative contribution of genetic and epigenetic mechanisms to tumor formation is an important goal of current research and should be facilitated by the whole cancer genome and epigenome approaches. Depending on the cancer type, each mechanism can operate early, late, or continuously in the development of the tumor

analysis methods, it was estimated that hundreds of CpG islands may be aberrantly methylated in any given tumor, though the range of methylation across individual tumors varies significantly [27]. Similar to mutation spectra, only a subset of these methylation events are sufficiently recurrent to qualify as nonrandom events, potentially arising through selection of cells harboring a methylation-mediated silencing event that confers a growth advantage. Large-scale integrated genomic and epigenomic tumor profiles have shown that the majority of loci affected by aberrant methylation are in fact independent of recurrent deletions [78, 79, 185]. Genes such as *WNK2*, encoding a serine/threonine kinase that negatively regulates MEK1, are largely subject to epigenetic silencing in one tumor type, but by genetic point mutations in other tumor types [79]. Taken together, these data suggest that genomic and epigenomic approaches are complementary for cancer gene discovery and that their integration could provide an ideal and more comprehensive platform for interrogating the cancer genome (Fig. 15.1).

15.6 Discovery of Cancer Genes via Methylome Analysis

As discussed above, no single current genome-wide DNA methylation approach can assay the entire cancer methylome. Thus, more focused and integrative approaches exploiting the cooperation between genetic and epigenetic mechanisms have been adopted in efforts to identify new cancer genes, many with promising results. Recently, for example, transcription factor 21 (*TCF21*) was identified as a putative tumor suppressor in head and neck and lung cancers by specifically screening a known region of LOH for aberrant DNA methylation [155]. Interestingly, this gene is located in a 9.6-Mb chromosomal domain known to suppress metastasis in melanoma cell lines [174]. However, no candidate gene had been proposed for this region, since mutations in *TCF21* are infrequent [174]. A similar strategy was utilized to identify oligodendrocyte transcription factor 1 (*OLIG1*), a frequently methylated gene and prognostic factor in human lung cancer located in a region of chromosomal loss [11, 108], and also for *HIC1* and others [169]. Like *TCF21*, *OLIG1* also was methylated at a much higher frequency than the existing LOH data would have suggested [107, 155], indicating that aberrant DNA methylation is likely to be the main mode of inactivation for these genes in the tumor types analyzed. Other putative tumor suppressor genes also located in regions of frequent LOH, such as *DLEC1*, *PAX7*, *PAX9*, *HOXB13*, and *HOXB1*, have been identified via the use of affinity columns to enrich methylated DNA sequences [131]. Given their specific technical limitations, these studies indicate that the integration of several experimental strategies will be required in order to maximize the discovery of new cancer-related genes. These studies illustrate the discovery potential of combined approaches, though the current cast of candidate cancer genes derived from methylation screens alone is far larger than can be discussed here.

15.7 Computational Analysis of the Methylome

Aberrant DNA methylation exhibits tumor-type-specific patterns [27]. However, it is unclear how these patterns are established and why a large number of CpG islands seem to be refractory to DNA methylation, while others are aberrant methylated at high frequency [30, 97, 132, 138, 181]. A functional explanation for this observation could be that all CpG islands may be equally susceptible to DNA methylation, but only a fraction is detected in tumors because of selection pressures. This hypothesis, though probably true for some genes, is unlikely to explain the mechanism responsible for aberrant methylation of all CpG island-associated genes.

Sequence-based rules derived from cancer cell methylation data have also been explored as a way to predict the pattern of aberrant methylation in cancer genome-wide [9, 44, 50, 51]. These studies have identified consensus sequences, proximity to repetitive elements, and chromosomal location as potential factors influencing or perhaps determining the likelihood that a CpG island might become aberrantly methylated. If the sequence context in which a CpG island is located influences its likelihood of becoming aberrantly methylated, the convergence of different computational analyses is likely to find commonalities that could help explain this phenomenon. An important goal in these studies will be to distinguish sequence rules that predict pan-cancer methylation from those that predict tumor-type-specific methylation, as these rules could be mutually exclusive. An intriguing and particularly striking association between a subset of genes susceptible to aberrant promoter methylation in adult human cancers and a subset of genes occupied or marked by polycomb group proteins in human embryonic stem cells has been reported independently by three groups [122, 144, 176]. These and earlier studies [135, 167] offer important new insight into possible mechanisms by which certain genes might be susceptible to methylation in cancer, and epigenetic support for the theory that human tumors arise from tissue stem cells (Fig. 15.2). Comparison of the sequences associated with PcG occupancy and those derived from the computational analysis of methylation-prone and methylation-resistant loci described above might be particularly revealing.

15.8 Histone Modifications and Chromatin Remodeling in Cancer

A second epigenetic mechanism of transcriptional regulation and chromosomal functioning (e.g., DNA repair, DNA replication, chromatin condensation) involves reversible histone modifications [63, 102, 103]. Eight histone proteins, two each of histone H2A, H2B, H3 and H4, along with 146 bp of DNA comprise a single nucleosome. Interaction of neighboring nucleosomes can be altered by the complex combinations of covalent modifications on the histones, which may represent a "histone code." Different types of histone modifications include phosphorylation, acetylation,

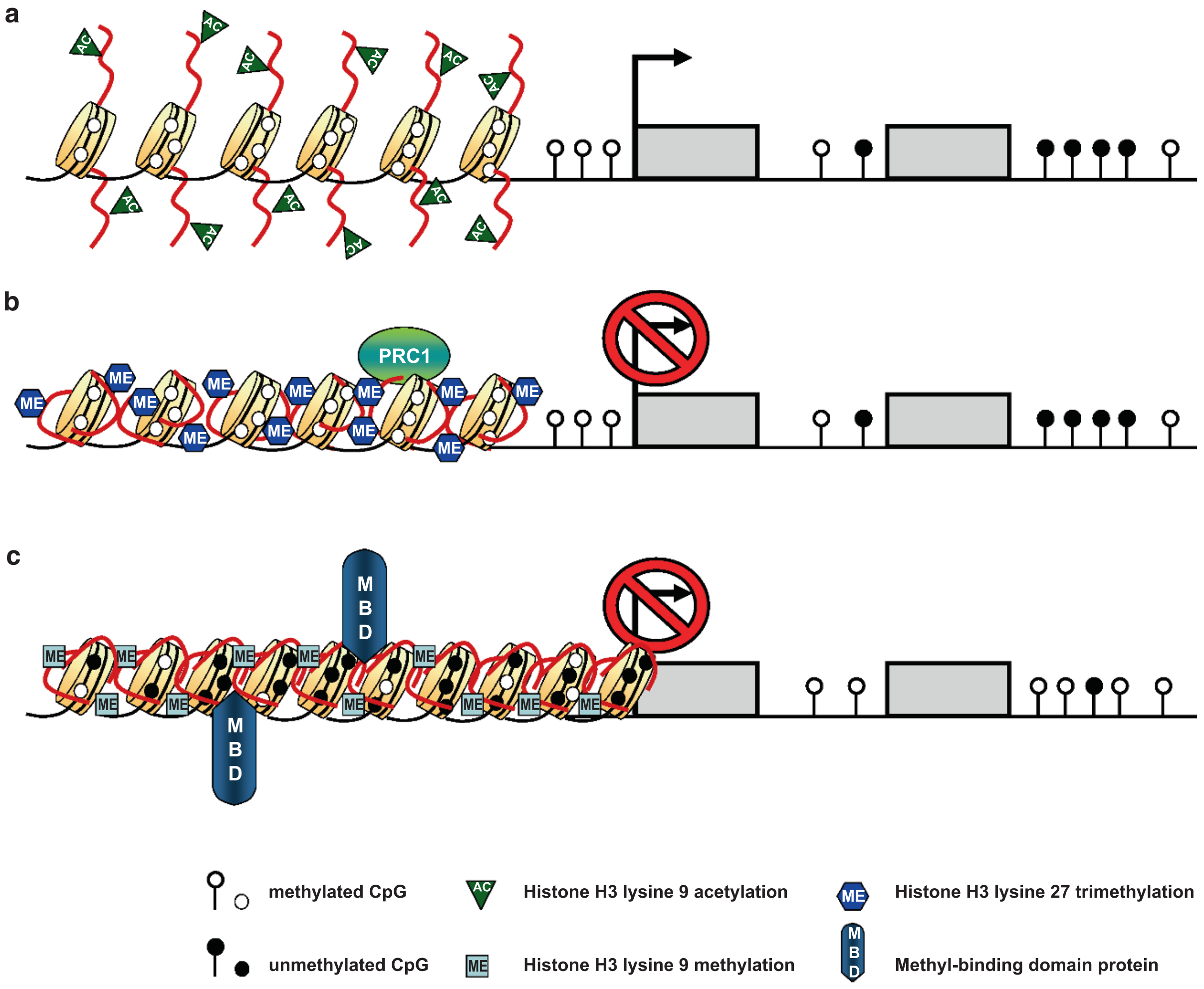
a
AC
b
PRC1
ME
c
MBD
ME
methylated CpG
unmethylated CpG
AC
Histone H3 lysine 9 acetylation
ME
Histone H3 lysine 9 methylation
ME
Histone H3 lysine 27 trimethylation
MBD
Methyl-binding domain protein

Fig. 15.2 (**a–c**) Role of DNA methylation and histone modifications at promoter CpG islands in normal cells and cancer. In normal cells, most promoter CpG islands do not exhibit DNA methylation. Thus, the expression status of a CpG island containing genes is primarily determined by the presence or absence of transcription factors and by histone modifications around the promoters of such genes. (**a**) In general, transcriptionally active CpG island loci exhibit unmethylated DNA, high levels of histone H3 lysine 9 acetylation, and/or H3K4 trimethylation, which are accompanied by an open chromatin configuration. (**b**) Transcriptionally silent genes, however, are marked by histone H3 lysine 27 trimethylation, a modification catalyzed by EZH2 (enhancer of zeste 2), a member of the Polycomb repressor complex 2 (PRC2). Following H3 lysine 27 trimethylation, these promoters are often bound by members of the Polycomb repressor complex 1 (PRC1), which together prevent transcription initiation by RNA polymerase II. This is also a mechanism of abnormal gene silencing in cancer, in the absence of aberrant DNA methylation. (**c**) In cancer, a large number of CpG islands are hypermethylated at their DNA, which generally correlates with transcriptional repression of the associated genes. These CpG islands generally exhibit a closed chromatin configuration, marked by histone H3 lysine 9 methylation, loss of acetylation, nucleosome occupancy around the transcription start site, and various types of methyl-binding domain proteins. Altogether, these modifications render the chromatin nonpermissive for transcription initiation

methylation (mono-, di-, and trimethylation), ubiquitylation, ADP ribosylation, deimination, proline isomerization, and sumoylation. The modifications may directly alter protein–histone interactions, or can indirectly influence protein–histone or protein–DNA interactions by attracting other proteins that bind specifically to modified histones. The enzymes responsible for these modifications, and for demodification or reversal, have significant specificity for the type of mark, the particular amino acid, and the position of the amino acid in the histone subunit. Histone modifications can be very dynamic in nature, changing rapidly in response to stimuli. Mapping individual histone modifications genome-wide is now possible with chromatin immunoprecipitation applied to tiling path microarray chips, although the resolution is not yet at the level of single nucleosomes and depends heavily on the quality of the antibody that recognizes the modification [7]. Because of the complexity of histone marks on a given nucleosome, new tools and approaches for testing the functional significance of individual modifications will be particularly useful, such as the synthesis of nucleosomes with pure, single modifications added in vitro, as well as new analogues of modified lysines [148, 149]. The interaction between, and inter-dependence of, DNA methylation and histone modifications is the subject of a large number of studies, particularly in cancer [14, 55, 89, 117, 157]. Alterations in the pattern and overall amount of each histone modification have also been reported in human cancers and cancer cell lines [90]. For example, again the silencing mark H3K27 trimethylation in promoters has been reported in association with gene silencing. These and other silencing marks may co-occur with aberrant DNA methylation and function synergistically in gene silencing, and also have been observed in the absence of aberrant DNA methylation. Experimental models using cancer cell lines suggest a relative order of silencing events involving both histone and DNA methylation, but this may be gene and cell type dependent. More globally, two characteristic changes of histone modifications in cancer are a decrease in acetylation of Lys16 and trimethylation of Lys20 on histone H4, in large part from repetitive portions of the genome and in association with hypomethylation of these DNA sequences [54].

15.9 Epigenome–Genome Interations in Human Cancer and Mouse Models: Gene Silencing Vs. Gene Mutation

Genetic and epigenetic mechanisms both contribute to, and probably interact during, tumorigenesis. In genetic mouse models of tumors, disruption of DNA methylation dramatically modifies the incidence of tumor formation and the spectrum of tumor types [107, 133, 165]. Methylation imbalance alone is also sufficient to induce tumors in mice [61, 77]. These studies illustrate a functional role of epigenetic imbalance in tumorigenesis, and also emphasize the interaction of genetic and epigenetic mechanisms in determining tumor incidence and tumor type.

In human tumors, genetic and epigenetic mechanisms can cooperate directly or indirectly. For example, direct cooperation includes complete inactivation of tumor suppressors by methylation of one allele and either deletion or mutation of the other [65, 120]. Epigenetic mechanisms can also cause genetic alterations, and *vice versa*. For example, aberrant methylation-associated silencing of *MLH1* leads to microsatellite instability in colon cancer [74, 91]. Similarly, methylation and silencing of the *MGMT* gene, which encodes a DNA repair enzyme, is significantly associated with G:C to A:T transition mutations in the tumor suppressor gene p53 in colorectal tumors [42]. Indirectly, aberrant loss of methylation in the pericentromeric regions of chromosomes 1 and 16, followed by cell division, is associated with abnormalities of these chromosomes, including loss and gain of whole chromosome arms, in cancer and in ICF (immunodeficiency, centromere instability, and facial anomalies) syndrome patients. Alternatively, translocations of *PML* and *retinoic acid receptor* can create a fusion protein that abnormally recruits the DNA methyltransferase and causes aberrant methylation at specific promoters in leukemia [34]. More global epigenetic defects described as a CpG island methylator phenotype [162, 173] are tightly associated with genetic mutations of the oncogene BRAF, potentially suggesting a common genetic-epigenetic course for these tumors. This candidate gene approach suggests there are important interactions between these two major mechanisms of tumorigenesis, but the extent to which these individual observations can be extrapolated to the whole cancer genome is unknown. Efforts that integrate different technologies, as described above, promise a more complete understanding of the genomic and epigenomic contribution to tumorigenesis.

15.10 Epigenetics and Response to Cancer Therapy

Aberrant methylation of particular CpG islands may also alter the response of a cancer cell to therapeutic agents, or serve as a clinically useful marker of clinical outcome. For example, normal expression of the DNA repair gene, *O*-6-methylguanine DNA methyltransferase (MGMT), is associated with resistance to therapy, whereas aberrant methylation of the MGMT 5′ CpG island, and presumably MGMT silencing [25, 26, 68], is associated with significantly improved antitumor response of alkylating agents such as temozolomide [41, 70]. In contrast, cisplatin-resistant cancer cells can be sensitized by relieving repressive histone H3 K27 methylation and DNA methylation, presumably by reactivating silenced tumor suppressors and modulators of cisplatin response [1a]. Efforts directed at finding DNA methylation-based markers for early detection of tumors and predicting tumor response to therapy are underway in research laboratories worldwide [10, 28, 146]. Assays are currently available to detect aberrant DNA methylation in minute samples that are obtained with minimally invasive procedures, such as sputum, blood, feces, urine and nipple aspirates, and which are likely to contain tumor cells and tumor DNA shed from a primary tumor mass [13, 104]. In contrast, loss of methylation from normally methylated promoters of the MAGEA gene family followed by MAGEA gene activation may elicit production of anti-MAGEA antibodies, which are detectable in the blood of patients with melanoma and other cancers [19].

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Population Genetic Principles and Human Populations

16

Emmanouil T. Dermitzakis

Abstract Human genetic variation has been at the core of recent interest for geneticists. The initial studies of human variation were done using mitochondrial DNA in a few individuals, but recent technologies produced large amounts of genetic data in large numbers of individuals. In this chapter we will discuss the nature and structure of genetic variation in humans, and the causes of this variation. We review some recent advances in the detection of human genetic variation, the caveats and issues when studying genetic data from human populations, and the prospects of applying such information for identification of functional genetic variants in the human genome.

Contents

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16.1 Introduction

In the early days of human genetics much of the research into levels and patterns of genetic variation in human populations was done using highly polymorphic genetic markers such as mitochondrial DNA [93] and microsatellites [101]. This was due to technological limitations, and only a few individuals could be interrogated; markers with high information content were necessary for the elucidation of recent human population history. With the completion of the human genome [59, 97] and the almost complete availability of annotated genes much of the focus has shifted towards the interrogation and characterization of complete genetic variation within human populations. Natural phenotypic variation can be

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measured at various levels and dimensions, such as variation in whole-organism phenotypic characteristics, levels of gene expression or protein expression, cellular response to stimuli, and more. However, the heritable component of phenotypic diversity in human populations is naturally based on the differences of the DNA sequence among individuals [12, 71]. Although we have a very good understanding of the four-letter code of DNA and now have advanced technologies to measure this genetic variation on a large scale, the properties and the fine structure of functional genetic variation as well as its causes are not clear to date.

The key forces that shape patterns of genetic variation are:

- *Mutation*: small and large changes of the nucleotide sequence.
- *Recombination*: exchange of genetic information between homologous chromosomes, mostly during meiosis.
- *Genetic drift*: the variance in sampling of genetic information from generation to generation that has particularly large effect in small populations.
- *Natural selection*: forces that increase or decrease the probability of inheritance of certain variants due to difference in fitness.
- *Migration*: exchange of genetic information between partly or fully isolated subpopulations.
- *Other demographic parameters*: structure of genetic variation in a population depends on a number of complex and frequently unknown demographic models such as inbreeding, assortative mating and others.

DNA sequence variation can come in various flavors and forms, from fine scale single nucleotide differences [12, 13, 71] to large-scale variations that affect millions of nucleotides at once [31]. There are, of course, many intermediate level variants in the human genome that can affect genome function also to various degrees of severity and form and under certain conditions. Another aspect of genomic variation is the degree to which populations share it. Some of this genetic variation is responsible for phenotypic variability present in all populations or phenotypic variability that is responsible for differences between populations [82]. The distribution of phenotypic variability in human populations and evolutionary properties of human genetic variation inform us about the history of the human species, the forces that have shaped its current phenotypic state of the human species and the basis for our differentiation from other closely related species.

Although genetic variation in human populations is an intriguing topic in itself and worthy of proper and in-depth interrogation from the basic science perspective, its contribution to disease, disease susceptibility or protection, and drug response has attracted a lot of the attention [2, 96]. In recent years, new technologies and methodologies have been developed specifically to explore the role of genetic variation to health and disease. The deep and proper understanding of the properties of human genetic variation is essential for the correct interpretation of the link between DNA sequence variation and disease [80, 81] and how this information can assist in the development of diagnostic tests as well as effective treatments.

Some of the key parameters to estimate in human populations are:

- Levels of nucleotide and structural variation (e.g., heterozygosity, density, and frequency of variants)
- Patterns and correlation of sequence variation (e.g., linkage disequilibrium)
- Degree of population subdivision (see also Chap. 20) and consequences for the flow of genetic variation
- Impact of natural selection on patterns of sequence variation

The aim of this chapter is to provide the framework and basic principles of human population genetics. Some of the issues raised here will be addressed in more detail in other chapters of this book. We will approach the topic from the points of view both of mutational processes and the forces that shape the history of such variation, such as natural selection and genetic drift, and of population structure and behavior. We will explore the range of sequence variants, the technological and statistical methodologies used to reveal its history and functional consequences, and the recent efforts to develop the infrastructure for the efficient exploration of large numbers of variants in large population samples.

16.2 Processes Shaping Natural Variation

16.2.1 Mutation and Polymorphism

Genetic variation in human as well as other species is primarily generated by mutational mechanisms that introduce new variants to the population. These variants can come in various forms from single nucleotide changes to multi-megabase-pair (Mbp) deletions, insertions, inversions, and translocations. The different types of variants also have different underlying mechanisms of mutation and therefore have different probabilities of occurring and also different regions in which they are more prevalent, and this depends on the DNA sequence context.

The most common and best-studied variants in populations are:

1. *Single nucleotide mutations (or point mutations):* These are mutations that change the nucleotide base at a particular position in the genome without affecting the length of the chromosome (Fig. 16.1). The average mutation rate is 10^{-8} per generation per site. These kinds of mutations are categorized as transitions (purine to purine or pyrmidine to pyrimidine or A–G or C–T mutations) and transversions (all others). Owing to structural and molecular similarity, transitions are more likely to occur with approximately two-fold higher probability than transversions [73]. Other factors that have an effect on the rate of mutation are the local nucleotide composition, overall nucleotide compositions of the genome and other context-dependent effects (e.g., CpG sites). Models that aim to estimate substitution rates of sequences frequently attempt to estimate and account for these parameters [35]. In most cases there is an assumption of the infinite sites model, which theoretically states that every new mutation in the population will affect a new site not previously mutated [54]. This model becomes impractical when one looks at complete genome sequence data in large numbers of individuals.
2. *Small insertions and deletions*: These are mutations that contribute to the addition or deletion of small numbers of nucleotides to the sequence (Fig. 16.1). The mutation rates and patterns of variation of such mutations have been studied to some extent in interspecies comparisons, but they remain largely unexplored in terms of intrapopulation variability. Projects that currently explore complete sequencing of multiple human genomes, such as the 1,000 Genomes Project, will reveal many aspects of their pattern and potentially allow the proper estimation of their mutation rates.
3. *Variable number tandem repeats and short tandem repeats*: See below.
4. *Large structural variants (SVs) including copy number variants, inversions, and translocations*: These are large insertions or deletions, which for operational purposes are defined as larger than 1 kb or rearrangements and translocations of large segments of genomic DNA that alter the genomic landscape of a chromosome [31] (Fig. 16.2).

16.2.2 Historical Perspective of DNA Polymorphisms and Their Use as Genetic Markers

Historically there have been a number of different types of DNA markers used for this purpose. Various efforts [51, 58] showed that a set of several hundred such polymorphisms, distributed over the entire genome, would allow the mapping of genes on all chromosomes provided that a sufficient number of informative families were available. Over the years several such markers were used and are still being used in some settings:

1. Restriction length fragment polymorphisms (RFLPs). These polymorphisms were discovered first, and they are usually the result of single-base-pair changes in the DNA sequences inherited by Mendelian transmission. Such mutations lead either to removal or, less commonly, to the introduction of a recognition site for a restriction enzyme, causing increases or decreases in the length of restriction

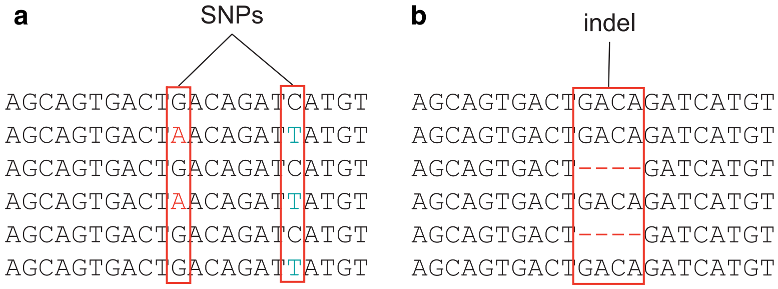

Fig. 16.1 Example of SNP and indel variations. On the *left panel* the two SNPs appear to be in perfect linkage disequilibrium with respect to historical recombination (D¢ =1), but the correlation coefficient *r*<1; there a large number of short-length differences between sequences denoted as insertions or deletions (indels). The *panel* on the *right* shows an example of small insertion/deletion (indel) variation

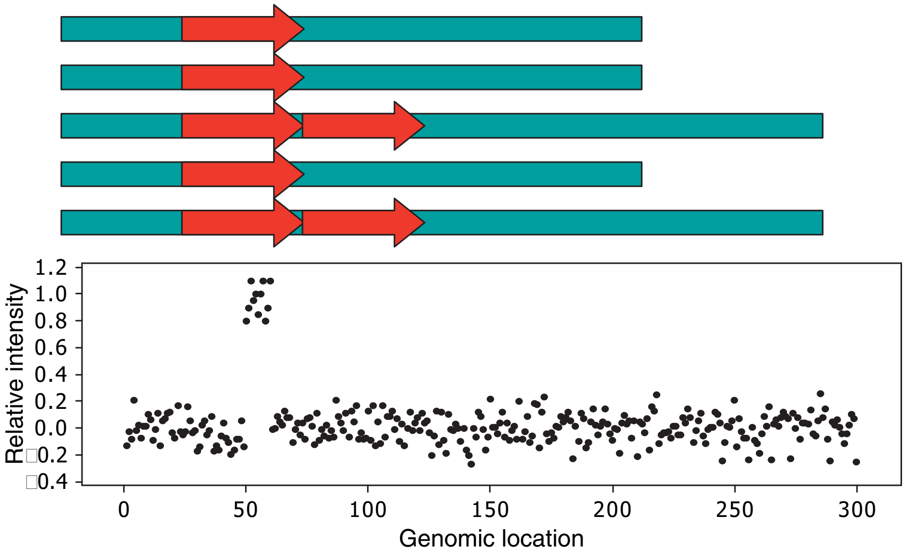

Fig. 16.2 Example of copy number variation. The *top panel* indicates the different haplotypes observed in the population with one or two copies of a piece of DNA. The *bottom panel* shows the hybridization-relative intensity in $\log_2$ units (*Y*-axis) for a sample homozygous for one copy vs a sample homozygous for two copies of the variant

fragments that have been previously amplified with PCR. These DNA variants are therefore referred to as restriction fragment-length polymorphisms. They are caused by a difference in the number of cleavage sites that are cut by a certain restriction endonuclease in different areas of the genome. They have previously served as proxies for single-nucleotide changes and in some sense represent the first genotyping assay of SNPs. Their use is not very frequent now, although they can still be used as a validation for the detection of a variant or for a high-quality assay in a medical setting.

2. Minisatellites (variable number of tandem repeats, VNTR). These were discovered by Jeffreys et al. [45–47] and are found very frequently in noncoding, repetitive areas of the genome. Sequences of about 9–60 base pairs are repeated in tandem order and may be present in a varying number of repeats per chromosome. One property, however, diminishes their utility for some applications: each of these polymorphisms has a relatively high mutation rate—a small percentage per generation. This is not entirely surprising. The similarity of base composition between multiple sequences creates ideal conditions for meiotic pairing of structure homologous but not position-homologous DNA segments, leading to unequal crossing over.
3. Microsatellites (short tandem repeats, STRs). These were discovered by Weber and May [101]. For example, pairs of two bases $(GT)_n$ may be repeated from a few to very many times. There may be up to about 30 different alleles in a population for one such polymorphism. The best method for their study is the PCR reaction. Two primers attached to the DNA on both sides of the polymorphism are required; this means that short base sequences outside the polymorphism must be known. Information on primers can now be gathered from a genome data base. In addition to the two-base-pair STRs, others comprising variable repeats of three to five base pairs have been described. The common STR markers have become the DNA variants of choice in the study of human and mammalian linkage, as well as in the early of human population studies [94]. The availability of the human genome sequence has made the identification and use of such sequences very common and a standard process.

16.2.3 Demography

16.2.3.1 Effective Population Size

An important parameter in population genetics is the effective population size, usually symbolized as N_e. This does not refer to the actual size of a population under study. The effective population size is: "[T]he number of breeding individuals in an idealized population that would show the same amount of dispersion of allele frequencies under random genetic drift or the same amount of inbreeding as the population under consideration" [104]. In other words, the effective population size represents the idealized population size that has the same genetic parameters as the observed population. The effective population size is usually lower than the observed size. The discrepancy between the effective and observed population size is due to many demographic phenomena: population expansion, bottlenecks (periods of radical reduction of population size), natural selection, and others.

16.2.3.2 Genetic Drift and Isolated Populations

There are specific assumptions and expectations behind the dynamics of polymorphisms in populations. If one considers a population of N diploid individuals (i.e., $2N$ chromosomes) in a population in which most theoretical assumptions above are met, then a new mutation in the population will have a frequency of $1/2N$. Assuming no selection in favor of or against such alleles, most of the mutations will oscillate around values close to their initial frequency ($1/2N$) and eventually disappear (go to zero frequency) from the population. Occasionally, such variants can increase in frequency and eventually either disappear or go to fixation (go to 100% frequency) in the population (Fig. 16.3). It can be shown that under assumptions of neutrality and random mating the probability of fixation of a new mutation is its initial frequency $1/2N$ [36]. In fact, it can be shown that at all times the frequency of a variant at a given time point is its probability of fixation. In populations with large size a lot of mutations are introduced in each generation (owing to the large number of chromosomes available to accumulate mutations), but each one of them has a low probability of fixation owing to a very low initial frequency. On the other hand, a population with small sample size can introduce a small number of mutations, but each one of them has a high probability of fixation owing to its high initial frequency. However, if one accounts for both mutation rate and probability of fixation after the mutation has occurred (and assuming no selection) the probability and rate of fixations are the same in small and large populations. This overall phenomenon is essentially a result of the sampling variance effect from generation to generation and is referred to as "genetic drift."

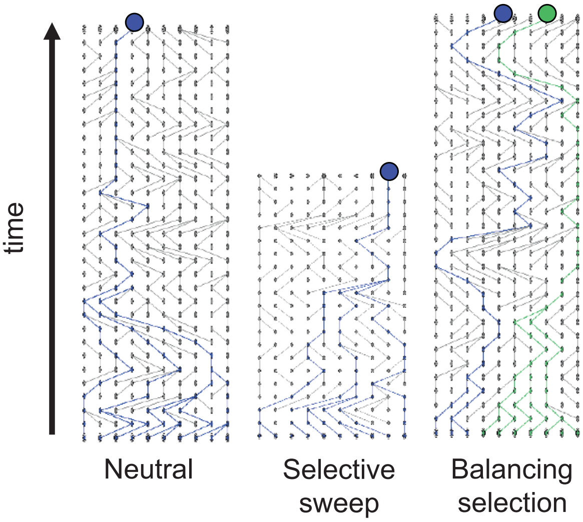

Fig. 16.3 Three models and their impact on coalescent tree structure. The *left panel* is the neutral pattern where the MRCA is the *blue dot* and the *lines* indicate transmission of the allele from one generation to the other. In the *middle panel* we show an example of a selective sweep scenario where the MRCA is more recent than in the neutral model (*blue dots* and *lines*). In the *right panel* we show an example of balancing selection, where even at the same number of generations as in the neutral model two major alleles (*blue* and *green dots* and *lines*) are segregating in the population, suggesting the MRCA is older than in the neutral model

Genetic drift is an important consideration in human populations because of the nature of human population dynamics. In particular, this is has become relevant in the studies of isolated and small founding populations such as those in Iceland and Finland. The fact that such populations have small effective population sizes (a metric that also represents the degree of genetic variation segregating in the population) makes it more likely that variants that are otherwise rare in the parent population can become quite common by chance, even in the presence of selection acting against them, making these population very useful for the mapping of common and rare disease variants [37, 72].

16.2.3.3 Migration

The segregation and transmission of genetic information does not only occur vertically from generation to generation, but also horizontally between population subgroups that are partly isolated. When the exchange of genetic information between subpopulations is completely free the two subpopulations will eventually become a single panmictic population within a few generations. However, in most cases the exchange of genetic information between subpopulations happens with low rates of migration of individuals either in one direction or in a bidirectional way. The rate by which new mutations or old variants brought to high frequency or fixation by way of either genetic drift or natural selection combined with the rate of migration between subpopulations determines the degree of differentiation between subpopulations after a number of generations.

16.2.3.4 Inbreeding/Nonrandom Mating

Even if individuals live in the same geographic region, the size of the population and also behavioral processes and choices result in distinct patterns of variation. When the population size is small or individuals tend to mate with related individuals more than reflected by the average pairwise degree of relatedness observed, then this leads to increased levels of homozygosity. The degree of this effect can be described by the inbreeding coefficient.

A more generalized pattern of nonrandom mating involves choices that individuals make that are either based on phenotypic similarities between the pair of individuals (assortative mating) or even more general choices based on phenotypic characteristics, such as height, eye color and others. If these phenotypic characteristics have a genetic basis this can lead to deviation from patterns of genetic variation beyond that caused by genetic drift.

16.2.4 Recombination

Another key property of the genome that was mentioned above is the landscape of recombination. In the past, recombination was considered a property of the genome that varied between large genomic regions but was pretty uniform on the fine scale. Pedigree studies have revealed the degree of variation in recombination rates between large genomic regions, and these were largely correlated with properties of genomic sequence, such as GC content, location relative to the centromere, and others [9]. Such recombination rates were based on the observation of recombination events from one generation to the next. There have also been fine-scale experimental studies on recombination, and those have hinted that recombination rates do not only vary across large genomic regions but also on a finer scale [48, 49]. These studies have observed recombination events by sperm typing and have shown that there exist hotspots of recombination within short multi-kilobase-pair regions of the genome. More recently, inferential methodologies, using dense population variation data and appropriate modeling under the coalescence model (see below), have revealed that recombination is distributed in a highly nonuniform way, with 80% occurring in hotspots of recombination, which comprise only a small fraction (about 20%) of the genome [60, 66, 69] (Fig. 16.4). This observation has become common knowledge, changing the way we view genomic variation and correlations between variants. As discussed above, the assumption that recombination is uniform in genomic regions is violated and increases the expected variance of statistics being used for population genetics inference.

16.2.5 Natural Selection

All the above considerations about human populations were not only necessary to understand the specific characteristics of human populations, but also to introduce the reader to some basic principles useful to the interpretation of patterns of sequence variation. The neutral theory of molecular evolution [53] had been proposed as the main process by which DNA sequences evolve. However, it is widely accepted that for a fraction of a genome one of the main forces that influence patterns and levels of sequence variation is natural selection. Natural selection makes some variants in the population have higher or lower probability of fixation relative to what could be estimated from their population frequency [3].

There are three main forms of selection with many versions and alternatives, but we will focus here only on the main points. We will try to avoid detailed statements and we advise the reader to consult the primary literature, since this is an ever-developing area (see Fig. 16.5). The first and most intuitive form of natural selection is purifying selection. Purifying selection

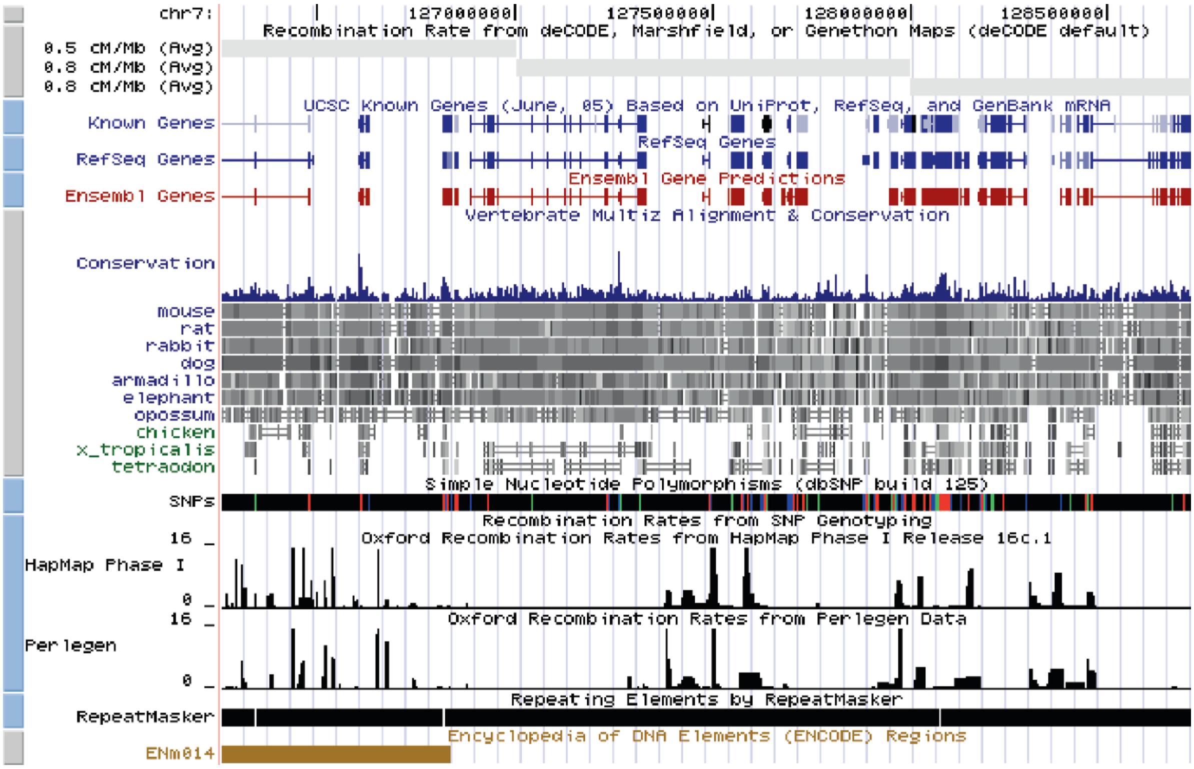
chr7:
127000000|
127500000|
128000000|
128500000|
Recombination Rate from deCODE, Marshfield, or Genethon Maps (deCODE default)
0.5 cM/Mb (Avg)
0.8 cM/Mb (Avg)
0.8 cM/Mb (Avg)
UCSC Known Genes (June, 05) Based on UniProt, RefSeq, and GenBank mRNA
Known Genes
RefSeq Genes
RefSeq Genes
Ensembl Gene Predictions
Ensembl Genes
Vertebrate Multiz Alignment & Conservation
Conservation
mouse
rat
rabbit
dog
armadillo
elephant
opossum
chicken
x_tropicalis
tetraodon
Simple Nucleotide Polymorphisms (dbSNP build 125)
SNPs
Recombination Rates from SNP Genotyping
Oxford Recombination Rates from HapMap Phase I Release 16c.1
16 _
HapMap Phase I
0 _
16 _
Oxford Recombination Rates from Perlegen Data
Perlegen
0 _
Repeating Elements by RepeatMasker
RepeatMasker
Encyclopedia of DNA Elements (ENCODE) Regions
ENm014

Fig. 16.4 Rates of recombination along the genome. A UCSC genome browser (genome.ucsc.edu) shot in which many genomic elements are shown. At the *bottom* of the *panel* two tracks with population-based estimated recombination rates are shown, based on HapMap SNP data and Perlegen SNP data

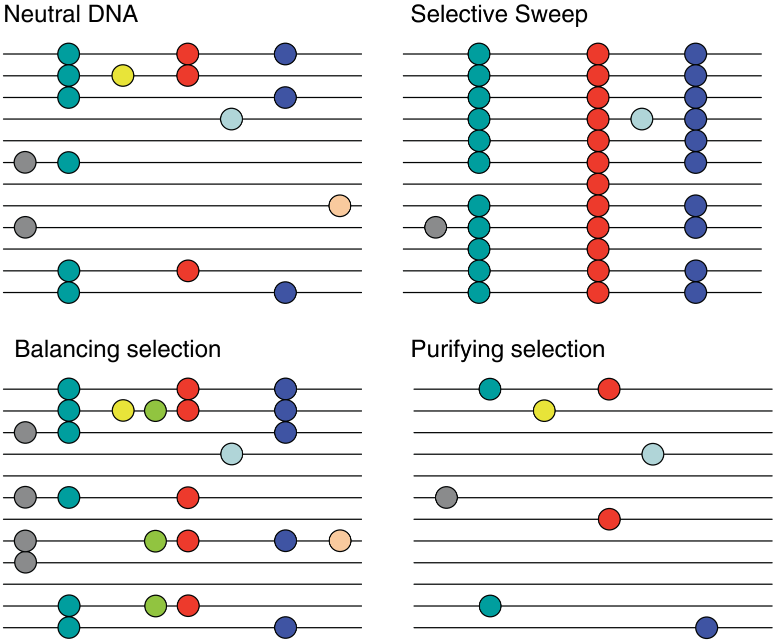

Fig. 16.5 Schematic of patterns of SNP variation. *Colored dots* indicate the new (derived) allele in the population in regions (each *color* indicates an independent mutation) undergoing neutral evolution, positive selection (selective sweep), balancing selection, and purifying selection

(or background selection) is based on the principle that when a new mutation occurs in a functional sequence, and if this new mutation disrupts the pre-existing function, then the individual carrying the new mutation may be at a disadvantage. Mutations that undergo purifying selection tend to disappear from the population quite rapidly. However, depending on whether the effect is co-dominant (or dominant in some cases) or recessive, this "waiting" time can be very short or long. Co-dominant mutations will directly confer a disadvantage to the individual even when in heterozygosity, so that they will disappear faster. Recessive mutations will only start being impacted by purifying selection when in homozygosity, which means that the mutation has to reach appreciable frequency to have a decent probability of being in homozygosity in individuals. They will likely have a long asymptotic process of elimination similar to that of neutral mutations, because of the normal phenotype in heterozygous individuals. Regions that are under purifying selection have an excess of rare variants in their site frequency spectrum relative to neutrally evolving sequences, mainly resulting from the suppression of new mutations reaching high frequency [14, 15].

It is possible that some of the mutations occurring mostly in functional sequences confer an advantage on the individual carrying them. This mode of selection is called positive selection and can occur either if a new mutation is advantageous or if an extant mutation becomes advantageous following changes in the environment. The effect of such an advantageous mutation is that it goes to fixation rapidly and can drag many other new, mostly neutral, closely linked mutations to fixation or near-fixation as well [4, 27–29]. This process is called "selective sweep," [91] and the allele frequency spectrum after a sweep is characterized by an excess of high-frequency derived alleles as a result of new rare variants in the vicinity of the positively selected variant reaching high frequency [26]. Patterns can become quite complex and less interpretable when multiple variants in the same genomic region undergo selective sweep at the same time or within short evolutionary time periods.

Finally, another mode of selection is balancing selection, where a region maintains high levels of variation and a large number of variants at intermediate frequencies. This type of selection is caused by conditional effects of selection of alleles to the frequency of other alleles. The presence of antagonizing advantages between alleles leads to an optimal equilibrium point of frequencies in the population. Examples are heterozygote advantage or frequency-dependent selection. A very common example of heterozygote advantage are the mutations of sickle-cell anemia and the thalassemias [103]. These were kept at high frequency because, although disadvantageous in homozygosity, they made their heterozygote carriers more fit than the wild type homozygotes, because they conferred protection from malaria. Balancing selection is a complex type of selection, and one of the characteristic patterns is that there is an excess of intermediate frequency variants and the tree of the population for such regions has very deep branches (see coalescent theory below), representing very old variation. Methodologies used for detecting possible natural selection acting on certain regions of the genome will be discussed below.

16.3 Patterns of Genetic Variation

16.3.1 Hardy–Weinberg Equilibrium

One of the key principles of population genetics is the Hardy–Weinberg equilibrium (HWE). If one considers a locus with two alleles, A1 and A2, then the frequency of allele A1 can be denoted as p and that of allele A2 as q. If one assumes random mating and no selection acting on the alleles (which in most cases is an approximation), the probability of observing an individual will genotype AA is the product of the frequency of allele A or p^2 and that of aa is q^2. The frequency of the heterozygote individuals Aa is $2pq$ (which stems from the fact that there are two ways to sample a heterozygote, Aa and aA). Of course, owing to finite population size this expectation is never realized exactly and there is always some variance around it. In order to test for deviations from HWE, the standard method is to perform a Chi-square test to compare the frequencies of observed vs expected counts of the three genotypes. Deviations from HWE can exist for many reasons, including population subdivision, recent migration at a high rate, strong natural selection, and others.

Under HWE, the genotypic frequencies of the three different genotypes are described by the equations below:

$$P_{AA} = p^2 \tag{16.1}$$

$$P_{Aa} = 2pq \tag{16.2}$$

$$P_{aa} = q^2 \tag{16.3}$$

where p is the allele frequency of A and q is the allele frequency of a and $p+q=1$.

16.3.2 Coalescent Theory

In most studies we sample a set of individuals from a population with the main goal of inferring the properties and population genetic parameters of the whole population from this sample under study, with certain assumptions. The history of a set of 2*N* chromosomes that we sampled from the population can be modeled within the coalescent framework that predicts that all these chromosomes have a common ancestor at some point in the past. This is called the most recent common ancestor (MRCA), and the tree leading back to MRCA represents the likely relationships of sequences observed at present into the past (Fig. 16.3). This was first described and formalized by Kingman [55] and was further developed by Hudson [38]. The coalescent theory states that the sample of chromosomes can be viewed as a phylogenetic tree of sequences from the same species, and the distribution of branch lengths of the tree can be estimated by the expected model by which the population has evolved. Current methodologies can accommodate recombination between the sequences, selection, various population events such as bottlenecks, or population expansions as well as gene conversion.

Coalescent models have become popular in the last two decades to describe population genetic processes and infer parameters. There are two key applications of coalescent models. One of them is to facilitate the interpretation of potential processes that have contributed to an observed pattern of genetic variation in a population sample. In such cases, thousands of coalescent simulations are run using parameters estimated from the sample. Different processes that could have led to the observed pattern are tested and followed by an assessment of which of the models tested fits best with the observed pattern. The coalescent model can also be used to estimate certain parameters from the population sample, such as population recombination rates (see below). Overall, the coalescent model has been established as one of the most reliable ways to model the relationships of sampled sequences from a population.

Coalescent models are also being used to look for the impact of natural selection. The pattern of branch lengths is correlated with the type of selection that has acted on the sequence. Let us consider a sample of chromosomes and a neutral coalescent tree for these chromosomes. Under a scenario of purifying selection (or background selection) the coalescent tree of an allele will on average have shorter branches, since many branches will have been lost as a result of deleterious mutations. With a scenario of positive selection, sampled chromosomes will converge to a MRCA faster, since the extant sample will represent a chromosome that was rapidly fixed. Finally, in the case of balancing selection the MRCA will converge much further back in the past owing to the maintenance of old polymorphism at intermediate frequency (see Fig. 16.3, and [3] for a review).

16.3.3 Population Differentiation

There are currently more than 6 billion people on the planet, but the amount of genetic variation we carry as a species is much lower than would be expected from a panmictic population of 6 billion people in equilibrium. The human population has undergone a radical expansion very recently (approximately the last 100,000 years), and this has not allowed for genetic variation to catch up. For this reason, in humans the effective population size has been estimated to be approximately 10,000, a number very small relative to the observed population size [95].

In addition to the small effective population size, human populations have undergone bottlenecks; they have been subdivided for long periods of time; and they have undergone admixture afterwards. This has created a very complex pattern of variation and patterns of correlations of variants in the genome (see Sect. 16.3.6, below) that appear to be unique and potentially a result of natural selection. However, these are artifacts of population structure and can be mistakenly inferred as natural selection. The main point is that the history of human populations has violated most of the standard assumptions made when we apply standard population genetic models, so that great caution is needed when one applies those models in human population data [62].

Methodologies have been developed to deal with the degree of population differentiation and substructure to facilitate genome-wide association studies. One of them uses a two-step scheme to correct for population structure in case-control studies by detecting associations that are significant within population subgroups detected. This methodology is implemented in the softwares STRAT and STRUCTURE [76–78]. Other methodologies estimate the degree of inflation of association P-values attributable to population substructure and apply a genome-wide correction to the distribution of P-values, otherwise known as genomic control [23, 24]. Finally, recent methods make use of the idea of describing genetic data with principal component analysis (Fig. 16.6), as was originally proposed by Menozzi et al. [67], with some modifications, and they use the pattern to correct for population substructure. This methodology has been implemented in the tool EIGENSTRAT [74, 75]. Although these methods can be very useful, such procedures may lead to loss of power, which results in population genetic inferences being more difficult and less accurate in human populations. The only thing that compensates for this is that there is a lot more data generated for human populations, usually in the thousands of individuals, so power and accuracy are recovered from the large amounts of data we use for the estimates.

16.3.4 Patterns of Single-Nucleotide Variation in the Human Genome

The complexity of the issues in population genetics becomes higher once we start evaluating multiple variable positions, especially if they are physically linked. One of the predominant types of sequence variation in the human genome is the large number of single-nucleotide polymorphisms (SNPs), also found in the literature as "segregating sites" or "polymorphic sites." These are single-base differences (Fig. 16.1) between chromosomes and can exist in various frequencies in human populations. According to the infinite sites model [54], each of these SNPs has only two alleles (two alternative nucleotides). Although this is largely the case, it is an approximation and obviously a function of how deep our interrogation of sequence variation is. The availability of nearly complete sequencing of hundreds of individuals from the 1,000 Genomes Project and large-scale sequencing from other projects allows detection of sites for which three alleles segregate in human populations. Conventionally, SNPs are the polymorphisms whose minor allele is present in at least 1% of the chromosomes of a given population. Most of population genetic theory has been developed around the pattern, density, and frequency distribution of SNPs. The average number of differences one can expect when comparing two homologous human chromosomes has been estimated at around 8–10 per 10,000 bps, with these levels being lower in functionally constrained sequences and higher in hypermutable regions (e.g., CpG sites). One can derive many of the historical properties of a given genomic region using the characteristics of SNP data within that region.

There are a number of methods that are being used to assess the degree of nucleotide diversity in sequences. Some of these metrics rely on the number of observed variable (segregating) sites in the sample of haplotypes

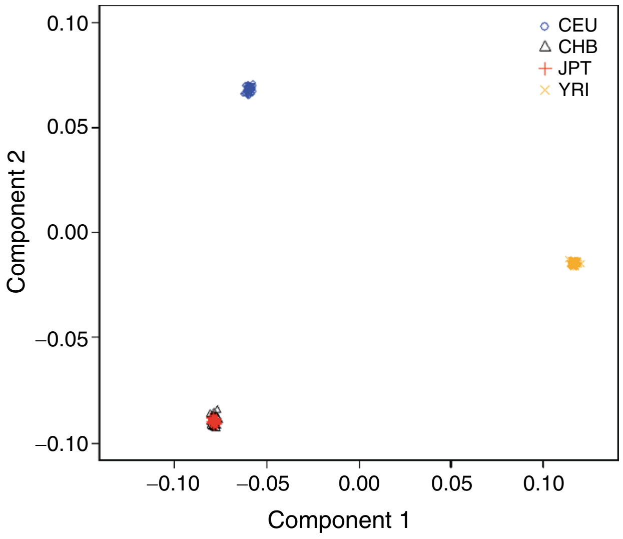

Fig. 16.6 Principal component analysis of SNP data among population samples. This figure shows the multidimensional scaling of SNP data for the four Hapmap populations for the first two principal components using EIGENSTRAT. The figure shows that the Caucasian (*CEU*), African (*YRI*), and Asian (*CHB* and *JPT*) samples cluster away from each other. The differences between CHB and JPT are too small to be detectable in the scale of the first two principal components

from the population and derive assumptions about the expected diversity assuming no selection. One of them is Waterson's q [100], estimated by the following equation:

$$\theta = \frac{S}{\underset{n=1}{\overset{i=1}{\square}}\ 1\square i} \tag{16.4}$$

where *S* is the number of variable sites and *i* is the number of chromosomes compared.

Other metrics directly count the number of mismatches between any two haplotypes and derive the average expected heterozygosity per nucleotide [70].

$$\pi = \underset{ij}{\square}\ xixj\pi i = \overset{n}{\underset{i=1}{\square}}\ \overset{i}{\underset{j=1}{\square}}\ xixj\pi ij \tag{16.5}$$

where x_i and x_j are the respective frequencies of the *i*-th and *j*-th sequences, *pij* is the number of nucleotide differences per nucleotide site between the *i*-th and *j*-th sequences, and *n* is the number of sequences in the sample.

In fact, the difference in inference between these two metrics was used by Tajima to detect patterns of variation that deviate from neutral patterns of natural selection with the statistic D [92].

$$D = \frac{\theta \square \pi}{Var} \tag{16.6}$$

However, one has to be very cautious about such deviations from expected patterns with metrics using nucleotide diversity inferences in order to detect selection. Such metrics are very sensitive to small deviations in expectations about the population structure, such as random mating and also levels and uniformity of recombination [87], and this leads to false interpretations.

Before we go deeper into the interpretation of signals derived from SNP data it is essential to discuss some basic assumptions that population genetic theory makes. When analyzing data of this type there are caveats one needs to be aware of when using "out-of-the-box" methodologies developed under theoretical assumptions in human data. Many of the standard population genetics models assume panmixia (equal probability of each individual to mate with any other individual), constant population size across generations, nonoverlapping generations, and uniform recombination rates across a region or no recombination at all. Given these assumptions one can contrast patterns of variation expected under a certain set of predefined parameters with those observed in real data and infer realistic population genetic parameters. As discussed previously and below, almost none of the above assumptions are true for human populations. Therefore it is essential to implement more sophisticated models that allow for these parameters to vary in order to interpret human variation data. In this chapter we do not attempt to explore the "deep" statistical modeling necessary for such analysis, but to give pointers to the caveats and suggest methodologies that will either bypass such effects or take them into account.

The frequency distribution of SNPs, otherwise called the site frequency spectrum, is usually informative of the forces or processes that have acted on a given genomic region. In general, it is expected that when a sequence evolves under a neutral model there is always a large number of rare SNPs. This is mainly due to the large number of variants that are new in the population, some of which are single observations (singletons) on individual chromosomes. The degree of this excess relative to the neutral expectation may indicate forces that have distorted the patterns of variation, such as natural selection or population bottlenecks. These issues have been discussed earlier in this chapter.

All the above discussion was based on the assumption that we have full sequencing data for a number of individuals for a given genomic region. However, this has not been possible until recently for human populations. Sequencing was very expensive and only recently new technologies (also called second-generation sequencing technologies) have provided the appropriate framework for rapid and low-cost resequencing of large genomic regions or full genomes [6, 100]. Much of the data and inferences about population-genetic parameters had to rely on genotyping data, i.e., the interrogation of the allelic state of known polymorphic position in the human genome. Why is genotyping not as good as sequencing? The main reason is that in order for a genotyping assay to be designed one needs to know that a nucleotide site is variable in at least one population sample. This means that variation is interrogated and inferred in a small number of individuals and then assayed in a larger number. This biases the frequency spectrum to common variants, since we only know about the rare variants of the individuals who were sequenced and are missing all the rare and singleton variants of the individuals who were just genotyped and not sequenced. This bias can become even worse if the ascertainment of variable sites is not uniform and is highly variable across regions. This was the case in the HapMap project discussed below [41–43]. Methodologies have been developed that can correct for the ascertainment bias if the ascertainment scheme is known [17]. However, these problems and the correction required highlight the fact that we urgently need resequencing data in order to elucidate some of the historical parameters of human population variation, and until we do this many of the signals will be confounded by ascertainment biases.

16.3.5 Patterns of Structural Variation in the Human Genome

Until a few years ago, much of the attention paid to human variation had been devoted to single nucleotide variation data, assuming that each haploid human genome has the same genome size and internal organization (i.e., gene order and number). However, if we want to get a full and complete picture of variants in the human genome that may have functional effects, we should not ignore insertion/deletion (indel) polymorphisms (Fig. 16.1). In this section, and for purely operational reasons, we mean the indels that are small (from one to a few tens of base pairs) and can be identified by simply comparing sequencing reads. Other, large-scale, variants are discussed below. There is a small number of studies that have carefully characterized the patterns and distribution of indels in the human genome. The few that have performed such analysis have shown that indels behave in a similar way to SNPs, with their density being lower in functional portions of the genome. Studies that have used interspecific distribution of indels have shown that one can detect strong signals of selective constraint [61], but such studies have not been applied in human variation on a large scale.

In the last 4–5 years it has become obvious that a newly rediscovered type of variation is also very common in human populations and is likely to contribute to phenotypic diversity and disease. These are the copy number variants (CNVs) and other structural variants (SVs, e.g., inversions, translocations), which are currently attracting a lot of attention [18, 40, 64, 84]. Once again, for operational reasons CNVs are defined as large (more than 1 Kb) [31] regions of the genome that are present in more or less than the expected copies in the genome (more or less than the two copies expected in a diploid individual), and this copy number is variable among individuals (Fig. 16.2). The mutational nature and population genetic properties of CNVs has not yet been fully elucidated, and their contribution to complex disease is still largely unexplored, but the examples we have in monogenic disorders suggest that such variants will be important for the understanding of human diversity and it is therefore worth exploring in human populations. Some initial studies have suggested that CNVs may be single mutations and therefore tractable by LD with SNPs in human populations [64], but some other CNVs are more complex in nature and likely a result of recurrent mutations, and these are unlikely to be easily tractable by LD [79]. CNVs and other structural variants also have consequences for the pattern of SNP variation in the human genome. Inversion and translocations lead to suppression of recombination and create artifacts of increased correlation structure in the human genome. Similarly, CNVs can create distorted patterns of SNP variation owing to naïve assumptions about the genotyping assays.

Two major recent studies have looked closely at patterns of copy number as well as some degree of other structural variation in human populations. The two approaches employed are quite different in resolution and confidence in inference and provide both overlapping and complementary results. The Genome Structural Variation Consortium initially employed a BAC CGH (comparative genome hybridization) array approach by which they assayed 270 individuals from the HapMap project in order to detect regions of the genome that are variable in copy number [79]. They detected more than 1,200 CNV regions in these populations. These were large regions of the genome (owing to resolution of the array), and they turned out to be a result of structural changes in dynamic regions of the genome such as segmental duplications. Another approach employed was that of sequencing both ends of medium-sized clones (fosmids) and mapping them back to the genome [52]. Using the size distribution that these clones were selected for, they were able to find regions of the genome in the eight assayed individuals that are either larger or smaller than the reference and therefore may contain insertions or deletions. In addition, by making use of the orientation of the sequencing reads they were able to assign potential inversions and translocations. A similar approach has been applied using second-generation sequencing technologies [56]. These and other studies have made it very clear that the study of structural variants in the genome is not going to be as straightforward as studies of SNP variation. Nevertheless, there is substantial evidence that CNVs and other structural variants contribute to common diseases from HIV resistance [34] to psychiatric disorders [19, 85, 99], and the drive to properly interrogate the degree and amount of copy number variation in human populations has been strong.

16.3.6 Haplotype Diversity—Linkage Disequilibrium

Variants in genomic regions of the human genome are correlated, because of common history and lack of historical recombination between them. Mutations land on a given haplotype and within a given framework of variants, and unless recombination is given enough time to shuffle them they co-segregate from generation to generation. The correlation of variants is called linkage disequilibrium (LD), to indicate the nonrandomness of alleles linked on the physical space of the chromosome (Fig. 16.7).

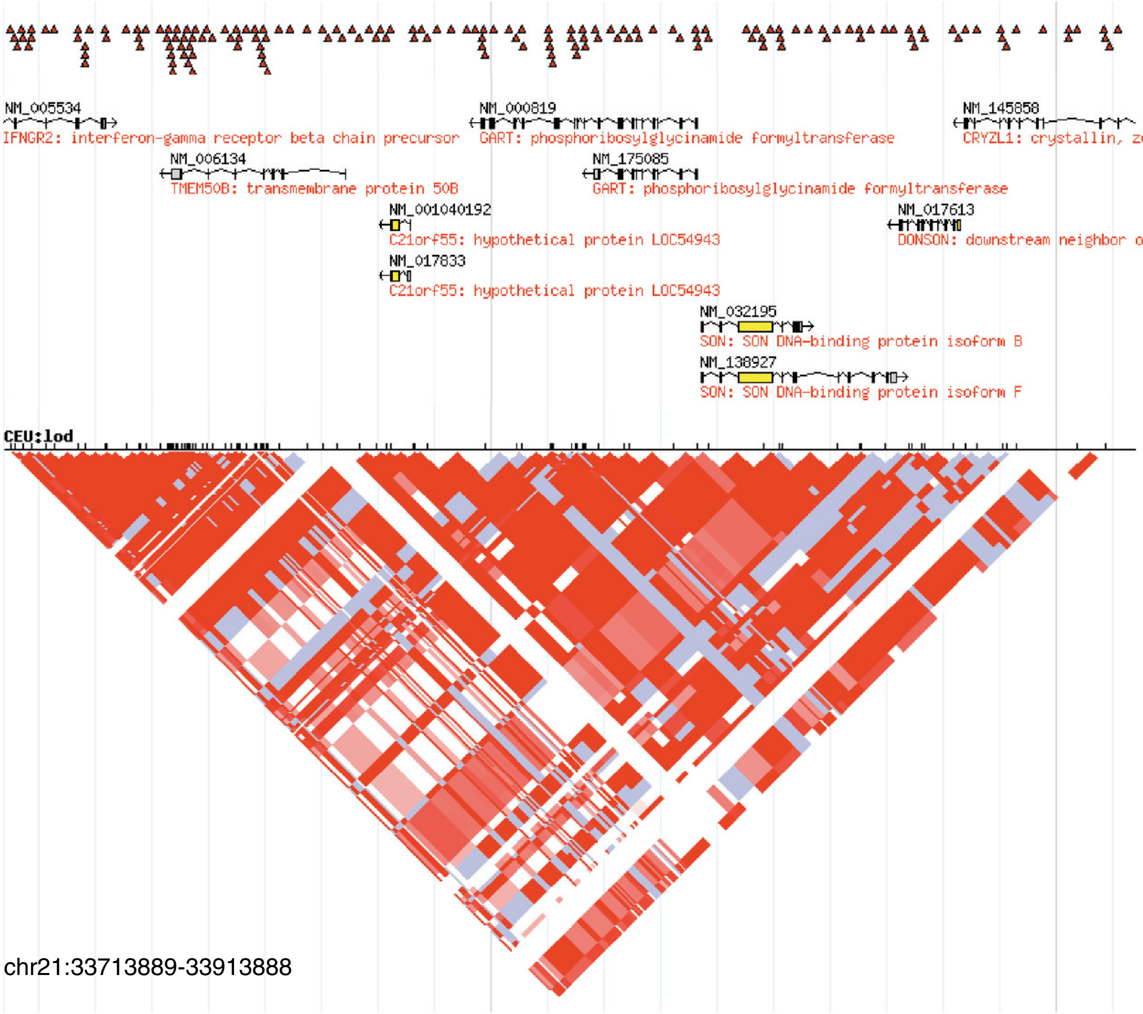

Fig. 16.7 Linkage disequilibrium plots in the context of annotation from the HapMap website browser (www.hapmap.org). The figure shows the annotation and SNP density for a short region of human chromosome 21. The *large inverted triangle below* indicates the pairwise linkage disequilibrium between SNPs. *Strong red* indicates significant linkage disequilibrium, *light red* LD of intermediate significance and *light gray* and *white*, low LD

There are various metrics for pairwise LD that have different properties, such as *D*, *D¢*, and *r*. *D* is an absolute measure of LD, *D¢* is a measure that corrects for the frequencies of the variants considered, and *r* is the statistical correlation between the variants.

$$\mathrm{D = P_{AB} = p_A{*}p_B} \tag{16.7}$$

where $\mathrm{p_A}$, $\mathrm{q_a}$, $\mathrm{p_B}$ and $\mathrm{q_b}$ are the allele frequencies of alleles and A, a, B and b and $\mathrm{P_{AB}}$ are the frequencies of AB haplotypes.

$D_{\max}$ is the maximum D value two variants can obtain if there has been no historical recombination between them.

$$D\square = \frac{D}{D_{\max}} \tag{16.8}$$

$$r = \frac{D}{\sqrt{p_A p_B q_a q_b}} \tag{16.9}$$

where $\mathrm{p_A}$, $\mathrm{q_a}$, $\mathrm{p_B}$ and $\mathrm{q_b}$ are the allele frequencies of alleles, A, a, B and b.

As discussed in a previous section, we tend to consider the history of human chromosomes in the framework of the coalescent model that assumes that any extant sample of chromosomes has a common ancestor at some time in the past [38]. The human genome has particularly large regions that are correlated, and LD decays very slowly with distance [1, 20, 21]. This is because the depth of the coalescent tree is shallow, especially in non-African populations, which means that there has not been enough time for recombination to break the correlations. The combination of shallow history and spotty recombination (owing to recombination hotspots) has created a pattern of blocky LD in the human genome where variants in large segments of the genome show very high LD within the segment but very low LD with neighboring regions [33]. This blocky structure has led to some ideas in the past that the human genome is divided into "haplotype blocks" that are discrete genomic segments. Although the general principle that the genome is blocky with respect to LD structure is true, it has now become obvious, especially since publication of the HapMap project, that the correlation structure of variants in the genome is much more complex and is not simply defined by physical boundaries on chromosomes [42, 43]. Nevertheless, this pattern of high LD between variants has proved very valuable, since one can interrogate only a subset of genetic markers (in most cases SNPs) to capture the majority of genetic variation of a human population. It is estimated that depending on the origin and history of the population we need 500,000 to 1 million SNPs to capture 70–80% of common genetic SNP variation [57].

A methodological issue when we work with diploid genetic data is to determine the arrangement of alleles on homologous chromosomes, otherwise called "phasing." Most of the data we usually obtain in human populations is in the form of genotyped data, so that the phasing is not known. Sophisticated statistical methods have been developed that make use of prior information, pedigree data, and LD to infer the phasing of SNP or other variants [88, 89]. Some of these methodologies and their performance are reviewed in a paper that compares their performance and efficiency [63]. Such methodologies have become essential in human genetic data analysis. Extensions of such data to accommodate variants such as microsatellites or CNVs have been developed and already being extensively used.

16.3.7 Detecting Natural Selection

There is a variety of methods that test for footprints of natural selection, some of which are more appropriate for human population data than others. Standard tests that look for the shape of the frequency spectrum (distribution of variants in frequency classes) [27, 32, 92] can be useful for human data, but these tests are also sensitive to demography and more empirical approaches are necessary to account for these effects by considering the distribution of the statistics in observed data of neutral sequences. Some other methodologies make use of the contrast of divergence to polymorphism data (HKA [39], MK [65]), but these are also sensitive to human demography. Finally, recent studies have taken the approach of using empirical distributions of such statistics to sample the tail of the distribution of the statistic and then take the regions or genes to another orthogonal (independent) set of analysis. All the methods above require re-sequencing data that is not currently available for human sequences but will soon become so under the framework of the 1,000 Genomes Project and other projects using new sequencing technologies.

Given the absence of full re-sequencing data and the availability of SNP genotype data, haplotype-based tests have become useful in human data. These tests are designed to partly account for human demography and are also fit for analyzing genotyping data of SNPs from a given region [83, 98]. Haplotype-based tests are particularly sensitive to recent events of positive selection. The main principle behind these tests is that recently positively selected variants take other neutral variants to high frequency, generating specific patterns of haplotypic diversity and variant correlation. The signal these tests detect is extended haplotype homozygosity around a potential region that has undergone recent positive selection.

16.3.8 Historical Perspective of Population Genetic Studies

Applications of DNA Marker Studies. The use of DNA markers extended the theoretical and practical applications of genetic linkage work considerably. For example, the high degree of individuality of DNA patterns together with the fact that DNA can be extracted from all nucleated cells, and even minute amounts can be amplified with the PCR reaction, makes DNA polymorphisms excellent tools for identifying individuals even if very little material is available. Thus, forensic applications for the identification of blood and sperm residues have come into common use [45]. While there is no controversy about the conceptual basis of this DNA technique, much discussion has been devoted to statistical issues that arise in calculating the probabilities that a suspect's DNA pattern comes from the same person. It is almost certain that using multiple markers appropriately makes it possible to demonstrate a unique DNA pattern for every person, except for identical twins.

Mitochondrial DNA Polymorphisms. Mitochondria are transmitted only from mothers to all of each mother's sons and daughters; there is no diploidy, no meiosis, and no recombination. Polymorphisms of mitochondrial DNA are especially useful in population genetics, mainly for the analysis of relationships between population groups and population history and most of the mutations do not appear to be subject to selection pressures. Therefore comparison of maternally inherited mtDNA restriction patterns between population groups gives an unbiased picture of the population's genetic history [11].

Y-Chromosome Polymorphisms. The other side of mitochondrial DNA is the Y-chromosome. The Y-chromosome is transmitted only by the father to a son. There is only a small portion of the Y-chromosome, called the pseudoautosomal region, that undergoes recombination with the X-chromosome. Variations on the Y-chromosome are very useful, in particular when combined with mtDNA analysis to reveal patterns of population history and behavioral aspects of ancestral populations [50].

16.4 Current Themes

16.4.1 International Efforts to Detect and Describe Sequence Variation

Cataloguing and proper recording of human variation is necessary to support the infrastructure of the search for human disease variants. A major international effort called the International HapMap Project was launched in 2003 to formally record this information in a number of human populations [41–43]. The project initially aimed at the genotyping of a few tens of thousands of SNPs stored in the SNP database of NCBI (dbSNP) in four chosen populations, a Caucasian population of Northern European origin from Utah (CEU), an African population from Ibadan, Nigeria (YRI), a Han Chinese population from Beijing (CHB), and a Japanese population from Tokyo (JPT). The need for large-scale genotyping technologies and the obvious breakthroughs that such technologies would make in disease gene hunting allowed the project to genotype more than 1 million SNPs in the first phase of the project and almost 4 million SNPs after the second phase. The datasets from the HapMap have some ascertainment problems, which are mainly due to rapid change of SNP selection during the project, and it is not clear yet how much the LD information obtained from the four populations will be applicable to other global populations. However, it is a resource that is beginning to serve the scientific community in a substantial way (Fig. 16.7), and we have already experienced the impact in disease genetics with associations of common variants with common diseases [102].

Another project that is expected to have a major impact on the exploration of variation in human populations is the 1,000 Genomes Project (www.1000genomes.org). This project aims at the re-sequencing of thousands of individuals from a number of diverse global locations, in order to elucidate variants that were previously undetected, in particular those with low frequencies [44]. This has been the result of major advances in genome sequencing over the last 4–5 years, and it is considered only the beginning of genome exploration at this level of detail. Accessibility to rare variants and availability of full genome sequencing is expected to spawn a new set of methodological advances that will provide a lot of new insights into population history as well as functional variation.

16.4.2 Prospects for Mapping Functional and Disease Variation

As described above, mutations can occur in any place in the genome, and depending on where they occur their fate in the population may be different. The majority of the genome is neutral DNA, with a high content of repeats and other nonfunctional sequences. But a substantial fraction is functional, and some of this is shared among all mammals or among vertebrates. In the past, much of the attention was devoted to variation in protein-coding DNA, and most of the functional variation was thought to lie within protein-coding sequences. In recent years, attention has been focused on noncoding DNA and it has been inferred that more than two thirds of the human functional DNA is noncoding [5, 22, 68, 86]. The consequences of nucleotide variants in coding DNA are pretty well understood, with nucleotides changing amino acid sites carrying an overall higher probability of having a functional effect than silent (non-amino acid-changing) sites [8, 10]. However, the code of noncoding DNA is not known, and even if we interrogate all sequence variants in the human genome it will be hard to assign a priori probabilities of which ones may have a functional effect. One way to deal with this is to use interspecific sequence conservation to partition variants within and outside of conserved sequences. This is a logical categorization and has been informative in some disease studies [25], but even then we rarely know the exact functional effect, because we do not understand the biochemical reasons underlying the conservation of the sequence. Furthermore, many of the functional variants in the human genome are not found in conserved sequences and the elucidation of such variants will be more difficult.

A very ambitious project, called the ENCODE (ENCyclopedia Of Dna Elements) project, has been initiated and is aimed at the identification of all functional elements in the human genome [30]. In its pilot effort, this project has generated large catalogs of functional elements in 30 Mb (1%) of the human genome. In addition, the HapMap Project has generated re-sequencing data for 48 individuals for 5 of the 30 Mbp. There was a great opportunity to intersect these two types of data and for the first time describe the patterns and levels of variation in a large set of functional elements (Fig. 16.8). The results of this effort [7] will provide a substantial framework for the interpretation of functional variation in the human and other mammalian genomes.

Another way of interrogating functional variation is to condition it on phenotypic effects. We can choose a phenotype with large enough genetic and heritable variation in human populations, such as cellular functions and responses. By using standard QTL mapping approaches we can map the location of the variants that explain the phenotypic variation. These approaches are generally used in humans for disease mapping in case-control studies, but in this case we apply it to a continuous trait. Such approaches have been used to map functionally variable regulatory regions by studying gene expression phenotypes from EBV-transformed lymphoblastoid cell lines of HapMap populations and using the SNPs genotyped in those populations as markers. Some studies have managed to map such variation [16, 90], but the resolution of the methods is low owing to the high LD in human populations (Fig. 16.9). Additional, first-order, assays are necessary (e.g., binding assays chip-seq, open chromatin assays) to multiple variable haplotypes in humans to reveal the amount, nature and degree of functional noncoding variation in humans.

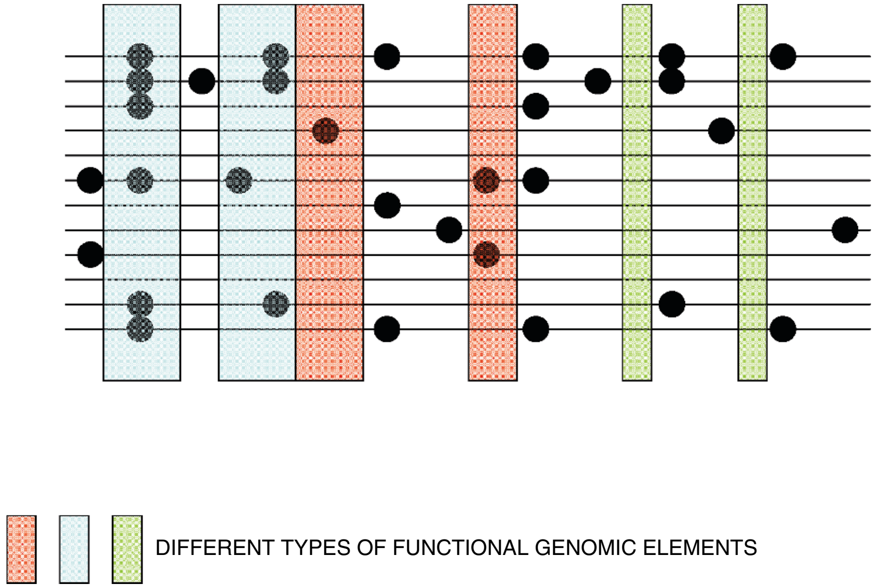

Fig. 16.8 Differential frequency spectrum depending on the type of functional element of a SNP. Patterns of SNP variation in functional DNA elements (*colored boxes*). Note the different patterns of SNP variation in each of them (both density and frequency), indicating the different modes of selection acting on the different types of functional elements

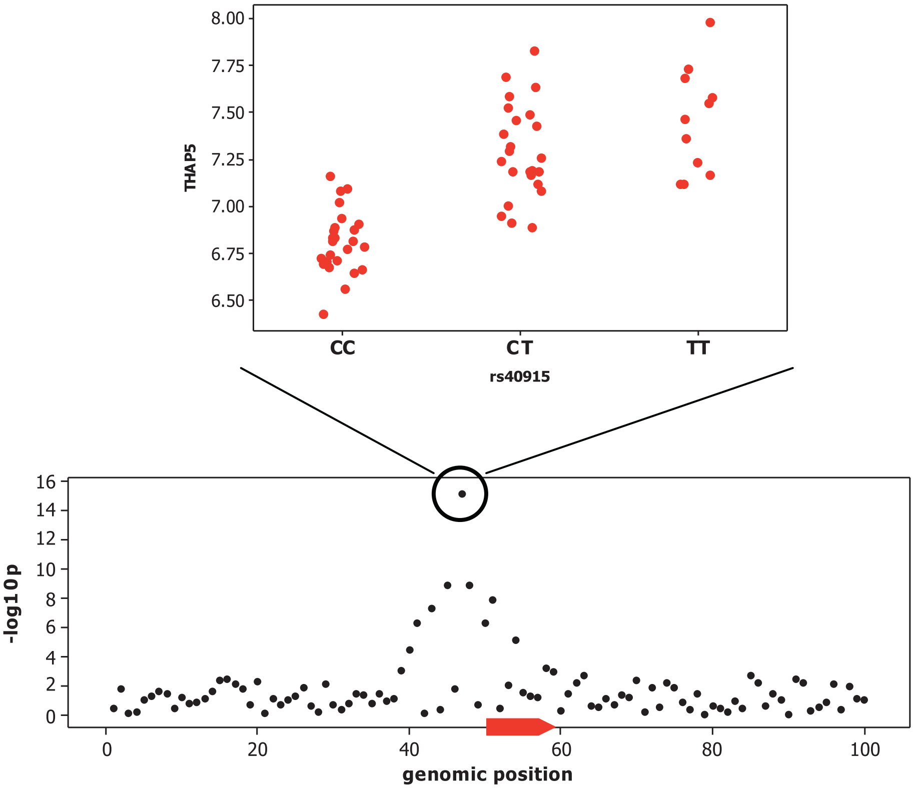

Fig. 16.9 *Cis*-association of gene expression variation in a population. The *panel below* shows the $-\log_{10}$pvalue (*Y*-axis) of the association test of SNPs with gene expression values of gene THAP5 in a given sample of individuals. The *X*-axis is the genomic region, and the *red arrow* is the gene. The *panel above* shows the scatterplot of $\log_2$ expression values (*Y*-axis) for each of the three genotypic classes (*X*-axis)

16.5 Summary

We have discussed some basic principles of human genetic variation, its nature and some characteristics that are specific to human populations. We like to think of our species as special, and our population genetics are not straightforward, but this is not a property of our species only. The study of human genetic variation requires a deep understanding of the population history. Although we have some incomplete historical records we are still not able to develop models that account for all the historical effects, and this is a serious obstacle to our getting a complete picture of our history, what has shaped our genetic diversity, and how different we are from our ancestors. This knowledge extends to the understanding of the causes of disease, some of which are a combination of our genetic makeup and our recent changes in lifestyle. The development of genotyping and sequencing technologies promises that we will soon have steadily improving data to address these issues.

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Consanguinity, Genetic Drift, and Genetic Diseases in Populations with Reduced Numbers of Founders

17

Alan H. Bittles

Abstract In western countries, consanguineous marriage often arouses curiosity and prejudice in approximately equally measure, despite the fact that until the mid-nineteenth century cousin marriages were quite common in Europe and North America. Attitudes to consanguinity remain very different in other parts of the world, in particular north and sub-Saharan Africa, the Middle East, Turkey and central Asia, and south Asia, where between 20% and over 50% of current marriages are contracted between biological relatives, with first-cousin unions especially common. Besides intra-familial marriage, in these regions a large majority of marriages also occur within long-established male lineages, e.g., clans and tribes in Arab societies and castes in India. Through time these lineages effectively become separate breeding pools, with founder effect, mutation, genetic drift and bottle-necking separately and collectively influencing gene pool composition. The present chapter first considers the concepts of random and assortative mating and then examines demographic, social, economic, and religious variables that influence the prevalence of preferred types of consanguineous marriage. The effects of consanguinity on human mate choice, reproductive success, and reproductive compensation are identified, and the impact of consanguinity on morbidity and mortality in infancy, childhood and adulthood are discussed and quantified. Three detailed case studies are then used to illustrate the influence of endogamy and consanguinity on human genetic variation and genetic disease: the Finnish Disease Heritage; inter- and intra-population genetic differentiation in India; and the distribution of specific disease alleles in Arab Israeli communities. The scale of global migration during the last two generations, with many millions of individuals, families, and occasionally entire communities moving within and between continents, has created an entirely new scenario in human population genetics. Against this background, consanguinity has re-emerged both as an important feature of community and public health genetics, and as a topic of general interest.

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Contents

17.1 Genetic Variation in Human Populations

The concepts of race and ethnicity often are highly controversial topics, and the use of supposed racial characteristics in differentiating between human populations has been strongly censured. At the same time, genomic microarray studies have convincingly demonstrated significant differences between major human populations living in different parts of the world, with common genetic variants playing an important role in inter-ethnic gene expression [86]. However, microarray studies also have shown that 93–95% of the total genetic variation was intrapopulation rather than interpopulation in origin [75]. While the proportionally minor genetic differences between populations and the attendant race/ethnicity/ancestry controversy are widely discussed and argued, an obvious and potentially more significant question arises with respect to the origins and causes of the very high level of intra-population genetic variation. How and why did this variation arise, how and why is it maintained, and what, if any, are the consequences in terms of biological fitness, and more especially genetic disease?

Throughout recorded human history, marriage between a male and female has been the predominant institution within which procreation occurred and genes were transmitted. Therefore a key initial step in investigating intra- and inter-population genetic differences is to examine how and why marriage partners are chosen in different societies. Virtually all traditional societies are divided into long-established communities, with limited inter-community marriage. Indeed, genome-based association studies conducted in industrialized Western societies have revealed similar, if less pronounced sub-divisions, and even in countries with large immigrant communities, such as the USA, Canada and Australia, recent arrivals typically marry within their own ethnic and/or religious community during the first and second post-migration generations. Although offering strong social advantages, this tradition has important genetic implications, since it is probable that couples from the same national, ethnic or religious sub-community will have a significant proportion of their genes in common, and therefore that their progeny are more likely to be homozygous for a detrimental recessive disorder [14].

17.1.1 Random Mating and Assortative Mating

One of the theoretical cornerstones of human population genetics, the Hardy–Weinberg principle, incorporates the provisos of infinite population size and random mating. Even cursory consideration of the growth rate of the global human population through time would indicate that blanket assumptions of this nature are seriously flawed. Thus it has been estimated that the total global population in 1,000 AD was some 310 million, increasing approximately 20-fold during the course of the second millennium to 6,070 million, with an additional 4,420 million humans in the twentieth century alone.

Likewise, rather than random mating, in many Western countries first cousin unions were both popular and highly prized up to the mid-nineteenth century and, for example, not only did Charles Darwin marry his first cousin Emma Wedgewood, Darwin's sister Caroline married Emma Wedgwood's brother Josiah, following intermarriage between the Darwin and Wedgwood families in the previous generation. However, in modern Western societies there is a strong belief that marriage between close biological kin is genetically disadvantageous, which has led to a marked decline in the prevalence of consanguineous marriage in these populations.

This does not mean that marriage partner choice has become an essentially random process, and even in societies where consanguinity is regarded with disfavor, positive assortative mating is the rule rather than the exception. Thus despite greater personal mobility, the choice of a marriage partner remains strongly influenced by geography and ethnicity, and by essentially social factors, such as religion, education, economic status, and political beliefs. Under these circumstances the strict concept of random mating does not apply, since it is probable that the marriage partners will have inherited identical alleles at a proportion of gene loci.

17.1.2 Genetic Drift and Founder Effect

The phenomenon of genetic drift is most simply defined as the influence of chance on gene frequencies in successive generations, and the probability of genetic drift is greatest in communities with small effective population sizes, i.e., with restricted numbers of potential mating couples. In evolutionary terms this situation can arise in several ways, for example, through founder effect, when a subgroup of a population establishes a new breeding colony; via a demographic bottleneck following major disease- or disaster-related mortality; and in subdivided populations with multiple, strictly endogamous subcommunities.

Where there is restricted marriage partner choice, genetic drift can lead to random inbreeding, with unions contracted between individuals not known to be biological relatives but drawn from the same confined gene pool. The net effect is similar to positive assortative mating, and the main outcome is a higher probability of homozygosity at some gene loci, resulting in an increased likelihood of recessive gene expression. This is important from a medical genetics perspective, since a recessive founder or de novo mutation can rapidly increase in frequency within a small community by chance alone, resulting in the birth of an affected child whether the parents are known to be consanguineous or believe themselves to be nonrelatives [104].

17.2 Consanguineous Matings

The origin of the term consanguineous is the Latin *consanguineus*, meaning 'of the same blood.' In a human genetics context, a couple are said to be consanguineous if they share one or more common ancestors. Since most pairs of individuals living in the same location will have a common ancestor somewhere in their family trees, for practical purposes the search for a shared ancestor generally does not extend back more than three or four generations. In medical genetics, the definition of consanguinity is usually restricted to a preferential union between a couple related as second cousins or closer, although as discussed in Sects. 17.2.1 and 17.4, important exceptions can and do arise.

17.2.1 Coefficient of Relationship and Coefficient of Inbreeding

Two basic measures are employed to quantify genetic relationships. The first is the coefficient of relationship (r), which is the proportion of genes identical by descent (IBD) shared by two individuals. The coefficient of relationship is calculated from the formula:

$r = \{(1/2)^n\}$

where n is the number of steps apart on a pedigree for these two individuals via their common ancestor. Thus for two persons related as first cousins:

$r = \{(1/2)^4\} + \{(1/2)^4\} = 1/8$

The coefficient of inbreeding (F) is the proportion of gene loci at which an individual is homozygous by descent (Table 17.1). Incestuous relationships, i.e., between father–daughter, mother–son or brother–sister are the closest form of human mating, with the partners sharing half of their genes ($r = 0.5$), and so any offspring would be homozygous at 1/4 of gene loci ($F = 0.25$). The closest legally permissible consanguineous unions are between an uncle and niece, which occur mainly in South Indian Hindu communities, or between double-first cousins, as in Muslim populations in the Middle East and Pakistan. In both of these types of marriage the partners share one fourth of their genes ($r = 0.25$) and the coefficient of inbreeding in their progeny is $F = 0.125$. Double-first cousins have both sets of grandparents in common, whereas in first-cousin marriage the couple shares two common grandparents (Fig. 17.1).

Table 17.1 Human genetic relationships

Biological relationship	Genetic relationships	Coefficient of relationship	Coefficient of inbreeding
Incest[a]	First degree	0.5	0.25
Uncle-niece Double first cousin	Second degree	0.25	0.125
First cousin	Third degree	0.125	0.0625
First cousin once removed Double second cousin	Fourth degree	0.0625	0.0313
Second cousin	Fifth degree	0.0313	0.0156
Second cousin once removed Double third cousin	Sixth degree	0.0156	0.0078
Third cousin	Seventh degree	0.0078	0.0039

[a] Incest is defined as a sexual relationship between father–daughter, mother–son or brother–sister

Second cousins have inherited 1/32 of their genes from a common ancestor ($r = 0.0313$), and so the offspring of a second-cousin union would be expected to be homozygous (or more strictly autozygous) at 1/64 of their gene loci, i.e., $F = 0.0156$. In populations with restricted marriage partner choice, couples who are not second cousins may be related through multiple pathways involving more remote ancestors. Under such circumstances the coefficient of inbreeding for an individual is calculated by summing each of the known pathways of inheritance. Thus, for an individual whose parents are third, fourth and fifth cousins ($r = 0.0078$, 0.0039 and 0.00195), the corresponding coefficient of inbreeding is ($F = 0.0039 + 0.00195 + 0.00098$), i.e., a composite coefficient of inbreeding of $F = 0.00683$.

In many societies, specific subcommunities or families have a long and unbroken tradition of consanguineous marriage, resulting in a cumulative coefficient of inbreeding that can greatly exceed the genetic influence of consanguinity in a single generation. To quantify this situation a correction term can be applied using the formula:

$$F = \Sigma(1/2)^n (1 + F_A)$$

where F_A is the ancestor's coefficient of inbreeding, n is the number of individuals in the path connecting the parents of the individual, and the summation (Σ) is taken over each path in the pedigree that goes through a common ancestor. In small endogamous communities with limited numbers of marriage partners, cumulative inbreeding via multiple consanguineous pathways can result in a significant build-up of homozygosity, even within a few generations.

17.2.2 Global Prevalence of Consanguinity

From a global perspective the lowest rates of consanguinity are found in Western Europe, North America and Oceania, where less than 1% of marriages are consanguineous, i.e., they are contracted between couples related as second cousins or closer ($F \geq 0.0156$). In some parts of Southern Europe, South America and Japan approximately 1–5% of current marriages are consanguineous, depending on local geography and

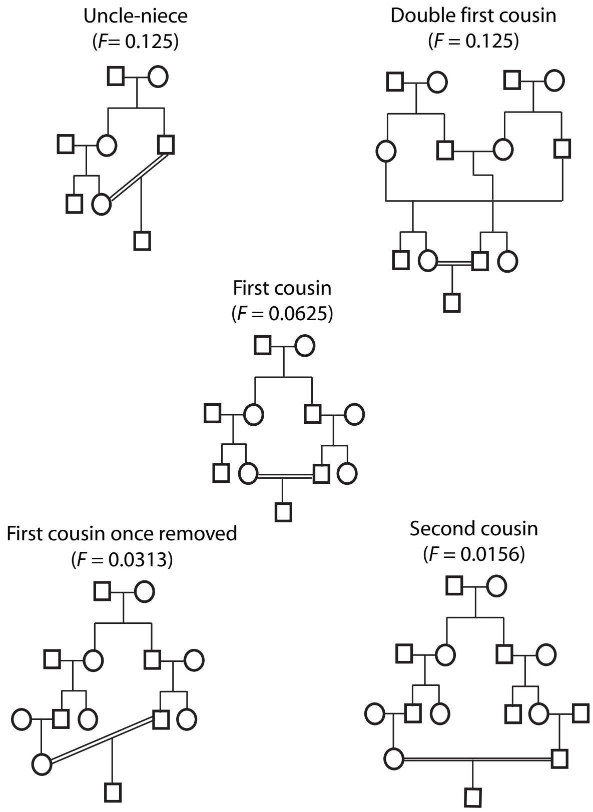

Fig. 17.1 Consanguineous pedigrees

social customs. The highest recorded rates of consanguinity are in North and sub-Saharan Africa, the Middle East, Turkey and Central Asia, and parts of South Asia, where unions between couples related as second cousins or closer account for 20% to over 50% of all marriages (www.consang.net). While a recent decline in the prevalence of consanguineous marriage has been reported in some Middle Eastern countries, such as Jordan [40], increases have been reported in the neighboring Arab states of Qatar [10], and the United Arab Emirates [5]. In the major South Asian countries of India [22], Pakistan [4], and Iran [78] little change appears to have occurred in the prevalence of consanguinity during the latter half of the twentieth century, although there is some evidence that attitudes towards consanguineous marriage are influenced by contemporary political regimes.

Data on consanguinity remains at best partial for many populous countries in Asia, including Bangladesh and Indonesia. Anthropological research in Africa has indicated that cousin marriage is common in many specific communities, but there is little information on its prevalence or the particular types of cousin union that are favored. Although consanguinity has been rare in Western societies since the early twentieth century, most Western countries are now home to large migrant communities which traditionally have contracted consanguineous unions, with all evidence pointing to continued preference for intrafamilial marriage in their newly adopted countries [11, 68]. For this reason, the summary country and regional data on consanguineous marriage presented in Fig. 17.2 are best considered as lower bound estimates of the overall global picture.

If the specific types and frequencies of consanguineous marriage are known, the mean coefficient of inbreeding (α) can be calculated to provide a measure of the intensity of inbreeding in the population, according to the formula:

$$\alpha = \sum \mathrm{p}_{\mathrm{i}} F_{i}$$

where *Σ* is the summation of the proportion of individuals *pi* in each consanguinity category *Fi*. As indicated in Table 17.2, the values for α vary widely between

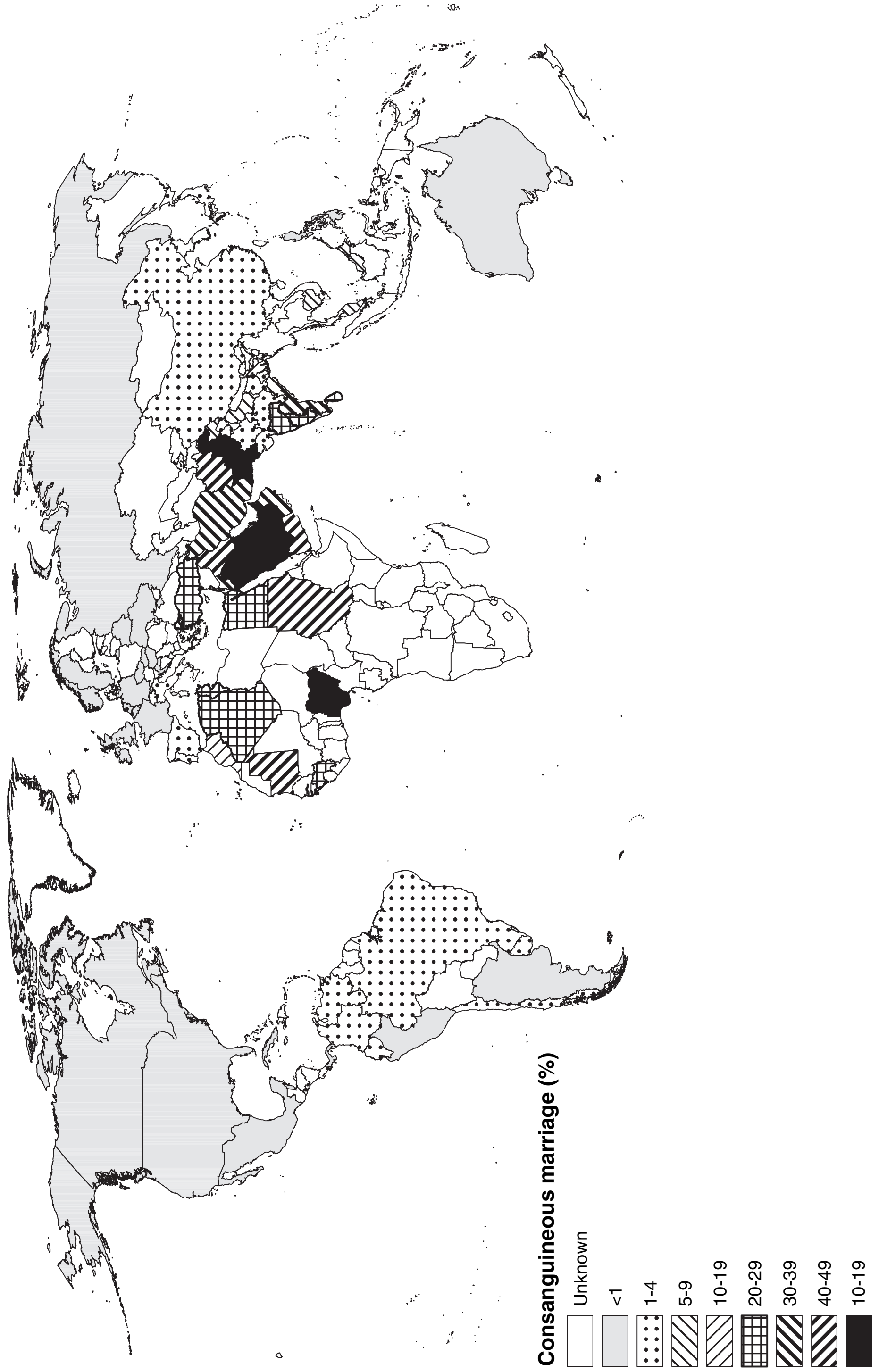

Fig. 17.2 Global distribution of consanguineous marriages

Table 17.2 Prevalence (%) and types of consanguineous marriage in different regions and populations

Region	Study setting	Participants	Number	%	Relationships[a]	Inbreeding (α)	Reference
Western Europe							
Great Britain	Birmingham	Obstetric inpatients	2,431	0.2	1C,2C	0.0001	[23]
Norway	All-Norway	Civil registration	893,941	0.7	1C,2C	0.0002	[56]
Spain	Sigüenza-Guadalajara	RC dispensation	5,315	12.6	1C,$1^{1/2}$C,2C	0.0029	[24]
North America							
Canada	Québec	RC dispensation	21,874	1.3	UN,1C,$1^{1/2}$C,2C	0.0003	[31]
USA	National	RC dispensation	133,228	0.2	1C,$1^{1/2}$C,2C	0.0001	[36]
South America							
Argentina	All-Argentina	Civil registration	212,320	0.4	1C	0.0002	[26]
Brazil	Rio Janeiro	RC dispensation	4,070	1.5	UN/AN,1C,$1^{1/2}$C,2C	0.0008	[30]
	Paraiba	RC dispensation	9,521	12.8	UN/AN,1C,$1^{1/2}$C,2C	0.0058	[30]
Northern Africa							
Tunisia	North	Obstetric inpatients	5,767	26.9	>1C,1C,$1^{1/2}$C,2C	0.0213	[73]
Egypt	National	Household/school/school/workplace	26,554	29.0	D1C,1C,$1^{1/2}$C,2C	0.0101	[39]
Sudan	Khartoum	Blood donors	4,833	52.0	1C,2C	0.0302	[79]
West Asia							
Saudi Arabia	National	Household survey	3,212	40.6	IC,2C	0.0241	[34]
UAE	Al Ain	Health centre	1,502	37.4	D1C,1C,$1^{1/2}$C,2C	0.0245	[5]
Turkey	National	Household survey	5,257	21.1	1C,2C	0.0096	[92]
South Asia							
India North	Lucknow (Hindu)	Obstetric inpatients	7,955	0.1	UN,1C	0.0001	[2]
- South	Karnataka (Hindu)	Obstetric inpatients	86,448	33.5	UN,1C,2C	0.0333	[22]
Pakistan	National	Household survey	6,611	61.2	1C,2C	0.0332	[4]
East Asia							
China - *Han*	Zejiang	Household survey	15,762	1.1	UN,1C,$1^{1/2}$C,2C	0.0006	[100]
Kirgiz	Xinjiang	Household survey	2,863	45.2	D1C,1C,$1^{1/2}$C,2C	0.0274	[99]
Japan	National	Household survey	9,225	3.9	1C,$1^{1/2}$C,2C	0.0013	[45]
Oceania							
Australia	Western Australia	Civil marriages	62,376	0.2	1C,$1^{1/2}$C,2C	0.0001	[67]

[a] Categories of consanguineous marriage: *UN* Uncle-niece, *AN* Aunt-nephew, *D1C* Double first cousin, $F = 0.125$; *1C* first cousin, $F = 0.0625$; *$1^{1/2}$C* first cousin once removed, $F = 0.0313$; *2C* second cousin, $F = 0.0156$

populations and regions, from 0.0001 in Western Europe and North America to 0.0241 in Saudi Arabia, 0.0332 in Pakistan and 0.0333 in South India, where consanguineous marriage is widely preferential. In the two latter countries the average level of inbreeding is thus equivalent to all marriages being contracted at the level of first cousin once removed ($F = 0.0313$).

Detailed global estimates of consanguineous marriage in the current generation are available by continent, country, region and population at www.consang.net. However, as indicated in Table 17.2, interpopulation comparability is difficult to achieve because of differences in the numbers of subjects recruited and in the types of study population, e.g., whether based on dispensations granted by the Roman Catholic church for consanguineous couples to marry, compulsory civil marriage registration, or records relating to obstetric inpatients. Likewise, the levels at which data have been collected vary, with some studies counting only first- or second-cousin marriages, while in other populations uncle-niece and aunt-nephew, double-first-cousin, and first-cousin once removed marriages also were recorded. What is, however, clearly apparent from Fig. 17.2 is that consanguineous marriage is not restricted to geographically remote communities or to specific religious, ethnic, or social isolates, as has been popularly believed.

17.2.3 Specific Types of Consanguineous Marriage

The specific patterns of consanguineous marriage contracted in different populations largely reflect their traditional customs and beliefs. The highest levels of consanguineous marriage so far reported in a single generation are from the former French colony of Pondicherry in South India, with 54.9% consanguinity (mean coefficient of inbreeding, α, 0.0449) [70], and among army recruits in the province of Punjab, Pakistan with 77.1% consanguinity ($\alpha = 0.0414$) [41]. The fact that the mean coefficient of inbreeding was actually higher in Pondicherry than Punjab despite a lower total percentage consanguinity is explained by the fact that most consanguineous marriages in Punjab were between first cousins ($F = 0.0625$), whereas in Pondicherry uncle-niece marriages ($F = 0.125$) predominated.

Local custom also dictates the specific types of first-cousin unions, so that in Arab Muslim communities a marriage between a man and his father's brother's daughter (FBD) is preferred, as opposed to the mother's brother's daughter (MBD) pattern of first-cousin marriage found in such disparate populations as Dravidian Hindus of South India, Han Chinese, and the Tuareg of North Africa [13]. A further factor to be considered is that in communities where consanguinity is preferential, couples in marriages categorized as nonconsanguineous very probably have inherited a significant proportion of their genes from one or more common ancestor, even though they themselves are unaware of any close genetic relationship.

Although the coefficient of inbreeding for FBD and MBD offspring is the same at autosomal loci ($F = 0.0625$), at X-chromosome loci $Fx = 0$ for FBD progeny but 0.125 for children born to MBD couples. Therefore, the specific forms of first cousin union favored and contracted within particular populations can have an important influence on the expression of X-linked disease genes.

17.2.4 The Influence of Religion on Consanguineous Marriage

The major world religions exert a strong influence on consanguineous marriage, both directly in terms of the types of marriages permitted and via the enactment of civil legislation. As indicated in Table 17.3, most of

Table 17.3 Religious attitudes towards consanguineous marriages

Religion	Subcommunity	Attitude
Judaism	Sephardi	Permissive
	Ashkenazi	Permissive
Christianity	Greek and Russian Orthodox	Proscribed
	Roman Catholic	Diocesan approval required
	Protestant	Permissive
Islam	Sunni	Permissive
	Shia	Permissive
Hinduism	Indo-European	Proscribed
	Dravidian	Permissive
Buddhism		Permissive
Sikhism		Proscribed
Confucianism/ Taoism		Partially permissive
Zoroastrian/Parsi		Permissive

the major religions sanction consanguineous unions, although there are quite marked differences within each religious tradition. Judaism and Islam largely follow the guidelines provided in Leviticus 18:7–18, but there is a Quranic prohibition on uncle-niece marriages, which are permitted within Judaism. Despite the Judaic core of Christianity, the Orthodox and Roman Catholic Churches restrict close-kin marriage. However, the strictures requiring dispensation for consanguineous unions were substantially relaxed by the Roman Catholic Church in the early twentieth century and now apply only to couples related as first cousins or closer [12]. By comparison, as part of the sixteenth century Reformation, the Protestant denominations basically reverted to the Levitical proscriptions on marriage, with first-cousin unions permissible. A similar divergence occurs within Hinduism, and while many Dravidian South Indians regard consanguineous marriage as preferential, in North India consanguinity is prohibited under the Indo-European Hindu tradition. A further detailed description of Hindu marriage practices and prohibitions is given in Sect. 17.8.2.

17.2.5 Civil Legislation on Consanguineous Marriage

While consanguineous unions are largely avoided in regions such as Western Europe and Oceania, first-cousin marriage is permissible under civil law in virtually all countries, and since 1987 marriage between half-sibs ($F = 0.125$) may be permitted in Sweden under specific circumstances. The situation is quite different in the USA. Until 1861 first-cousin marriage was legal, but through time legislation to ban different types of consanguineous marriage was gradually introduced at state level, the most recent example being a ban on first-cousin marriage adopted by the state of Texas in 2005. This means that first-cousin unions are a criminal offense in 10 states, and are illegal in a further 22 states, despite a Federal recommendation in 1970 that all state laws on first-cousin marriage should be rescinded [15]. The USA is one of the few countries to have enacted legislation of this type, alongside the People's Republic of China and the Democratic People's Republic of Korea.

17.2.6 Social and Economic Factors Associated with Consanguinity

Consanguineous unions have been most frequently reported within the ruling classes and land-owning families of Western societies, and also within powerful mercantile dynasties, such as the Rothschilds, whose family members have worked cohesively across national boundaries for many generations. The picture is quite different in other less economically developed parts of the world, with the highest rates of consanguineous marriage commonly reported among poor, rural, and largely illiterate communities in societies throughout Asia and North Africa [12, 43].

As indicated in Table 17.4, the preference for consanguineous marriage is primarily social in nature, since it is believed that family ties will be strengthened, family honor will be optimally maintained, and health or financial uncertainties that may be encountered following marriage with a partner from another family or community are avoided [15]. Also, in societies where males and females are segregated from late childhood, potential marriage partners are more likely to know each other if they are biological relatives, since they would have been able to meet at family social functions. Premarital arrangements are simplified in a consanguineous union, and the relationship of a couple and their in-laws is expected to be more congenial, which is particularly important for female autonomy in the patrilocal societies typical of most Asian countries (Table 17.4).

As in Western societies, economic considerations are an important facet of marriage partner choice, and in

Table 17.4 Social and economic advantages of consanguineous marriage

The assurance of marrying within the family and the strengthening of family ties
The assurance of knowing one's spouse prior to marriage
Simplified premarital negotiations, with conditions and marriage arrangements agreed in the partners' early or late teens
Greater social compatibility of the bride with her husband's family, in particular her mother-in-law, who also is a relative
Lower risk of undeclared health problems in the intended spouse
Reduced requirement for dowry or bridewealth payments, with consequent maintenance of the family goods and monies
For land-owning families, maintenance of the integrity of family land-holdings, which otherwise might be subdivided by inheritance

countries in which dowry payments are the norm, marriages within the family reduce or even negate the potential financial costs to the bride's family [12, 57]. Problems arising from marriage to a close relative have been cited in a minority of cases, especially where there is a large age gap between spouses. But in most instances marital stability appears strong and divorce is uncommon, possibly reflecting the family disunity that could arise if a marriage between cousins were to fail [13].

17.3 Inbreeding and Fertility

The prevailing suspicion of consanguineous unions in Western societies is centered on the belief that the offspring of a close-kin marriage will be physically and/or mentally disadvantaged. However, it also has been suggested that consanguineous relationships are less fertile than unions between nonrelatives. An influential early example of spiritual guidance on the inadvisability of consanguineous marriage was provided by Pope Gregory I in the late sixth century. Besides rather dubiously citing Leviticus 18:6 as the basis in Holy Scripture for the avoidance of cousin marriage, 'None of you shall approach to any that is near of kin to him, to uncover their nakedness,' and thus avoiding the specific guidelines provided in Leviticus 18:7–18, the Pope also claimed that unions between consanguineous spouses were infertile [38]. Where empirical information has been collected in human populations, the studies often have relied on small sample numbers, a shortcoming that makes the results difficult to assess [21]. However, in general, reduced levels of pathologic sterility have been reported among consanguineous couples [33, 72] with no evidence of an increase in fetal loss rates [21, 48], and indirect indicators of fetal survival, such as multiple birth rates and the secondary sex ratio, also failed to show an adverse inbreeding effect.

17.3.1 Genetically Determined Factors Influencing Human Mate Choice

It has been proposed that an olefactory mate-choice system operates in humans and other mammals. For example, in studies on the Hutterites, a highly endogamous Anabaptist sect resident in the USA, there was a lower than expected incidence of HLA haplotype matches between spouses, which was interpreted as evidence for the instinctive avoidance of partners with similar human leukocyte antigen (HLA) haplotypes [64].

The phenomenon of mate choice also was investigated in Swiss university students, with female students asked to conduct blind smell-testing of cotton T-shirts previously worn by male students and score the resultant body odors in terms of pleasantness and 'sexiness.' It was claimed that level of attractiveness of the male body odors was proportional to the degree of major histocompatibility complex (MHC) dissimilarity between the male subjects and the female testers, although follow-up studies indicated a high level of scoring variance [94]. Quite different results were obtained when a similar experiment was conducted with unmarried Hutterite women. In this case, the women were more likely to favor the odor of a 'donor' with whom they shared an intermediate number of HLA alleles. Furthermore, the positive preference appeared to be based on HLA alleles inherited from the subject's father but not her mother [49].

From the viewpoint of actual marriage partner choice, it can be convincingly argued that the findings of each of these studies have limited relevance in communities where consanguineous unions are strongly preferential, since in these more traditional societies marriage contracts are generally subject to parental decision-making [21].

17.3.2 Inbreeding and Fetal Loss Rates

Enhanced genetic compatibility would be expected between mother and fetus in consanguineous unions owing to their greater proportion of shared maternal and paternal genes. In keeping with this premise it has been claimed that intrauterine mortality is reduced in the pregnancies of consanguineous couples, with lower rates of conditions such as Rhesus (Rh) incompatibility [87] and pre-eclamptic toxemia [88]. Conversely, the fetal allograft hypothesis proposes that antigenic disparity between mother and fetus is beneficial to fetal development [29, 62], which would suggest higher losses in consanguineous pregnancies.

Table 17.5 Average number of live-born children by coefficient of inbreeding (*F*) (from [21])

	Coefficient of inbreeding (*F*)[a]				
	Uncle-niece/ double first cousin	First cousin	First cousin once removed	Second cousin	Nonconsanguineous
	$F=0.125$	0.0625	0.0313	0.0156	0
Mean number of live-born children	3.26	3.43	3.18	2.96	2.57
Number of studies	17/30	30/30	19/30	20/30	30/30

[a]The patterns of consanguineous marriage assessed varied between individual studies, with comparative data on fertility in first-cousin and nonconsanguineous matings available for all 30 studies analyzed, for uncle-niece/double first cousin unions in 17 studies, for first cousins once removed in 19 studies, and for second cousins in 20 studies

A positive association between parental HLA sharing at allele loci and recurrent abortion has been reported, with negative selection against individuals homozygous at HLA loci [51]. Unfortunately, retrospective data on pregnancies and prenatal losses may be subject to significant levels of recall bias, resulting in data of dubious reliability and significant underestimation of the levels of prenatal losses [95, 96].

Studies based on sequential human chorionic gonadotrophin (hCG) assays are more reliable, with on average some 40+% of all post-implantation conceptions lost. This figure rises with advancing maternal age, and in a hCG-based study in Bangladesh, while 45% of the pregnancies detected among women at 18 years of age spontaneously miscarried, among women aged 38 years fetal losses increased to 92% [65]. Since these levels of spontaneous abortion/miscarriage are very much higher than generally reported in inbreeding studies, there must be a strong suspicion that early pregnancy losses have been undetected or were underreported in investigations that relied solely on women's recall.

17.3.3 Comparative Fertility in Consanguineous and Nonconsanguineous Couples

A majority of comparative studies into fertility have shown a positive correlation between consanguinity and the number of live-born children. Data analyzed in a meta-analysis of 30 studies conducted in Asian and African countries are summarized in Table 17.5, with a higher mean number of children born in all categories of consanguineous marriage when compared with nonconsanguineous couples. Since the structure of each study varied according to the locally preferred types of consanguineous marriage, complete data were available only for first-cousin and nonconsanguineous couples, with first cousins showing the highest mean number of children ($n=3.43$). But even among the uncle-niece and double-first-cousin marriages ($F=0.125$), information on the numbers of live-born children had been published for 17 of the 30 populations, with mean fertility ($n=3.26$) higher than among nonconsanguineous spouses ($n=2.57$).

Typically, maternal age at marriage is negatively associated with consanguinity, resulting in a younger maternal age at first birth [22]. In addition, a higher mean age of motherhood has been reported among consanguineous couples [91], which supports the belief that early marriage, the earlier commencement of reproduction, and maximization of the maternal reproductive span by consanguineous couples are critical biosocial factors in determining family size.

The uptake of contraception may be lower in consanguineous couples [44], and reproductive compensation has been advanced as an additional explanation for the positive association between consanguinity and fertility, with infants dying at an early age rapidly replaced [63, 83]. Reproductive compensation could involve a conscious decision by parents to achieve their desired family size, but at the same time a further pregnancy following the death of a breast-fed infant may mainly be a consequence of the cessation of maternal lactational amenorrhea. The relationship between consanguinity, fertility, and reproductive compensation is however complicated, since the greater the number of children born to parents who are carriers of one or more detrimental recessive alleles, the higher the expectation that at least some of their progeny will be affected and so could die in early childhood.

17.4 Inbreeding and Inherited Disease

A significant positive association has been repeatedly demonstrated between consanguinity and early mortality, with disorders involving the expression of detrimental recessive genes especially involved. But since the poorest sections of all populations are most disadvantaged in terms of health and health care provision, overrepresentation of poorer and less educated families among consanguineous couples creates problems in assessing the effects of consanguinity on morbidity and mortality.

The first structured study into the medical effects of inbreeding was organized by Dr. Samuel Bemiss of Louisville, Kentucky [9], who in 1858 examined reports forwarded by medical colleagues on the health outcomes of unions ranging from incest ($F=0.25$) to third-cousin marriages ($F=0.0039$). Hundreds of further studies have been undertaken since that time, based on a variety of sampling techniques including pedigree analysis, household surveys and questionnaires administered to hospital in- and outpatients. In populations where uncle-niece or double-first-cousin and first-cousin marriages are preferential, unions beyond second cousins ($F<0.0156$) are of limited medical significance [13]. By comparison, where consanguineous unions generally are rare, biologically remote relationships in the present generation, such as third cousins and beyond ($F\leq0.0039$) may nevertheless be of clinical importance in families where cumulative inbreeding at differing levels of consanguinity has occurred through time, with a consequent build-up of homozygosity. A similar phenomenon can arise in communities in which close-cousin unions have been proscribed on religious grounds but marriages between couples who are related to a lesser degree are permissible.

17.4.1 Consanguinity and Deaths in Infancy and Childhood

Data on the relationship between consanguinity and birth measurements have been mixed, with some studies suggesting that babies born to consanguineous parents are smaller and lighter, and therefore less likely to survive, whereas others have failed to detect any significant difference. By comparison, there is a general consensus that postnatal mortality and morbidity are higher among the progeny of consanguineous unions, and the rarer the frequency of a deleterious recessive gene in a population, the greater the proportional disadvantageous effect of inbreeding on its expression [13]. Estimates of the overall adverse effects of consanguinity have been highly variable, and it is generally accepted that earlier surveys may have produced spuriously high values due to inadequate control for important non-genetic variables that are known to influence childhood health, including maternal age and education, birth order, and birth intervals.

In developing countries, excess consanguinity-associated deaths are largely concentrated during the 1st year of life, but in many cases no specific cause of death is determined because of inadequate diagnostic facilities and parental reluctance to sanction prenatal diagnosis or autopsy examinations [16, 68]. Where a diagnosis has been possible, a clear link between consanguinity and autosomal recessive disorders is apparent, with multiple deaths reported in a proportion of consanguineous families, the effect being proportional to the level of parental genetic relatedness [13, 90].

17.4.2 Consanguinity and Childhood Morbidity

By definition, studies into the prevalence of birth defects are dependent on the diagnostic criteria employed and, in less developed countries, recognition of the symptoms of congenital disorders can often overlap with and reflect late fetal and neonatal survival rates. In developed countries, on average 4–5% of newborns have some form of birth defect [28]. A significant excess of major congenital defects has been diagnosed in consanguineous offspring, especially disorders with a complex etiology and a higher rate of recurrence, but the reported rates of birth defects associated with consanguinity have varied quite widely. Thus, in an Arab community in Israel first-cousin progeny had 3.8% excess major malformations [47], whereas a 26-year study based on the Medical Birth Registry of Norway reported 1.9% excess birth defects in Norwegian first-cousin couples and 2.4% among Pakistani migrant couples [89]. According to the Latin America Collaborative Study of Congenital

Malformations (ECLAMC) which examined 34,102 newborn infants for congenital anomalies, a significant association with consanguinity was found only for hydrocephalus, postaxial polydactyly, and bilateral cleft lip with or without cleft palate [74].

From these data it is difficult to identify major categories of disease that are specifically overrepresented in consanguineous progeny. Cognitive impairment is more common in consanguineous offspring, and a study of Arab schoolchildren in Israel indicated a 0.8- to 1.3-point decrease in mean IQ scores among first-cousin progeny by comparison with the children of unrelated parents, with a 2.6- to 5.9-point decline in the mean IQ scores of double-first-cousin progeny [8]. There also was a significantly higher level of variance in the IQ scores of the double-first-cousin progeny, suggesting the expression of detrimental recessive genes in some of these children. In Pakistan mild and severe intellectual and developmental disability also have been associated with consanguinity [32], although as with cognitive impairment poor social conditions may play significant causative roles in such cases.

As large, inbred pedigrees offer a cost- and time-effective strategy to locate disease mutations, the technique of homozygosity mapping in consanguineous families [52] has been widely adopted to identify the causative loci for disorders such as autosomal recessive nonsyndromal hearing loss, and blindness caused by early onset retinal dystrophies and childhood glaucoma, each of which has been reported at increased prevalence in specific consanguineous communities.

17.4.3 Consanguinity and Adult Mortality and Morbidity

Although potentially the most intriguing and challenging age range during which the adverse effects of consanguinity on health could be expressed, morbidity in adulthood has been underinvestigated. There is some preliminary evidence that certain cancers, especially breast cancer [54, 85], and specific forms of early-onset cardiovascular disease [46] are more prevalent in consanguineous individuals. The adult progeny of consanguineous unions also are overrepresented in institutions caring for persons with intellectual disability [13].

A major difficulty in assessing many of the findings obtained with adult-onset diseases is that they were derived from composite studies based on investigations conducted across discrete breeding populations, with little control for sociodemographic variables. Because of a lack of precise information on the composition and structure of the consanguineous and nonconsanguineous study groups, and appropriate matching for nongenetic variables, the comparisons drawn often prove to be irreproducible. An exception is the high prevalence of Alzheimer disease diagnosed in an Israeli Arab community, with more than one-third of the cases diagnosed members of a single clan (*hamula*) [35]. This supports an earlier study from the demographically well-characterized Saguenay area of Québec, Canada, which found that cases of late-onset cases of Alzheimer disease associated with the apolipoprotein (APOE) ε4 allele were significantly more inbred than controls [93].

Long-term studies conducted on the Dalmatian Islands, Croatia have suggested that inbreeding is a strong predictor for a wide range of late-onset disorders, including hypertension, coronary heart disease, stroke, cancer, uni-/bipolar depression, asthma, gout and peptic ulcer [76, 77]. At least in the short term, studies which concentrate on subcommunities of this type are more likely to provide information on disease-predisposing alleles than ethnically mixed populations. Although, even in population isolates with extensive pedigree data, failure to allow for the influence of distant genealogical loops can result in false positives in homozygosity mapping [55].

17.5 Incest

Incest is the most extreme example of human inbreeding, with the partners having a coefficient of relationship, *r*, of 0.5, so that any progeny born of an incestuous union would be expected to have a coefficient of inbreeding (*F*) of 0.25. Incest also differs from all other forms of inbred union since, in contemporary societies, it is universally regarded as both a criminal and a moral offense. Brother–sister marriages were recorded in Pharaonic and Ptolemaic Egypt, Zoroastrian Persia, the Inca Empire, and other historical dynasties, and they also were noted among nonroyal families in Roman Egypt from the first to the fourth centuries AD [81, 82]. Perhaps because of the high level of disapproval that incest attracts in modern societies, there are very few credible data sets on the outcomes of incestuous pregnancies. Yet the numbers of reported prosecutions on grounds of incest, usually involving father–daughter relationships, suggest that incest may be more common than is generally supposed, with brother–sister incest especially underreported.

In many instances where a child is born to a very young mother, the father of the child is not identified, even though incest may be suspected. If the child is healthy it is probable that no further action will be taken even if the child is offered for adoption. But when a sick child is born there is a greater imperative to investigate the cause of the illness, which in turn may lead to incest being identified. Under these circumstances significant overestimation of the adverse outcomes of incest could result, suggesting that considerable caution needs to be applied in the interpretation of incest data.

17.5.1 Mortality and Morbidity Estimates for Incestuous Matings

As shown in Table 17.6, according to data on 213 children collated from the four best-known studies of incest, conducted in the USA, UK, Czechoslovakia, and Canada over some 50 years [1, 6, 25, 84], only 46.0% of incestuous pregnancies resulted in the birth of a healthy infant. Follow-up ranged from 0.5 to 37 years, and among the incestuous offspring 39.4% had a recognized autosomal recessive disorder or a congenital malformation, had succumbed to sudden infant death, or had severe nonsyndromic intellectual disability, with deaths in 14.1% of cases. A further 14.6% of subjects had a mild disorder, including intellectual and developmental disability. By comparison, just 8.0% of the 113 nonincestuous controls died or were diagnosed with a serious defect, suggesting a mean level of excess mortality or serious defect in the incestuous progeny of 31.4%.

It should be stressed that in many cases the incestuous mothers were very young, with gynecological immaturity a possible adverse factor in the pregnancy, and in a substantial percentage of these cases either the mothers or the fathers, and sometimes both, had serious pre-existing physical or mental disorders [17]. Therefore, it is probable that the adverse pregnancy outcomes may, in part, have been due to causes other than detrimental recessive gene expression. Clarification of this issue will be dependent on additional data becoming available, but as already observed, the collection of unbiased information on the health sequelae of incest is extremely difficult.

17.6 Genetic Load Theory and Its Application in Consanguinity Studies

All humans are heterozygous for a number of detrimental recessive genes, and the term ‘genetic load’ refers to the decrease in the average fitness of a population caused by the expression of genes which reduce survival. Lethal gene equivalents are defined as the number of detrimental recessive genes carried by an individual in the heterozygous state which, if homozygous, would result in death. Therefore, by comparing death rates in the progeny of consanguineous and unrelated couples, it is possible to estimate the numbers of lethal gene equivalents in a community or population.

The number of lethal gene equivalents in a population can be calculated according to the formula:

$$-\log_e S = A + BF$$

where S is the proportion of survivors in the study population, A measures all deaths that occur under random mating, B represents all deaths caused by the expression of recessive genes via inbreeding, and F is the coefficient of inbreeding [58]. By plotting a

Table 17.6 Mortality and morbidity estimates for incestuous progeny. (From [55, 77, 81, 82])

Number studied	Follow-up (yr)	Autosomal recessive disorders	Congenital malformations/ sudden infant deaths	Nonspecific severe intellectual disability	Others, including mild intellectual disability	Normal
213	0.5–37	11.7%	16.0%	11.7%	14.6%	46.0%

weighted regression of the log proportion of survivors (*S*) at different levels of inbreeding (*F*), *A* can be determined from the intercept on the Y-axis at zero inbreeding ($F=0$), and *B* (the number of lethal gene equivalents) is given by the slope of the regression.

Since consanguineous individuals have a greater probability of inheriting the same mutant allele(s) from a common ancestor, their progeny will be at a higher risk of expressing one or more recessive disorders. By calculating the number of lethal gene equivalents, the results of inbreeding surveys could be transformed into a meaningful and reproducible format, which then could be comparatively applied to the results of surveys in different populations. A multinational meta-analysis conducted on over 600,000 pregnancies and live births collated from 38 study populations indicated 4.4% excess prereproductive mortality in first-cousin progeny (measured from approximately 6 months gestation to a median age of 10 years) [20]. This level of excess mortality equates to 1.4 lethal equivalents per zygote, and a subsequent study of first cousin versus nonconsanguineous marriages in Italy from 1911-1964 produced equivalent results, with 3.5% excess deaths at $F=0.0625$, i.e., 1.2 lethal equivalents per zygote [27].

17.7 Genomic Approaches to Measuring Inbreeding at Individual and Community Levels

The direct estimation of an individual's inbreeding coefficient by reference to genomic data offers many advantages, since it can include the influence of historical levels and patterns of inbreeding that may not be identifiable within a pedigree. A maximum-likelihood method of analysis has been developed using simulated whole genome data which permits inference of the identity by descent (IBD) status of both alleles of an individual at each marker along the genome. The method also provides a variance measure for the estimates and, for example, it was shown that while the mean value for IBD status for first cousins was 0.0625, at individual loci the calculated values ranged from 0.03 to 0.12 [53].

Microsatellite analysis of DNA samples obtained from UK ethnic migrants showed that in the Pakistani Muslim community, in which consanguineous marriage is widely favored and practiced, the observed *F* values were much higher than in a co-resident Indian Sikh community which avoided consanguinity [66]. This study also indicated significant genetic substructuring, which could interfere with estimates of the frequency of recessive disease genes. Using both SNP and microsatellite analysis, a subsequent study of UK Pakistani consanguineous individuals with a range of autosomal recessive diseases showed that, on average, persons whose parents were first cousins ($F=0.0625$) were actually homozygous at 11% of the loci tested, with a range of 5-20% [98].

The findings of these studies indicate the influence of cumulative inbreeding on genome structure at both individual and community levels. In addition, they confirm the desirability of a prior understanding among researchers and clinicians of the social structure of communities, in particular their marriage patterns, since information of this nature could have a major role in determining the patterns and frequencies of specific genetic disorders.

17.8 The Influence of Endogamy and Consanguinity in Human Populations

Inter- and intrapopulation fluctuations in the frequencies of coding genes are well recognized and documented, and it seems probable that similar variations will be demonstrated in the control of gene expression. Three quite different, representative human populations, Finland, India, and Israeli Arabs, will be used to illustrate the impacts of founder effect, random drift and consanguinity on genetic structure and the prevalence and expression of recessive disease genes.

17.8.1 The Finnish Disease Heritage

Finland is a small and formerly quite isolated country with a unique genetic history. The original inhabitants are thought to have been arctic northern European Uralic speakers who settled the territory of Finland some 6,500 years ago after the decline of the last Ice Age.

Somewhat later arrivals included peoples from south-eastern and western Europe between 5,000–6,000 and 4,500 years ago, respectively, with later minor waves of German, Scandinavian, and Baltic peoples [60].

The initial population settlement was concentrated in the south and west of the country, and at the start of the twelfth century the total number of inhabitants was less than 50,000. Commencing in the sixteenth century there was internal migration northward, and by the mid-seventeenth century the total numbers had increased to 400,000–450,000. But in the Great Famine of 1696–1697 approximately 25–33% of the inhabitants died, and additional major population losses occurred owing to plague at the beginning of the 1700s, and famine following crop failures in 1866–1868 [60].

The concept of the Finnish Disease Heritage (Table 17.7) was introduced in 1973 to describe some 36 mostly autosomal recessive diseases that are typical of the Finnish population while rare in other populations [59, 61]. Conversely, disorders which are common in most other northern European populations, such as cystic fibrosis and phenylketonuria, are very rare in Finland. The causative genes have been identified for 29 of the Finnish diseases [61], and four main groups of disorders can be categorized according to their patterns of geographic distribution in the current population of 5.3 million (www.findis.org).

For the most common diseases, such as congenital nephrosis, cartilage hair hypoplasia, and aspartylglucosaminuria, a lysosomal storage disease which causes intellectual and developmental disability, the birthplaces of the grandparents of affected individuals in the present generation are widely distributed throughout the country. With a second larger group of disorders, e.g., Mulibrey nanism, Usher syndrome type 3, and nonketotic hyperglycinemia, there is clustering in geographic subregions, usually areas initially populated from the sixteenth century onward. A third group, typified by Meckel syndrome and diastrophic dysplasia, is found predominantly in the western early settlement area. While the fourth group of disorders, comprising Northern epilepsy and the Finnish variant of late infantile neuronal ceroid lipofuscinosis, originated locally in the Kainuu region close to the eastern border and in Southern Ostrobothnia, respectively. Seven other autosomal recessive, autosomal dominant, and X-linked disorders have been included in the Finnish Disease Heritage, and a further five diseases are under investigation and may be incorporated in future years [61].

Table 17.7 The Finnish disease heritage. (From [61])

Disease	Incidence in Finland
Autosomal recessive	
Congenital nephrosis	1:8,000
Infantile neuronal ceroid lipofuscinosis	1:14,000
Meckel syndrome	1:15,000
Unverricht-Lundborg disease	1:17,000
Aspartylglucosaminuria	1:18,000
Cartilage-hair dysplasia	1:18,000
Spielmeyer–Sjögren disease	1:19,000
Hydrolethalus syndrome	1:22,000
Diastrophic dysplasia	1:22,000
Autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy	1:27,000
Lethal congenital contracture syndrome (Herva)	1:29,000
Congenital chloride diarrhea	1:33,000
Mulibrey nanism	1:37,000
Usher syndrome type 3	1:42,000
Salla disease	1:42,000
Cornea plana congenita	1:46,000
Congenital lactase deficiency	1:48,000
Muscle-eye-brain disease	1:52,000
Nonketotic hyperglycinemia	1:52,000
Lethal arthrogryposis with anterior horn cell disease (Vuopala)	1:53,000
Jansky–Bielschowsky disease variant	1:59,000
Hyperornithinemia with gyrate atrophy of choroid and retina	1:63,000
GRACILE syndrome (Fellman)	1:64,000
Selective malabsorption of vitamin B_{12}	1:68,000
Nasu–Hakola disease	1:71,000
Lysinuric protein intolerance	1:76,000
PEHO syndrome	1:78,000
IOSCA syndrome	1:90,000
Cohen syndrome	1:105,000
Rapadilino syndrome	1:105,000
Follicle stimulating hormone-resistant ovaries (Aittomäki)	1:127,000
Northern epilepsy	1:176,000
Autosomal dominant	
Meretoja disease	~1:6,000
Tibial muscular dystrophy	~3/year
X-chromosome	
Choroideremia	~2/year
Retinoschisis	~1:17,000

Given the dispersed nature of much of the population, and the small numbers of individuals, it might have been expected that consanguinity contributed substantially to the prevalence of the various recessive disorders. In fact, except for some parishes in northern Finland with a substantial Sami minority, first-cousin marriage was historically rare in the country, in part because of the dispensation requirement for such marriages that remained in place until 1872, with fees payable to the King. Thus, even among the Swedish-speaking Lutheran minority of Finland the attitudes towards consanguinity differed from those in neighboring Sweden, where first-cousin marriage was freed from civil law restrictions in 1844, leading to an increase in first-cousin unions during the remainder of the nineteenth century [19]. Instead, the historical population profile of Finland was characterized by conditions under which founder effects, genetic drift, and demographic bottlenecks occurred, and it is these factors that have shaped and determined the present-day national and regional profiles of genetic disease [60].

17.8.2 Inter- and Intra-population Differentiation in India

The present-day population of India is estimated at some 1,200 million, having increased from 271 million in the year 1900 and 361 million in 1950, resulting in a greatly enlarged overall effective population size. From a genetic perspective a further significant aspect of the Indian population is that, in common with Middle Eastern and North African populations, and neighboring Pakistan and Afghanistan, where tribal and clan marriage boundaries are in place, marriage in India is contracted within highly endogamous castes [18].

Caste membership is hereditary and defines an individual's position within Indian society. The caste system is believed to have been in existence for at least 2,000 years, and in the past it appears to have been somewhat more flexible, with the emergence of new castes and subcastes recorded during the eighteenth and nineteenth centuries. As an example of the current level of demographic and genetic complexity within India, there are seven major religions, and 299 different languages spoken by 4,635 officially recognized ethnic communities, which in turn are composed of an estimated 50,000–60,000 highly endogamous subpopulations [37].

The majority Hindu population, which accounts for approximately 80% of the national population and so currently numbers over 1,000 million, is structured into four major hierarchical groups (*varna*), Brahmins, Kshatriyas, Vaishas, and Sudras, The four *varna* in turn are subdivided into numerous castes (*jati*) and subcastes, and virtually all Hindu marriages continue to be contracted within hereditary caste boundaries. As these institutions are reputed to have been in existence for some 2,000 years, there has been ample opportunity for intercaste genetic differentiation to have occurred via founder effect and genetic drift, especially given the much smaller, and multiply subdivided, population of India in historical times. Therefore, it would be expected that, through time, caste-specific genetic disease profiles would have developed.

There also is a major dichotomy between the majority Indo-Europeans of north India, who avoid consanguineous marriage, and the Dravidian Hindus of south India, where first-cousin and uncle-niece marriage is widely popular and in many communities preferential (Fig. 17.2). This subdivision is believed to date back to the Codes of Manu compiled around 200 BC [50], and it continues to the present day. Given the long-term preference for close consanguineous marriage it was proposed that the endogamous and largely consanguineous populations of south India would have purged lethal recessive genes from their gene pools [80]. Empirical evidence of the range and prevalence of genetic disorders in the current South Indian population has indicated that this outcome is improbable [71], probably due to reproductive compensation, which would effectively delay if not nullify the elimination of deleterious recessives from the gene pool(s).

17.8.3 Consanguinity and the Distribution of Disease Alleles in Israeli Arab Communities

Arab populations in Israel typify a third major form of human genetic organization. For some 500 years prior to the early twentieth century, Arab communities in the Holy Land were part of the Ottoman Empire. As such, members of these communities were able to mix freely with other neighboring Arab populations, although in most cases marriages were contracted within tribes and frequently at the level of the clan (*hamula*). In addition, consanguineous unions were widely favored, in particular father's brother's daughter-first-cousin marriage (termed *ibn amm*), with double-first-cousin and second-cousin unions also quite common. Conditions favoring village endogamy increased markedly in the years following establishment of the state of Israel in 1948, with the initial movement of an estimated 700,000 people to other neighboring countries and the effective closure of the borders between Israel and the surrounding Arab states. Since 1948, the Arab population of Israel has undergone very rapid natural expansion and now totals over 1.2 million.

Autosomal recessive disorders were found to be more common in the progeny of consanguineous parents [101], and the prevalence rates of congenital malformations were higher in Palestinian Arab and Druze communities, where clan endogamy and consanguinity were strongly favored, than in the more exogamous Jewish and Christian communities [102]. As in Finland, some diseases, such as β-thalassemia, familial Mediterranean fever, and deafness are frequent in the whole Arab population, whereas others are restricted to specific regions or villages. For a specific, rare inherited disease, a single founder mutation would ordinarily be expected within a small geographic area. However, in the case of the lysosomal storage disorder metachromatic leukodystrophy caused by a deficiency of arylsulfatase A, multiple causative mutations were identified within a restricted region, suggesting the occurrence of a number of founder mutations at this disease locus [42]. Subsequent studies have further demonstrated some 19 mostly chronic autosomal recessive disorders in a village with 8,600 inhabitants, i.e., a prevalence for these disorders of approximately 1/70 [103].

A detailed investigation of 12 recessive mutations affecting the inhabitants of a single village has indicated both founder effects and de novo mutations, and the transfer of mutations between families via marriage. Under such circumstances, a single family with one or more family members diagnosed with a specific recessive disorder would usually indicate a recent event, whereas a rare disease affecting members of several families would be more convincingly interpreted as an older mutation [104]. But in all such cases, a thorough understanding of past and present marriage patterns is an essential prerequisite, and given the demographic history of founder effects, migration, population bottlenecking, and rapid expansion, in combination with clan endogamy and preferential consanguinity, the resultant overall picture becomes kaleidoscopic.

17.9 Evaluating Risk in Consanguineous Relationships

The three preceding examples illustrate some of the unexpected complexities that can be encountered when dealing with actual human populations, and the importance of at least a basic knowledge of the demographic structure of a population. They also highlight the difficulties that may be encountered in some populations in differentiating between random inbreeding, brought about by founder effect, endogamy and genetic drift, and preferential consanguinity. Yet this differentiation is critical in accurately assessing the outcomes of consanguineous unions, and in providing risk estimates in settings such as a genetic counseling clinic.

The importance of recognizing and controlling for remote levels of consanguinity in gene association studies has already been noted [55], as have the combined roles of consanguinity and population subdivision in many clinical situations [18]. Although it has been claimed that statistical methods such as principal components analysis can be employed to correct for population stratification in genome-wide studies [69], their successful application in societies as multiply subdivided as India would be a very major challenge. Greater care is therefore warranted in the selection of cases and controls for gene association studies and, if properly conducted, greater reproducibility in their outcomes should follow.

From a practical perspective, the ability to purvey risk in an unambiguous and readily understood manner is an all-important issue in human and medical genetics. Risk estimates expressed as relative risks, odds ratios, or attributable risks, i.e., the fraction of cases in a population that can be attributed to a particular risk factor, are very useful in epidemiological studies. However, in a genetic counseling setting the probability of an adverse outcome needs to be presented in as uncomplicated a manner as possible, taking into account factors such as the background population risk, degree of consanguinity, and relevant family history [11].

When dealing with a topic as potentially sensitive as consanguineous marriage, the avoidance of any potential misunderstanding or misinterpretation by clients and their families is critical.

17.10 Concluding Comments

The development of society-compatible education programs on consanguineous marriage, in combination with evidence-based screening and genetic counseling guidelines, is of paramount importance. It has been proposed that communities in which consanguineous marriage is preferential can be at an advantage when screening for deleterious mutations, for example, in the case of β-thalassemia in Pakistan [3], as there is a high probability that all family members will be homozygous for the same mutation. Under these circumstances, identification of the specific mutation in an affected individual can serve as a diagnostic marker for an extended family group at high genetic risk. Some caution is, however, needed in this approach, since past intermarriage with other unrelated families and communities could have led to significant gene admixture and hence increased the likelihood of compound heterozygosis. But in general the concept is valid and useful, as indicated by a successful screening program for autosomal recessive nonsyndromic intellectual disability in an Israeli Arab community [7].

The future status of consanguineous marriage is a matter of conjecture. Currently an estimated 1,000 million people live in countries where from 20% to over 50% of marriages are consanguineous [18], and it seems highly improbable that a form of marriage which remains so widely popular would rapidly decline in popularity. But strenuous semiofficial efforts are being made to lessen the appeal of consanguinity in many developing countries, often to the distress and embarrassment of consanguineous couples. The situation for migrant communities in western societies is different again, since there is both an attraction to continue with a form of marriage that has been undertaken for many generations in their countries of origin, and at the same time a desire among some younger members of migrant families to adopt the social mores of their new homeland, including exogamous marriage customs.

Within migrant communities there is a much greater awareness of genetic disease than would have been the case in their homeland, and of the increased risk of an affected child being born to a consanguineous couple [13]. Ultimately, declining family sizes and a consequent reduction in the availability of potential marriage partners within the immediate family may prove to be the major factor in determining the future prevalence of consanguineous unions. However, it also has to be acknowledged that the presence of several family members with a major disabling disorder may severely limit the marriage opportunities of other family members, thus increasing the probability of further intrafamilial unions [7, 18].

While medical genetics is a relatively new subject, consanguineous marriage has been, and remains, a core feature of many successful human societies. A recent World Health Organization Report on Medical Genetics Services in Developing Countries advised that: ‘Preference for consanguineous marriage is a feature of the socio-cultural context within which medical genetic services must work’ [97]. Adoption of this eminently sensible and nonjudgmental approach should ensure that the health needs of families and communities in both the developing and developed world can best be met.

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Human Evolution

18

Michael Hofreiter

Abstract The study of human evolution is as old as evolutionary biology itself. Despite this long history, progress was slow in many fields. Until the 1980s, neither the closest living relative of our species nor the geographical origin of modern humans was known. However, since then a flood of new data has provided detailed insights into many aspects of human evolution. Thus, population genetic analyses of DNA sequences unequivocally identified Africa as the continent of modern human origin and provided information about the colonization of the whole globe by modern humans. A whole plethora of recently discovered fossil remains of hominid species show a detailed picture – albeit not yet well understood – of modern human ancestors and side branches. And ancient DNA analyses have revealed our relationship to our closest relatives, the extinct Neanderthals, with the prospect of the complete Neanderthal genome being sequenced soon. Finally, first candidate genes have been identified that may have been of critical importance in the evolutionary process of becoming human. At the same time, traits such as cultural tradition and tool use have been discovered in other primate species, especially our closest relatives, the great apes, leaving few traits that may be exclusively human. Identifying these traits, revealing their genetic basis and understanding the evolutionary forces that lead to their selection will be the challenges to research in human evolution with the aim of eventually understanding what makes us human.

Contents

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18.1 Historical Overview

18.1.1 Before and Around Darwin

Humans are a unique species – there can be little doubt about this statement, as it is true for any of the some million species living on earth. However, humans are indeed special in some ways. For example, contrary to all other species, for the human species *Homo sapiens*, no type specimen, i.e., a specimen that uniquely identifies the species exists [59]. Moreover, no other species lives in such complex societies or has such a sophisticated culture. However, maybe one of the traits defining humans best is the fact that humans are capable of thinking about their origin – and do so extensively. This is testified by the fact that the origin of humans has interested people in all parts of the world; almost every culture has some creation myth, trying to explain how humans originated. In the Christian societies of western civilization humans were seen as directly created by God as described in the Bible. Interestingly, this belief did not keep Carl von Linné in his *Systemae Naturae*, published as early as 1758, from putting humans, great apes, and monkeys together in the order Primates. Yet it was another 100 years before this formal placement was provided with a theoretical underpinning by the joint publication of Charles Darwin's and Alfred Russel Wallace's essays on the causes of evolution [28, 153]. Although its principle was laid out in these publications, the theory of evolution – and its implications for humans' place in nature – were not widely recognized for another year, when Darwin published *The Origin of Species* in 1859 [29]. Although the book was, as Darwin himself wrote in the Introduction, just "a long argument" to support evolution as a concept, Darwin did not fail to mention that all his arguments certainly also applied to the human species. Thus, humans did not only look similar to other primates, but this similarity could be explained by the fact that they were distant relatives. This idea dethroned humans from the special position they had occupied in western thinking until then, making them the simple product of a process that also applied to any other living species.

However, its application proved extremely fruitful. Just 3 years before Darwin's publication, the Neanderthal type specimen had been discovered [49] and the theory of evolution now allowed it to be placed in a meaningful context [61]. Thus, in 1863 Huxley, "Darwin's bulldog" wrote an entire book [61] on human evolution, and 8 years later, Darwin himself published *The Descent of Man* [30], in which he not only lays out anatomical similarities but also argues for the differences in mental capabilities between humans and animals being only gradual rather than principal nature. Although erroneous in details, the book is an impressive collection of facts showing beyond reasonable doubt that humans were the product of a long evolutionary process.

18.1.2 Sarich and Wilson

After the publication of *The Descent of Man*, progress in the study of human evolution was quite slow for almost 100 years. A close relationship between humans and great apes was undisputed, and some important fossils such as *Homo erectus* and *Australopithecus africanus* were discovered. However, an important question remained unresolved. This concerned the time-scale of human evolution. In many aspects humans are so different from great apes that estimates of when humans had split from their relatives varied from 5 to 25 million years. This lack of knowledge effectively prevented any meaningful discussion about the process of human evolution. At the same time the fossil record was too sketchy and the dating of fossils too unreliable to provide more detailed information. However, this situation changed radically with the work published by Sarich and Wilson in 1967 [125]. They had used an immunological comparison to put a time-scale on human evolution by immunizing rabbits against human blood serum and then using the antibodies obtained to test their cross-reactivity to serums from different ape and monkey species. Quantification of the reactivity and the use of a calibration point, with the baboon assumed to have split from the human lineage some 30 million years before present, allowed them to estimate that humans and chimpanzees had separated probably as recently as only about 5 million years ago. This result had major implications for the study of human evolution. There was no extended time-span with a lack of fossils as believed by many researchers at that time. Rather the differences between humans and chimpanzees had evolved within a comparatively short period. In a way this result was also the beginning of a different view on these differences, which we know today, are nowhere near as big as many researchers liked to believe only a few decades ago. Thus, the work of Sarich and Wilson opened the door to a completely new understanding of human evolution that has developed during the four decades since their discovery and is based on many new fossils and a whole range of new techniques and data.

18.1.3 From a Straight Line to a Bush of Hominid Species and Beyond

Twelve years after the work by Sarich and Wilson one of the last dogmas in human evolution was destroyed. In a seminal paper Johanson and White [69] finally buried the idea that since the divergence of humans and chimpanzees from a common ancestor a straight line had led to modern humans by unequivocally showing – although strong evidence pointing in this direction had been brought forward some years earlier – that during the Pleistocene at least two hominid species had existed contemporaneously (Fig. 18.1). Since then an ever-growing number of hominid species has been discovered (see Sect. 18.2), with sometimes four or even more species living at the same time (Fig. 18.2; e.g., [15]). Consequently, the history of human evolution now looks like a bushy tree with many dead ends and unclear relationships between the various species – and in fact there is a lot of discussion about which fossils to recognize as different species at all. However, progress has not only been made in palaeoanthropology. The sequencing of DNA made it possible not only to investigate the relationship of humans to their primate relatives but also to investigate the population history of the human species in great detail, including the age of human diversity, the geographical origin of modern humans [14, 62, 148], and their various migrations (e.g., [117, 119]). More recently sequence analyses also resulted in the discovery of genes that were positively selected during human evolution, such as *FOXP2*, a gene involved in language evolution, or the gene coding for human sarcomeric myosin *(MYH16)*, a muscle protein, which may have influenced human skull morphology. Studies of these genes allowed first clues to which genetic changes are responsible for specific human traits (e.g., [40, 51, 134]). Tremendous progress in DNA sequencing has lead to the launch of a Neanderthal genome project [52], which has the potential to reveal how we differed genetically from our closest, albeit extinct, relatives. New techniques have also allowed first insights into not only differences in DNA sequences but also the extent to which the expression of genes differs between or has evolved in parallel in humans and their relatives [77]. Finally, progress has been made in understanding to what extent other animal species show behavioral features which were so far thought to be unique to humans. It has been shown that tool use in free-living animals not only exists in chimpanzees – a fact that was already known to Darwin [30] – but also in evolutionarily distant animals, such as crows and dolphins. Even more strikingly, cultural traditions, long thought to be a unique human trait, have been discovered in both chimpanzees [155] and orangutans [146].

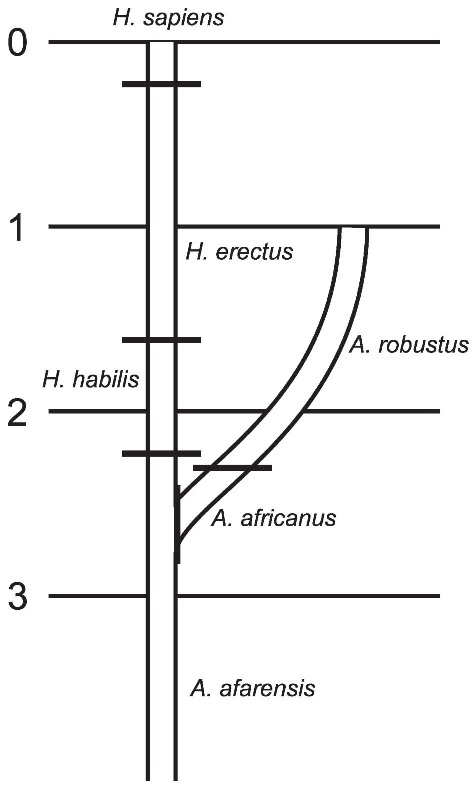

Fig. 18.1 Phylogenetic tree of human evolution from 1979. (After [69])

Progress in the study of human evolution has been substantial during the last 20 years and the future is likely to yield further insights to eventually reveal not only what makes us human but also how we got there.

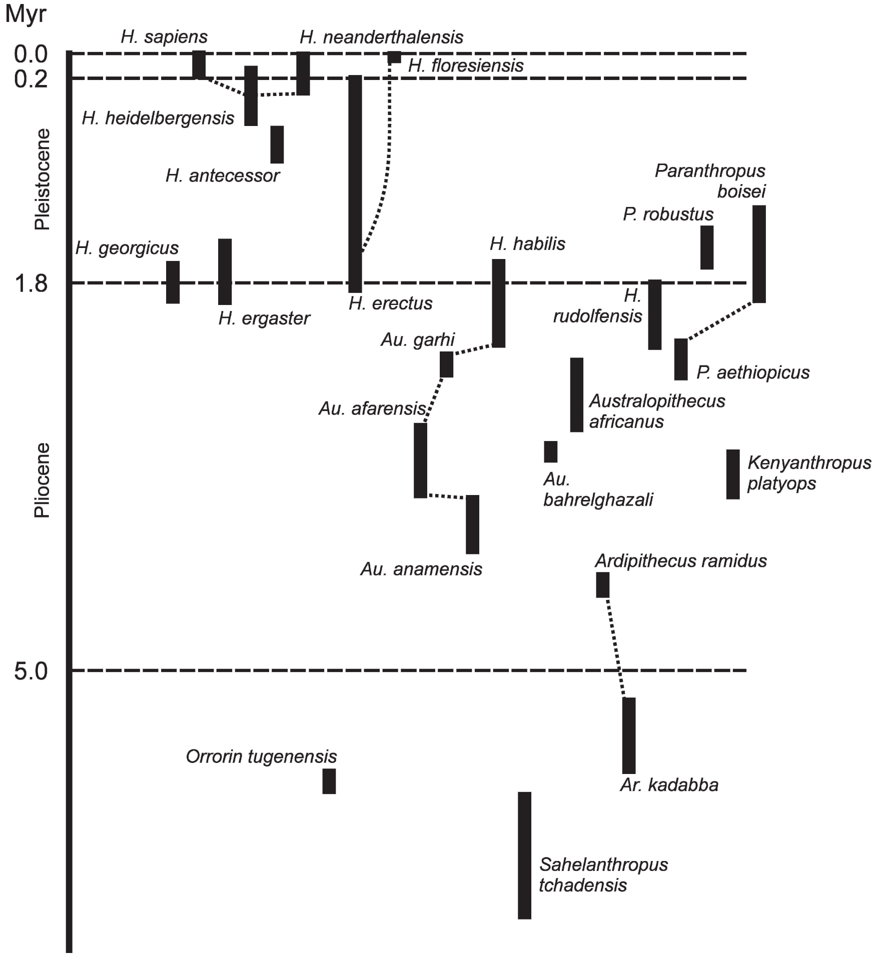

Fig. 18.2 Fossil record of human evolution. *Dotted lines* indicate tentative relationships. (After [15] and other sources)

18.2 The Fossil Record

18.2.1 Palaeoanthropology

18.2.1.1 The African Record

The fossil record testifying to human evolution has become almost fantastically diverse. The first fossil hominid to be described was the Neanderthal type specimen discovered in 1856 and scientifically described as *Homo neanderthalensis* in 1864 – although a Neanderthal skull from Engis in Belgium had already been discovered in 1829, but was not recognized as such until 1936. The next species to be discovered was *Homo erectus* in 1891, followed by *A. africanus* in 1925. In 1979, when the concept of the existence of two contemporaneous hominid lineages had gained substantial support, altogether six hominid species had already been described [69]. Today about 20 different hominid species are recognized. Many, such as the so far oldest hominid fossil *Sahelanthropus tchadensis* [12] and the Late Pleistocene dwarf species *Homo floresiensis* [11], have been discovered during the last 15 years. The former has extended the fossil record of human evolution back to almost 7 million years, bringing the fossil close to the divergence between humans and chimpanzees as calculated from molecular data. Despite the exceptionally good record of hominid fossils, the relationships between the various species are far from being clear (e.g., [157]). In part, this is due to the fact that many fossils, such as *Kenyanthropus platyops* or *Orrorin tugenensis*, are represented by only fragmentary material from few individuals. Despite many fossils, which have to span about 7 million years and a vast geographic area, the fossil record is still quite sketchy. Nonetheless, it is in many ways informative. The discovery of early hominid fossils from both East (*Orrorin tugenensis*, 5.7–6 million years and *Ardipithecus kaddaba*, 5.5–5.8 million years old) and West Africa (*Sahelanthropus tchadensis*, 6–7 million years old) shows that soon after the divergence from the chimpanzee, hominids had already spread widely across Africa. Interestingly, even these early fossils show evidence for bipedal locomotion although a recent finding of a juvenile *Australopithecus afarensis* skeleton suggests that arboreal behavior may still have played a part about 3.3 million years ago [2]. Thus, bipedal locomotion evolved very early in human evolution, much earlier than for example increased brain size, a pattern known as mosaic evolution [15]. Moreover, individual traits did not evolve in a linear fashion. For example, *Sahelanthropus tchadensis*, the earliest fossil species on the human lineage discovered to date, had a brain volume of 320–380 cm^3, similar to that in living chimpanzees. *A. africanus*, which lived at least 3 million years later, had a brain volume only 30% larger. However, within the next 3 million years, the brain volume tripled to an average of 1,355 in modern humans ([15] and refs therein; see also Fig. 18.3). Thus, the evolution of human-specific traits took place in both a mosaic and a nonlinear fashion.

Although it is not yet clear which fossils represent direct human ancestors and which represent side branches of the hominid tree that did not leave descendants, such as the robust Australopithecines *Paranthropus robustus, P. boisei, P. aethiopicus* and possibly *K. platyops*, there is no doubt that hominids proliferated in Africa for millions of years, occupying different ecological niches [69].

18.2.1.2 Fossils from Asia and Europe

In addition to the African fossil record, there have been a number of discoveries outside Africa, beginning with the recovery of a number of *Homo erectus* (or *Homo georgicus* as claimed by some authors) fossils from the site of Dmanisi in Georgia, dating back about 1.8 million years [50]. These findings, together with dating of the Indonesian *H. erectus* fossils to a similar age [138], showed that hominids left Africa quite early and were successful enough to settle in geographically distant places. Only slightly younger

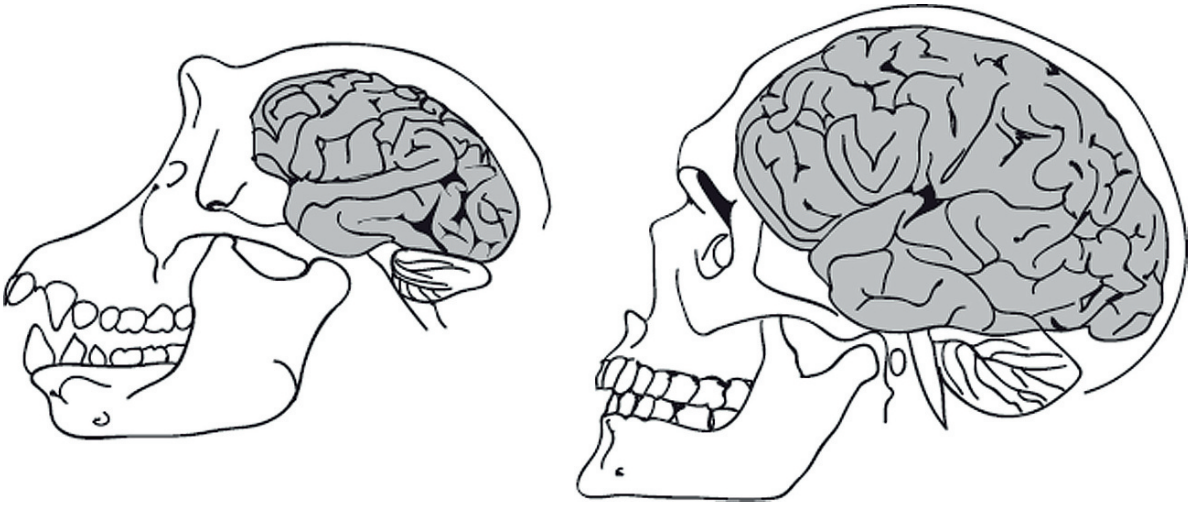

Fig. 18.3 Comparison of the brain of a chimpanzee (*left*) and that of a modern human (*right*). Note also the different shapes of the skulls in the two species. (After [15] and other sources)

artifacts from Majuangou at 40° north in China dating back between 1.66 and 1.32 million years show that hominids could survive in northern Asia over prolonged time periods as soon as the Early Pleistocene [165]. Moreover, discoveries in Spain and Italy showed that hominids occurred in Europe already as early as about 800,000 years ago. Although no hominid fossils of comparative age have been discovered north of the Alps, dating of flint artifacts from the UK indicates that hominids probably reached Europe north of the Alps as much as 700,000 years before present (B.P.) [108].

It now seems that before the dispersal of modern humans there were at least three waves of emigrations out of Africa between 1.8 and 0.5 million years ago and descendants of the different migration waves probably survived for quite a long time in both Europe and Asia. Thus, it has been argued that the Middle Pleistocene hominids from Sima de los Huesos in Spain were the ancestors of the Neanderthals [5], which disappeared about 30,000 years ago [99], and that *H. erectus* in Asia also survived until the Late Pleistocene.

Asia, with the recent discovery of *H. floresiensis*, with an estimated age of about 18,000 years, in fact yielded not only the youngest extinct hominid species to date [11] but also one of the most spectacular and controversial fossils in recent years [4, 66]. *H. floresiensis* has not only a substantially reduced body size; but it also has a very small brain size, a fact that has resulted in arguments about whether the individual discovered was healthy or rather microcephalic. This argument has been extended to the point where the species status of *H. floresiensis* has been questioned and the fossil has been interpreted as simply a microcephalic human pygmy [66]. However, most authors agree that *H. floresiensis* is indeed a separate species and most probably a descendant of *H. erectus* whose small size and specific features evolved as a result of the special evolutionary pressures on small islands [8]. This illustrates beautifully that hominids evolve according to the same evolutionary mechanisms as other animal species, a fact already known to Darwin [30].

Overall, the fossil record has been quite informative with regard to human evolution, but owing to the rapid decay of DNA nothing is known about genetic differences between us and our ancestors or cousins – with the one exception of the Neanderthals.

18.2.2 Neanderthals and Ancient DNA

18.2.2.1 Mitochondrial Sequences

In many ways Neanderthals (*H. neanderthalensis*) occupy a special position in the study of human evolution. They were the first extinct hominid species to be discovered; they are the closest relative of modern *H. sapiens*; and they are the first – and so far only – extinct hominid from which DNA sequences have been obtained [82]. Since the initial publication of DNA sequences from the Neanderthal type specimen almost 10 years ago, mitochondrial (mt) DNA sequences have been obtained from 13 additional specimens (Fig. 18.4) [81] all showing that with respect to mtDNA, Neanderthals fell outside the variation of modern humans, with the two lineages having diverged about 600,000 years ago [83]. While for some time it looked as though if Neanderthals carried very low levels of sequence diversity, several recent publications report somewhat more divergent mtDNA sequences suggesting that sequence diversity in Neanderthals was not that depleted and was probably similar to that in modern humans. Moreover, the identification of Neanderthal mtDNA sequences from the Altai Mountains also extended the geographical range of this extinct human group [81].

These data have not only been used to determine the phylogenetic relationship of Neanderthals and modern humans, they also made it possible – together with comparative analyses on fossils of modern humans – to estimate the maximum amount of mtDNA gene flow that could have been from Neanderthals to modern humans. Using a very conservative approach, Serre et al. [130] concluded that this contribution could not have been larger than 25%. With a more realistic migration model for modern human colonization of Europe a follow-up publication using the same data even concluded that no more than 0.1% gene flow could have occurred [27].

18.2.2.2 Palaeogenomics

While further work on more individuals would be desirable to clarify the extent and possible geographical structure of mtDNA sequence variation in Neanderthals, all the above analyses have the disadvantage that

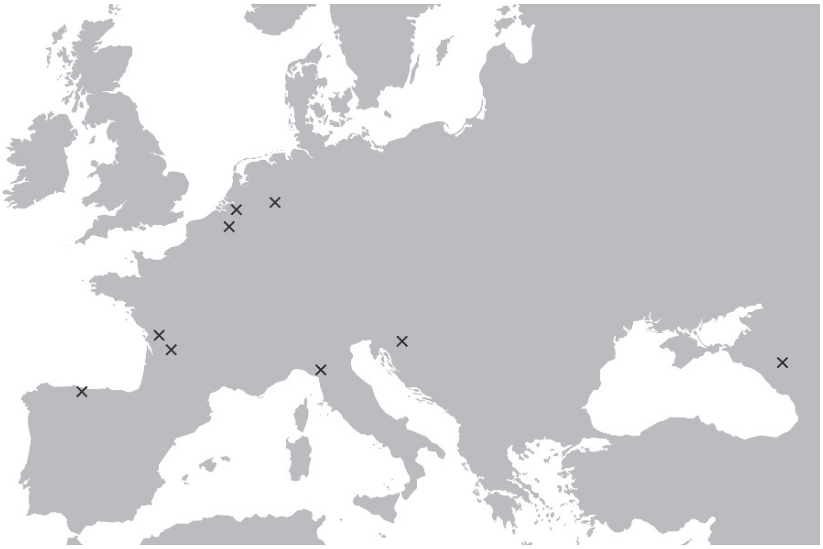

Fig. 18.4 Sites in Europe from where Neanderthal mtDNA sequences have been obtained. Recently Neanderthal mtDNA sequences were also found in fossils from Teshik-Tash, Uzbekistan, and Okladnikov Cave, Russian Altai [81], not depicted on this map

mtDNA represents just a single genetic locus, which is, moreover, inherited only through the maternal lineage. Although for a long time analyses of nuclear (nu) DNA from Neanderthal fossils seemed impossible, progress in the analysis of ancient DNA analyses has made it possible. Thus, two recent publications [52, 106] have reported sequencing and analyses of nuDNA. These two studies used rather different techniques. The first used ancient DNA extracts taken from a Neanderthal specimen from Vindija cave, Croatia, and put the DNA into the bacterium *Escherichia coli*. In this way a genomic library is constructed which can then be sequenced. The second study used a new technique allowing direct sequencing of hundreds of thousands of DNA fragments, which was applied to extracts from the same Neanderthal individual. However, what the two techniques have in common is that they are shotgun techniques, i.e., they do not target any specific sequence region but simply give a random cross section of the DNA present in the ancient DNA extracts. Unfortunately, only the minority of this DNA originates from Neanderthals. Thus, only about 6% of the sequences obtained were of hominid origin, with the remaining ones representing other organisms living in and on the bone, such as fungi, bacteria, and many unknown sequences, which are typically found in such metagenomics studies. Even so, the two studies reported 65,000 bp [106] and about 1 million bp [52] of Neanderthal nuDNA sequences, respectively. These data allowed some interesting insights. First, both techniques showed the Neanderthal sequences evenly distributed along the chromosomes, suggesting there is little bias with respect to different parts of the genome represented in the sequence reads. This is an important insight, as it shows that it is in principle possible to sequence the complete genome of *H. neanderthalensis*. Second, in both studies the divergence time between human and Neanderthal DNA sequences was estimated with somewhat different, albeit overlapping, results. Noonan et al. give a best guess of about 700,000 years (with the confidence interval spanning 450,000 to 1 million years), whereas Green et al., with their larger data set, arrive at 516,000 years with a smaller confidence interval of between 470,000 and 570,000 years. Strikingly, this is hardly older than the average sequence divergence between two modern humans. In addition, Noonan et al. also estimate the population divergence time between humans and Neanderthals, which with 370,000 years is even younger, as sequence divergence always predates population divergence (Fig. 18.5). Although Green et al. did not estimate population divergence, their younger sequence divergence date shows that they would have arrived at an even younger date. These results have several important corollaries.

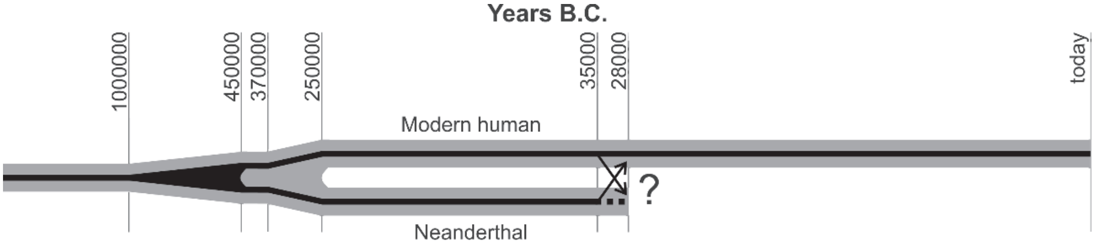

Fig. 18.5 Scheme of modern human–Neanderthal relationships. The *black lines* indicate DNA sequences and the *gray shades*, populations. Between 450,000 and 1 million years ago the nuclear DNA sequences separated, while Neanderthal and modern human populations split only about 370,000–250,000 years ago, according to data published by Green et al. [52] and Noonan et al. [106]. Modern humans entered Europe at least 35,000 years ago, while Neanderthals survived until 28,000 years ago or later, leaving at least 7,000 years for potential gene flow

If true, many hominid fossils, such as the older Atapuerca findings from Spain or the El Ceprano fossil from Italy, are too old to represent Neanderthal ancestors. Moreover, there will be few fixed differences between humans and Neanderthals. In fact, Green et al. found that at many DNA sequence positions where modern humans are polymorphic, Neanderthals show the derived sequence state, which the authors interpret as possible evidence for gene flow from modern humans to Neanderthals. This is extremely interesting as the Neanderthals from Vindija have previously been described as unusually gracile and as possible examples for hybridization between humans and Neanderthals. An alternative explanation would be that some sequences represent contamination of the experiments with modern human DNA, a common problem in ancient DNA studies (e.g., [58, 130]). Although in both studies the authors tested for contamination of the extracts with modern human mtDNA, which was found to be very low, it is not clear whether these results are also representative for nuclear DNA. Only more data if possible from several fossils, will clarify how much time and how many sequence differences separate us from Neanderthals.

However, it is noteworthy that population genetic studies on modern humans also found evidence for admixture of DNA sequences from archaic hominids to the gene pool of modern humans [111]. For this to be true, admixture would have to have occurred during a relatively restricted time frame. Although the presence of modern humans and Neanderthals in Europe overlapped temporarily [101], this overlap was probably shorter than previously believed. The oldest modern human remains are from Pesterca de Oase in Romania and have been directly dated to about 35,000 years [143], although there is evidence for the presence of modern humans at other sites, e.g., in south-western Germany around the same time [28]. Conversely, the youngest evidence for the presence of Neanderthals in Europe is from Gibraltar and suggests a terminal age for the existence of Neanderthals of 28,000 years B.P. or younger. Although this allows for 7,000 years of interaction between modern humans and Neanderthals, the sites for the oldest modern human fossils and the youngest Neanderthal fossils are geographically quite distant and the youngest dates for the geographically closer Vindija Neanderthals have recently been revised to about 31,000 years. Despite these revisions, there is little doubt that modern humans and Neanderthals not only met but also interacted ([99, 101] and refs therein). However, whether gene flow occurred during this process, and if so, to what extent and in which direction can only be revealed by future studies.

18.3 The Genetics of Human Evolution

18.3.1 The Genomes of Humans and Their Relatives

Phylogenetically, humans belong to the African great apes, as do gorillas, which represent the first branch diverging from a common ancestor some 8 million years ago, and chimpanzees and bonobos, two closely related sister species that diverged from the human lineage probably 6–7 million years ago (Fig. 18.6).

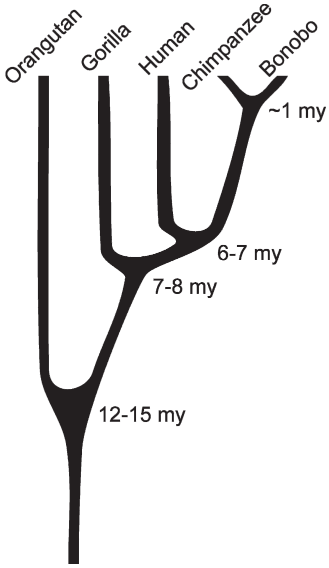

Fig. 18.6 A schematic phylogenetic tree showing the relationships and approximate time-scale for the evolution of humans and great apes. The time estimates for the different divergence events are averages from various studies. It can be seen that humans are evolutionarily as distant from bonobos as they are from common chimpanzees

Ultimately, human evolution is simply a long chain of changes in the genome that have taken place since our own lineage and that of the chimpanzee separated some million years ago. Thus, in a way, the key to human evolution lies in genome sequences. The first published genome sequence was that of the human mitochondrial genome [3] with a length of about 17 kb. This was followed by the homologous sequences from the closest relatives of humans, chimpanzee, gorilla, and orangutan, which allowed scientists to identify the chimpanzee as the human's closest relative [56, 57], a result soon corroborated by nuclear DNA sequence data [57]. However, later analyses have shown that the picture is more complex. An analysis of 53 nuclear loci showed that although for most regions humans and chimpanzees are most closely related, the authors found trees incongruent with that phylogeny for 22 of the 53 loci (42%, [22]). Thus, at many parts of the genome, gorillas are more closely related to either humans or chimpanzees than humans and chimpanzees are to each other. This is possible because in the nuclear genome recombination is a regular event, which breaks up the genetic linkage between regions on a chromosome. Thus, the history of different regions in the nuclear genome may be different. Moreover, the common ancestor of two species always harbors sequence variation. If two populations separate, and eventually evolve into two different species, for some time some of the sequence variants in one species may be more closely related to sequence variants in the other species than to different sequence variants within the same species. Eventually, each species will become fixed for one lineage and its descendants, and the sequence variation between the two species becomes reciprocally monophyletic, a process called lineage sorting. However, if one of the species splits again into two daughter species shortly after the first divergence, lineage sorting may not yet be complete and some sequence variation predating the first population divergence can persist. In this case lineage sorting may occur in a way that the genetic tree for certain regions does not reflect the population divergence tree (Fig. 18.7). As the speciation events of humans, chimpanzees, and gorillas took place within a short time, the ancestral population of humans and chimpanzees still harbored some sequence diversity that predated the divergence of the gorilla, allowing for some genetic regions being more closely related between gorillas and either of the two other species than between these two. Although a recent study [109] showed that the proportion of such regions is somewhat lower than previously assumed, with about 30%, it is still a substantial part of the genome. Even more interestingly, the authors found the divergence between human and chimpanzee sequences to vary substantially among

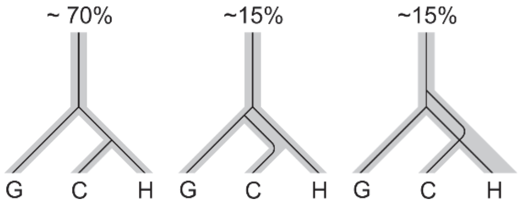

Fig. 18.7 Three possible ways in which genetic lineages can be related and their approximate proportions in genomes of humans, chimpanzees, and gorillas. *Black lines* represent genetic lineages; the *gray outline* shows the population divergence pattern. At each point in time, the populations harbor genetic diversity, which is not shown in the figure. Therefore, when the time between two divergence events is short, some of the genetic diversity in the population at the second divergence may actually predate the first divergence event resulting in the lineage sorting pattern shown in the *middle* and to the *right*. That way, there are three possible ways in which genetic lineages can be related, although there is only one pattern for the population divergence between the three species

different regions of the genome, corresponding to a time range of as much as 4 million years. They explained these results by a complex speciation history of humans and chimpanzees, including secondary hybridization between the two lineages [109]. However, the data could also be explained by a model with strictly allopatric speciation in combination with a large ancestral population size [7]. Although it is not yet clear which interpretation is correct, this example shows the power of genomic analyses to infer events going back to the roots of human evolution.

So far, complete genomes have been sequenced from a number of mammalian species, including mouse, rat, dog, human, and chimpanzee, and more are soon to come. The human genome was the first mammalian genome for which a draft sequence became available [89]. In 2004, the completion of the euchromatic genome sequence was published [35], which contained only 341 gaps, as compared to about 150,000 gaps in the draft sequence. However, the finished genome confirmed, with an estimate of only 20,000–25,000 protein coding genes, the conclusion from the draft sequence that the human genome contains much fewer protein coding genes than previously believed. Completion of the draft sequence of the chimpanzee genome [37] made it possible to estimate the sequence divergence between us and our closest living relatives with great accuracy. Surprisingly, only 1.06% or less of the positions represent fixed differences between the two species [37]. However, owing to insertions or deletions on either lineage, 1.5% (or 40 million basepairs) of the genome sequences are unique in each species. Segmental duplications on one of the lineages change the genomic landscape between humans, with 2.7% difference, more than nucleotide substitutions, with 1.2% difference. They also influence the expression of certain genes [23]. Moreover, a comparison of humans, chimpanzees, bonobos, orang-utans, and gorillas showed that more than 1,000 genes are not present in all species but are unique to one or several of these species and that 134 of these have been created by duplication on the human lineage [48]. How many of these new genes are under positive selection is not yet clear, but for a gene family called Morpheus this has been clearly shown [70]. This gene family expanded in both humans and great apes, with as many as 15 copies present in the human genome and between 25 and 30 copies in the chimpanzee genome. Strikingly, some of these new copies show extremely high Ka/Ks ratios, a strong indication of positive selection acting on these genes. Another gene family shows an even more extreme pattern. One of the 134 human specific genes found in the above-mentioned study encodes a primate-specific sequence domain (DUF1220) that has become extremely amplified on the human lineage with at least 212 copies present in the human genome [112]. In a similar way to Morpheus, many of these sequences show high Ka/Ks values indicating that positive selection has influenced these sequences. Moreover, many of the genes carrying a DUF1220 domain are expressed in neuronal cells in brain regions associated with higher cognitive functions [112]. Thus, gene duplications are likely to have contributed substantially to adaptive processes during human evolution.

There are also large-scale chromosomal differences between humans and great apes, the best known ones being the fusion that created human chromosome 2 and the human-specific pericentric inversions on chromosomes 1 and 18. A recent study probing 12% of the human genome found 63 large-scale, yet cytogenetically undetectable, sites of copy number variations among humans and the great ape species [92]. These rearrangements range in length between 40 and at least 175 kbp, and most of them were found to be located in euchromatic, gene-rich regions of the genome, adding substantially to the differences between humans and their closest relatives.

Thus, humans differ from their closest relatives in many aspects of their genome, ranging from the about 35 million single nucleotide substitutions that separate humans and chimpanzees [37] through small-scale insertion/deletion events and larger sequence duplications to the cytogenetically visible differences, such as the fusion of two ape chromosomes that created human chromosome 2. Yet, besides these differences between the species, there is also ample variation within species, including humans.

18.3.2 Diversity Within the Human Genome

The first studies investigating diversity within humans were done using proteins [16]. Even these early studies showed substantial genetic diversity within the human species. However, the extent of genetic diversity within the human genome could only be properly assessed when analyses of DNA sequences became possible. One of the first surveys investigated restriction fragment length polymorphisms in the beta-globin region from a world-wide sampling of 601 humans [152] and found greater diversity within African populations than within populations outside Africa. A key study with respect to such analyses was published in 1987, when Cann and colleagues presented restriction fragment analyses data from mitochondrial DNA of a world-wide sample of 147 humans [14]. In their survey, they found that 195 of 467 restriction sites were polymorphic in humans. Again, African populations showed the highest diversity. However, as a restriction site contains several nucleotides, where only one needs to be different to create a polymorphism, these data did not allow estimation of mtDNA sequence diversity within humans. The next step in this direction was taken when 189 humans were sequenced for 610 bp of the mitochondrial control region [148]. These samples yielded 135 different sequences, with 179 positions that were polymorphic, i.e., almost a third of the positions studied. As the control region is the most variable part of the mitochondrial genome, these data were also not representative for the mitochondrial genome. Eventually, in 2002, Ingman et al. [62] sequenced complete mitochondrial genomes of 69 humans from a world-wide sampling and found that 657, or about 4%, of the approximately 16,500 bp of the mitochondrial genome were variable in modern humans. Owing to this high within-species diversity, mtDNA has been used extensively in the study of modern human migrations (see Sect. 18.4.1).

Compared with mtDNA sequences, nuDNA sequences show much lower levels of diversity. The first world-wide study of human genetic diversity in the nuclear genome used the same sampling set as the above study on complete mtDNA genomes and surveyed 10 kb on the X-chromosome [72]. Strikingly, only 33 positions, i.e., 0.33% of the analyzed sequence, were found to be variable. Additional studies on the X-chromosome (e.g., [159]) and also on autosomes [163, 164] found similar, albeit somewhat higher levels of variation with between 44 and 75 variable positions within about 10 kb of nuclear sequence. Generally, all these studies indicate a low amount of mean pairwise sequence difference between two human chromosomes, averaging about 0.1% [163].

This low amount of sequence diversity within humans is in stark contrast to the extent of sequence diversity within our closest relatives, the great apes. Sequencing the same 10-kb region of the X-chromosome as in humans, Kaessmann et al. [73] found, with 84 variable positions, more than twice as many variable positions in only 30 chimpanzees than in the above 69 humans. On the same locus more variation than in humans was also detected in 11 gorillas (41 variable positions) and 14 orang-utans (78 variable positions; [74]). Similar results were found for the Y-chromosome, with both chimpanzees and bonobos showing higher levels of sequence variation than humans [135]. However, autosomal sequences show a somewhat different picture. Using about 23 kb from 50 different autosomal loci, Yu and colleagues [160] found bonobos to have a mean pairwise sequence difference (pi) that is even lower, at 0.078%, than that in the corresponding sequences in humans (0.088%). Although chimpanzees show a somewhat higher pi-value (0.132%), the difference between humans and chimpanzees in within-species diversity is much smaller in this study than in the above studies on mtDNA, X-chromosome, and Y-chromosome. Although western lowland gorillas show somewhat higher sequence diversity for these loci, with 0.158% it is only about twice as high as in humans [161]. Taken together, sequence data currently indicate that diversity within the human nuclear genome is somewhat lower than in some great ape species, but comparable to that within bonobos and the western chimpanzee subspecies [160].

However, the term ‘low diversity’ should not be misunderstood. An average sequence diversity of 0.1% between two individuals sums up to 3 million differences across the complete genome. As polymorphic positions can be used for various purposes, including the mapping of genetic diseases, efforts are under way to create extensive data bases of such single-nucleotide polymorphisms (SNPs). In a recent study report the results of the first phase of the HapMap project, a collection of high-quality SNPs in the human genome, was published [36]. This study involved more than 1 million SNPs with a frequency of the minor allele higher than 5%. In addition, 10 regions with 500 kb in length were sequenced in 48 humans, indicating that the overall frequency of SNPs is about 10-fold higher, with one SNP occurring every 279 bp [36]. In fact, given the number of about 6.5 billion people currently living on earth and the substitution rate of nuclear DNA, every mutation that is compatible with life should be present in at least one individual of the global human population.

In addition to single nucleotide variation there are several other forms of within-human genetic diversity. For example, there are repetitive elements in the human genome that occur in extremely large copy numbers. Two classes of these repetitive elements are SINEs (short interspersed elements, less than 500 bp in length) and LINEs (long interspersed elements). Both LINEs and SINEs increase in numbers by retrotransposition (see, e.g., [8, 132] for reviews). In other words a copy is transcribed into RNA, which is then reverse-transcribed into DNA and inserted somewhere in the genome. The most common SINE element in the human genome is the primate specific Alu family, a 300-bp-long sequence element with altogether more than 1 million copies, which comprise about 10% of the human genome [8]. Alu elements tend to insert in gene-rich regions and can therefore both influence the expression of genes and interrupt genes, resulting in genetic disorders. Owing to their high sequence similarity, Alu elements can also cause recombination between two Alu elements in different regions of the genome, resulting in deletions, duplications, or translocations, which may among other things cause cancer (for a comprehensive review see [8]).

As only a few Alu elements are multiplied at a time, Alu elements can be divided into different families. Although most of these insertions are fixed in humans, there are also families that are quite polymorphic, such as the subfamilies Alu Yc1, Yc2, and Yb9 [122], for which about a third of the members within each family have been found to be polymorphic for presence / absence in humans. Altogether about 5,000 Alu elements inserted into the human genome after the divergence between humans and chimpanzees, but not all of these are polymorphic [8]. Polymorphic Alu elements were also identified on the human X- and Y-chromosomes and represent excellent markers for population genetic studies [122], as Alu element insertions at a specific site in the genome are almost certainly unique events, with very little chance of reversal. Thus, parallel or back-mutations hardly ever occur [8], making them unusually robust markers for inferring evolutionary events.

LINE elements are in many ways similar to SINEs, but they are present in lower copy numbers of about 100,000 in the human genome [132]. However, as they are longer, they also comprise about 15% of the total human genome. LINEs have expanded in numbers in the human genome during the last 100 million years of mammalian evolution, but analogously with Alu elements, there are LINEs that are human specific, such as the L1 Ta subfamily, for which 12% of 249 loci were found to be polymorphic in humans [150]. Together with polymorphic Alu elements, LINE elements of the L1 subfamily are likely to comprise as many as 2,000 polymorphic markers in the human genome, comprising a large amount of genetic diversity within humans and at the same time providing a rich source of information for population studies [132].

Finally, another source of within-species diversity has recently been discovered in the human genome, so-called large-scale copy number polymorphisms (CNPs; reviewed in [21, 47]). These regions range in size from about 1 kb to as much as 3 Mb [47] and have been found to be surprisingly common in the human genome. Thus, an initial screening of 20 individuals revealed 221 CNPs, with an average length of 465 kb, and individuals differing by an average of 11 CNPs. Other studies found similarly high levels of structural variation, indicating that this type of variation may contribute substantially to genetic diversity in humans. As the pieces involved are large, the similarity between two humans may indeed be less than the usually assumed 99.9% [25]. Moreover, as these duplications contain large numbers of genes, they are of substantial clinical relevance [21, 47]. A recent genome-wide study on copy number variations (CNV) in 270 individuals from a world-wide sampling found 1,447 CNVs en-compassing a total of 360 Mb, or 12% of the genome [115]. Interestingly, CNVs encompass, genome-wide, more nucleotides than SNPs [115]. Thus, in a way, CNVs represent more genetic diversity among humans than do SNPs. Moreover, there is evidence that certain regions are hotspots for CNVs, as CNVs have been observed at corresponding loci in both humans and chimpanzees.

Overall, the human genome contains substantial variations between individuals, ranging from individual nucleotides to large-scale structural variations. During human evolution, this variation has been subject to both positive and negative selection.

18.3.3 Positive and Negative Selection in the Human Genome

A key feature of Darwinian evolution lies in the fact that selection works on random variation, i.e., mutations occur in the genome and these are either selectively neutral or positively or negatively selected. In 1968, Kimura proposed that most amino acid substitutions that become fixed in a species are selectively neutral [78], i.e., they have neither a positive nor a negative effect on the fitness of their carrier. It is important to note that the neutral theory does not claim that most mutations are neutral – in fact Kimura assumed that most newly arising mutations are deleterious, but will be removed quickly from the population via negative selection and therefore have no chance of getting fixed. The critical point of the neutral theory is that it assumes that very few substitutions become fixed due to positive selection. To what extent this is true is still a matter of debate. For example, Fay and colleagues estimated that as many as 35% of the amino acid substitutions that were fixed on the human lineage were driven by positive selection [46], a value similar to that estimated for the *Drosophila* genome, for which up to 50% of the substitutions are estimated to become fixed as a result of positive selection [44]. Extrapolated to the human genome, this would result in about 70,000 amino acid substitutions having been under positive selection at some time in human history [15]. If this number is correct, it will be extremely difficult to identify genes that have been under positive selection during human evolution, as each protein would on average have accumulated two or more positively selected amino acid substitutions during the last 5–7 million years. At the same time this number translates into one adaptive substitution getting fixed on the human lineage every 100 years. However, more recent estimates have led to quite different results. Some studies have put the proportion of genes in the human genome that have been under positive selection close to zero [37, 162], and the highest recent estimate for genes under positive selection is about 6% [13]. Thus, the neutral theory seems to apply well to the human genome. Still, if we take a range of estimates between 0.4 [105] and 6% [13] for the proportion of substitutions fixed as a result of positive selection, the number of adaptive substitutions in the human genome still ranges between 800 and 12,000.

Therefore, even with these reduced numbers, searching for genes that have been under positive selection is somewhat like searching for a needle in a haystack. Yet several candidate genes have been identified. Among the first genes claimed to be under positive selection were several proteins critical for male reproduction [158]. Although this claim has been questioned, it does not seem too far fetched, as male reproductive genes are under positive selection in many species and more recent studies have confirmed that male reproductive genes are a primary target of positive selection in humans [124]. Thus, this does not represent an example for selection on a human-specific trait such as upright walking, increased brain size, or language capability, to name just a few (see [15] for more examples). Research focusing on the genetic basis of such human-specific traits is of great interest, as it has the potential to provide insights into what makes us human.

One of the most widely recognized findings along these lines was the identification of a gene that, when defect, causes language disorders, the forkhead protein FOXP2 [86]. Strikingly, even though FOXP2 is a highly conserved transcription factor comprising only one amino acid difference between chimpanzee and mouse, two additional amino acid changes have occurred in the human lineage [40]. Sequencing of 14 kb around the two amino acid substitutions differentiating humans and chimpanzees showed evidence for a recent selective sweep sometime during the last 200,000 years having led to their fixation [40]. While it is unlikely that a complex trait such as language evolved via only two amino acid changes in a single transcription factor, further evidence has been found that FOXP2 does indeed play a crucial part in vocalization. First, additional mutations in FOXP2 resulting in language disorders have been identified in humans [95], and the regions of expression of FOXP2 in human and mouse brains during embryonic development correlate well with the regions of pathology in adult human brains of individuals carrying a defect allele of FOXP2 as suggested by neuroimaging [87]. Second, and much more intriguing, FOXP2 seems to play a critical part in song learning in birds. The first evidence that FOXP2 may also have a role in vocalization in birds' was provided by the fact that its regional expression in the bird's brain is highly similar to that in human brains. Moreover, in song-learning birds such as zebra finches, FOXP2 expression in brain regions critical for song learning varies over time [55]. Thus, in zebra finches it is up-regulated at 35–50 days after hatching, when song learning occurs, and in canary birds FOXP2 expression varies seasonally [55]. These results are strong evidence that FOXP2 has a critical role in vocal learning and may indeed have played an important part in language evolution in humans.

Another human-specific trait that is of prime interest is brain size. Brain size increased only moderately during early human evolution but started to increase rapidly with the appearance of the genus *Homo* in the fossil record about 2.4 million years ago [15]. This increase in brain size was paralleled by a substantial decrease in masticatory muscles even in early representatives of the genus *Homo*. Intriguingly, Stedman and colleagues identified a myosin gene that is expressed only in the muscles of the head and contains an inactivating mutation in humans [134]. Application of molecular clock dating yielded an age of about 2.4 million years for the inactivating mutation, temporally almost exactly coinciding with the appearance of *Homo* in the fossil record and the beginning of accelerated brain size increase in human evolution. However, whether these results indeed provide evidence that inactivation of this gene removed a selective constraint on the increase of brain size, as speculated by the authors, is not clear.

In contrast, there is little doubt that genes influencing the nervous system, and especially the nervous system development, have played a critical part in human evolution. A faster rate of protein evolution has been found for such genes for primates than for rodents, and the effect is most pronounced on the lineage leading to humans [33]. Genes influencing brain development and size seem to show particularly rapid protein evolution on the human lineage [51], although some of these genes also show similarly rapid evolution in other primate lineages, such as the gene ASPM on the gorilla lineage [80]. Thus, the first steps in identifying the genetic changes underlying human-specific traits have been taken, and more results can be expected in the future.

However, research on positive selection in humans has not been restricted to differences between humans and the great apes; the large amounts of SNP data [36] available for different human populations have also resulted in renewed interest in local adaptive evolution in humans, i.e., selection of certain traits in some geographical regions but not in others. Even given these large data sets, identifying candidate genes for local adaptive selection is not straightforward, and even if candidate genes are identified, understanding their function and the selective forces driving their evolution is not a trivial task (reviewed in [124]). To screen these data for possible signs of recent positive selection, several new tests have been developed. One of the most commonly used ones is the extended haplotype homozygosity test (EHH;[123]) and its variants. This test relies on the assumption that recent positive selection leaves a signature of a selective sweep in the form of reduced diversity around a selected site. Thus, if selection has been acting on one population but not on others, the former population is expected to have significantly extended homozygosity around the selected position relative to the later ones. Using the HapMap data [36] it has been shown that recent local adaptation has been taking place in all three major continental groups (Asia, sub-Saharan Africa, and Europe) generally studied for SNPs [151].

The example for local adaptation that is probably best known and understood is sickle-cell anemia which occurs at high frequency in certain regions of Africa where malaria is also prevalent. Despite strong selection against the sickle cell allele in its homozygous state, heterozygous individuals have a sufficient selective advantage in regions with high malaria prevalence to keep the sickle cell allele at high frequency via balancing selection [84]. Another genetic change conferring malaria resistance, a mutation at the *G6PD* gene, was recently found to have been under balancing selection long before malaria became prevalent in Africa, and it may thus originally have had a different adaptive function [147]. Another trait that has long been suspected of being under local positive selection in humans is skin color. There is wide geographical variation in human skin color, but whether these differences are adaptive has long been a matter of debate. In several studies Jablonski and Chaplin systematically investigated the extent of exposure to UV radiation and of skin reflectance in a particular population [64]. In their studies they found a strong correlation between UV radiation and skin reflectance of the exposed population. They explained these results by a trade-off between vitamin D synthesis – for which UV radiation is necessary – and photolysis of folate by UV radiation. Thus, in regions of high UV radiation, vitamin D synthesis is still possible with comparatively dark skin, which at the same time limits folate photolysis, whereas in regions of low UV radiation skins have to be light to ensure sufficient vitamin D synthesis and folate photolysis is not a severe problem. Although additional factors are likely to influence skin color in human populations, Jablonski's and Chaplin's data clearly provide a solid basis for studies on the evolution of skin color in different human populations. Recently progress has also been made in understanding the genetic basis of skin color differences in humans. Skin color in mammals is influenced by a number of genes [65] and differences in skin color can therefore be due to various genetic changes. It has been known for some time that the gene *MC1R* influences both hair and skin color and is under negative selection for full activity in Africa, whereas a number of loss-of-function mutations were found in Europeans at a high frequency, due to either loss of constraint or to positive selection for loss-of-function [116]. A stronger case for positive selection for light skin color in Europeans was made for a gene called *SLC24A5*. Initially found to be locally selected in humans in a whole genome screen [36], its function was at that time unclear. However, the same gene was later identified as a key pigmentation gene in zebra fish, and analyses of human populations have shown that it accounts for about a third of skin color variation between Europeans and West Africans [88]. Moreover, this gene shows signals of recent positive selection in Europeans, supporting the hypothesis that light skin color was indeed selected for in Europe.

Another classic example for recent local selection in humans is lactose tolerance. Most humans, like most other mammals, express the enzyme lactase, which allows digestion of the milk sugar lactose, only during infancy and lactase expression rapidly declines after weaning. However, it has been known for a long time that some humans continue to express lactase also as adults, and the frequency of this trait closely correlates with the consumption of unprocessed milk [137]. Thus, lactase persistence, and consequently lactose tolerance, is high in northern Europe and declines toward the south (Fig. 18.8). Yet, formal genetic evidence for positive selection on this trait had not been provided for a long time. By typing more than 100 SNPs in a region of 3.2 Mb around the lactase gene, Bersaglierie and colleagues finally found strong evidence that one lactase allele was indeed positively selected in Europeans [9]. Moreover, they found the selective signal to be one of the strongest observed for any gene in the human genome. Also, molecular dating suggests that strong selection on this allele started about 5,000–10,000 years ago, which is consistent with dairy farming being the cause of a selective advantage of lactase persistence. Apart from Europeans, several other populations in the world show lactase persistence, but the nucleotide position likely to be responsible for this trait in Europeans (C13910T) is found at very low frequency in these populations. Intriguingly, two independent studies in 2006 identified several SNPs that are associated with lactase persistence in Western African populations [63, 140]. Thus, lactase persistence seems to have evolved at least two times independently in human populations, following the same evolutionary pressure.

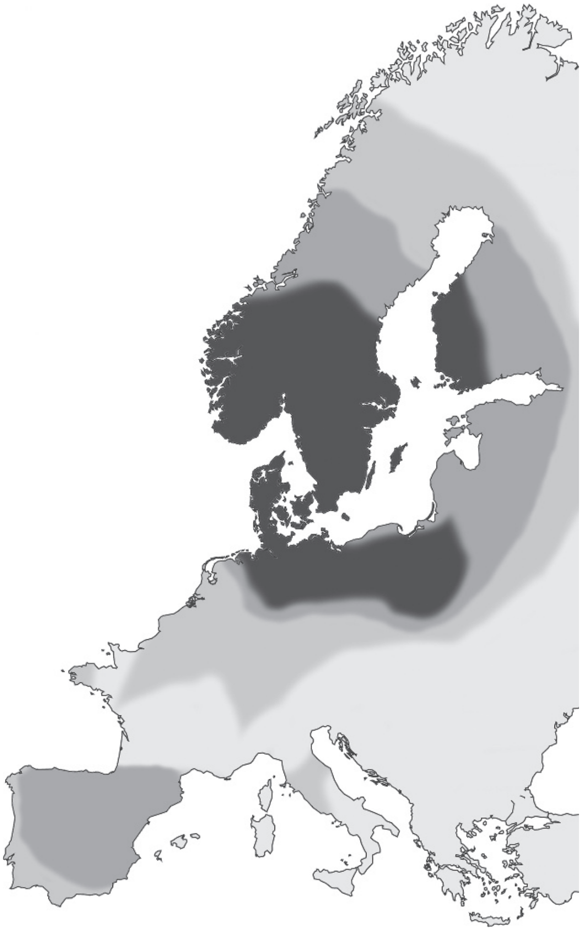

Fig. 18.8 Differences in allele frequency of lactose tolerance in Europe. *Darker shading* indicates higher frequencies of lactose tolerance

Finally, some studies have led to the provocative claim that local adaptive selection also affected genes influencing brain development [42, 98]. The authors studied two genes known to influence brain size and to have been under positive selection during human evolution, the microcephalin gene (*MCPH1*; [42]) and the *ASPM* gene (abnormal spindle-like microcephaly associated; [98]). For both genes, they found alleles that showed strong evidence of having been under recent positive selection, such as extended haplotype homozygosity. Intriguingly, these haplotypes are not fixed in the human population and their frequency differs between regions from 0 to 60% for *ASPM* and from 3.3 to 100% for *MCPH1*. Additional analyses suggest that the selected variant of *MCPH1* arose only about 37,000 years ago in the human population [42] and that of *ASPM* even more recently, i.e., about 5,800 years ago [98]. Whether these alleles confer any functional differences and which evolutionary forces have been driving their increase in frequency are so far unanswered questions. However, a recent study found no association between either of the supposedly selected variants and neither brain size nor measures of cognitive performance, calling into question the initial interpretation that the two genes have been selected for brain-related effects [139]. Yet the *MCPH1* story contains another interesting twist. Although the presumably selected haplotypes have a coalescent age of only 37,000 years, their divergence to the nonselected haplotypes dates back as early as 1.1 million years [43]. Applying simulations to obtain data similar to the ones observed, Evans and colleagues concluded that the best explanation for the observed pattern is admixture from an archaic hominid population such as the Neanderthals. However, whether this is true and modern humans thus may have benefited from genetic contributions of archaic hominids needs to be tested in further studies.

18.3.3.1 Human Evolution and Medical Genetics

It has long been hoped that the knowledge about human evolution, and especially human genetics, will improve the possibilities of treating or curing human diseases, especially widespread ones, such as cancer, heart diseases, or type 2 diabetes. Enormous hopes have been set in the decoding of the human genome. As mentioned above, the genetic factors known to influence susceptibility to malaria have long been known and studied in great detail. Yet, a widely effective treatment for malaria has still not been found and malaria remains one of the most deadly diseases in tropical regions, with more than 1 million victims every year. Thus, knowledge about genetic risk factors does not necessarily result in better treatment, and this is true not only for infectious diseases but also for cancer and heart disease to name just two groups.

With the advent of ever-cheaper DNA sequencing and the resources of millions of SNPs (see Sect. 18.3.2) in the human genome, two areas of medical genetics have been the focus of increased research efforts: personalized (sometimes also called Darwinian) medicine and genome-wide association studies searching for risk factors for common diseases. As these two fields are especially linked to human evolution only these topics are discussed in this chapter. However, it should be noted that there are many more research areas in medical genetics, which are beyond the scope of this chapter.

The investigation of DNA sequences has been completely transformed during the last 2 years. Instead of ~70,000 bp that are obtained in a single run on a capillary sequencer using standard Sanger DNA sequencing, new sequencing techniques allow between 20 million and 1 billion basepairs to be sequenced in a single run [60]. Thus, the idea that complete genomes may be sequenced from individual patients to allow medical treatment to be specifically tailored, a concept known as "personalized" medicine, may soon become reality [17, 60]. However, despite increasing interest in the interplay between genetic differences among individuals and both disease susceptibility and drug response [25, 85], there are major obstacles on the road to this aim. First, sequencing techniques are still too expensive to allow the so-called $1,000 genome, which is commonly seen as a prerequisite for clinical use of individual genome sequences. Second, the interplay between individual genetic differences and disease susceptibility or drug response is likely to be complex, owing both to modifying genes elsewhere in the genome and to environmental factors that are different for every patient [128]. Thus, even if genome sequencing becomes cheap enough for complete genome sequences to become a realistic option for every patient, it will be important to ensure that other factors are not simply discounted and that patients rather than genomes are treated [128]. However, if it does indeed become possible to obtain complete genome sequences from each and every patient such information will be of great value for both understanding and treating diseases, including major complex diseases such as cancer or heart disease.

While complete genome sequences for many individuals are still out of reach, technical progress has already made typing of thousands of individuals for large numbers (up to 1 million) of single nucleotide polymorphisms (SNPs) possible. Applied to cohorts of patients and controls, such data can be used to search for genetic regions that are associated with certain diseases [20]. A recent large-scale study identified potential disease linked genetic regions for seven common diseases (bipolar disorder, coronary artery disease, Crohn's disease, hypertension, rheumatoid arthritis, type 1 diabetes, and type 2 diabetes; [38]). While such studies are becoming increasingly popular among medical geneticists, they are unfortunately fraught with problems [20]. First, owing to the high number of tests made in such a study without correction for multiple testing a large number of false positives will be detected. This problem was recently highlighted by an international study that failed to replicate any of 13 previously detected SNPs claimed to be linked to Parkinson's disease [39]. Although in other cases it was possible to confirm linkage between a SNP and disease susceptibility, in many cases replicate studies failed to confirm the results of initial studies, leading to the proposition that association between a SNP and a certain disease should only be considered if it has been replicated in at least two independent studies [20]. Another problem with association studies investigating complex diseases lies in the fact that many loci detected in large-scale studies may increase the individual risk only marginally, as found for a number of new breast cancer susceptibility loci [34]. Thus, only the combination of a large number of genetic loci in combination with environmental factors is likely to increase individual disease risk to a measurable effect, which is not an unexpected result for so-called complex diseases [133]. Finally, even if a SNP is associated with a certain disease, this by no means indicates that the SNP is in any way causative. Establishing a causal relationship between a genetic variant and a disease requires extensive biochemical experiments and further investigations. And once this is established it is still a long way until a medical treatment for a certain genetic susceptibility will be available.

Owing to the complexity of the genetics of disease, another approach, called "The genetics of health" has recently been suggested. This idea is based on the fact that it has been estimated that each individual inherits ~300 mutations with deleterious effects [104]. Despite this, there are individuals who stay healthy until late in life, which is possibly due to the effect of protective alleles for certain genes. A possible alternative route for using evolutionary knowledge in medical genetics would therefore be to search for such protective alleles and modifier genes and – if possible – use the corresponding proteins in medical treatments. As these proteins occur in healthy individuals, adverse side effects are rather unlikely. While this is an interesting and potentially promising alternative idea, as with the other uses of evolutionary insights in medical genetics, applications of this approach are not yet in sight.

Despite all these problems, it is clear that a better understanding of the evolution of the genes that make humans susceptible or resistant to diseases is likely also to lead to better treatments in the long run.

18.4 Recent Events in Human Evolution

18.4.1 Out of Africa into New-found Lands

18.4.1.1 Out of Africa

One of the earliest and maybe most significant findings from human genetics is the insight that modern humans share a recent common origin in Africa [14, 148, 152]. Also controversial in the beginning, it has been supported by numerous studies using markers across the whole genome. The first studies on mtDNA using restriction fragment length polymorphism (RPLPs) [14] and control region sequences [148] have relatively recently been supported by sequencing of a worldwide sample of complete mtDNA genomes [62]. This study not only supported the early studies with respect to a topology of the human mtDNA tree with a lot of diversity in Africa and the rest of the world displaying

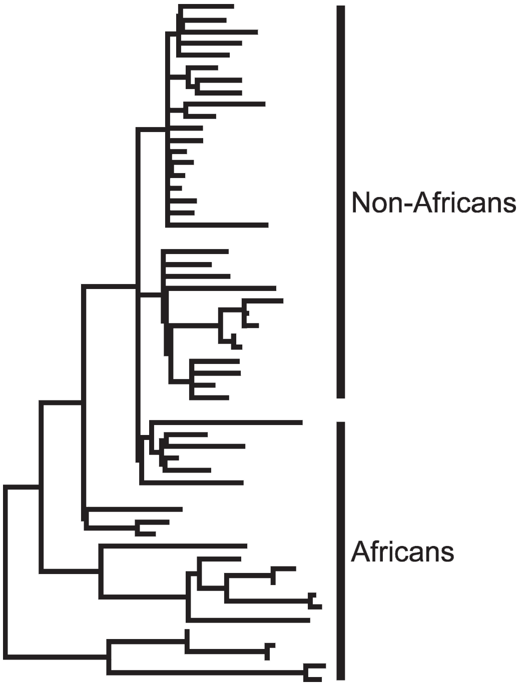

Fig. 18.9 Phylogenetic tree of a worldwide sample of complete human mtDNA sequences. As can be seen, all non-African sequences are nested within African sequence diversity. (After [62])

a subset of this diversity (Fig. 18.9), but also showed that the early studies were surprisingly accurate with respect to the most recent common ancestor (MRCA) of human mtDNA. Thus, while Cann and Vigilant postulated ages for the MRCA of 200,000 and 166,000–249,000 years, respectively, Ingman et al. arrived at a very similar age of 172,000 ± 50,000 years. Interestingly, the oldest modern human fossils have been dated to around 195,000 years B.P. [97]. However, this is a mere coincidence as most sequence regions have far older coalescent dates and should not be taken as evidence that modern humans are direct descendants of these fossils. Apart from determining the relationship of modern mtDNA sequences and the date of their MRCA, Ingman and colleagues were also able to estimate the time when modern humans started to expand in population size to about 38,500 years ago, close to the time when substantial cultural changes occurred in human evolution. This notion of a recent human population expansion was supported by a number of studies using nuclear DNA (e.g., [163, 164]). However, other work indicates that the picture of human population history is more complex and may not be explained by a single population expansion [18, 159]. For example, data from the X-chromosome indicate that before the population expansion detected using mtDNA sequences, there had already been another population expansion in modern humans 100,000 – 200,000 years ago [74]. A general problem with these analyses lies in the fact that often several alternative models describe the data equally well, so distinguishing between models of long-term growth and those assuming a bottleneck followed by rapid population growth is difficult. Moreover, population expansions seem to have taken place in different geographical regions at different times. Thus, there is evidence for an expansion in Africa as much as 100,000 years ago, whereas the expansions in Asia and Europe date to 52,000 and 23,000 years ago, respectively, [140].

Independently of these signals of population growth, nuclear DNA sequences also support the notion of a recent human origin in Africa (reviewed in [140]). One of the earliest studies using long sequences was on a 10 kb region on the X-chromosome and showed that also for nuclear DNA more sequence diversity is found in Africa [72]. This result, together with the conclusion that humans emigrated relatively recently, has been confirmed by numerous studies, using DNA sequences from autosomes, X- and Y-chromosomes, and other markers, such as Alu insertions from all parts of the genome (e.g., [8, 18, 163, 164]). Most notably, genome-wide analyses of both microsatellites [113] and haplotype structures [26] show a strong negative correlation between the geographical distance of a population from Africa and its genetic diversity. This pattern is best explained by a recent emigration of modern humans out of Africa, with limited gene flow between populations afterwards [113]. Dating this emigration has been somewhat more problematic. However, the molecular estimates center more and more on a date of 60,000–65,000 years B.P. (see e.g., [94, 140, 142). Intriguingly, Y-chromosomal analyses arrive at a somewhat younger date for the MRCA for all non-African Y-chromosomes, with an age of 44,000 years [145]. This discrepancy can either be explained by Y-chromosomal introgression into Africa subsequent to the initial emigration or, as seems more likely, by uncertainties in the molecular dating. Extensive studies of mtDNA sequences recently also indicated that the initial emigration of modern humans out of Africa took place along a southern coastal route and not through the Levant, as

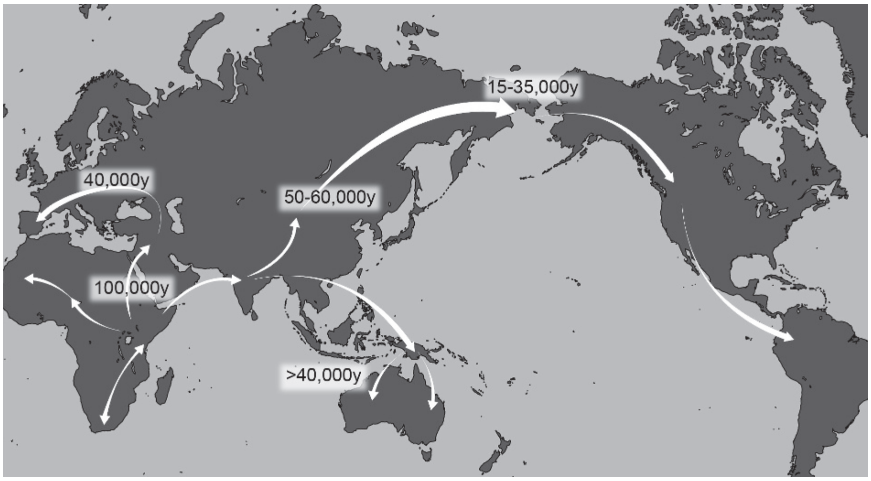

Fig. 18.10 Migration of modern humans out of Africa. The *arrows* indicate suspected routes and the numbers estimated years before present when the various areas were colonized by modern humans. Note that some of the time estimates, especially the timing of the settling of the Americas, are still widely debated

previously believed [94]. This idea is supported by archeological data that indicate an occupation of the Red Sea region together with use of marine food by modern humans around 125,000 years ago [154]. Thus, humans may have emigrated out of Africa from this region along the southern coast of Asia, reaching Australia (Fig. 18.10), as evidenced by archeological findings, as much as 50,000–60,000 years ago [136], which is consistent with the molecular data that hint at an emigration out of Africa about 65,000 years ago [94]. These data also indicate that human migration must have been relatively rapid at about 4 km/year [94]. It is still a matter of debate what triggered the emigration of modern humans out of Africa. Klein [79] has argued for an Upper Paleolithic revolution with some major cognitive or behavioral change having taken place in humans about 45,000 – 50,000 years ago. However, this notion has been contested with the argument that the archeological evidence provides no strong indication for such a rapid revolution, but rather shows a continuous development [96]. Recently, Mellars [100] argued that rapid climatic changes in Africa about 70,000–80,000 years ago resulted in similarly rapid economic and social changes in African societies, resulting in a population increase that then triggered the emigration out of Africa. Although all these models are intriguing, it should be kept in mind that hominids had emigrated out of Africa several times before, in the absence of sophisticated technology and without any evidence for major sociocultural changes in the African hominid populations preceding these events.

Despite the emigration out of Africa about 65,000 years ago, it was some time before modern humans started colonizing Europe (Fig. 18.10). The earliest modern human fossils in Europe are from Romania, dating back to about 35,000 radiocarbon years B.P. [143]. However, the archeological evidence and differences between uncalibrated carbon dates and true age indicate that modern humans settled in Europe about 40,000 years ago (e.g., [24], reviewed in [101]). Intriguingly, in the Ural region, humans had also managed to settle at the Arctic Circle as early as almost 40,000 years B.P. [110], suggesting that humans reached the Arctic only shortly after they had settled in Europe.

18.4.1.2 Migrations to Europe

Since the initial colonization, several subsequent events have shaped human genetic diversity in Europe, such as recolonization of middle and Northern Europe after the last glacial maximum (e.g., [142]) and the expansion of Neolithic farmers from the Near East north-westward into Europe [19, 103]. To what extent Neolithic farmers really contributed to the modern human gene pool in Europe has been a matter of

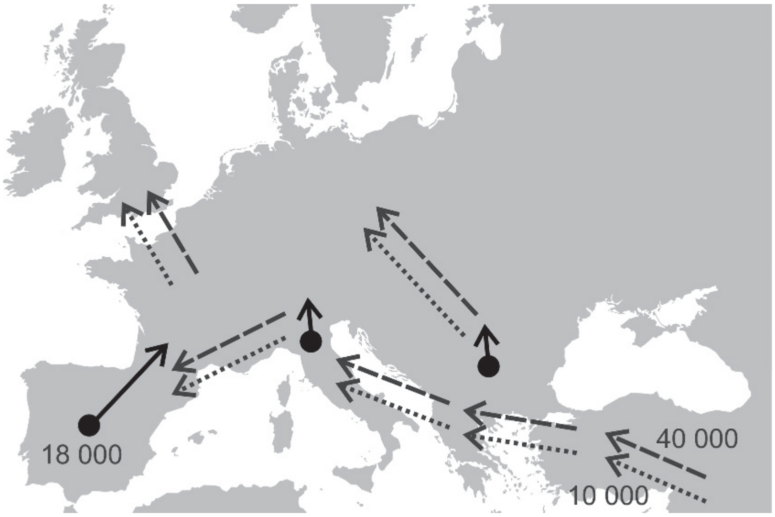

Fig. 18.11 Timing and direction of the three major migration waves that formed the structure of genetic diversity in modern Europeans. (After [6])

debate, ranging from the notion that the contribution was major [19] to the claim that it was less than 25% [117, 142]. However, there is a strong signal of an allele frequency cline from the Near East to northwestern Europe both for autosomal markers [19] and for Y-chromosomal ones [121, 129], making it the predominant pattern of genetic diversity within Europe [6]. At the same time, there is evidence of a postglacial expansion from the Iberian Peninsula [1]. Altogether, the data indicate that at least three major migrations influenced the structure of the modern European gene pool (Fig. 18.11): the initial immigration some 40,000 years ago, a postglacial expansion from southern refugia about 18,000 years ago and the demic diffusion of Neolithic farmers about 10,000 years ago [6].

Analyses of ancient DNA from Neolithic human fossils have added another twist to this complex picture. Haak and colleagues [54] studied a large number of skeletons from Linear pottery culture (LPC) sites and, surprisingly, found a group of haplotypes (N1a) at a frequency of 25%, although this haplogroup is rare (~0.2%) in modern populations all around the world. From these data they concluded that the contribution of Neolithic settlers to the modern European gene pool was only minor. However, it should be noted that LPC is not the same as Neolithic and that the spread of the Neolithic across Europe was not a homogenous event [142].

18.4.1.3 Colonizing America and Oceania

Finally, there are two major regions of the world that have been colonized by humans relatively recently, the Americas and Oceania. The debate about the timing of the settling of the Americas can be summarized in the question whether the initial immigration took place before the appearance of Clovis lithic sites around 13,000 years B.P. or not [127]. Proponents of the Clovis-first hypothesis still contend that there is no solid evidence for the presence of humans in the Americas before the Clovis people. However, there is increasing evidence that human presence in the Americas predated the appearance of the Clovis lithic culture [45]. One of the most important sites in this debate is Monte Verde in Chile, which has been dated to about 14,500 years B.P., clearly predating the Clovis culture [102, 127]. Late Pleistocene sites of human occupation have also been found in Amazonia, and there is an increasing number of sites that predate Clovis culture [45]. Moreover, Y-chromosomal data indicate an entry of humans into the Americas anywhere between 10,000 and 20,000 years B.P. [68, 127], most likely predating the appearance of the Clovis culture but at the same time making a very early entry into the Americas (>25,000 years B.P.) rather unlikely. Further data on both archeological sites and from population genetic studies will hopefully resolve this controversy in the near future.

Oceania was the last major region of the world to become colonized by modern humans, with the earliest human migrations dating to only 800–3,200 years ago [114]. Linguistic evidence points to an Asian origin for Polynesians, whereas archeological evidence argues for a Melanesian origin. The spread of Polynesians is associated with the Lapita cultural complex, but the timing and process of this expansion has been controversial and several competing hypotheses have been presented. Among these is the "slow-boat" hypothesis [75], which proposes that Polynesians originated around the region of Taiwan but moved through Melanesia only slowly, interacting with the population there and also picking up a substantial amount of genetic diversity. This hypothesis is based on the fact that the majority of Polynesian Y-chromosomal haplotypes originated in Melanesia [75]. Further work confirmed this conclusion and at the same time found more than 90% of the mtDNA haplotypes to be of Asian origin [76], suggesting a dual origin of Polynesians. The much larger Melanesian contribution to the Y-chromosome than to mtDNA is hypothesized to be due to the generally matrilocal social structure of Polynesian societies [76]. Other studies have also confirmed a Taiwanese origin for Polynesian mtDNA, and the slow-boat hypothesis is now widely accepted [156]. Moreover, the mtDNA and Y-chromosomal data also show a west-to-east decrease in genetic diversity, which not only indicates a west-to-east direction of settlement in Polynesia but also argues for regular voyaging rather than haphazard settlements [76]. This result is in line with results on the genetic diversity from New Zealand Maoris, which is relatively wide and therefore indicates that settlement of New Zealand was intentional rather than the result of shipwrecks [156]. However, the success story of human settlements in Polynesia also has a dark side. Several Polynesian societies, such as those on Easter Island or Magareva, collapsed because they completely deforested their islands. A comparison of a large number of environmental factors showed that deforestation or lack thereof was not dependent on the respective societies but rather on environmental factors that support or slow down reforestation [118]. In other words, human societies may have a tendency to cause their own collapse by degrading their environment, and the authors suggest that environmental factors may also explain similar deforestation-associated collapses (e.g., Fertile Crescent, Maya, and Anasazi) or the lack thereof (Japan and highland New Guinea) in other parts of the world [118].

18.4.2 Domestication

Possibly the most important event in human history during the last 13,000 years was the beginning of domestication of animals and plants [31]. Domestication was initiated in a maximum of nine regions of the world [31] and resulted in a replacement of hunter-gatherer societies by farmers all around the world although it is not clear to what extent this happened, owing to population replacement, or to what extent assimilation of the farming life-style was involved. The rapid adoption of a farming life style was due to several advantages that farmers have over hunter-gatherer societies. First, farming can support much higher population densities than is possible for hunter-gatherer societies. Second, farming societies are sedentary, which allows accumulation of food stocks, and this is essential for complex technology and centralized states, two hallmarks of modern complex societies [32].

Some of the earliest domestications took place in the Fertile Crescent in the Near East, dating back to about 11,000 years, as evidenced by both archeological and genetic data. Here, several crop species, such as wheat, rye, and barley were domesticated. Moreover, at least one of two independent cattle domestications [10, 93] also took place in this region [144] resulting in modern European cattle (*Bos taurus*). Interestingly, genetic data also show that European aurochs (*Bos primigenius*) were probably never domesticated as a number of ancient DNA sequences from fossil British bones differed substantially in their sequence from all modern European cattle sequences [144]. A second cattle domestication took place in India, leading to the zebu lineage (*Bos indicus* [10, 93]), whereas African cattle are a hybrid population that have a zebu-like appearance, which is consistent with genetic data from their nuclear genome, but have derived their mitochondrial DNA from European cattle. Deep divergences between groups of sequences have been observed for many domesticated species (apart from cattle also for sheep, goat, and donkey) and have usually been interpreted as evidence for independent domestication events. However, this interpretation has to be treated with caution, as deeply diverging lineages may occur in a single wild population, as shown for the yak [53] and are thus in themselves not convincing evidence for independent domestication events. The same is true for high levels of genetic diversity in a domesticated species which has been taken as evidence for domestication from multiple populations of the horse [67]. This interpretation may be correct, but in the absence of knowledge about the genetic diversity of the underlying wild population it is also possible that domestication took place from a single, highly diverse population, as shown for the yak. So far the highest number of domestication events has been claimed for the pig with about a dozen [90]. It remains to be seen whether this is true or rather an artifact of subsequent – intentional and unintentional – back-crossing of domesticated animals with their local wild relatives, which it is suggested, has been a rather frequent practice [149].

Interestingly, the first animal to be domesticated was not a life-stock species but the dog, whose domestication took place about 15,000 years ago, probably in China [126]. Moreover, studies of fossil dogs from America, predating European contact, showed that these Native American dogs were not independently domesticated but rather brought over by humans immigrating from Asia [91]. Thus, domestic animal species may be transported rapidly and over large distances by humans, possibly blurring signals of their geographic origin. A very early (10,000 years B.P.) transportation from Asia to America has also been postulated for another utility species, the bottle gourd [41].

Finally, it should be noted that the effects of domestication on human societies have not all been positive. For example, it has been argued that the African malaria parasite *Plasmodium falciparum* rapidly increased in population size about 10,000 years ago with the onset of agriculture [71]. Thus, domestication did not only improve the nutritional situation for human societies, but also exposed them to new parasite loads. However, domestication was clearly a major step in human evolution and a prerequisite for modern human societies.

18.4.3 Modern Human Population Structure

Human evolution was clearly a dynamic process and the current human population is the result of numerous migrations and population size changes, as shown above. So, the question of how we are to view modern human genetic diversity remains open. As noted before, humans carry relatively little genetic diversity compared with their closest relatives, the great apes, and all modern humans outside Africa share a recent African origin, so that we all seem to be Africans, either living on that continent or in recent exile" [107]. What is a matter of debate is how much structure the modern human gene pool contains. In 2002 Rosenberg and colleagues [119] argued that given a sufficient number of markers, humans can be placed into clusters that correspond to a geographical origin, implying – albeit most probably inadvertently – that major genetic differences exist between continental groups such as Africans, Asians, and Europeans. This conclusion has been contested (e.g., [131]) with the argument that the result of Rosenber et al. [119] was an artifact of the study design, and human genetic diversity is best explained by a clinal model of isolation by distance, without any major jumps in genetic distance over short geographical distances. Thus, rather than being made up of several distinct human groups that are genetically well separated, human genetic diversity changes continuously, with humans living in geographical proximity also being more closely related genetically and humans living at greater geographical distance also being genetically more different. This later view has been confirmed by additional studies that detected a strong signal for isolation by distance in both the data set of Rosenberg et al. [113] and in other data sets [30]. However, expanding their data set, Rosenberg and colleagues reaffirmed that human populations indeed cluster by geographical origin [120]. At the same time, they do find a pattern of isolation by distance, with genetic distance generally increasing with geographical distance. The reason why they nevertheless detect clusters lies in the fact that small jumps in genetic distances occur across short distance of geographical barriers. Thus, the clusters are real but they explain only a small proportion of the genetic differences between humans, a result also seen in other data sets. For example, in a compilation of about 1 million SNPs most are shared between Africans, Europeans, and Asians and only a very few represent fixed differences between the continental groups. So despite some phenotypically obvious differences between human populations, such as skin color, across the whole genome there is very little genetic differentiation between human populations. Given that modern humans originated in Africa only about 200,000 years ago and humans are a notoriously migratory species, it is not surprising that we are all very close relatives.

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Glossary

Clinal model
A model describing the change in genetic diversity across geographical distance. In the clinal model, genetic differences among populations increase more or less constantly with increasing geographical distance.

Clovis culture
Clovis describes a Native American cultural horizon, dated to ~ 11,000 radiocarbon years B.P. Characteristic for this culture are the beautiful bifacial spear points made from stone, also known as Clovis points.

Hominid
A member of the mammalian family Hominidae, which includes humans and the great apes (orangutans, chimpanzees and gorillas).

Lapita culture
A Pacific Ocean culture, dated to ~ 3,500 to 2,500 B.P., which might be ancestral to cultures in Polynesia, Micronesia, and Melanesia.

Linear pottery culture
The linear pottery culture is a Neolithic archeological horizon from Central Europe, dated to about 7,500–6,500 B.P.

Melanesia
The part of Oceania north and north-east of Australia.

Neolithic
The last part of the Stone Age, starting approximately 10,000 years ago. The beginning of the Neolithic also marks the start of farming. It ended when the use of metal tools became common, at different times in different regions.

Oceania
The island region in the Pacific east and north-east of Australia.

Pericentric inversion
An inversion of a part of the chromosome including the centromere.

Polynesians
Inhabitants of Polynesia, the island region roughly in the triangle from Hawaii, New Zealand and Easter Island.

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Comparative Genomics

19

Ross C. Hardison

Abstract Comparative genomics harnesses the power of sequence comparisons within and between species to deduce not only evolutionary history but also insights into the function, if any, of particular DNA sequences. Changes in DNA and protein sequences are subject to three evolutionary processes: drift, which allows some neutral changes to accumulate, negative selection, which removes deleterious changes, or positive selection, which acts on adaptive changes to increase their frequency in a population. Quantitative data from comparative genomics can be used to infer the type of evolutionary force that likely has been operating on a particular sequence, thereby predicting whether it is functional. These predictions are good but imperfect; their primary role is to provide useful hypotheses for further experimental tests of function. Rates of evolutionary change vary both between functional categories of sequences and regionally within genomes. Even within a functional category (e.g. protein or gene regulatory region) the rates vary. A more complete understanding of variation in the patterns and rates of evolution should improve the predictive accuracy of comparative genomics. Proteins that show signatures of adaptive evolution tend to fall into the major functional categories of reproduction, chemosensation, immune response and xenobiotic metabolism. DNA sequences that appear to be under the strongest evolutionary constraint are not fully understood, although many of them are active as transcriptional enhancers. Human sequences that regulate gene expression tend to be conserved among placental mammals, but the phylogenetic depth of conservation of individual regulatory regions ranges from primate-specific to pan-vertebrate.

Contents

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19.1 Goals, Impact, and Basic Approaches of Comparative Genomics

Comparative genomics uses evolutionary theory to glean insights into the function of genomic DNA sequences. By comparing DNA and protein sequences between species or among populations within a species, we can estimate the rates at which various sequences have evolved and infer chromosomal rearrangements, duplications and deletions. This evolutionary reconstruction can then be used to predict functional properties of the DNA. Sequences that are needed for functions common to the species being compared are expected to change little over evolutionary time, whereas sequences that confer an adaptive advantage when altered are expected to have greater divergence between species. Furthermore, sequence comparisons can help in predicting what role is played by a particular functional region, e.g., coding for a protein or regulating the level of expression of a gene.

These insights from comparative genomics are having a strong impact on medical genetics, and their role is expected to become more pervasive in the future. When profound mutant phenotypes lead to the discovery of genes in model organisms (bacteria, yeast, flies, etc.), the human genome is immediately searched for homologs, which frequently are discovered to be involved in similar processes. Control of the cell cycle [76] and defects in DNA repair associated with cancers [24, 47] are particularly famous examples. In studies of the noncoding regions of the human genome, conservation has become almost a proxy for function [20, 26, 64], and we will explore the power and limitations of this approach more in this chapter. The mapping and genotyping of millions of polymorphisms in humans [32] coupled with the availability of genome sequences of species closely related to humans [14, 70] has stimulated great interest in discovering genes and control sequences that are adaptive in humans, which may provide clues to the genetic elements that make us uniquely human (see Chaps. 8 and 16). As more and more loci are implicated in disease and susceptibility to diseases, identifying strong candidates for the causative mutations becomes more challenging. Research in comparative genomics is helping to meet this challenge by generating estimates across the human genome of sequences likely to be conserved for functions common to many species as well as sequences showing signs of adaptive change. Finding disease-associated markers in either type of sequence could rapidly narrow the search for mutations that cause a phenotype.

19.1.1 How Biological Sequences Change Over Time

All DNA sequences are subject to change, and these changes provide the fuel for evolution. Replication is highly accurate but not perfect, and despite the correction of many replication errors by repair processes during S-phase, a small fraction is retained as altered sequences. Mutagens in the environment can damage DNA, and some of these induced mutations escape repair. In addition, DNA bases can change spontaneously, for example, oxidative deamination of cytosine to produce uracil. The *mutation rate* is the number of sequence changes escaping correction and repair that accumulate per unit of time. The average mutation rate in humans has been estimated to be about 2 changes in 10^8 sites per generation [43, 57]. Thus for a diploid genome of 6×10^9 bp, about 120 new mutations arise in each generation. As will be discussed later in more detail, the mutation rate varies among loci and depends on the context, with transitions at CpG dinucleotides occurring about ten times more frequently than other mutations.

Mutations can be substitutions of one nucleotide for another, deletions of strings of nucleotides, insertions of nucleotides, or rearrangements of chromosomes, including duplications of DNA segments. Substitutions are about ten times as frequent as the length-changing alterations, with transitions greatly favored over transversions.

Mutations occur in individuals, and it is instructive to consider how an alteration in a single individual can eventually lead to a sequence difference between two species, which we call a *fixed difference*. Of course, only mutations arising in the germ-line can be passed along to progeny and have some possibility of fixation. Initially, the allele carrying a mutation has a low frequency in the population, i.e., $1/(2N_e)$ for a diploid organism, where N_e is the effective population size. All the mating individuals in a population contribute to the pool of new alleles. Mutant alleles that are disadvantageous will be cleared out of the population quickly, whereas those that confer a selective advantage rapidly will go to fixation (occurrence in most members of a population). However, many of the new mutations will have no effect on the individual; we call these mutations with no functional consequence *polymorphisms* or *neutral changes*. The frequency of these polymorphisms will increase or decrease depending on the results of matings and survival of progeny. The vast majority will be transitory in the population, with most headed for loss. However, the stochastic fluctuations in allele frequencies will allow some to eventually increase to a high frequency. Thus, some of the neutral changes lead to fixed differences. In fact, Kimura [40] and others have argued that such neutral changes are the major contributors to the overall evolution of the genome.

In order for a sequence change to have an effect on an organism, the change has to occur in a region that is involved in some function. Examples of such regions are an exon encoding part of a protein or a promoter or enhancer involved in gene regulation. The rapid removal of disadvantageous alleles results from *negative* or *purifying selection* (Fig. 19.1). The rapid fixation of advantageous alleles is *adaptive evolution* resulting from positive selection. Biological function is inferred from evidence of selection. Thus, the aim of comparative genomics to identify functional sequences can be stated as a goal of finding DNA sequences that show significant signs of positive or negative selection.

In addition to mutations of single bases, strings of nucleotides can be inserted or deleted as a result of replication errors or recombination. Often, the direction

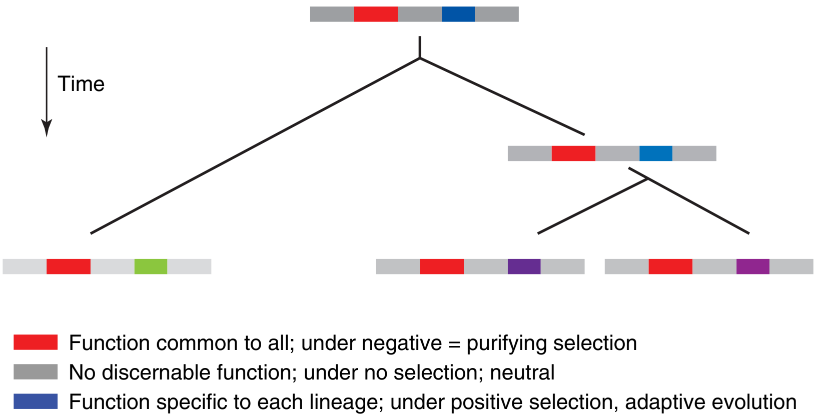

Time
🟥 Function common to all; under negative = purifying selection
⬜ No discernable function; under no selection; neutral
🟦 Function specific to each lineage; under positive selection, adaptive evolution

Fig. 19.1 Three modes of evolution, two of which are associated with function. The red line indicates a functional DNA sequence whose role has remained the same from ancestor to contemporary sequences, and thus it has been subject to purifying selection. The blue line represents a sequence that was functional in the ancestor, but changes in separate lineages (illustrated by different shades of blue, green, and purple) are adaptive and hence are subject to positive selection. The gray lines represent sequences of no known function, i.e., neutral DNA

of the event is not known because it is inferred from a gap in an alignment of only two sequences. In these cases the event is called an *indel*. Adding a third sequence to the alignment as an outgroup allows one to conclude with some confidence whether the event is an insertion or a deletion. Indels are less frequent than nucleotide substitution, and their frequency declines sharply with the size of the insertion or deletion. However, a single insertion or deletion can involve tens of thousands of nucleotides. Thus, they account for the majority of the nucleotides that differ between closely related species.

Rearrangements of chromosomes, such as intrachromosomal duplications and inversions or interchromosomal translocations, also lead to large-scale changes both in contemporary populations and over evolutionary time. Some chromosomal rearrangements are associated with human disease (see Chap. XX). In comparisons over evolutionary time, e.g., between mammalian orders, the history of chromosomal rearrangements can be reconstructed with some accuracy.

19.1.2 Purifying Selection

DNA sequences that encode the same function in contemporary species and in the last common ancestral species have been subject to *purifying* selection. The DNA sequence carried out some function in the ancestor, and any changes to this successful invention are more likely to break it than to improve it. Mutations in the sequence tend to work less well than the original one, and those mutations are cleared from the population. Hence the selective pressure to maintain a function prevents the DNA sequence from accumulating many changes, and the selection is referred to as purifying. This type of selective pressure tends to decrease the number of changes observed, and thus it is also called *negative* selection. The sequence under purifying selection is *constrained* by its function to remain similar to the ancestor. Saying that a sequence is subject to constraint is the equivalent of saying that it is subject to purifying selection. Examples of sequences under constraint include most protein-coding regions and many DNA sequences that regulate the level of expression of a gene.

In this chapter, we distinguish between conserved and constrained elements. A feature (e.g., a segment of DNA, a protein, an anatomical structure) that is found in contemporary species and that is inferred as being derived from a similar feature in the last common ancestor is conserved. In particular, a DNA sequence that reliably aligns between two species is considered to be conserved. That does not necessarily mean that it is functional. Evidence of *constraint*, i.e., alignment with a level of similarity greater than expected for neutral DNA, is taken as an indicator of function common to the two species.

The hallmark of purifying selection is a rate of change that is slower than that of neutral DNA. The next section (Sect. 19.2) will delve more deeply into how rates of evolution are determined, but for now assume that we can align related sequences with reasonable accuracy and can use that alignment to measure how frequently mismatches occur. Then the problem of finding sequences under purifying selection becomes one of determining the substitution rate in a segment that is a candidate for being functional and comparing it to the rate in neutral DNA. DNA segments whose inferred rate of evolutionary change is significantly lower than neutral will show a peak of similarity for comparisons at a sufficient phylogenetic distance (e.g., human versus mouse in Fig. 19.2).

In order to distinguish neutral from constrained DNA, sequences of divergent species must be compared. The choice of species to compare will depend on the questions being examined, but enough sequence change must have occurred to distinguish signal from noise. In practical terms, human comparisons with chimpanzee are too close (too similar) to effectively find constrained sequences, but multiple alignments among many primates do have considerable power [8]. Many studies have used comparisons between mammalian orders, such as primate (human) with rodent (mouse), to see the constrained sequences (Fig. 19.2).

19.1.3 Models of Neutral DNA

Although the concept of DNA that has no function is very useful and has led to much insight in molecular evolutionary genetics, it is difficult to establish that any DNA is truly neutral. Several models for neutral DNA are in common use. One of the earliest is the set of nucleotides in protein-coding regions that can be altered without changing the encoded amino acid [41]. The nucleotides are called *synonymous* or *silent* sites. They are neutral with respect to coding capacity, but alterations in particular synonymous sites can affect

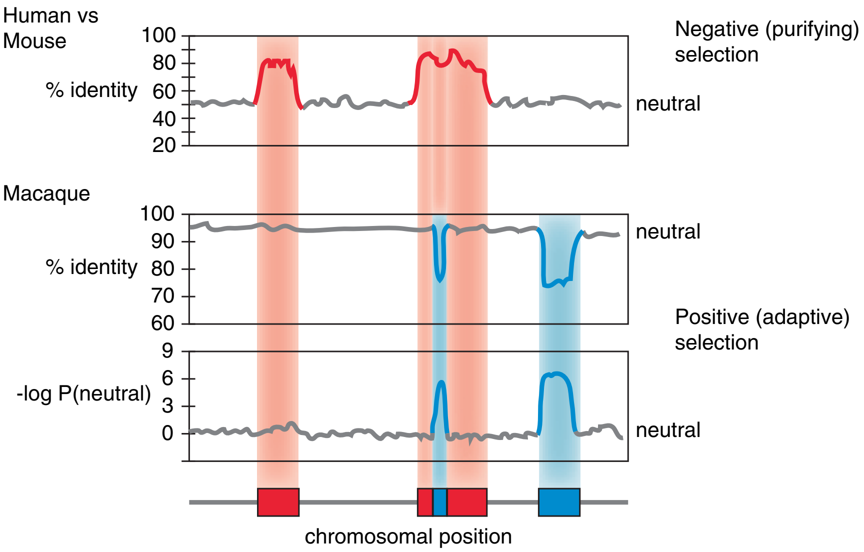


Fig. 19.2 Ideal cases for interpretation of sequence similarity. Idealized graphs of levels of sequence similarity (as percent identity) for a segment of a human chromosome compared with mouse (top) and rhesus macaque (middle), and of the likelihood that the DNA interrogated by the human-macaque comparison is not neutral (negative logarithm of the probability that the sequence similarity comes from the distribution of values for comparisons of neutral DNA, third graph). In the graphs, values that are close to those observed for a model of neutral DNA are shown in gray, those that indicate the action of negative selection are red, and those that indicate positive selection are blue. The bottom map is an interpretation of the graphs as discrete segments of DNA either under negative (red boxes) or positive (blue boxes) selection on a background of neutral DNA (gray line). Note that one segment shows evidence of negative selection since the separation of primates from rodents (red in top graph) but positive selection since the separation of human and Old World monkey (macaque) lineages (blue in middle and bottom graphs)

translation efficiency, splicing, or other processes. The latter appear to be a minority of synonymous sites, and as a group the synonymous sites are the most frequently used neutral model.

Another useful model for neutral DNA are *pseudogenes*. These are copies of functional genes, but the copies no longer code for protein because of some disabling mutation, such as a frameshift mutation or a substitution that generates a translation termination codon. For the period of time since the inactivating mutation, the pseudogene has likely been under little or no selective pressure. The rate of divergence of pseudogenes after inactivation is clearly higher than that of the homologous functional genes, and they have been used successfully as neutral models in many studies of particular gene families (e.g., [48]). One limitation of using pseudogenes as a neutral model is the uncertainty of determining when the inactivating mutation(s) occurred. Also, they are rather sparse for genome-wide studies.

For comparisons in mammalian genomes, *ancestral repeats* (Fig. 19.3) have proved effective, albeit imperfect, models for neutral DNA [27, 85]. The interspersed DNA repeats in the genomes of humans and other mammals are derived from transposable elements, mostly *retrotransposons* that move via an RNA intermediate. Members of an interspersed repeat family generated by recent transposition (on an evolutionary time-scale) are quite similar to each other because they have not had sufficient time to diverge. These are restricted to particular clades, such as the *Alu* repeats that are prevalent in primate genomes. Considerably more differences are observed among members of repeat families that are derived from transposons active in an ancestral species because of the longer divergence time. The members of these older repeat families are present in all the descendant species. Examples include *LINE2* and *MIR* repeats, which are present in the genomes of all eutherian mammals examined. Interestingly, all the members of these ancestral repeat families are quite divergent from each other, indicating that they have not been actively transposing since the separation of the descendant species. Thus, most ancestral repeats appear to be relics of ancient transposable elements, and they are not active even for

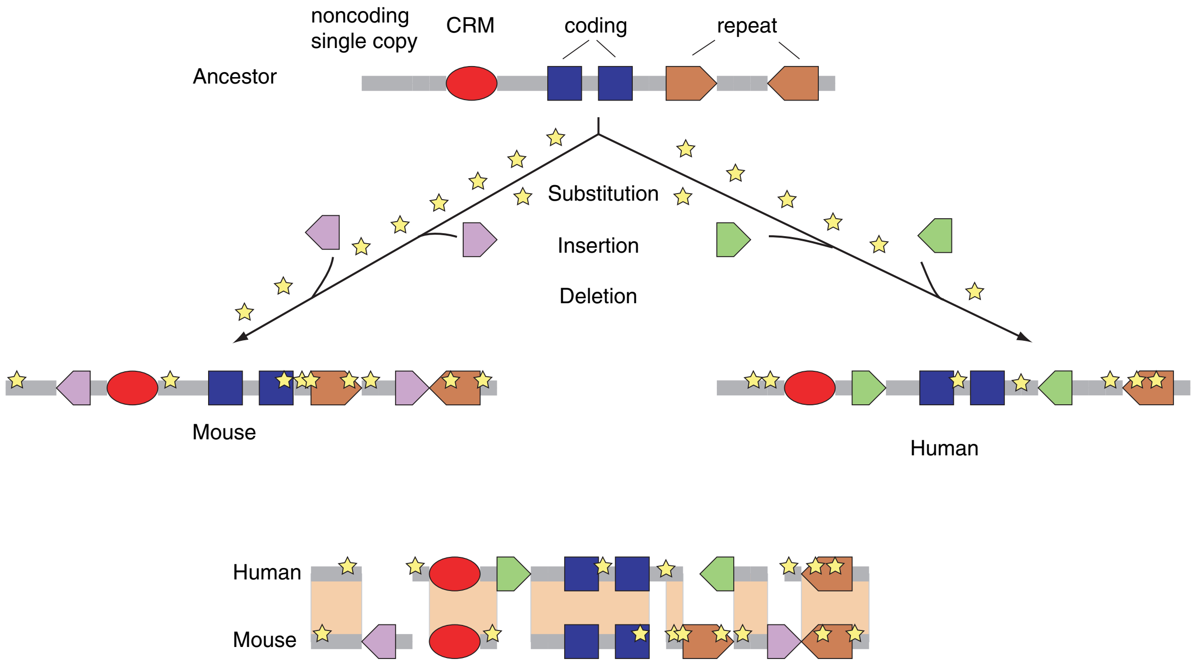


Fig. 19.3 Substitutions, insertions of transposable elements and deletions in the evolution of genomes. (**a**) Illustration of functional regions such as protein-coding exons (blue boxes), cis-regulatory modules (CRMs, red ovals), such as enhancers and promoters, and ancestral repeats (brown pointed boxes). After divergence of rodents and primates, sequences diverge by substitutions (gold stars), insertion of lineage-specific transposable elements (purple and green pointed boxes), and deletions. (**b**) Alignments of the contemporary species allow some of the evolutionary history to be reconstructed, including deletions inferred from the nonaligning portions

transposition. The vast bulk of these ancestral repeats have no apparent function. They are found frequently in eutherian mammals, and thus provide a neutral model with many sites.

When interpreting any measurement or study involving a comparison with a neutral model, it is important to keep in mind that the deduced absence of function is limited by contemporary knowledge. Experimental tests and molecular evolutionary studies have shown that some individual synonymous sites and ancestral repeats are not neutral. They do not constitute the bulk of the sites in these neutral models, and of course the known functional sites can be removed from the neutral set. However, future studies could reveal additional function, which will affect interpretations based on these neutral models.

19.1.4 Adaptive Evolution

The functions of some DNA segments and proteins have changed along the evolutionary lineages to contemporary species. Some sequence changes confer a new function on the DNA or protein that helps the organism adapt to a new environment or condition. These advantageous mutations increase in frequency in a population, leading to fixation (i.e., becoming the predominant allele in the population). The selective pressure favoring these changes is called *positive* selection, since it tends to increase the frequency of changes. This leads to *adaptive evolution*, i.e., a change in a DNA or protein sequence that favors survival and procreation of an organism. The positive selection for new functionality is also referred to as *Darwinian* selection.

The hallmark of adaptive evolution is a rate of sequence change that is faster than that of neutral DNA. Sequences subject to adaptive evolution may change so much that they will not align reliably at greater phylogenetic distances (Fig. 19.1). Also, the selective pressure leading to adaptive changes may apply only recently or in limited clades, such as among humans or among humans and great apes. Thus, sequence comparisons to find adaptive changes are usually done for closely related, recently diverged sequences (Fig. 19.2). The signal for positive selection may be captured as a significant decrease in similarity between species or an increase in the probability that a sequence has not evolved neutrally (Fig. 19.2).

19.2 Alignments of Biological Sequences and Their Interpretation

Biological sequence comparisons are most commonly done with protein sequences (strings of amino acids) or DNA sequences (strings of nucleotides). The comparisons begin with an alignment, which is a mapping of one sequence onto another with insertions of gaps (often indicated by a dash) to optimize a similarity score (Fig. 19.3). The score can be determined in a variety of ways, but in all cases matching symbols (for amino acids or nucleotides as appropriate) are favored, whereas mismatches are not favored and gaps are penalized. The gap penalty frequently takes the form of a gap-open penalty plus an additional, smaller penalty for each position included in the gap. The latter are referred to *as affine gap penalties*.

19.2.1 Global and Local Alignments

A *global* alignment maps each symbol in one sequence onto a corresponding symbol in another sequence. The result is an alignment of the two (or more) sequences from their beginnings to their ends, with any length differences accommodated by gaps that are introduced. This is an appropriate strategy for sequences are related to each other over their entirety. That is the case for many proteins and many mRNAs. The earliest computer program for aligning two biological sequences, written by Needleman and Wunsch [58], generates global alignments. Popular contemporary programs for aligning proteins, such as *ClustalW* [80], also compute global alignments. Global aligners for DNA sequences include *VISTA* [54], *MAVID* [9], and *LAGAN* [10].

A frequent task in comparative genomics is to find matches between two or more sequences that are not related over their entire lengths. For instance, two protein sequences may be related only in one or a few domains, but be different in other parts. The protein-coding portions of genes are frequently divided into short exons that are separated by introns. Exons tend to be under constraint, whereas much of the intronic DNA may be neutral, and thus at a sufficient phylogenetic distance introns can be so divergent that they no longer align, whereas exons will match well. The most common use of comparative genomics is to search a large database of all compiled DNA or protein sequences with a query sequence of interest. In this case, the goal is to find a match that may comprise only one part in billions of the database. When a match between only a portion of two or more sequences is desired, then a *local* alignment should be generated. One of the earliest computer programs for finding local alignments came from Smith and Waterman [75]. The *blast* family of programs (Basic Local Alignment Search Tool, [1]) is used for database searches. One variant, called *blastZ*, has been adapted to compute local alignments of long genomic DNA sequences [72].

19.2.2 Aligning Protein Sequences

Proteins are composed of 20 amino acids, so that for any position in one sequence the possibilities for alignment with a position in a comparison sequence are 1 match, 19 mismatches, or a gap. However, the likelihood for each of the 19 mismatches is not the same. Replacement of an amino acid by a chemically similar amino acid occurs much more frequently than does replacement with a distinctly different amino acid. These different frequencies of amino acid substitutions can be captured as a *scoring matrix*, in which matches are given the highest similarity score and mismatches that occur frequently in protein sequences are given positive scores, decreasing with declining frequencies of the substitution. These scoring matrices are determined by the frequency with which mismatches are observed in well-aligned sequences. Several effective matrices have been generated, beginning with the pioneering work of Dayhoff et al. [18] and continuing on to the BLOSSUM matrices of Henikoff [29].

Alignments can be used to organize relationships among the large number of sequenced proteins. Large compilations of aligned protein sequences are analyzed to find clusters of proteins that appear to share a common ancestor and to find blocks of aligned sequences that are distinctive for various protein domains. Indeed, when genes and their encoded proteins are predicted or identified in genome sequences, the primary basis for making inferences about their function is sequence similarity to known proteins.

Sequence similarity between proteins can be found with considerably greater sensitivity than can be found using a DNA sequence. The reason is that the 20 amino acids found in proteins constitute a much more complex group of characters, or alphabet, than the four nucleotides found in DNA. Thus, alignments between distantly related proteins may only match at a very small percentage of positions, but these are still statistically significant and they can be biologically meaningful.

19.2.3 Aligning Large Genome Sequences

The smaller alphabet for DNA sequences, consisting of only four nucleotides (A, C, G, T), means that the threshold for statistical significance is considerably higher than that used for protein sequences. For random sequences of equal nucleotide composition, any position in one sequence should have a 25% chance of matching any position in the other. However, sufficiently long runs of matching sequences are much less likely, and reliable alignment can be generated between related sequences. Just like for alignments of protein sequences, some substitutions are more likely to occur than others. For example, transitions are much more frequent than transversions. These preferences can be incorporated into the alignment process by using scoring matrices that were deduced from the empirical frequencies of matches and substitutions in reliable alignments, similar to the process that generated scoring matrices for protein alignments.

The portions of DNA sequences that code for proteins tend to be more similar and to have many fewer indels than the rest of a genome for comparisons at a sufficient phylogenetic distance. Hence these are relatively easy to align and different alignment strategies tend to give similar results for coding regions. Other parts of the genome are more likely to have mismatches or to have undergone insertion or deletion, which requires introduction of gaps into the alignment. In these noncoding regions, choice of an alignment strategy is expected to have an impact on the result. Global aligners are expected to have somewhat greater sensitivity, but they may include more inaccurate alignments. Local aligners will not align sequences that are too dissimilar, even if they occur in analogous positions in the two genomes. More calibration of the various methods is needed to clarify these issues, but at this point there is no consensus on whether the regions that fail to align by local aligners are not homologous, or whether they are homologs that have changed so much that the similarity is not recognizable by these programs [53].

Chromosomal rearrangements complicate the construction of comprehensive alignments between genomes. Genes that are on the same chromosome in one species are *syntenic*. Groups of genes that are syntenic in humans are frequently also syntenic in mouse, and thus these groups of genes display *conserved synteny*. In addition, they frequently maintain a similar order and orientation, indicating *homology*, which is similarity because of common ancestry. The homologous segments between distantly related species rarely extend for entire chromosomes, but rather one human chromosome will align with several homology blocks in mouse, many of which are on different chromosomes in mouse (Fig. 19.4). For genome comparisons, the goal is to find all the reliable alignments within the homology blocks and deduce how the various homology blocks are connected in the genomes of the species being compared. This requires additional steps to the alignment procedure. For local aligners, it means that the large number of individual alignments needs to be organized along chromosomes. For global aligners, it means that homology blocks must be identified prior to execution of a global alignment.

Local alignments are restricted to the DNA segments between rearrangement breakpoints. A collection of local alignments can be organized into *chains* to maintain the order of DNA segments along the chromosome. In this case, local alignment A is connected to local alignment B in a chain if the beginnings of the aligned sequences in B follow the ends of the aligned sequences in A. The chains can be nested in a group, called a *net* [39], and these are used to navigate local alignments through rearrangements (Fig. 19.4). On a large scale, these nets can be used to illustrate chromosomal rearrangements between species, and on a smaller scale they can reveal multiple events associated with rearrangement breakpoints.

Global aligners can be used in genomic regions that have not been rearranged. In practice, for whole-genome alignments, homology blocks are initially identified using a rapid local alignment procedure. Then a global

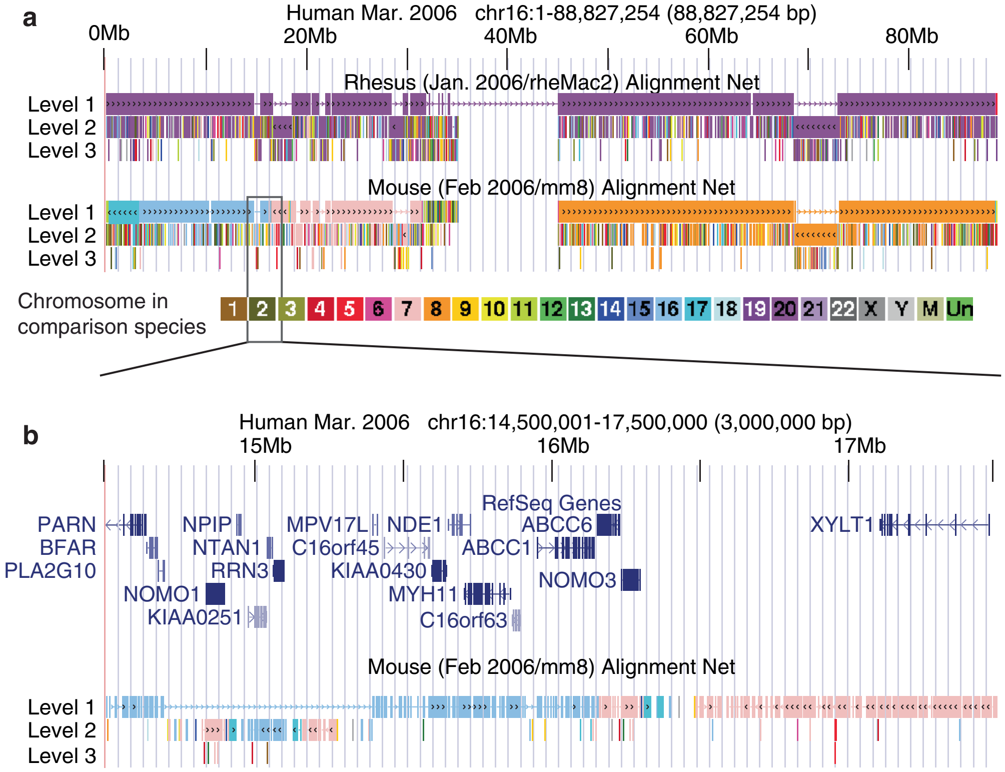


Fig. 19.4 Blocks of conserved synteny and chromosomal rearrangements with human chromosome 16 as the reference sequence. (**a**) Almost all of human chromosome aligns with rhesus chromosome 20, indicated by the purple boxes, but portions of human chromosome 16 align to different chromosomes in mouse, which are color coded by the aligning chromosome in the comparison species. For both comparisons, levels 1, 2, and 3 of a nested set of chained alignments (called a net) are shown. Local alignments form a chain when the start positions of the sequences in one alignment follow the end positions of the sequences in the preceding alignment. The level 1 chain is the highest scoring (usually longest) set of local alignments; the level 1 chain with rhesus covers almost all of rhesus chromosome 20. Gaps in the level 1 chain are filled with the highest scoring additional chains to make level 2 chains, and so on for up to six levels. Inversions are evident by changes in the directions of the arrowheads on the chain maps. (**b**) A higher resolution view of a portion of human chromosome 16 that encompasses a major change in conserved synteny from mouse chromosome 16 (light blue) to mouse chromosome 7 (pink). The diagram illustrates the results of a complex rearrangement history, including an inversion and interlacing of matches to the two mouse chromosomes. Many genes are present in this region despite the complex rearrangements of the chromosome between human and mouse

aligner such as *LAGAN* is run on the sequences in the regions that have not been rearranged [10].

Several powerful Web-servers are available for running these alignment programs on chosen sequences. Often it is prudent to use precomputed alignments because of the complexity of these alignment pipelines and the need for careful adjustment of alignment parameters for different comparisons. Nets and chains of local alignments generated by *blastZ* are available from the UCSC Genome Browser [45] and Ensembl [31]. Precomputed alignments of whole genomes generated by pipelines using *LAGAN* and *VISTA* are also available. As discussed in the next section, analyzes of these alignments can be used to predict function in genomic DNA sequences. Table 19.1 lists a selection of network servers for making and viewing alignments.

19.3 Assessment of Conserved Function from Alignments

Many of the sequences that are conserved between species can be found in the portions of genomes that align. As discussed above, alignment algorithms are good, but imperfect, and no one can guarantee that all

Table 19.1 Selected network servers for making and viewing alignments of genome sequences

Program or pipeline	Name	URL
blastZ, nets and chains	UCSC Genome Browser	http://genome.ucsc.edu/
blastZ, nets and chains	Ensembl	http://www.ensembl.org/
VISTA	VISTA Tools	http://genome.lbl.gov/vista/index.shtml
LAGAN	LAGAN alignment toolkit	http://lagan.stanford.edu/lagan_web/index.shtml
MAVID	MAVID Server	http://baboon.math.berkeley.edu/mavid/
blastZ and others	DCODE.org NCBI	http://www.dcode.org/
blastZ	PipMaker	http://pipmaker.bx.psu.edu/pipmaker/

the conserved sequences will align, especially as the phylogenetic distance between the species increases. Nevertheless, the portions that align should have much of the conserved DNA. Within that conserved DNA is a subset that has a function common to the species being compared; that is the portion that shows evidence of constraint, i.e., purifying selection. Thus, searching genome alignments for evidence of constraint is a major, powerful approach for finding functional DNA sequences.

19.3.1 Phylogenetic Depth of Alignments

The longer two species have been separated, the more divergent their genomes become, and thus one indicator of constraint operating on a sequence is that it aligns with sequences in distantly related species. Several insights can be gleaned by examining the phylogenetic distance at which a particular sequence or class of genomic features continues to align.

As expected, most of the human genome aligns with the genomes of our closest relative, the chimpanzee, and an Old World monkey (the rhesus macaque). The genomes of the comparisons species are not finished for the most part, and thus the values for portion aligning (Table 19.2) will be underestimated, but

Table 19.2 Portions of the human genome conserved and constrained between various species

	Distance from human		Fraction of human aligning to comparison species[d]			
Comparison species[a]	Divergence time (Myr)[b]	Substitutions per synonymous site[c]	Total genome[e]	Coding exons[f]	Regulatory regions[g]	UCEs[h]
Chimpanzee	5.40	0.015	0.95	0.96	0.97	0.99
Macaque	25.0	0.081	0.87	0.96	0.96	0.99
Dog	92.0	0.35	0.67	0.97	0.87	0.99
Mouse	91.0	0.49	0.43	0.97	0.75	1.00
Rat	91.0	0.51	0.41	0.95	0.70	1.00
Opossum	173	0.86	0.10	0.82	0.32	0.95
Chicken	310	1.2	0.037	0.67	0.06	0.95
Zebrafish	450	1.6	0.023	0.65	0.03	0.76
Number			2.858×10^9 nucleotides	250,607	1,3	481

Notes:

[a] Sources of genome sequences are: human: [33]; chimpanzee: [14]; macaque: [70]; dog: [49]; mouse: [85]; rat: [25]; opossum: Broad Institute; chicken: [30]; zebrafish: Zebrafish Sequencing Group at the Sanger Institute

[b] Divergence times for separation from the human branch to the branch leading to the indicated species are from [46]

[c] Estimated substitutions per synonymous site are from [53]

[d] The human genomic intervals in each dataset were examined for whether they aligned with DNA from each comparison species in whole-genome *blastZ* alignments [42]. An interval that is in an alignment for at least 2% of its length was counted as aligning, but in the vast majority of cases the entire interval was aligned.

[e] The number of nucleotides in the human genome that align with each species was divided by the number of sequenced nucleotides in human (given on the last line)

[f] Coding exons are from the RefSeq collection of human genes [68]

[g] Putative transcriptional regulatory regions were determined by high-throughput binding assays and chromatin alterations in the ENCODE regions [79]; the set compiled by King et al. [42] was used here

[h] Ultraconserved elements (UCEs) are the ones with at least 200 bp with no differences between human and mouse [4]

they are still informative. Since almost all of the genome aligns, of course virtually all known functional regions align between human and apes or Old World monkeys. This includes coding exons [68] and putative transcriptional regulatory regions, which are deduced from high-resolution studies on occupancy of DNA by regulatory proteins [79].

When the comparison is made with genomes of eutherian mammals outside the primate lineage, considerably less of the human genome aligns (Table 19.2). Within the 37–57% of the genome that does align, however, we find almost all of the coding exons (95–97%) and putative regulatory regions (74–89%). Even less of the genome aligns with the marsupial opossum (about 13%). At this phylogenetic distance, the alignments of coding exons tend to persist, but only 39% of the putative regulatory regions still align. Only a small fraction of the human genome aligns to more distant species, such as chickens and fish. At this distance, the estimated substitution rate in neutral DNA (synonymous sites) is so high that a segment of neutral DNA is no longer expected to align, and thus it is highly likely that all the alignments between human and chicken or fish are in functional regions.

The insights about conservation of functional elements are easier to visualize when presented as a function of phylogenetic distance (Fig. 19.5). No single comparison is adequate for all goals. Some are particularly good for one purpose, such as using human-opossum alignments for examining coding regions. Almost all the coding regions still align at this distance, but only 13% of the genome aligns. Most comparisons involve a trade-off between sensitivity (the ability to find the desired feature) and specificity (the ability to reject undesired sequences). One may want to examine alignments at a sufficient distance such that no neutral DNA is aligning, but at that distance (e.g., human-chicken) a third of the coding exons and about 90% of the putative regulatory regions no longer align. This means that the specificity is excellent but the sensitivity is lower than usually desired. In practice, it is common to examine comparisons among multiple species that have given good sensitivity, such as alignments among eutherian mammals, and to apply some discriminatory function to better ascertain the regions that are constrained or show some other evidence of function. Alignments to more distant species can be included as well, but they should not be used as an exclusive filter.

The utility and limitations of examining multiple eutherian species has been studied extensively. About 1,000 Mb align among human, mouse, and rat [25], illustrated by the central portion of the Venn diagram in Fig. 19.6. A similar study of human, dog, and mouse revealed about 812 Mb conserved in all three [49]. This approximately 1 Gigabase of genome sequence found in common can be considered the core of the genome of placental mammals. The DNA sequences needed for functions common to all eutherians are expected to be in this core, and indeed virtually all coding exons and putative regulatory regions are found in it (Table 19.2). However, it seems unlikely that this entire core is under constraint. About 162 Mb of the core consists of repetitive DNA

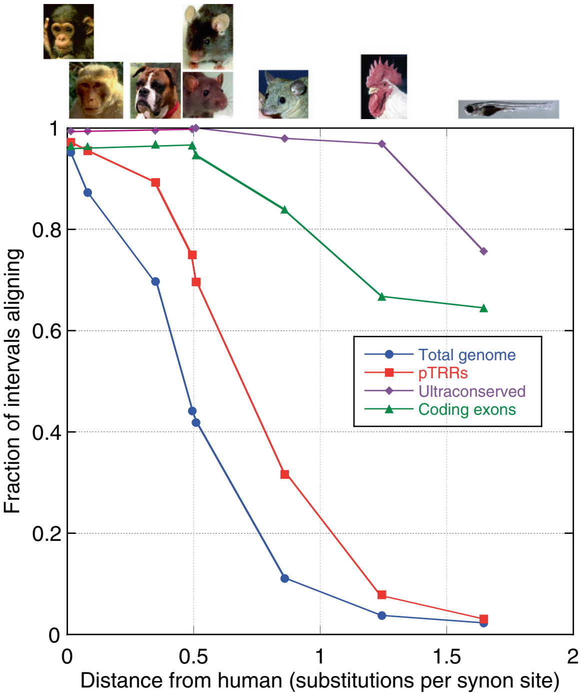


Fig. 19.5 The fraction of genomic intervals that align with comparison species at increasing phylogenetic distance. The fractions of intervals in putative regulatory regions (pTRRs, red squares), coding exons from RefSeq (green triangles) and ultraconserved elements (purple diamonds) substantially exceed the fraction of the human genome (blue circles) that aligns with each species in almost all comparisons. The comparison species in increasing order of distance from human are chimpanzee, rhesus macaque, dog, mouse, rat, opossum, chicken, and zebrafish (pictured above the graph). The distance is the estimated number of substitutions per synonymous site along the path in a tree from human to each species [53]. This measures takes into account faster rates on some lineages, and thus it places mouse and rat more distant from human than dog, despite the earlier divergence of carnivores

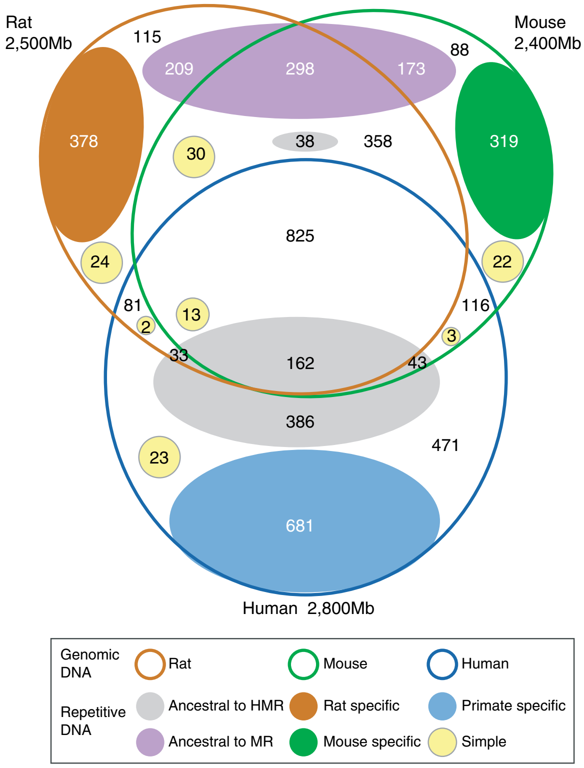


Fig. 19.6 Venn diagram showing common and distinctive sequences in humans and two rodents. As summarized in the key (box under the diagram), the outlined ellipses represent the DNA in each genome, and the overlaps show the amount of sequence aligning in all three species (rat, mouse, and human) or in only two species. Portions of the ellipses that do not overlap represent sequences that do not align. Different types of repetitive DNA are shown as colored disks, and are classified by their ancestry. Those that predate the divergence between rodents and primates are gray, and those that arose on the rodent lineage before the divergence between rat and mouse are lavender. Disks for repeats specific to each species are colored orange for rat, green for mouse, and blue for human; and disks for simple repeats are colored yellow. The disks for the repeats are placed to illustrate the approximate amount of each type in each alignment category. Uncolored areas represent nonrepetitive DNA; the bulk is assumed to be ancestral to the human–rodent divergence. The numbers of nucleotides (in Mb) are given for each sector (type of sequence and alignment category). (Reprinted from Gibbs et al. [25], with permission from Nature Publishing Group)

that is ancestral to primates and rodents (Fig. 19.6). As discussed above, most of this ancestral repetitive DNA can be considered neutral. Granted that some of these ancestral repeats may indeed be functional, it is unlikely that all of them are. Hence, even in the approximately 800 Mb of the core that is nonrepetitive, it is expected that some, and maybe much, also lack a function conserved in all eutherians. This illustrates the need for further discrimination of constrained sequences from those that are conserved but are apparently neutral. Figure 19.6 also shows that the rat and mouse genomes share many DNA sequences that are not in human, and about 358 Mb are nonrepetitive. One may expect to find rodent-specific functional sequences in these portions of the mouse and rat genome. Genomic DNA sequences that are found only in rat or only in mouse are dominated by lineage-specific interspersed repeats.

19.3.2 Portion of the Human Genome Under Constraint

Within the subset of the human genome that aligns with other species, we want to know what fraction of it appears to be under constraint (covered in this section), and then to be able to identify the constrained sequences (covered in the next section). One way to estimate the portion of the human genome under constraint is to evaluate all the segments that align with a comparison species for a level of similarity higher than that seen for neutral DNA. This would be a straightforward approach if we knew all the neutral DNA (which we do not; see Sect. 19.1.3), and if the neutral DNA diverged at the same rate at all positions in the chromosome (illustrated by the ideal case in Fig. 19.2). However, the estimated neutral rates show substantial local variation across the human genome (Fig. 19.7). This has been seen for comparison of the human genome with mouse [27, 85], dog [49], and chimpanzee [14]. Thus, estimates of constraint need to take into account the local rate variation.

For comparison of the human and mouse genomes [85], alignments throughout the genomes were evaluated for a level of similarity that exceeds the similarity expected from the amount of divergence in ancestral repeats in the vicinity. The distribution of similarity scores in ancestral repeats is normal, and many similarity scores in the bulk of the genome overlap with those in the neutral distribution (Fig. 19.8). Notably, a pronounced shoulder of alignments presents a score higher than the scores for a vast majority of ancestral repeats. The broad distribution of alignment scores through the genome can be interpreted as the combination of two distributions, one for neutral DNA and one for DNA that is under constraint. Various models lead to the conclusion that about 5% of the human genome falls into the latter distribution. A similar estimate has been obtained for alignments of the human and dog genomes [49]. In support of the idea

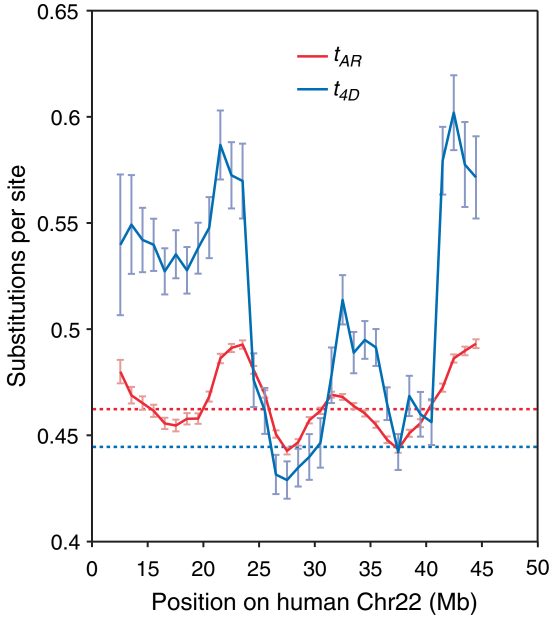


Fig. 19.7 Variation in the rate of human-mouse divergence in neutral DNA along human chromosome 22. The substitutions per site in ancestral repeats (t_{AR}, red) and in and in the subset of synonymous sites that are fourfold degenerate (t_{4D}, blue) were estimated in 5 Mb windows, overlapping by 4 Mb. The horizontal dotted lines indicate the estimates of t_{AR} and t_{4D} across the entire human genome. The confidence intervals are shown as brackets; the places where the confidence interval lies outside the genome-wide estimate are those with significant differences in evolutionary rate. (Reprinted from Waterston et al. [85], with permission from Nature Publishing Group)

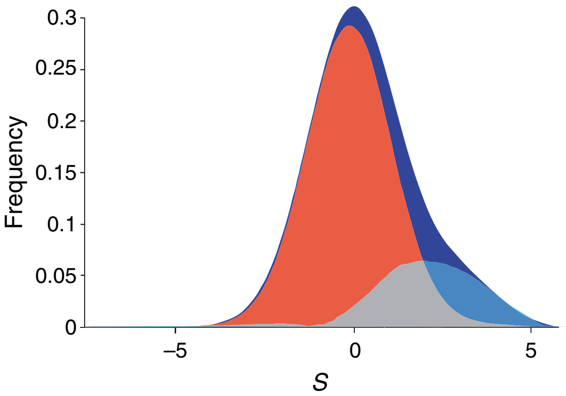


Fig. 19.8 Decomposition of conservation score into neutral and likely selected portions. *S* is the conservation score adjusted for variation in the local substitution rate. The frequency of the S scores for all 50 bp windows in the human genome, after alignment with mouse, is shown as the blue distribution. The frequency of S scores for ancestral repeats is shown in red. The inferred distribution of scores for regions under constraint is shown in light gray and light blue. This represents about 5% of the human genome. (Reprinted from Waterston et al. [85], with permission from Nature Publishing Group)

of a conserved eutherian core genome that encompasses the sequences with common function, the human sequences inferred to be under constraint are the same whether the comparison is with dog or mouse [49].

This result tells us that about 5% of the human genome has been under continuous purifying selection since the divergence of primates from carnivores and rodents, approximately 85–100 million years ago. The functions that would be subject to the continuous selection are those that were present in a eutherian ancestor and continue to play those roles in contemporary primates, rodents, and carnivores (and likely all eutherians). This is a lower bound estimate of the portion of the human genome that is functional. DNA sequences that have diverged for new functions in different lineages are not included in this estimate, nor are sequences that have acquired function recently through adaptive evolution. Thus, the portion of the human genome that is functional is certainly higher than 5%, but it is not possible with current knowledge to place an upper bound on the estimate.

The lower bound estimate of the portion under continuous constraint is a remarkable number. The portion of the human genome needed to code for proteins has been estimated at about 1.2%, with another 0.7% corresponding to untranslated regions of mature mRNA [33], giving an estimate of about 2% of the genome devoted to coding for mRNA. This leaves about 3% of the human genome with sequences that do not code for protein but still carry out functions common to eutherian mammals. Among these additional sequences under constraint should be genes for noncoding RNAs and DNA sequences that regulate the level of expression of genes. It is striking that the fraction of the genome devoted to the conserved noncoding functions is greater than the fraction needed to code for proteins.

19.3.3 Identifying Specific Sequences Under Constraint

In order to find particular functional sequences, it is necessary to identify specific sequences whose alignments are likely to be in the portion under constraint. In principle, it is a matter of finding segments with a similarity score above the neutral background (Fig. 19.2). Of course, it is important to adjust the analysis for variation in local substitution rate, as just discussed. For example, from the distribution of *S* scores in ancestral repeats (Fig. 19.8) based on pairwise human–mouse alignments, one can compute a probability that a given alignment could result from the locally adjusted neutral rate. Those that are unlikely to result from neutral evolution between humans and nonprimates are likely to be under constraint.

Other measures have been developed to utilize the greater amount of information in multiple sequence alignments to identify constrained sequences. One measure is based on modeling the genome as having two states of "conservation," one that is effectively neutral and one that is the slowly changing, constrained state. By combining phylogenetic models with Hidden Markov models of those states, a score called *phastCons* is computed, which gives the posterior probability that any aligned position came from the constrained state [74]. This measure is routinely computed genome-wide for several sets of genome alignments, and is accessed as the "Conservation" track on the UCSC Genome Browser (Fig. 19.9). Note that it has a form similar to the idealized case in Fig. 19.2, with higher peaks associated with a greater likelihood of being constrained.

A constrained sequence is one that had an opportunity to change because it was mutated in an individual in a population, but the mutation was not fixed in the genome sequence of the species because of selective pressure against the change. Thus, there could have been a substitution, but purifying selection rejected it. Another measure of constraint, called genomic evolutionary rate profiling or GERP [16], explicitly models this process and estimates the number of "rejected substitutions" (Fig. 19.9). Another method, binCons, models the substitution frequency as a binomial distribution, with the contribution of alignments of different species weighted according to their phylogenetic distance from the reference species [52].

In a region evaluated by these methods, some segments are identified as being under constraint by all three, and others are found by only one. Each approach has value, and each has some unique advantages and some idiosyncratic problems. Thus, it is useful to combine the output of each to generate sets of "multispecies conserved sequences" [53, 79]. The strict, moderate, and relaxed sets correspond to the MCSs found by intersection, inclusion in at least two, or the union of the three sets. The example shown in Fig. 19.9 illustrates strong constraint not only in the coding exons but also in the introns. Experimental tests on two of these intronic constrained elements show that they affect the level of expression from a linked promoter [71].

19.4 Evolution Within Protein-Coding Genes

Comparative analysis of protein-coding genes requires several steps. First, a set of protein-coding genes must be defined in each species, and then a set of orthologous genes shared among the species is examined. With this, the rates of change among proteins can be computed and then one can study how those differences in rates correlate with function. Most protein-coding genes are under significant constraint over the course of mammalian evolution. However, genes whose products have roles in reproduction, chemosensation, immunity, and metabolism of foreign compounds are found consistently to be changing more rapidly than other genes. Thus, these are some of the functional classes that determine species-specific functions.

19.4.1 Comparative Genomics in Gene Finding

One of the most important tasks in genomics is to identify the segments of DNA that code for a protein. As covered in Chap. XX, most eukaryotic genes are composed of *exons*, which code for mRNA, and *introns*, which are transcribed but spliced out of the mature mRNA. Most internal exons encode a portion of the protein product of the gene, whereas the initial and terminal exons also contain untranslated regions of the mRNA. Most protein-coding exons can be identified by a variety of approaches. However, combining the exons into genes, including accurate determination of the initial exon (or multiple initial exons), is more of a challenge.

The several approaches for finding exons and genes can be divided into two categories: evidence-based and *ab initio*. Evidence-based methods find genomic DNA segments that align almost exactly with known protein sequences (after translating the genomic sequence) or complete mRNA sequences. Most evidence-based methods also incorporate data on

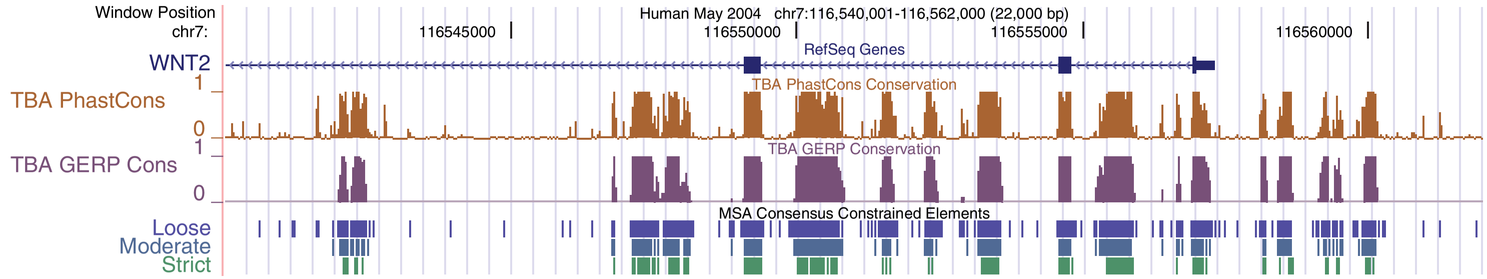


Fig. 19.9 PhastCons and GERP in a portion of ENCODE region ENm001. The first three exons (blue boxes) and introns (lines with arrows showing the direction of transcription from right to left) of the gene *WNT2* are shown on the top line. The next two panels plot the *phastCons* and *GERP* scores, respectively, with higher values indicating a higher probability that a sequence is under constraint. The bottom panel shows the levels of multispecies conserved sequences (see text)

expressed sequence tags (ESTs), which are short sequences containing portions of a very large number of mRNAs, and tags of sequence derived from the 5′ capped ends of mRNAs. The mRNA-coding segments of genomic DNA are grouped, using rules about pre-mRNA splicing signals, to find strings of exons that after splicing gives the mRNA sequence, or after splicing and translation gives the protein sequence. In order to find likely exons of genes whose mRNA sequences are not in the databases, *ab initio* methods based on models derived from basic knowledge about gene structure are applied. The genetic code and rules for splice junctions (Chaps. XX) provide the rules that make up the basic grammar for encoding proteins. Hidden Markov models such as those in the programs *genscan* [11] and *genmark* [28] are used to find likely exons and likely arrangements for these exons in genes.

Adding alignments of sequences of other species can improve gene prediction. Two commonly used methods are *Twinscan* [89] and *SGP* [87]; these build on the models in *genscan* but also apply rules from comparative approaches, such as allowing mismatches at degenerate sites in the genetic code. Another program, *exoniPhy* [73], uses the grammar of protein coding and a phylogenetic analysis of multispecies alignments to improve exon finding.

Often the initial and final exons do not code for protein, and thus the *ab initio* predictors no longer benefit from the well-known rules for encoding proteins. Furthermore, it is not uncommon for a gene to have multiple initial exons, with some used at particular times of development or in certain tissues. Thus, the accuracy of fully assembling genes from exons is enhanced by evidence such as mRNA sequences and tags derived from the 5′ ends of mRNA. Powerful pipelines for gene annotations have been developed that combine both evidence-based and *ab initio* methods; one of the most widely used is the Ensembl automatic gene annotation system [17].

In the current assembly of the human genome (NCBI build 36, March 2006, hg18), the Ensembl pipeline predicts 270,239 exons. These are arranged into 44,537 mRNAs from 21,662 genes. Most genes code for multiple mRNAs, thereby greatly increasing the diversity of proteins encoded in the human genome. Of these exons and genes, how many are found in other species, and which contribute to lineage-specific characteristics?

19.4.2 Sets of Related Genes

When discussing genes that are shared among species, we usually want to find the genes that are derived from the same gene in the last common ancestor. Homologous genes that separated because of a speciation event are *orthologous*. When there is a simple 1:1 relationship between orthologous genes, such as for *RRM1* in Fig. 19.10a, then any differences between the genes can be interpreted as changes since the time of divergence of the species.

When homologous genes are members of multigene families, then it is important to distinguish genes that have separated as a result of gene duplication (*paralogous* genes) from the orthologous genes, which separated by speciation events (Fig. 19.10a). For instance, the beta-like globin genes in humans arose by duplication in mammals. Within this gene family, each gene is paralogous to the other. For example, *HBE1* and *HBB* are paralogs that resulted from an earlier duplication, whereas *HBG1* and *HBG2* are paralogs that duplicated recently. Each of the four beta-like globin genes in chickens is paralogous to the other three, again because of the duplication history.

When gene duplications have occurred independently in both lineages, then all the duplicated genes in one species are orthologous to each of the genes in the other lineage. This is a many-to-many orthologous relationship. The human *HBB* gene is equally distant from each of the chicken beta-like globin genes, and it is orthologous to each.

Frequently a comparison will involve multigene families in species that share a duplication history, such as the beta-like globin gene clusters in human and macaque (Fig. 19.10b). The gene duplications outlined in panel A pre-date the catarrhine ancestor (ancestor to Old World monkeys, apes and humans). Thus, the *HBB* gene in humans is orthologous to the *HBB* gene in macaque, but it is paralogous to the other macaque beta-like globin genes, such as *HBD*, *HBG1*, etc. Likewise, the human *HBE1* gene is orthologous to the *HBE1* gene in macaque, but paralogous to the others. Comparisons between the orthologs reflect changes that have occurred since the separation of Old World monkeys and humans, whereas comparisons between the paralogs will reflect changes over a much greater phylogenetic distance, i.e., back to the gene duplications that generated the ancestors to the genes being

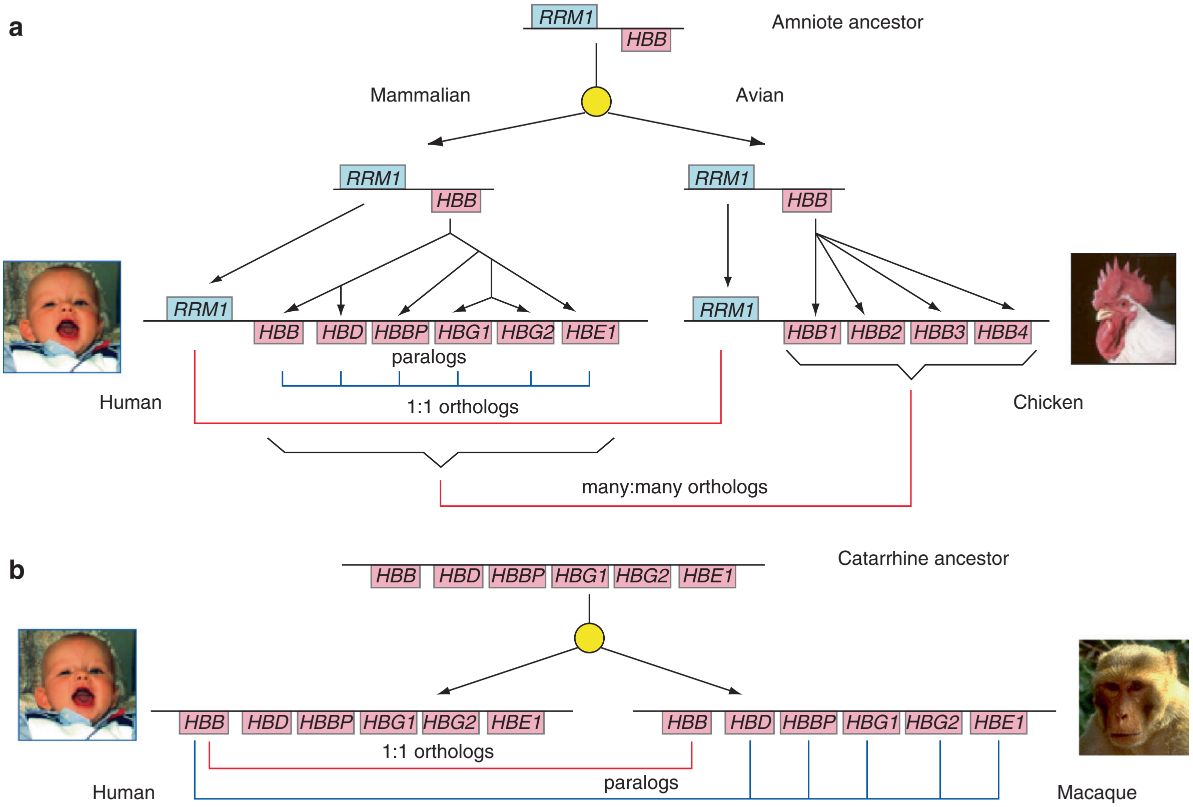


Fig. 19.10 Orthologous and paralogous relationships among genes. Speciation events are shown as yellow disks, and gene duplications are denoted by bifurcating arrows or multiple arrows with a single source. Red lines between genes in contemporary species connect orthologous genes, whereas blue lines connect paralogous genes. (**a**) Illustration of the phylogenetic history of the *RRM1* gene (encoding ribonucleotide reductase M subunit) and the *HBB* gene (encoding beta-globin) and genes related to it by duplication since the divergence of mammalian and avian lineages from the amniote ancestor. The gene duplications in the beta-like globin gene family occurred separately in the mammalian and avian lineages, leading to paralogous relationships within a species and many-to-many orthologous relationships between the species. (**b**) Illustration of the the phylogenetic history of the beta-like globin gene cluster over the much shorter time since humans and macaques (an Old World monkey) diverged from the catarrhine ancestor. The gene duplications predate the ancestor, and thus the speciation event resulted in 1:1 orthologous relationships between human and macaque *HBB*, human and macaque *HBD*, etc. Other relationships, e.g., between human *HBB* and macaque *HBD* are paralogous

compared. In this situation, correct assignments of paralogous and orthologous relationships are particularly important. For instance, an incorrect assignment of paralogous genes as being orthologous between human and macaque would lead to a conclusion of greater sequence change since speciation than would a truly orthologous comparison.

Once gene sets have been defined in two or more species, then orthologous gene sets can be determined. For the cases of 1:1 orthologs, reciprocal highest similarity is a good guide to orthologous relationships. The more complicated cases for multigene families can be summarized as many-to-many orthologous relationships. Figure 19.11 shows the results of comparisons of protein-coding genes among human (*Homo sapiens*), chicken (*Gallus gallus*), and the teleost fish *Fugu rubripes* [30]. Of the almost 22,000 genes annotated in humans in this study, about a third are in 1:1:1 orthologous relationships with chicken and *Fugu*, and about 5% are in many-to-many relationships. About a third of the genes have clear homologs but cannot be definitively assigned as orthologous. Intriguingly, about 4,000 human genes do not have a clear homolog in either chicken or fish. These may encode mammal-specific functions.

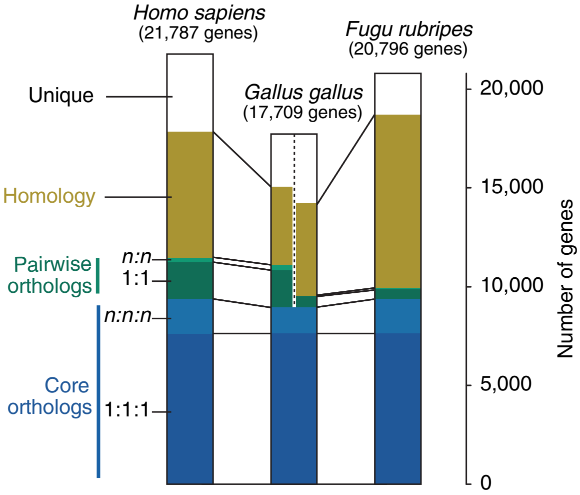


Fig. 19.11 Homology relationships among protein-coding genes in human (*Homo sapiens*), chicken (*Gallus gallus*), and the fish *Fugu rubripes*. Genes in the three species are grouped by their orthology relationships among the three species (1:1:1 or *n:n:n* for many:many:many) or between two species if the gene is not detected in a third species. Genes that are clearly related between species but for which clear orthology relationships cannot be determined are placed in the 'Homology' class. Genes not falling in the orthology or 'homology' classes are considered 'Unique'. (Reprinted from Hillier et al. 2004, with permission from Nature Publishing Group)

19.4.3 Rates of Sequence Change in Different Parts of Genes

Within the set of 1:1 orthologous genes, the amount of sequence similarity can be determined in each of the basic parts of a gene. One of the first genome-wide studies in mammals compared human genes with mouse genes [85], and it confirmed many insights from smaller scale studies. The protein-coding exons are the most similar between human and mouse, showing about 85% identity (Fig. 19.12). The regions adjacent to the splice junctions show peaks of higher identity, reflecting the selection on both coding potential and on splicing function. The introns have the lowest similarity, but they are considerably more similar than is DNA in ancestral repeats (the neutral model in this study), which are about 60% identical. The untranslated regions of exons are about 75% identical. The higher percent identity in the untranslated regions and introns, than in the neutral model, indicate that some portion of these sequences is under constraint. Intronic regions that provide important functions include splicing enhancers and transcriptional enhancers. In the 3′ untranslated region can be found targets for regulation by miRNAs as well as the polyadenylation signals. These short segments can be subject to stringent constraint. If all the intronic and untranslated sequences were subject to such stringent constraint, then their overall percent identity would be closer to that of the coding regions. Thus, one interpretation of these results is that intronic and untranslated regions contain short constrained segments interspersed within larger regions with little or no signature of purifying selection.

19.4.4 Evolution and Function in Protein-Coding Exons

From the earliest comparisons of homologous protein sequences, it was recognized that some proteins change little between species. A classic example is histone H4,

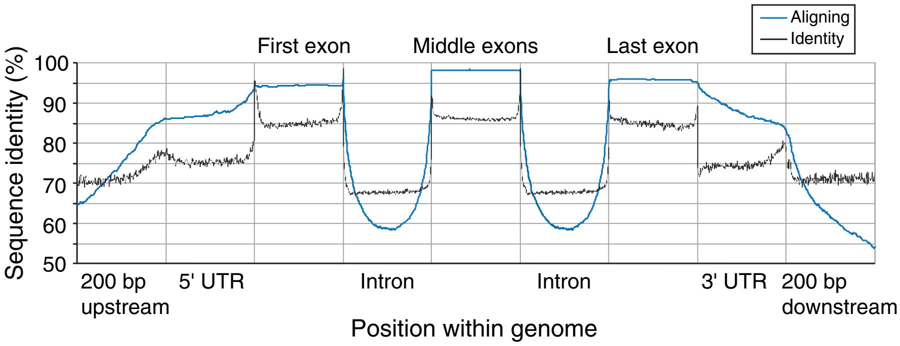


Fig. 19.12 Sequence identity between human and mouse in a generic gene. Within a group of 3,165 RefSeq genes that aligned between the mouse and human genomes, 200 evenly spaced bases across each of the variable-length regions were sampled between human and mouse. The blue line shows the average percentage of bases aligning and the black line shows the average base identity. (From Waterston et al. [85], with permission from Nature Publishing Group)

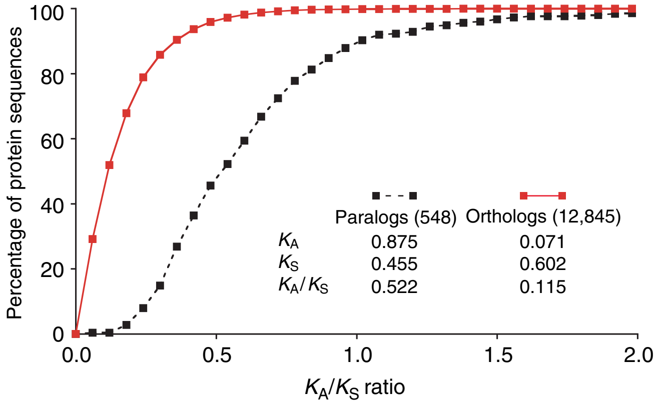


Fig. 19.13 Cumulative distribution of K_A/K_S values for mouse proteins compared with human homologs. The distribution of scores for proteins that are clearly orthologous between human and mouse is shown by the red points and line. The distribution of scores for proteins encoded by locally duplicated, paralogous mouse-specific gene clusters is shown by the black points and line. (From Waterston et al. [85], with permission from Nature Publishing Group)

which has only one amino acid replacement between peas and cows. Other proteins change rapidly. Among the most rapidly changing proteins are the fibrinopeptides, which are segments of fibrinogen molecules that are cleaved off by thrombin during blood clotting. It appears that the amino acid sequence of the fibrinopeptides is not critical for their function, and they are under little or no selective pressure. Interspecies comparisons of even a modest number of proteins showed that the rate of changes in amino acids ranged over 100-fold [60]. Some proteins, such as histones, are under stringent selection over most of their sequence, whereas others seem to be free to change extensively – or have been adapted to new function.

Comparisons of the protein-coding genes for entire mammalian genomes provide the opportunity to examine these issues more comprehensively. The sets of related genes between species can be analyzed to show which genes are under strong purifying constraint and which show signs of adaptive evolution. For protein-coding genes, it is common to consider substitutions at synonymous sites to be neutral. The number of synonymous substitutions per synonymous sites in two species is called K_S. This can be used as an estimate of the neutral rate. Then the number of nonsynonymous substitutions per nonsynonymous site, or K_A, can be compared with K_S to obtain an estimate of the stringency of the purifying selection or the strength of adaptive evolution. As a rule of thumb, a K_A/K_S ratio of 0.2 for human–mouse comparisons is indicative of constraint, whereas ratios of 1 or greater indicate adaptive evolution.

In a study of orthologous genes aligned between mouse and human [85], about 80% show an overall signal for constraint (Fig. 19.13). Very few show evidence of positive selection over their entire length. Thus, at the phylogenetic distance of mouse and human, evolution of protein-coding sequences in orthologous genes is dominated by constraint. This result indicates that the matching, orthologous segments code for proteins that provided a function in the ancestor, and their descendant sequences provide a similar function in contemporary species. Many changes in the encoded amino acid sequences have been selected against because they did not improve the function of the protein. We note that short segments or single codons under positive selection would not be detected in this test.

In contrast, the set of paralogous genes compared between mouse and human are shifted to higher K_A/K_S ratios. Thus, the paralogous genes are more likely to be undergoing adaptive evolution (positive or diversifying selection) than are the orthologous genes. The multigene families are major contributors to lineage-specific function. Duplication of genes leaves at least one copy free to accumulate changes that can provide an adaptive advantage. In contrast, genes that remain as single copies are constrained to fulfill the role that they have played since they arose in some distant ancestor.

19.4.5 Fast-Changing Genes That Code for Proteins

The families of fast-changing genes appear to be adapting to new pressures in a lineage-specific manner. An examination of the types of gene families with this property should provide insights into the types of pressures that lead to adaptive changes. A remarkably consistent result has been found in multiple studies

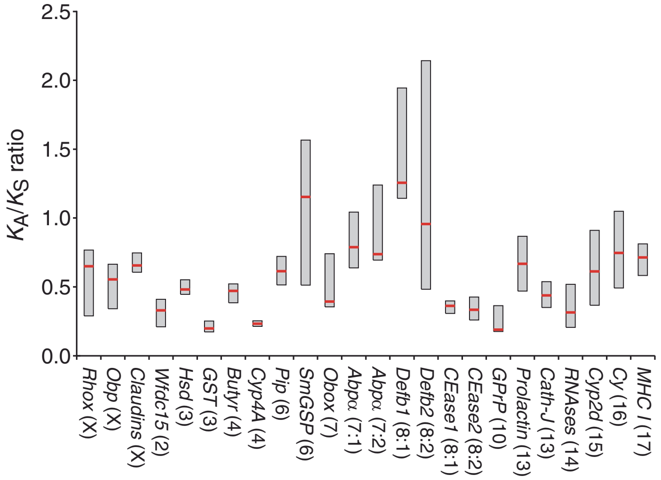


Fig. 19.14 Distributions of K_A/K_S values for duplicated mouse-specific gene clusters. The chromosome on which the clusters are found is indicated in brackets after the abbreviated cluster name. The K_A/K_S values for each sequence pair in the cluster were calculated from aligned sequences. The box plots summarize the distributions of these values, with the median indicated by the red horizontal line and the boxes extending from the 16th and 83 rd percentiles and hence covering the middle 67% of the data. (From Waterston et al. [85], with permission from Nature Publishing Group)

of this question. The four general categories of reproduction, chemosensation, immune response, and xenobiotic metabolism (breakdown of drugs, toxins, and other compounds not produced in the body) encompass many of the genes and gene families subject to positive selection. Thus, these are the major physiological functions in which rapid sequence change leads to adaptive evolution.

For example, the locally duplicated gene families with relatively high K_A/K_S values fall into distinct functional classes (Fig. 19.14). Members of the major categories for adaptive evolution (reproduction, chemosensation, immune response, and xenobiotic metabolism) are apparent. For example, the mouse *Rhox* genes on chromosome X are homeobox genes expressed in male and female reproductive tissue, and targeted disruption of the *Rhox5* gene leads to reduced male fertility [51]. Another example is the oocyte-specific homeobox gene *Obox* on mouse chromosome 7. The *Obp* gene cluster encodes odorant-binding proteins such as lipocalins and aphrodisin, involved in both chemosensation and reproduction. Immune response genes include the *MHC I* genes on chromosome 17, which regulate the immune response, the *Wfdc15* gene, which encodes an antibacterial protein, and the *Defb* genes on chromosome 8 encoding beta-defensins. Several adaptive genes are involved in xenobiotic metabolism, including members of the cytochrome P450 gene family, *Cyp4a* and *Cyp2d*, and a glutathione-*S*-transferase gene (*GST*).

Additional studies of lineage-specific expansions of gene families in comparisons of rat and mouse [25] and of humans and chickens [30] identify the same general categories of reproduction, chemosensation, immune response, and xenobiotic metabolism. Thus, along multiple lineages, these gene families are implicated in adapting to unique pressures on each species. Enrichment of these functional categories for genes implicated in adaptive evolution can be readily rationalized. Changes in genes involved in reproduction and chemosensation could lead to or maintain the differences that cause divergence of species. Adaptation of immune function and the ability to metabolize foreign compounds are important for survival in the distinctive environment of each species. Other families with rapid changes between species include keratins, which are involved in making feathers in birds but hair in mammals.

19.4.6 Recent Adaptive Selection in Humans

In addition to improving our understanding of the evolution of humans within the context of other vertebrates, comparative genomics also provides insights into recent adaptive changes that may eventually tell us what genome sequences make us distinctively human. Comparisons to close relatives such as the chimpanzee and analysis of human polymorphisms drive these new studies.

As was the case for human–mouse comparisons discussed above, the K_A/K_S ratio was computed in genome-wide comparison of the human and chimpanzee gene sets [12, 14, 15, 61]. The ratio for human–chimpanzee comparisons is significantly higher than that seen for mouse–rat comparisons, showing more changes in amino acids in proteins (normalized to synonymous substitutions) in the hominid lineages than in rodents. This does not, however, indicate an overall stronger positive selection in hominids, but rather it reflects the relaxation of purifying selection in species with a small population size. Estimates of effective population size for rodents far exceed those for humans and chimpanzees, and it is well recognized that the severity of selection increases with population size. However, despite this relaxed selection, examination of the orthologous genes with the most extreme ratios of amino acid-changing substitutions to presumptive neutral changes reveals interesting candidates for hominid-specific adaptive evolution. One is the gene for glycophorin C, which is the membrane protein used for invasion of the malarial parasite *Plasmodium falciparum* into human erythrocytes. Others include granulysin, which is needed for defense against intracellular parasites, and semenogelins, which are involved in reproduction. A stronger signal for positive selection can be observed when genes are grouped together, either by physical proximity (often as duplicated genes) or by functional category. For human-chimpanzee comparisons, the sets of genes changing most rapidly include the now-familiar categories of reproduction (e.g., spermatogenesis, fertilization, and pregnancy), chemosensation (olfactory receptors, taste receptors), immunity (immunoglobulin lambda, immunoglobulin receptors, complement activation), and xenobiotic metabolism, plus additional categories such as inhibition of apoptosis.

The distribution of human polymorphisms along chromosomes and their frequency in populations can be analyzed for insights into very recent selection (reviewed in [5, 44]). Positive selection is expected to drive mutations quickly to fixation, so loci under positive selection should be characterized by a skew in the allele frequency distribution toward rare alleles. One measure of that skew is Tajima's D [77]. Also, the rapid fixation of an advantageous allele will bring along linked polymorphisms. These polymorphisms will not have had time to be separated from the selected allele by recombination, and thus linkage disequilibrium will extend further around positively selected alleles than is expected from neutral evolution. Various tests of properties such as these have been developed, and have traditionally been applied to a small number of loci. A major limitation to these studies is that changes in population demographics can generate the same signals. For example, recent expansion in population size, such as that experienced by humans, will also lead to an excess of rare alleles or extended linkage disequilibrium. Thus, it is difficult to disentangle the confounding effects of population demographics and positive selection when only a few genetic loci are examined. However, the recent availability of genome-wide data on polymorphisms [32] provides one solution. Changes in population size should affect all loci in the genome, whereas selection should act on only a few. Thus, when the distribution of values for Tajima's D, long-range haplotype, or related measures are examined for a large number of loci, then it is likely that the outliers are undergoing adaptive evolution [5].

Recent genome-wide studies have identified significant outliers based on frequency of rare alleles (Tajima's D, [13, 37]) and linkage disequilibrium [82, 83]. For example, Carlson et al. [13] calculated Tajima's D in sliding windows across the human genome for populations descended from Africans, Europeans, or Chinese. Several extended regions with consistently negative values for Tajima's D were identified, with most observed in only one of the populations (Fig. 19.15). Negative values for Tajima's D are associated with positive selection if population expansion is not a factor, and the study design to identify outliers in a genome-wide analysis should greatly reduce the confounding effect of such an expansion. Thus, results such as those in Fig. 19.15 indicate that at least one genetic element in the roughly one megabase region with reduced Tajima's D has been under positive selection in humans of European ancestry. Resequencing of targeted genes within these regions has supported the conclusion of positive selection, and in some cases (e.g., *CLSPN* in Fig. 19.15) it has revealed a polymorphism that alters the encoded amino acid sequence [13]. Such a change in amino

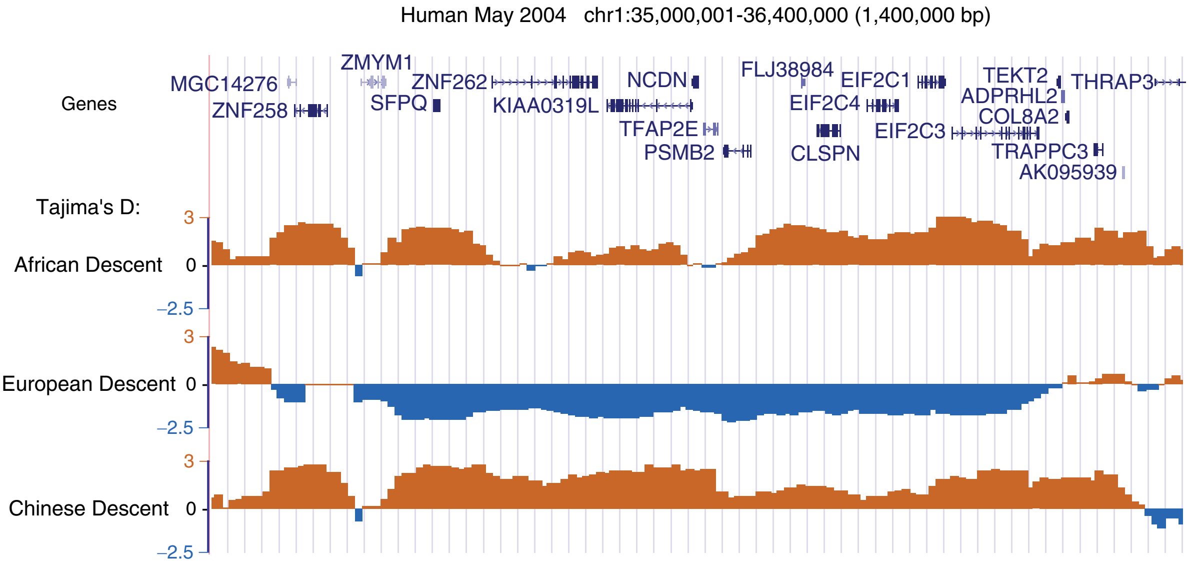


Fig. 19.15 An extended region with an excess of rare alleles indicative of positive selection. The region from human chromosome 1 is one of several identified in the study by Carlson et al. [13] showing an excess of rare alleles in at least one of three human populations (those of European descent in this case) as measured by Tajima's D [77]. Negative values of Tajima's D can be explained by positive selection or population expansion; the design of genome-wide studies favors the former explanation. The full data from the study are available on the UCSC Genome Browser [39]; this figure was generated from the Browser output

acid sequence is a candidate for the functional variant under selection.

A third type of test for recent selection utilizes both human polymorphism data and interspecies divergence between human and close relative, such as chimpanzee. The McDonald-Kreitman [55] test compares the ratio of polymorphisms to divergence (r_{pd}) at nonsynonymous sites (leading to amino acid changes in the protein product) with that ratio in synonymous sites, which do not change the amino acid sequence and are expected to be largely neutral. If the changes in nonsynonymous sites had no selective advantage or disadvantage, then r_{pd} at these sites would not be significantly different from r_{pd} at neutral sites. Deviation from neutral expectation can be evaluated with a chi-square or related statistic. Bustamante et al. [12] applied this test to over 11,000 human genes (with polymorphsims determined in three different populations) compared with chimpanzee. They found that 9% had a significant signal for positive selection and 14% had a significant signal for negative selection.

Each method for finding loci under recent selection in humans has its distinctive strengths and weaknesses. Much effort is currently devoted to examining overlaps and differences in the results. Among the several studies reviewed by Biswas and Akey [5], a total of 2,316 human genes were found to have at least one signature for positive selection. Almost a third of these, including *EDAR, SLC30A9*, and *HERC1*, are found in more than one genome-wide study. Other candidate genes for positive selection are found by only one approach, such as *TRPV5* and *TRPV6*. At least to some extent, the failure to overlap reflects the different types of selective events being assayed in the different tests. The features examined by one approach, such as low frequency alleles, are not contributing to other tests, such as linkage disequilibrium measurements based on common alleles [5].

Some genes that are candidates for human-specific selection lead to intriguing and exciting possibilities, such as alterations in *FOXP2* implicated in language acquisition [22] and *MCPH1* and *ASPM* implicated in brain size [23, 56]. Further studies of recent selection in humans should lead to critical new insights into human biology and disease.

19.4.7 Human Disease-Related Genes

Comparative genomics can be used to study the origins and implications of genetic variants associated with human disease. Disadvantageous mutations should be cleared from a population quickly, so why are some genetic diseases rather common?

One factor is the relaxed selection against mildly deleterious alleles resulting from population expansion. A common estimate of the effective population size of humans is about 10,000 individuals, and of course the population has expanded dramatically to the current level of over 6 billion. This would tend to favor the persistence of some deleterious mutations, and the results of a McDonald-Kreitman test [12] indicate that many of the amino acid polymorphisms in humans are moderately deleterious.

Another factor is positive selection in one region of the world driving an allele to high frequency, but that allele is pathogenic in other regions of the world. A classic example is the *HBB-S* allele of the gene encoding beta-globin. This allele encodes a mutant beta-globin that in combination with alpha-globin and heme constitutes HbS. This is the hemoglobin variant that causes red blood cells to form a sickled, inflexible morphology when deoxygenated, and thus leads to sickle cell disease. However, the *HBB-S* allele in heterozygotes reduces the susceptibility of humans to malaria, and thus it is a protective allele in regions of the world in which malaria is endemic. In fact, haplotype analysis has shown that the *HBB-S* allele has arisen independently multiple times in recent human history [2, 63]. This indicates a strong positive selection in the presence of the malarial parasite. Unfortunately, the negative consequence is that people who are homozygous for the *HBB-S* allele are highly prone to sickle cell disease.

A third factor is that some disease-associated variants were protective in the more distant past but are now detrimental for most contemporary human lifestyles. In the "thrifty genotype" hypothesis [59], the limited caloric intake and need for high activity levels in ancestral humans would have favored a thrifty genotype that made efficient use of food. However, many contemporary humans live in an environment with an excess of available food. Being "too thrifty" with energy metabolism could lead to problems such as diabetes. Disease-associated variants that were advantageous in the past should match the amino acid at that position in ancestor, and some of these will still be seen in related species. Indeed, human disease-related variants match with the amino acid in the corresponding position of chimpanzee [14] and rhesus macaque [70] in about 16 and 200 cases, respectively. Further studies of these candidates are needed, but the results suggest that retention of an ancestral state is also contributing to human disease alleles.

19.5 Evolution in Regions That Do Not Code for Proteins or mRNA

Despite the importance of protein-coding regions to genome function, these sequences account for about one-third of the sequences that have been under selection for a common function in eutherian mammals. Accounting for the remaining selection in noncoding regions is a major on-going effort in genomics and genetics. Two functional categories are the focus of much attention: genes that do not code for proteins, such as microRNA (miRNA) genes, and gene regulatory regions. An equally important question is to what phylogenetic depth functional noncoding regions are conserved. These issues will be examined in this section.

19.5.1 Ultraconserved Elements

The level of constraint on genomic sequences spans a wide range, and it likely that different functions are subject to distinctive levels of constraint. The most intense constraint is revealed in the human DNA segments called *ultraconserved elements*, or UCEs [4]. These are the 481 human DNA segments that are identical to mouse DNA for at least 200 nucleotides. Sequences that code for proteins have frequent mismatches between human and mouse at synonymous sites, so these UCEs are under stronger purifying selection than most exons. This pattern of conservation indicates that all nucleotides in the identical segment are critical for some function. The UCEs are broadly conserved in vertebrates, and they show the slowest rate of divergence over the period of vertebrate evolution of any known elements in the genome (Table 19.2, Fig. 19.5).

Determining the roles for the UCEs is currently a matter of intense interest. Only a small fraction (23%) overlaps with mRNA for known protein-coding genes. Thus, the majority is associated with some noncoding function. About half of those tested serve as tissue-specific enhancers in transgenic mouse embryos [64]. A small number are related to each other, and examination of these has revealed a family of sequences derived from an ancient transposable element that have been recruited for activity as a distal enhancer for one gene and part of an exon for another [3]. Another subset of very slowly changing regions (across most eutherians) was examined for rapid change along the human lineage since divergence from chimpanzee. These *human accelerated regions* include a gene that encodes an RNA that may function in cortical development [66]. A full explanation of the stringent constraint on each nucleotide within the UCEs remains elusive. Not only is the intensity of constraint beyond that seen for almost all protein-coding regions, but even RNAs with considerable secondary structure rarely show this resistance to substitution.

Another enigmatic aspect to UCEs is their restriction to vertebrates. Protein sequences, which evolve faster than UCEs in vertebrates, frequently show significant similarity between vertebrate and invertebrates species. Sometimes the similarity extends from vertebrates to eubacteria. In contrast, no homolog to a UCE sequence has been observed outside vertebrates. Worms (and possibly other invertebrates) have analogous highly constrained noncoding sequences, but they differ in sequence from the vertebrate UCEs [81]. Thus, this stringent constraint on noncoding sequences may have evolved in parallel in vertebrates and invertebrates. Finding the sources of the UCEs and explaining how they could be under such intense constraint are important goals for future work. Answers to these questions may reveal aspects of genome function that have yet to be imagined. The fact that the roles and origins of the most stringently constrained sequences in vertebrates are still unknown illustrates how much still needs to be accomplished in comparative genomics.

19.5.2 Evolution Within Noncoding Genes

Many genes do not code for protein, and these must account for some of the noncoding DNA that is under constraint. However, some of the better-known noncoding genes do not help explain the fraction under constraint, but for technical reasons. Consider the genes for RNAs utilized in the mechanics of protein synthesis, such as ribosomal RNAs (rRNA) and transfer RNAs (tRNAs). The rRNA genes are clustered in highly duplicated regions on the short arms of chromosomes 13, 14, 15, 21, and 22. These regions are not included in the assemblies of the human genome, and thus they do not contribute to the minimal estimate of 5% of the genome under constraint in mammals. The tRNA genes are small and contribute little to the selected fraction. Other RNAs, such as snRNAs involved in splicing and processing of precursors to mRNA, also tend to be encoded on small genes. Multiple copies of sequences related to the snRNA genes are present in the human genome, some of which may no longer be active. The contribution of snRNA genes to the fraction of the human genome under constraint needs further study.

The miRNAs do not code for protein, but they negatively regulate mRNA function or abundance. Hybridization of an miRNA to its mRNA target to generate a duplex with some mismatches leads to inhibition of translation of the mRNA. Hybridization of an miRNA to its target to generate a perfect duplex leads to degradation of the target mRNA (see Chap. XX).

The known miRNA genes are constrained, with many conserved from humans to chickens. However, the full set of miRNA genes is not known, and information is limited about the structure and conservation of genes encoding the precursors to miRNAs. Thus, the miRNAs clearly are important contributors to the fraction of the genome under purifying selection, and they could account for substantially more of the constraint that is currently known.

Members of another class of RNA that apparently does not code for protein are detected by hybridization of copies of cytoplasmic RNA to high-density tiling arrays of nonrepetitive human genomic DNA. These results show transcription of protein-coding genes as expected, but about half the transcribed regions are not associated with known genes [34]. These unannotated transcripts, referred to as *transfrags*, are often of low abundance and are expressed in a limited set of tissues. The contribution of transfrags to constrained sequences in human is a matter of current study (e.g., [67, 79]).

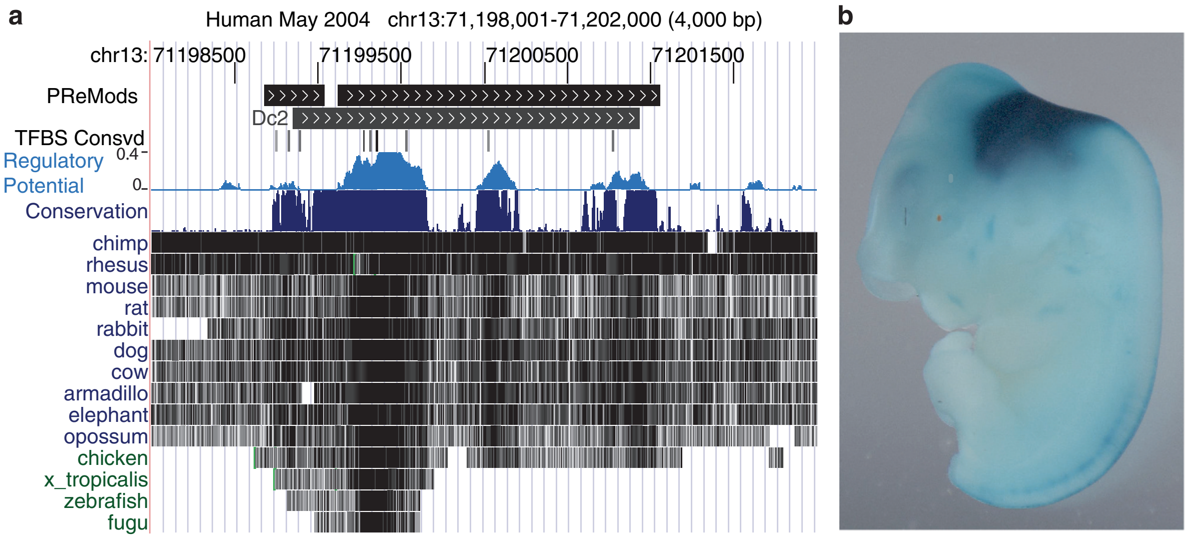


Fig. 19.16 An enhancer of the *DACH1* gene predicted by comparative genomics. This human gene is homologous to the *Drosophila* gene *dachshund*, and it is needed for development of the central nervous system and other organs. Within one of the very large introns of *DACH1* are some deeply conserved DNA segments. (**a**) Several features of the Dc2 region, including its conservation from humans to fish, high regulatory potential [78], and prediction as a regulatory module by the PReMod pipeline [6]. Examples of conserved matches to transcription factor binding site motifs are also shown. (**b**) This DNA segment is sufficient to enhance expression of a beta-galactosidase reporter gene in the hindbrain of a transgenic mouse embryo. The blue stain is a marker for beta-galactosidase activity. The Dc2 region was shown to be an enhancer by Nobrega et al. [62]; the image is from the Enhancer Browser (Table 19.3)

19.5.3 Evolution and Function in Gene Regulatory Sequences

DNA sequences needed to regulate the level, developmental timing, and tissue-specificity of gene expression include promoters that designate the correct start site for transcription, enhancers that increase the level of expression, silencers that decrease the level of expression, and insulators that separate genes and regulatory regions from the effects of neighboring regulatory regions. Many but not all of these regulatory regions are conserved among mammals [26, 64]. Some of the DNA sequences that regulate genes encoding developmental regulatory proteins are conserved from mammals to fish, indicative of strong constraint [62, 88]. One example is shown in Fig. 19.16. However, other regulatory regions show more rapid evolution, e.g., replacing one motif for binding a transcription factor with a similar sequence in another location [19, 50] or being present in only one lineage. Despite numerous studies of the extent of conservation of regulatory regions in individual loci, no clear consensus had emerged on the dominant pattern of conservation.

A major limitation to previous studies has been the small number of regulatory regions that have been identified experimentally. Establishing the role of a segment of DNA in regulation requires multiple experiments, and traditionally these were done in a highly directed manner that did not lend itself to high throughput. Now it is possible to enrich DNA for sites occupied by transcription factors (by chromatin immunoprecipitation or ChIP) and then hybridize this enriched DNA to high-density tiling arrays of genomic DNA (DNA chips). This ChIP-chip experiment [69] reveals sites bound by transcription factors in a high-throughput manner. Experiments by the ENCODE Project Consortium [79] evaluating sites occupied by several transcription factors have yielded a large set (over 1,000) of putative transcriptional regulatory regions in about 1% of the human genome. This large set of DNA intervals implicated in transcriptional regulation was identified by experiments that are agnostic to interspecies sequence conservation, and thus it is an ideal set in which to determine the phylogenetic depth of conservation [42]. As shown in Fig. 19.5 and Table 19.2, about two out of three of these putative transcriptional regulatory regions are conserved from humans to other placental mammals (but no further), and about one out of three are conserved to marsupials. Less than 10% are conserved from humans to birds. An equal fraction, about 3%, is found at the two extremes of conservation, viz., found only in primates or conserved from humans to fish. Thus, the bulk of the regulatory regions are conserved in placental mammals, and we expect that comparisons among these species will continue to be effective at finding and better understanding these regulatory regions. However, a particular phylogenetic depth of conservation is not a consistent property of gene regulatory sequences. Rather, the depth of conservation is a property that varies among the regulatory sequences. Ongoing studies may reveal whether particular functions of regulatory regions or their targets correlate with the depth of conservation.

Although it is not a property shared by all putative regulatory regions, many do have a significant signal for purifying selection. A small majority (about 55%) overlap at least in part with DNA segments that are in the 5% of the human genome that is under strong selection [79]. However, only about 10% of the nucleotides in the putative regulatory regions are under strong constraint, suggesting that small subregions of enhancers and promoters, e.g., binding sites for particular transcription factors, are under purifying selection. Thus, the putative regulatory sequences identified in the ENCODE project contribute only a small amount to the 5% under strong constraint [79].

19.5.4 Prediction and Tests of Gene Regulatory Sequences

Effective use of comparative genomics to find gene regulatory sequences is challenging for at least two reasons. The variation in phylogenetic depth of conservation is a major complication; some human regulatory regions will be observed only in alignments of primates, whereas others align with species as distant as fish. Although the large majority of regulatory regions are conserved in multiple placental mammals, even some apparently neutral DNA aligns reliably at this phylogenetic distance. Thus, the ability to align at this distance is not a property that identifies regulatory regions with good specificity.

Most efforts to detect candidate gene regulatory regions from aligned sequences also use some form of pattern information. For example, the known regulatory regions are clusters of binding sites for transcription factors. The binding sites are short (about 6–8 bp) and many allow degeneracy (e.g., either purine or either pyrimidine works equally well at some sites). Therefore, the binding site motifs themselves do not confer strong specificity. However, in combination with clustering and conservation, this set of criteria has good power to detect novel regulatory regions [6]. A set of about 200,000 regions, called *PReMods*, has been identified as predicted regulatory regions in the human genome using this approach.

The motifs for binding sites in regulatory regions are not known completely. These currently unknown motifs can be incorporated into the prediction of regulatory regions by using machine-learning procedures to find distinctive patterns of alignment columns that are common in a training set of alignments in known regulatory regions, but are less abundant in a set of alignments from likely neutral DNA. The statistical models describing these distinctive patterns are then used to score any alignment for its *regulatory potential*. One implementation of this approach has generated a set of about 250,000 regions of human DNA with a high regulatory potential [78]. Many of these overlap with the PReMods discovered as conserved clusters of transcription factor-binding motifs. Regions with high regulatory potential and a conserved binding site for an erythroid transcription factor are validated at a good rate as enhancers in erythroid cells [84].

In summary, several methods based on comparative genomics can be used with some success to predict gene regulatory sequences, but none achieves the level of reliability desired. Deep conservation of noncoding sequences, e.g., from human to chicken or human to fish, can be used without additional information about patterns such as binding site motifs. However, this approach will miss the majority of gene regulatory regions. For noncoding sequences conserved among placental mammals, clustering of pattern information should be incorporated. The pattern information can either be based on prior knowledge (such as binding motifs) or learned from training sets. Currently, in vivo occupancy of DNA segments by transcription factors is being determined comprehensively by ChIP-chip and related methods. Integration of this information with the comparative genomics should add considerable power to the identification of regulatory regions [21].

19.6 Resources for Comparative Genomics

The large amount and wide variety of data on comparative genomics of mammals and other species can be daunting to those who wish to use them. Also, as discussed throughout this chapter, the level of conservation of functional regions tends to vary from region to region. Detailed information needs to be readily accessible for individual regions and for classes of features across a genome. These needs are accommodated by genome browsers and data marts. Computational tools for further analysis of the data are also available, and one workspace for such tools will be described here.

19.6.1 Genome Browsers and Data Marts

Genome browsers show tracks of user-specified information for a designated locus in a genome. The major browsers for mammalian genomes are the UCSC Genome Browser [45], Ensembl [31], and MapView at NCBI [86] (Table 19.3). Comparative genomics tracks showing results of whole-genome alignments are available at the UCSC Genome Browser and Ensembl. As illustrated in Fig. 19.4, the regions of the human genome aligning with a comparison species can be seen as nets and chains. Inferences about severity of constraint are captured on the "Conservation" track (similar to that in Fig. 19.9), based on phastCons [74].

Often it is desirable to collect and analyze all members of a feature set across a genome or large genomic intervals. This requires the ability to query on the databases of features that underly the browsers. Two such "data marts" are the UCSC Table Browser [35] and BioMart at Ensembl [36]. Both provide interactive query pages to provide access to the data.

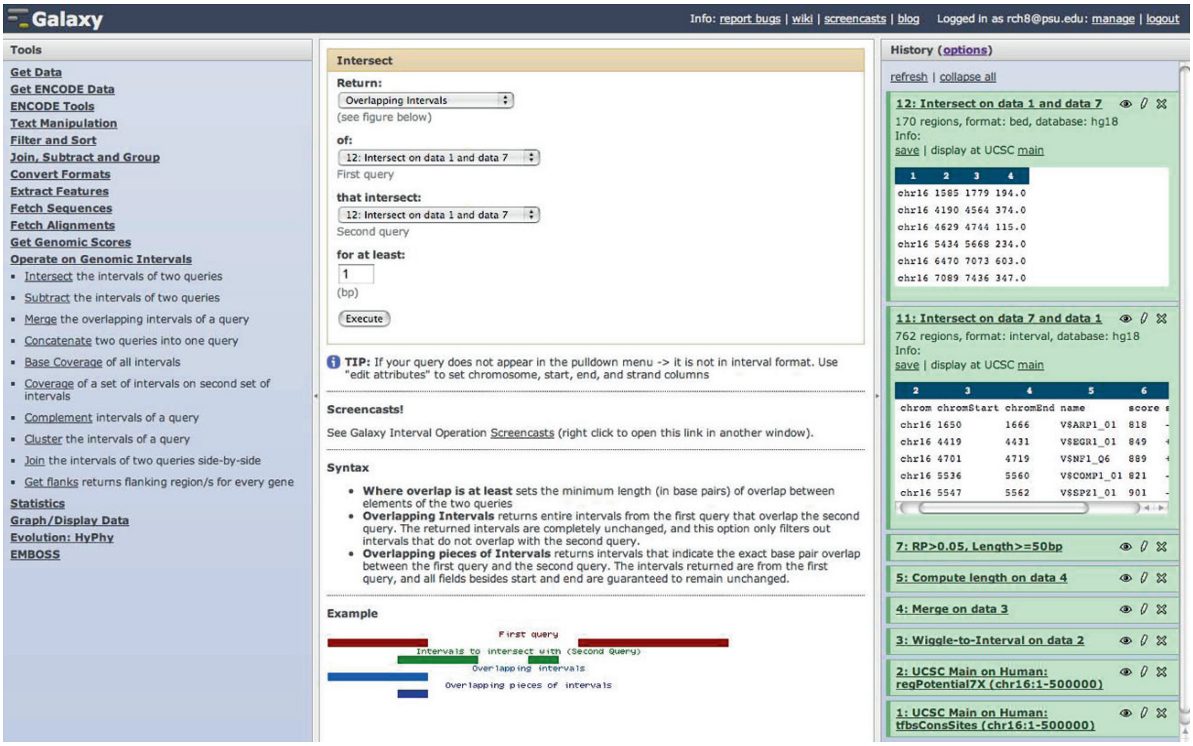
19.6.2 Genome Analysis Workspaces

Once the data have been obtained, users frequently need to analyze them further. Different data sets may need to be combined or compared. The level of constraint or regulatory potential may be needed. Estimates of evolutionary rates may be desired. Different tasks will require distinct sets of tools. Considerable progress can be made by acquiring the necessary computer programs and executing them on the user's computer system. However, this leaves it to the user to find or write the needed tools.

An alternative is to connect versatile data acquisition with integrated suites of computational tools in a common workspace such as Galaxy [7] (Table 19.3). This resource allows users to import data from various sources, such as the UCSC Table Browser, BioMart, or files from the user's computer. Once imported, a wide variety of operations can be performed on the data sets, such as edits, subtractions, unions, and intersections. Summary statistics can be computed and distributions can be plotted. Various evolutionary genetic analyzes can be performed.

Table 19.3 Data resources and analysis workspaces for comparative genomics

Name	Description	URL
UCSC Genome Browser	Sequences, comparative genomics, annotations	http://genome.ucsc.edu
Ensembl	Sequences, comparative genomics, annotations	http://www.ensembl.org/
NCBI MapViewer	Gene, EST and other maps of chromosomes	http://www.ncbi.nlm.nih.gov/mapview/
UCSC Table Browser	Query for genomic features	http://genome.ucsc.edu/cgi-bin/hgTables
BioMart	Query for features of genes	http://www.ensembl.org/biomart/martview/
VISTA Enhancer Browser	Data on conserved noncoding regions tested as developmental enhancers	http://enhancer.lbl.gov/
Galaxy	Interactive workspace for analysis of genome sequences, alignments and annotation	http://main.g2.bx.psu.edu/


Galaxy
Info: report bugs | wiki | screencasts | blog
Logged in as rch8@psu.edu: manage | logout
Tools
Get Data
Get ENCODE Data
ENCODE Tools
Text Manipulation
Filter and Sort
Join, Subtract and Group
Convert Formats
Extract Features
Fetch Sequences
Fetch Alignments
Get Genomic Scores
Operate on Genomic Intervals
Intersect the intervals of two queries
Subtract the intervals of two queries
Merge the overlapping intervals of a query
Concatenate two queries into one query
Base Coverage of all intervals
Coverage of a set of intervals on second set of intervals
Complement intervals of a query
Cluster the intervals of a query
Join the intervals of two queries side-by-side
Get flanks returns flanking region/s for every gene
Statistics
Graph/Display Data
Evolution: HyPhy
EMBOSS
Execute
History (options)
refresh | collapse all
First query
Intervals to intersect with (Second Query)
Overlapping intervals
Overlapping pieces of intervals

Intersect

Return: Overlapping Intervals
(see figure below)

of: 12: Intersect on data 1 and data 7
First query

that intersect: 12: Intersect on data 1 and data 7
Second query

for at least: 1
(bp)

TIP: If your query does not appear in the pulldown menu -> it is not in interval format. Use "edit attributes" to set chromosome, start, end, and strand columns

Screencasts!

See Galaxy Interval Operation Screencasts (right click to open this link in another window).

Syntax

- **Where overlap is at least** sets the minimum length (in base pairs) of overlap between elements of the two queries
- **Overlapping Intervals** returns entire intervals from the first query that overlap the second query. The returned intervals are completely unchanged, and this option only filters out intervals that do not overlap with the second query.
- **Overlapping pieces of Intervals** returns intervals that indicate the exact base pair overlap between the first query and the second query. The intervals returned are from the first query, and all fields besides start and end are guaranteed to remain unchanged.

Example

12: Intersect on data 1 and data 7
170 regions, format: bed, database: hg18
Info:
save | display at UCSC main

1	2	3	4
chr16	1585	1779	194.0
chr16	4190	4564	374.0
chr16	4629	4744	115.0
chr16	5434	5668	234.0
chr16	6470	7073	603.0
chr16	7089	7436	347.0

11: Intersect on data 7 and data 1
762 regions, format: interval, database: hg18
Info:
save | display at UCSC main

2	3	4	5	6
chrom	chromStart	chromEnd	name	score
chr16	1650	1666	V$ARP1_01	818
chr16	4419	4431	V$EGR1_01	849
chr16	4701	4719	V$NF1_Q6	889
chr16	5536	5560	V$COMP1_01	821
chr16	5547	5562	V$SP21_01	901

7: RP>0.05, Length>=50bp

5: Compute length on data 4

4: Merge on data 3

3: Wiggle-to-Interval on data 2

2: UCSC Main on Human: regPotential7X (chr16:1-500000)

1: UCSC Main on Human: tfbsConsSites (chr16:1-500000)

Fig. 19.17 Using Galaxy to find predicted regulatory regions. The user interface for Galaxy has three panels. Tools for obtaining and analyzing data are selected from the left panel, and the user selects input data and other parameters in the central panel. A history of previous results is maintained on the right panel. In this example, candidates for gene regulatory modules in a 500 kb region of human Chromosome 16 are obtained by queries to the UCSC Table Browser to obtain conserved matches to transcription factor binding motifs (query 1) and regions of of high regulatory potential (score >=0.05 in query 2; these results were converted to intervals, merged and filtered for length>=50 bp to obtain the results in query 7). Intersections reveal conserved motifs that are in regions of high regulatory potential (query 11) and *vice versa* (query 12)

Precomputed scores such as phastCons and regulatory potential can be aggregated on specified intervals. The interface at Galaxy for a series of operations that can predict gene regulatory regions is shown in Fig. 19.17.

19.7 Concluding Remarks

Comparative genomics brings considerable power but daunting challenges to the study of human genetics. No aspect of comparative genomics has been perfected; even the commonly used methods of aligning sequences and predicting protein-coding genes have room for improvement. However, considerable insight and functionality can be gleaned from the predictions and comparisons that are currently available. Real biological variation, for example, in the rate of evolutionary change at different loci or the phylogenetic depth of conservation of a feature class, means that no single threshold for a conservation-based score will be adequate to find all the features of interest. However, as the variation is better understood and as functional correlates of the variation are established, then the potential power of comparative genomics will be better harnessed. Current data can be readily accessed and evaluated. Additional types of data, such as genome-wide ChIP-chip results, coupled with tools for better integration of disparate data types, should lead to considerable future progress in the functional annotation of the human genome.

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Genetics and Genomics of Human Population Structure

20

Sohini Ramachandran, Hua Tang, Ryan N. Gutenkunst, and Carlos D. Bustamante

Abstract Recent developments in sequencing technology have created a flood of new data on human genetic variation, and this data has yielded new insights into human population structure. Here we review what both early and more recent studies have taught us about human population structure and history. Early studies showed that most human genetic variation occurs within populations rather than between them, and that genetically related populations often cluster geographically. Recent studies based on much larger data sets have recapitulated these observations, but have also demonstrated that high-density genotyping allows individuals to be reliably assigned to their population of origin. In fact, for admixed individuals, even the ancestry of particular genomic regions can often be reliably inferred. Recent studies have also offered detailed information about the composition of specific populations from around the world, revealing how history has shaped their genetic makeup. We also briefly review quantitative models of human genetic history, including the role natural selection has played in shaping human genetic variation.

Contents

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20.1 Introduction

Technological developments arising from the International Human Genome Sequencing and the International Haplotype Map (The International HapMap Consortium, 2003, 2005, 2007) [20–22] projects are transforming the study of human population genetics by dramatically reducing the cost of sequencing and genotyping. For example, as of early 2009, it costs about U.S. $500 per sample to genotype a million variable DNA sites (i.e., SNPs) and structural variants in the human genome and between $50,000 and $100,000 to sequence a human genome de novo. Recalling that the first human genome cost on the order of $1 billion dollars to sequence, this is a 10^4 gain in efficiency over less than a decade. Furthermore, by the time this book is published the costs we quote above may be reduced by another factor of two or three. In the next 5–10 years, therefore, we will likely see hundreds of thousands (if not millions) of human genomes sequenced, and the vast majority of variation within and among human populations cataloged and analyzed to answer fundamental questions in human and medical genomics.

The purpose of this chapter is to lay the groundwork for thinking about how we will begin to make use of this tremendous abundance of data. While these data will dwarf all that has come before, we will see that many of the questions we wish to answer are actually quite old – some as old as the field of human genetics, itself.

20.1.1 Evolutionary Forces Shaping Human Genetic Variation

Quantifying patterns of human genetic variation serves several important roles in genetics. First, it helps us understand human history and often gives us insights into time periods that have left no written record. For example, global patterns of human genetic variation suggest an African origin of modern humans approximately 150,000–200,000 years ago and are consistent with a "serial" founder model (see Sect. 20.5.1) for subsequent colonization and peopling of the world. Second, it helps us understand human *evolutionary* history. For example, patterns of human genetic variation allow us to delineate what genomic changes are unique to our species (i.e., shared by all humans to the exclusion of other apes), and which may be shared ancestrally (or recurrently) with other species. Likewise, patterns of human genetic variation can give us insight into regions of the human genome that may have experienced recent positive, negative, or balancing selection (see Nielsen et al. [39] for a recent review).

Understanding patterns of human genetic variation is also fundamental for the proper design of medical genomic studies, since population structure can often be a confounding variable in genome-wide association mapping. As the density of markers queried for association with disease increases and we begin to look at rare variants that may show limited geographic distributions, quantifying population structure at ever finer scales will be critical to the interpretation and analysis of experiments which aim to correlate patterns of genetic and phenotypic variation. In order to properly set the stage for our discussion, we will briefly review some key concepts from population genetics, anthropology, and genetics that may be unfamiliar to some readers.

The evolutionary dynamics of natural populations (be they human, plant, animal, or otherwise) are governed by a confluence of different evolutionary forces.

Chief among these is *mutation*, which is the ultimate source of variation. As this book illustrates, the process of mutation is a heterogeneous category of changes in DNA that come about through myriad pathways and ultimately induce changes ranging from single base pair alterations (i.e., single nucleotide polymorphisms or SNPs) to small insertion and deletions to large-scale structural rearrangments or even the addition or deletion of whole chromosomes. Most of the variation we will discuss in this chapter will be of the "small scale" variety, with a particular emphasis on understanding patterns of microsatellite, SNP, and haplotype variation.

We limit ourselves to these marker types largely due to practicality: assaying SNP and microsatellite variation has become standardized, and there are now a plethora of studies – such as those cited later on in this chapter – that have undertaken surveys using these markers across diverse human populations. Our hope is that, as the world of personalized genomics becomes a reality, large and micro-scale structural variation becomes cataloged and standardized in similar ways.

The second key force shaping patterns of human genetic variation is *genetic drift*. As you will recall from Chap. 16, genetic drift is a stochastic force that apportions variation by randomly subsampling variation from one generation to the next. Traditionally, we model genetic drift as simple binomial sampling of alleles. That is, if we consider a biallelic locus under no selection and represent the frequency of an allele *A* at time *t* in a given population of size *2N* as x_t, the frequency in the next generation (x_{t+1}) is binomially distributed with probability of success x_t and sample size *2N*. (It turns out this binomial distribution can be generalized and there is a rich treatment of this subject in theoretical population genetics.) This random sampling from generation to generation induces what is known as a "random walk," such that the collection of allele frequencies from the start of the population history until the current time ($x_0, x_1, \ldots, x_t$) as well as the distribution of long-term average frequencies across different sites can be modeled using a litany of theoretical tools.

For our purposes, we will focus on several qualitative impacts of this neutral evolutionary model. First, for a given population, the dynamics of genetic drift will be governed by the magnitude of *2N*, so that populations with a large number of individuals will "drift" more slowly or take smaller steps in frequency space from generation to generation than small populations. This model also suggests that if we were to follow lines-of-descent (i.e., the number of offspring left some time in the future by a given lineage today) with no difference in average offspring number among lineages, then the probability of a given lineage eventually overtaking the population is simply given by its current frequency. (For example, a lineage or allele at 20% frequency has a 20% chance of eventually getting fixed in the population, and an 80% chance of eventually getting lost.) Likewise, the model predicts that frequency is often a good proxy for age (at least for neutral alleles) so that a mutation at 25% frequency in the population is very likely to be older than a mutation at 5% frequency. For this reason, the distribution of SNP frequencies or the so-called allele-frequency spectrum contains a fair amount of information regarding the history of the population. Mathematically, we would define this quantity using an equation such as the following for a population with sample size of n_i individuals:

$$Y_i := \{\text{the number of SNPs where the sample frequency is } i/(2n_i)\}. \tag{20.1}$$

We will return to Y_i later in the chapter and discuss methods for inferring demographic history and selection from these frequencies. (Note: in the equation above we are assuming directionality as to which allele is the ancestral form and which is the derived. In practice, we infer this information from comparative genomic data, ideally, with correction for multiple mutations occurring at the site. See [17, 18] for a discussion of this problem).

The third force that will affect patterns of human genetic variation is *migration* or, more generally, *demographic history*. By this we mean that a given population (certainly for humans) is unlikely to reproduce as a fully endogamous unit. Rather, there is some probability every generation that new migrants from other populations may enter and contribute to the gene pool of the next generation. We also know that a given population is unlikely to remain the exact same size from generation to generation; it may increase or decrease in size, or go through boom/bust cycles. The number of demographic models one can construct is staggering, but certain general properties of models are described below.

For example, populations that have a closely shared evolutionary history – say they are exchanging migrants often or split from a common ancestral population a short time ago – will show a strong and positive correlation in allele frequencies both over time (i.e., the two populations' x_t values for a specific SNP will be correlated over time) as well as across the genome (i.e., the observed Y_i values will be correlated). We can also define a quantity such as the "joint allele frequency spectrum" to help us quantify this correlation and gauge the impact of different evolutionary forces on sets of populations. Mathematically, for a pair of populations *i* and *j* with sample size n_i and n_j this might take the form of a quantity Y_{ij} such that:

$$Y_{ij} = \{\textit{the number of SNPs where the sample frequency is } i/(2n_i) \textit{ in population } i \textit{ and } j/(2n_j) \textit{ in population } j\}. \tag{20.2}$$

As we will see throughout this chapter, the allele frequency spectrum both of a single population (i.e., Y_i) and for a pair (Y_{ij}) or more ($Y_{ijkl\ldots}$) contains a fair amount of information regarding the evolutionary history of the populations in question. Many of the commonly used statistics in population genetics such as Wright's F-statistics, defined in Sect. 20.2.1, are

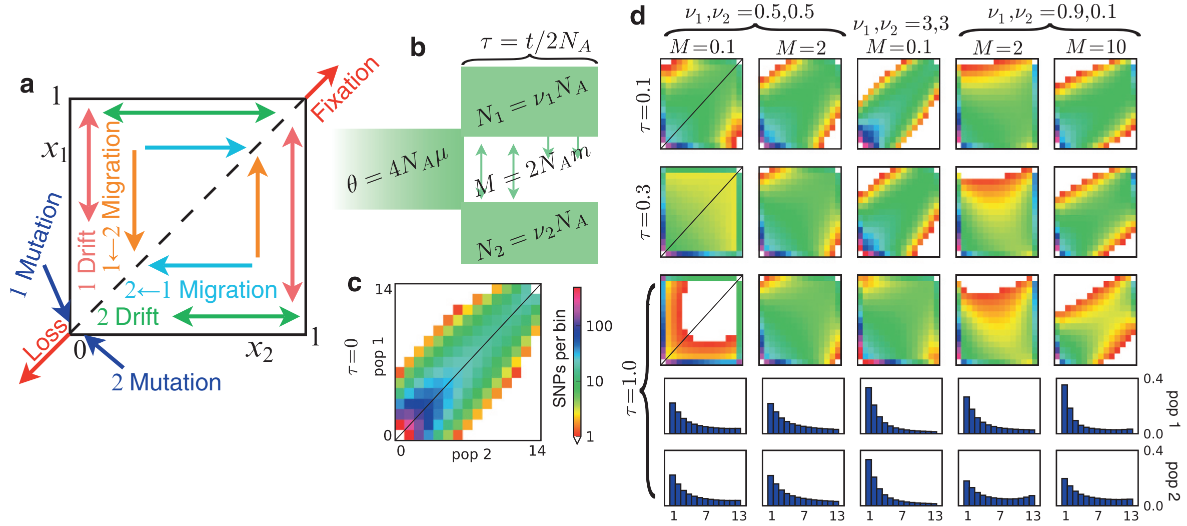


Fig. 20.1 Frequency spectrum gallery (adapted from Gutenkunst et al., manuscript submitted). (**a**) Impact of different evolutionary forces on shared patterns of genetic variation for a pair of populations, as defined by the density of alleles at relative frequencies x_1 and x_2 in populations 1 and 2. (**b**) Graphical description of an evolutionary model in which a pair of populations diverge and continue to exchange migrants. Specifically, an equilibrium population of effective size N_A diverges into two populations $2N_A\ \tau$ generations ago. Populations 1 and 2 have effective sizes $\nu_1 N_A$ and $\nu_2 N_A$, respectively. Migration is symmetric at $m = M/(2N_A)$ per generation, and the scaled mutation rate $\theta = 1{,}000$. (**c**) The allele frequency spectrum (AFS) at $\tau = 0$. Each entry is colored according to the logarithm of the number of SNPs with a given pairwise sample frequency, ranging from 0 to 14 copies of an allele in each population. (**d**) The AFS at various times for various demographic parameters, on the same scale as **c**. From the two-dimensional spectra, note that increased migration leads to more correlated SNP frequencies, and differences in population size lead to asymmetric genetic drift and thus an asymmetric AFS. For $\tau = 1$, the single-population spectra are also shown, where the scale is the fraction of polymorphisms observed at a given sample frequency. In these, note in particular that when populations experience growth, the spectrum is skewed toward rare alleles, particularly for the middle scenario.

summaries of these quantities. In Fig. 20.1 we show how different demographic forces acting on a population can impact their marginal (Y_i) and joint (Y_{ij}) site-frequency spectra.

The fourth force which contributes to the distribution of human genomic variation is natural selection. As was discussed in the chapter on population genetics (Chap. 16), selection works to decrease the frequency of deleterious alleles, increase the frequency of positively selected variants, and stabilize the frequency of variants subject to balancing selection. In human populations, it appears that selection is a much weaker force than genetic drift or demographic history in shaping global patterns of genomic variation. Nonetheless, there are some clear examples of positive and balancing selection on the human genome which have been recently reviewed (see [39]). Here, we will discuss selection briefly and mostly in light of selection against deleterious alleles, since this is the most prevalent form of selection operating on the human genome (see Sect. 20.6.2).

20.2 Quantifying Population Structure

In this section, we introduce several methods for quantifying and detecting population structure. We begin by introducing the classic *F*-statistics, which measure the degree of genetic differentiation among pre-defined and discrete subpopulations. We will then focus on model-based clustering methods, which aim to characterize latent and possibly nondiscrete population structure.

20.2.1 F_{ST} and Genetic Distance

Nonrandom mating in a population with substructure has two consequences: first, preferential mating between individuals from the same subpopulation is a form of *inbreeding*, and has the effect of reducing genetic diversity (measured as, say, heterozygosity) in the overall population; second, as the subpopulations experience independent genetic drift, allele frequencies at genetic markers tend to diverge. Originally introduced by Wright in 1921 [69] to quantify the inbreeding effect of population substructure, F_{ST} has become one of the most widely used measures of genetic differentiation between predefined subpopulations.

Consider the simple setting, in which a population consists of several subpopulations. F_{ST} is defined as the decrease in heterozygosity among subpopulations (H_S), relative to the heterozygosity in the total population (H_T):

$$F_{ST} = \frac{H_T - H_S}{H_T}, \qquad (20.3)$$

where H_S is the *expected* heterozygosity, computed under the assumption that mating is random within each subpopulation (Hardy-Weinberg equilibrium), while H_T is analogously computed assuming random mating in the entire population without population structure.

Alternatively, F_{ST} is often loosely interpreted as the proportion of variance in allele frequencies at a locus that is explained by the subpopulation level of organization. For example, suppose the frequency of an allele is 0 and 1 in two subpopulations, respectively, then $F_{ST}=1$, meaning the variance in allele frequency is completely explained by the population division. Under this framework, F_{ST} at a biallelic single nucleotide polymorphism (SNP) marker can be computed based on the allele frequencies:

$$F_{ST} = \frac{\sigma_p^2}{\overline{p}(1 - \overline{p})}, \qquad (20.4)$$

where σ_{p}^2 is the variance of allele frequencies among subpopulations and $\overline{p}$ denotes the average allele frequency in the pooled population. It can be shown that (20.3) and (20.4) are mathematically equivalent for biallelic markers, but (20.4) is often computationally more convenient.

F_{ST} is often taken as a genetic distance measure, with higher values of F_{ST} reflecting a greater level of genetic divergence. However, both (20.3) and (20.4) define F_{ST} for a specific locus; F_{ST} can vary considerably from locus to locus. Moreover, a locus that is under population- or environment-specific selection can also exhibit unusually high F_{ST}. For example, across globally-distributed human populations, functional polymorphisms in genes related to skin pigmentation show unusually high levels of F_{ST} (i.e., population differentiation) as compared to the genome-wide distribution (see [45]). To reduce the variance across the markers and the bias due to a small number of strongly selected loci, when F_{ST} is reported as an index for genetic distance among subpopulations, it is often calculated by averaging both the numerator and the denominator in (20.3) or (20.4) across loci.

When one is interested in quantifying the degree of substructure among predefined populations, F_{ST} is a simple and useful measure of genetic distance. However, it is often the case that we are interested in using the genetic data itself to define the populations. In particular, if we are interested in detecting cryptic or hidden population structure, then we need to resort to other approaches (see Sect. 20.4). One method for detecting latent population structure, principal component analysis (PCA), was introduced in Sect. 6.4.4. In the next section, we explain a complementary approach, which defines subpopulations based on statistical genetic models for the data.

20.2.2 Model-Based Clustering Algorithms

Cluster analysis refers to a large family of approaches, whose goal is to simultaneously define subsets (called clusters) and to assign observational units into these clusters, so that members in the same cluster are similar by some criteria. For a comprehensive survey of clustering approaches, readers are referred to Mardia et al. [32] or Hastie et al. [15].

In the context of inferring genetic structure, the data usually consist of individuals genotyped at multiple genetic markers (e.g., restriction fragment length polymorphisms RFLPs, microsatellites, or SNPs). In the discrete population model, all alleles in an individual are assumed to be drawn randomly from one of the subpopulations, according to a set of allele frequencies that are specific to each subpopulation. The goal of the analysis is to simultaneously estimate subpopulation allele frequencies and group membership (i.e., which individuals are drawn from which subpopulation). However, for many human populations, there is often no single group from which individuals derive their ancestry. That is, recent migration gives rise to genetically admixed individuals, whose genomes represent a mixture of alleles from multiple "ancestral" populations (see Sect. 20.5).

Mathematically, this means that an individual may have partial membership in more than one cluster. These clusters are biologically interpreted as ancestral populations for the admixed individuals. For example, African Americans in the United States are a recently admixed group, deriving ancestry from European and West African ancestral populations [65]. Under the admixture model, an African American individual's population membership is characterized by the *individual ancestry* (IA) proportion, which is a vector representing the probability that a randomly selected allele from this individual originates from a European (or alternatively, an African) ancestor.

Under either the discrete or the admixture model, individuals' memberships (or IA values) are jointly inferred with the allele frequencies in each subpopulation, using either maximum likelihood or Bayesian methods. We begin by explaining the maximum likelihood approach for the discrete subpopulation model, as this model illustrates the principles that underlie most of the model-based approaches [63]. Let $G_i^m = (a(i,m), b(i,m))$ denote the genotype of individual *i* at marker *m*, with *a(i,m)* and *b(i,m)* being the unordered pair of alleles. Let $Z_i \in (1,\ldots, k)$ indicate the subpopulation membership for individual *i*, and $P = \{p_{m_l}^k\}$ be the frequency of allele *l* at marker *m* in population *k*. Under the assumption that genotypes among markers are independent conditioning on an individual's membership, and that all markers are in Hardy-Weinberg equilibrium within each subpopulation, the likelihood function, treating *Z* and *P* as parameters, is simply the product of the probability of observing each allele:

$$L(P,Z;G) \propto \prod_i \prod_m p_{m_{a(i,m)}}^{z_i} p_{m_{b(i,m)}}^{z_i}. \qquad (20.5)$$

For the admixture model, one can substitute Z_i by $(Z_{i,m}^a, Z_{i,m}^b)$, the population origin of each allele, and model $Z_{i,m}^a$ and $Z_{i,m}^b$ as independent draws from the multinomial probability vectors of individual ancestry. The inference of population structure amounts to the inference on Z_i, or the genome-wide average of $(Z_{i,m}^a, Z_{i,m}^b)$.

In the maximum likelihood approach, the expectation maximization (EM) algorithm can be used to find the maximum likelihood estimates for the parameter values, (*P*,*Z*) [57, 63, 70, 74]. Alternatively, Bayesian approaches incorporate prior distributions into the likelihood, in order to evaluate the posterior distribution. The Bayesian methods offer a flexible framework for incorporating more complex population history models. For example, one of the widely used Bayesian programs, STRUCTURE, includes useful features such as modeling linkage among loci, and the ability to model correlated allele frequencies between evolutionarily related ancestral populations [14, 49].

20.2.3 Characterizing Locus-Specific Ancestry

For admixed populations, methods described in the preceding section can be used to infer individual ancestry, which represents the genome-wide average ancestry proportions in an individual. If admixture has occurred recently, the genome of an admixed individual resembles a mosaic of fairly long chromosomal blocks derived from one of the ancestral populations. With high-density genotype data, it is now feasible to delineate these ancestry blocks with relatively high accuracy. Figure 20.2 illustrates how ancestry blocks can be reconstructed. While numerous statistical methods have been proposed (e.g., [60, 62]), it is important to realize that the source of information underlying all methods is the different allele and haplotype frequencies among the ancestral populations. As such, the accuracy with which one can infer locus-specific ancestry depends on the genetic divergence between the ancestral populations. The distribution of the ancestry blocks also depends on the admixing history: ancient admixing events result in smaller ancestry fragments, while recent admixing events give rise to extended blocks. With any method, the ability to identify a switch in ancestral state deteriorates when the blocks are very small. Therefore, the accuracy of locus-specific ancestry inference depends on (at least) two aspects of the population history: the divergence between the ancestral populations, and the time of the admixing events. Simulation studies using HapMap data suggest that current high-density genotype data harbor sufficient information for accurate ancestry inference for African-Americans or Hispanics [62]. Locus-specific ancestry can provide information regarding the population history of an admixed

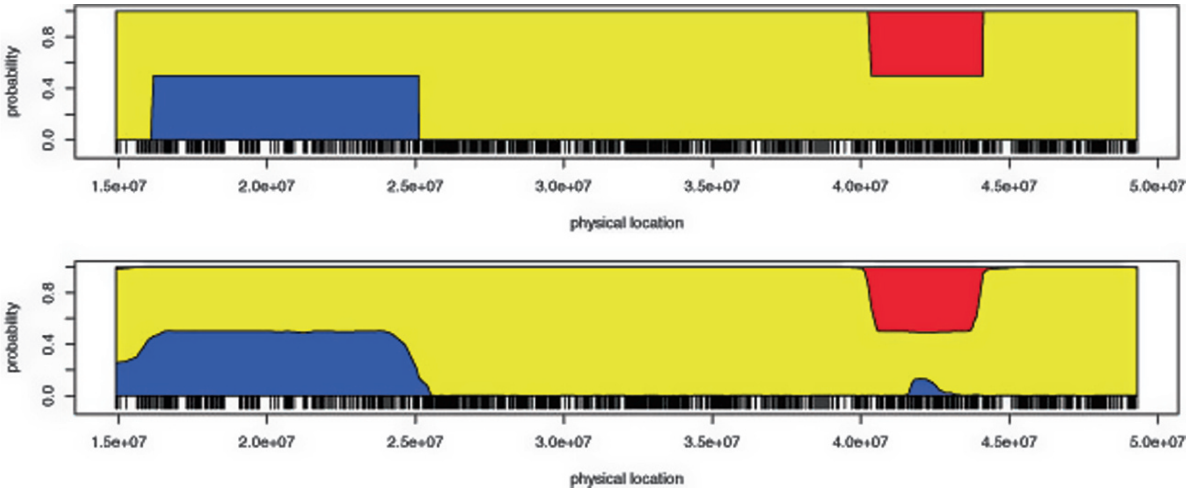


Fig. 20.2 Estimating ancestry along a chromosome. The top panel shows ancestry blocks along a simulated chromosome (*red*: African, *blue*: European; *yellow*: Asian). The bottom panel shows the reconstructed ancestry using high-density SNP markers, which are indicated by the black ticks at the bottom of each panel

population, as well as the finer-scale genetic structure within admixed groups. These are topics we will discuss in greater detail in Sect. 20.5.

20.3 Global Patterns of Human Population Structure

In this and the subsequent section, we begin a detailed exploration of empirical studies of human population genetic structure. First we explore major studies and datasets, now paradigms in the field of human population genetics, that compare human genetic variation at the level of multiple continents; the title of each subsection in this section is the title of a major paper or book in human population genetics. More recent studies of high-density genotyping data reveal patterns in genetic variation at fine geographic scales, as will be discussed after this section's historical perspective is presented.

20.3.1 The Apportionment of Human Diversity

Most studies of human population genetics begin by citing a seminal 1972 paper by Richard Lewontin bearing the title of this subsection [29]. Given the central role this work has played in our field, we will begin by discussing it briefly and return to its conclusions throughout the chapter. In this paper, Lewontin summarized patterns of variation across 17 polymorphic human loci (including classical blood groups such as ABO and M/N as well as enzymes which exhibit electrophoretic variation) genotyped in individuals across classically defined "races" (Caucasian, African, Mongoloid, South Asian Aborigines, Amerinds, Oceanians, Australian Aborigines [29]). A key conclusion of the paper is that 85.4% of the total genetic variation observed occurred within each group. That is, he reported that the vast majority of genetic differences are found within populations rather than between them. In this paper and his book *The Genetic Basis of Evolutionary Change* [30], Lewontin concluded that genetic variation, therefore, provided no basis for human racial classifications.

Lewontin's argument is an important one, and separates studying the geographic distribution of genetic variation in humans from searching for a biological basis to racial classification. His finding has been reproduced in study after study up through the present: two random individuals from any one group (which could be a continent or even a local population) are almost as different as any two random individuals from the entire world (see proportion of variation within populations in Table 20.1 and [20]).

An important point to realize is that Lewontin's calculation (and later work that confirms his finding) are based on the *F*-statistics introduced in Sect. 20.2.1 (see

Table 20.1 In this analysis of molecular variance, the total genetic variation observed is partitioned by that explained within populations in the same sample, among populations within regions, and among regions (from [53], reprinted with permission from AAAS)

			Variance components and 95% confidence intervals (%)		
Sample	Number of regions	Number of populations	Within populations	Among populations within regions	Among regions
World	1	52	94.6 (94.3, 94.8)	5.4 (5.2, 5.7)	
World	5	52	93.2 (92.9, 93.5)	2.5 (2.4, 2.6)	4.3 (4.0, 4.7)
World	7	52	94.1 (93.8, 94.3)	2.4 (2.3, 2.5)	3.6 (3.3, 3.9)
World-B97	5	14	89.8 (89.3, 90.2)	5.0 (4.8, 5.3)	5.2 (4.7, 5.7)
Africa	1	6	96.9 (96.7, 97.1)	3.1 (2.9, 3.3)	
Eurasia	1	21	98.5 (98.4, 98.6)	1.5 (1.4, 1.6)	
Eurasia	3	21	98.3 (98.2, 98.4)	1.2 (1.1, 1.3)	0.5 (0.4, 0.6)
Europe	1	8	99.3 (99.1, 99.4)	0.7 (0.6, 0.9)	
Middle East	1	4	98.7 (98.6, 98.8)	13 (1.2, 1.4)	
Central/South Asia	1	9	98.6 (98.5, 98.8)	1.4 (1.2, 1.5)	
East Asia	1	18	98.7 (98.6, 98.9)	1.3 (1.1,1.4)	
Oceania	1	2	93.6 (92.8, 94.3)	6.4 (5.7, 7.2)	
America	1	5	88.4 (87.7, 89.0)	11.6 (11.0,12.3)	

[67] for a discussion) averaged across single genetic loci. While it is an undeniable mathematical fact that the amount of genetic variation observed within groups is much larger than the differences among groups, this does not mean that genetic data do not contain discernable information regarding genetic ancestry. In fact, we will see that minute differences in allele frequencies across loci when compounded across the whole of the genome actually contain a great deal of information regarding ancestry. Given current technology, for example, it is feasible to accurately identify individuals from populations that differ by as little as 1% in F_{ST} if enough markers are genotyped. (See discussion below for a detailed treatment of the subject.) It is also important to note that when one looks at correlations in allelic variation across loci, self-identified populations and populations inferred for human subjects using genetic data correspond closely [12, 53].

20.3.2 The History and Geography of Human Genes

For more than 40 years, Luigi Luca Cavalli-Sforza and colleagues have worked to document and interpret patterns of human genetic variation. Along the way they have developed and perfected many of the statistical methods used to visualize and quantify patterns of variation and interpret their findings in light of human history and evolution. Their canonical book, *The History and Geography of Human Genes*, summarizes much of what they have learned about the pattern and process of human genetic variation across 1,800 indigenous populations.

Before delving into their findings, it is important to define two important concepts that permeate their work and those of the field a whole. The first is *treeness*, a concept introduced by Cavalli-Sforza and Piazza [5] to summarize population structure across multilocus data. Statistically, we can think of *treeness* as a way of summarizing "block structures" seen in matrices of pairwise genetic distances between populations. Specifically, block structures emerge when populations descended from a common ancestor are grouped together in these matrices, since closely related populations (say sister populations) will show similar levels of differentiation to a distant pair of closely related populations, the matrix will appear to show nearly duplicated rows and columns (or "blocks" of relatedness). By summarizing the blocks as arising from bifurcating trees, one can in theory build up a history of the population splitting events that gave rise to the sampled groups. It is important to emphasize that population trees are somewhat different from traditional phylogenetic (or species) trees since they are summarizing a reticulated history with often a great deal of gene flow among the terminal branches. The second concept that is important to discuss is the technique of principal component analysis (PCA). As we have already seen in Sect. 6.4.4, PCA is a general tool for exploratory data analysis that has found wide application in genetics. Cavalli-Sforza and colleagues were among the first to use PCA of population allele frequency matrices to identify major axes of variation in the data and interpret these axes in light of human history, as we will discuss below. One important distinction to emphasize is that much of the PCA work they carried out was done at the *population* level while much of the PCA that is carried out today is done at the individual level. (That is, PCA analysis of genotype value matrices where the entries are "0," "1," or "2" depending on how many copies of the "A" allele vs. the "a" allele, a given individual carries at a locus).

Using PCA Cavalli-Sforza and colleagues deeply explored human population genetics structure in *The History and Geography of Human Genes*. (A representative example of the PCA plots they generated is given in Fig. 20.3, which summarizes major axes of variation across the sampled populations they studied). A key emphasis of their work was on understanding how or whether language presented barriers to gene flow (i.e., quantifying how much of nonrandom mating in human populations is attributable to language) (see Fig. 20.4). The idea that languages and genes may evolve at similar rates and that a similarity in linguistic markers between two languages may likely reflect a recent shared genetic history among speakers of those languages remains controversial in the field of linguistics. However, Cavalli-Sforza et al. [8] underscored that human evolutionary genetics studies can rely on data and results from other fields – such as anthropology, archaeology, and linguistics – to synthesize inferences about human history.

The book by Cavalli-Sforza and colleagues is known for its numerous *synthetic maps* [34]. Synthetic

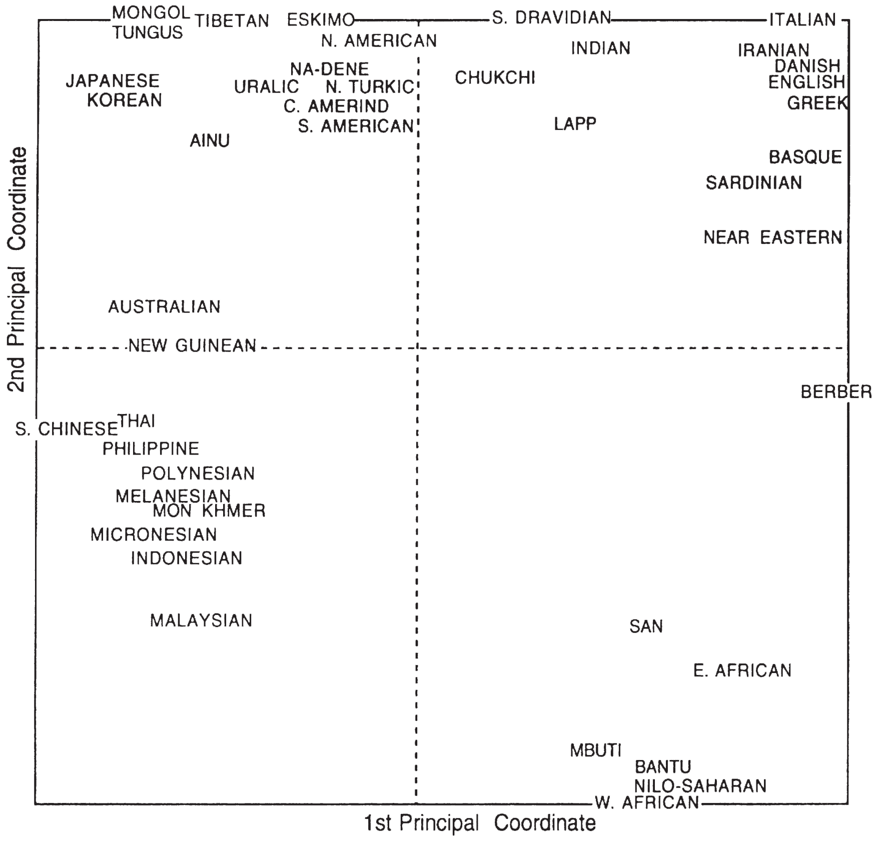


Fig. 20.3 Principal component map of 42 population studies by [6]. The first two PCs summarize 27% and 16% of the variation, respectively. Africans cluster in the lower right quadrant, with Europeans in the upper right, Southeast Asians in the lower left, Northeast Asians and Americans in the upper left. The first PC separates Africans and Europeans from the rest; the authors propose that the first PC does not separate Africans from non-Africans because there are only 6 African populations compared to 36 other populations. From [7]. From Cavalli-Sforza L., The History and Geography of Human Genes, copyright 1994 Princeton University Press. Reprinted by permission of Princeton University Press

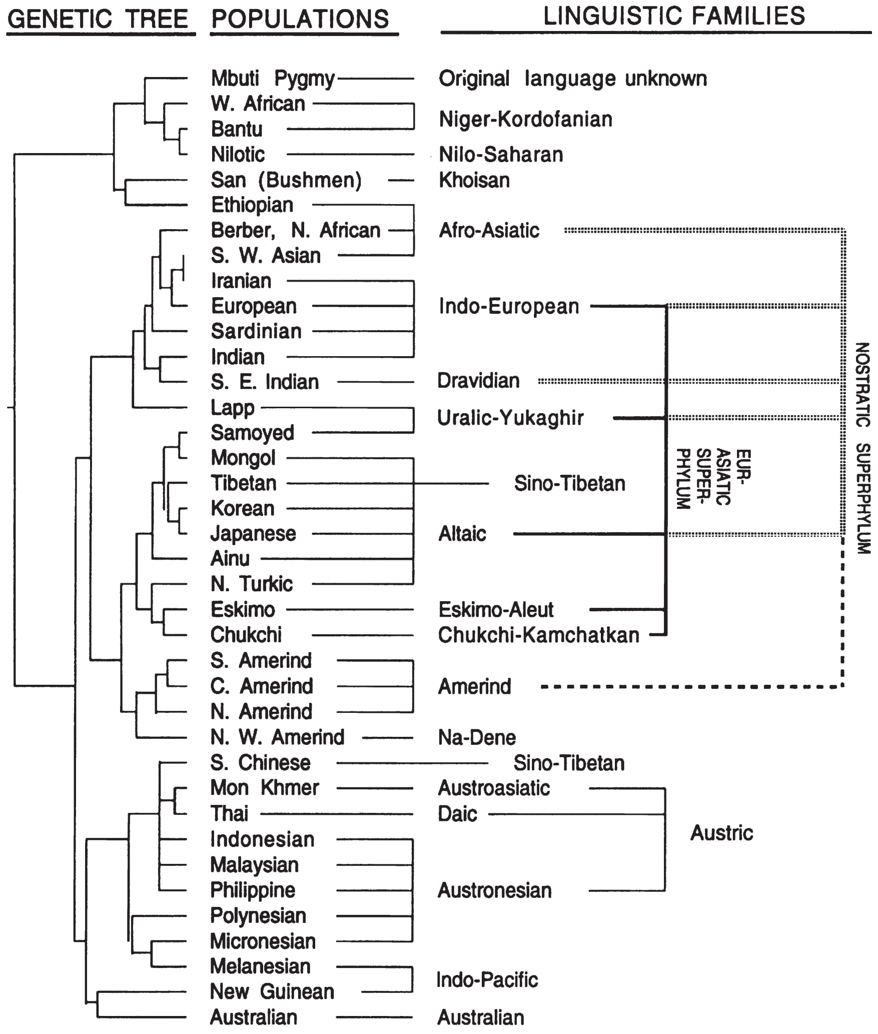


Fig. 20.4 Aligning the genetic tree with linguistic families and superfamilies, as in [8]. From [7]. From Cavalli-Sforza L., The History and Geography of Human Genes, copyright 1994 Princeton University Press. Reprinted by permission of Princeton University Press

maps overlap a single principle component onto a geographic map and are interpreted as revealing migration routes taken through the frequency and geographic spread of the allelic variant (see last three columns of Fig. 20.5). Many synthetic maps reveal north-south and east-west gradients in genetic variation, which might be interpreted as linking variation in a particular gene to climate or ecology. Recent work shows that PCA is expected to reveal axes of genetic variation that are orthogonal [41] and that multiple interpretations may be consistent with a given PCA representation of genetic variation.

20.3.3 Genetic Structure of Human Populations

An important and influential resource for studying human genetic variation has been the Human Genome Diversity Panel. Spearheaded by Howard Cann, Luca Cavalli-Sforza, and Jim Weber [4] (see Box 20.1), the HGDP is a collection of immortalized lymphoblastoid cell lines of over 1,000 individuals from 51 populations. By creating a renewable resource of DNA, the panel has afforded deep inferences on human evolutionary history, especially genomic signatures of

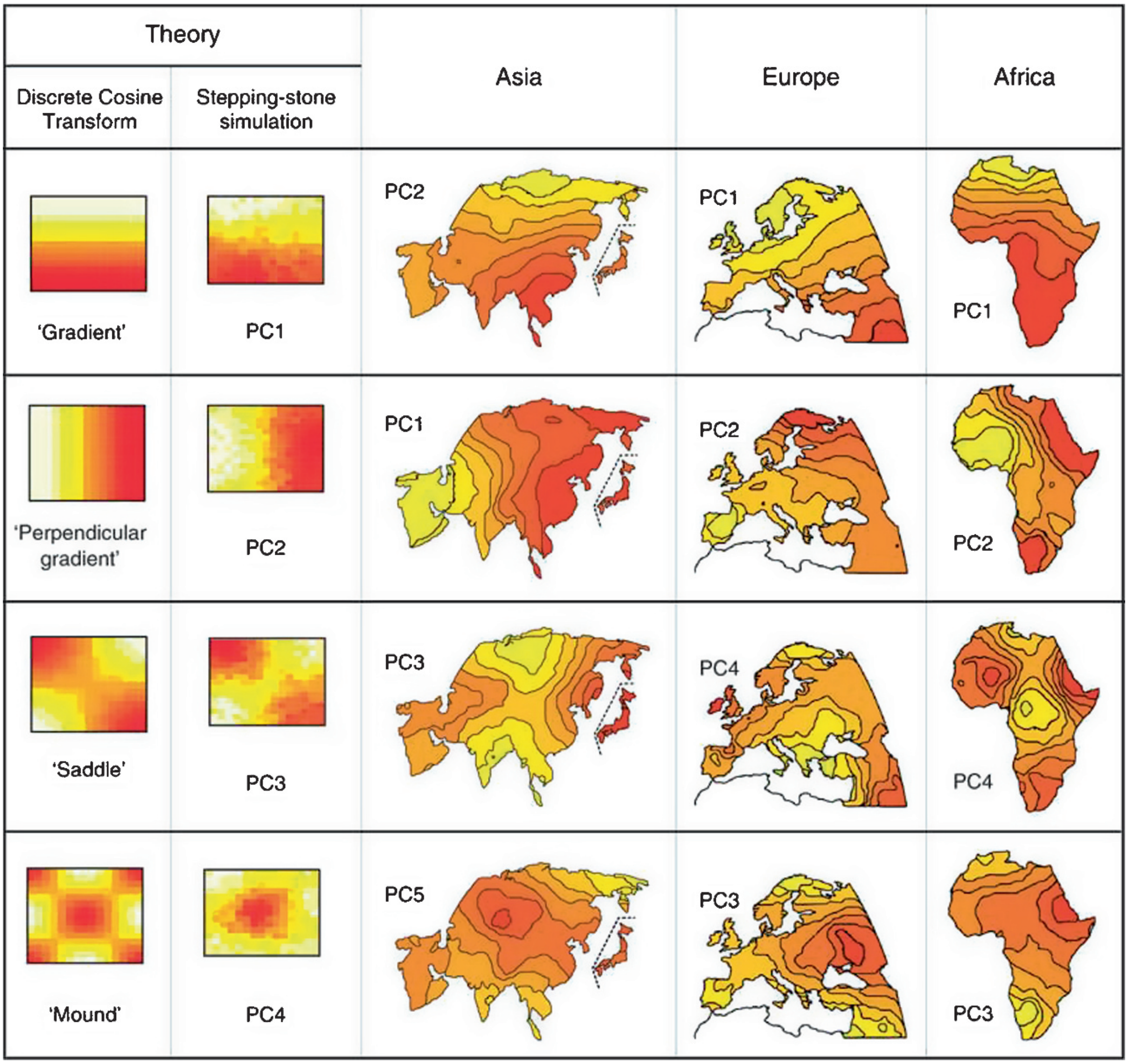


Fig. 20.5 Comparing synthetic maps from [34] with theoretical and empirical expectations. Menozzi et al. [34] performed principal component analyses on frequencies of 38 genes in various populations; the last three columns of this figure depict their original results. In the panel displaying PC1 in Europe, for example, the frequencies of certain alleles decrease (shown by more yellow colors) away from the Middle East; authors state that this pattern parallels the arrival of agriculture, which originated in the Middle East and then spread northward to Europe [6]. The Menozzi et al. [34] figures have been arranged to correspond with the shapes seen in the first two columns, which are based on theoretical and simulation results from [41]. Novembre and Stephens [41] simulated populations evenly-spaced in two-dimensional habitats with homogeneous migration rates arross time and space. Their PCA of these simulations found large-scale orthogonal gradients and "saddle" and "mound" patterns (see first column) when visualizing principal components even under this homogenous migration scheme. The second column displays PCA results from their simulations. The first column shows common structures seen in covariance matrices of population allele frequencies where genetic similarity decreases with geographic distance in a two-dimensional habitat, known as a stepping stone model. The regularity with which they observe these patterns runs counter to Menozzi et al.'s [34] claim that their PCA results are indicative of specific migration events.

historical relationships between populations, by providing a means to genotype (and ultimately sequence) genomes from diverse human populations. The first study of genetic variation in the HGDP scored polymorphism across 377 autosomal microsatellite loci in the panel [53] and recapitulated Lewontin's [29] result that the vast majority of human population genetic variation is found within local populations. However, the study also demonstrated individuals could be assigned to their continent of origin, and in some cases their population of origin using the model-based clustering algorithm STRUCTURE [49]. The authors reported "it was only in the accumulation of small allele-frequency differences across many loci that population structure was identified."

In a follow-up study (see Fig. 20.6), Rosenberg et al. [52] genotyped 993 total markers in the HGDP and demonstrated increased resolution of population structure as a result of increasing the amount of genetic data used. In particular, when the method is asked to identify two clusters (i.e., $K=2$), the authors found that STRUCTURE differentiates between indigenous American (purple cluster) and African (orange cluster) populations, with other populations having a gradient of membership in the African cluster that drops off with geographic distance from Africa. As the number of clusters K used in the STRUCTURE analysis increased, correlations in genotype data within continents of origin allowed Eurasia, East Asia, and Oceania to be identified as separate clusters as well. The structure that is identified is that of differences between continents, with a few notable exceptions. For example, the orange Africa cluster membership in the Mozabites reflects the gene flow this Middle Eastern population has had with Africa, due to the samples' location in North Africa. Similarly, membership in the blue Eurasian cluster in the Maya reflects gene flow with Europe during colonization that this American population experienced to a greater extent than other American populations in the HGDP.

The genotyping of 650,000 SNPs in these populations [31] allowed the detection of individual ancestry and population substructure with very high resolution within continents as well as across them (more examples of analyses with dense SNP maps will be discussed in Sect. 20.4). Li et al. [31] were further able to examine the distribution of ancestral alleles (nucleotides observed in chimpanzee) in HGDP populations by genotyping two chimpanzee samples at the same markers. The ancestral allele-frequency spectrum across loci can yield clues to the history of individual populations, because we expect populations with a small effective size and/or populations that have experienced a bottleneck to have more pronounced genetic drift, which can result in a relatively rapid increase in derived allele frequencies compared to populations with larger effective sizes or populations that have experienced expansion.

20.3.4 A Haplotype Map of the Human Genome

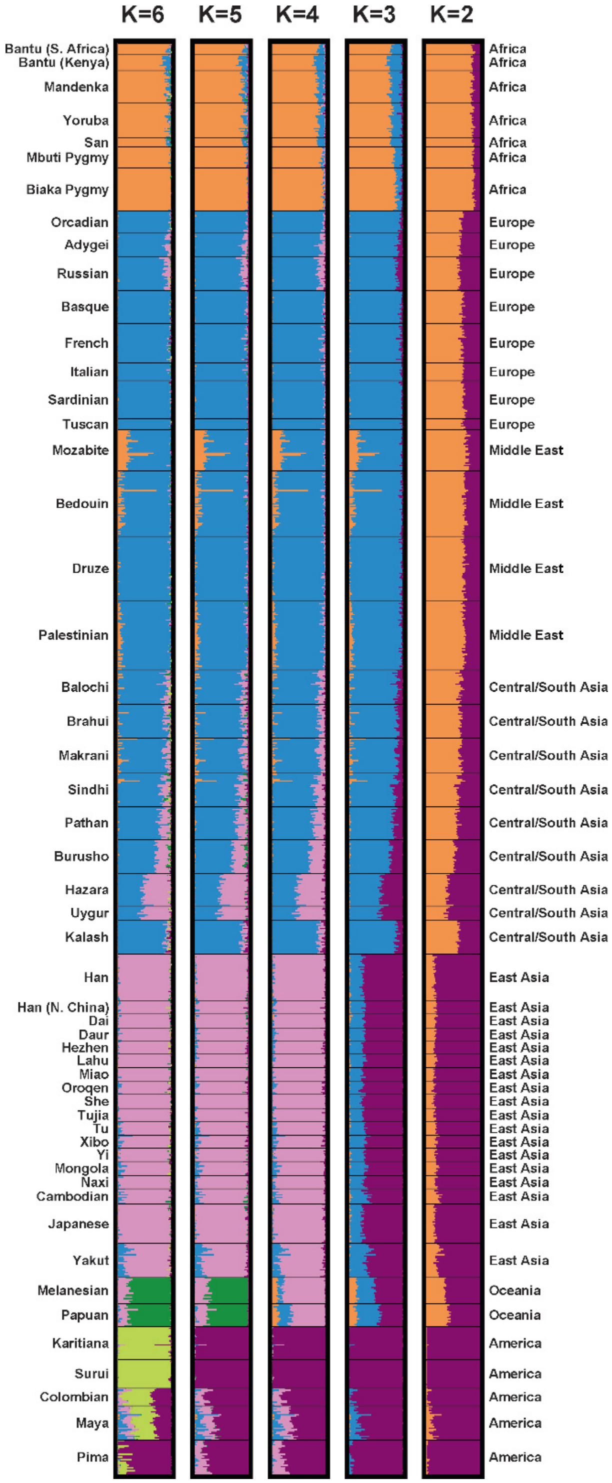
A comprehensive search for genetic causes of common diseases, such as type II diabetes or macular degeneration, requires examining genetic differences between a large number of affected individuals (i.e., cases) and matched controls. Key to facilitating this effort is knowledge about patterns of linkage disequilibrium (LD) or nonrandom association among SNPs in the genome. Such correlations between causal mutations and their haplotypes have long been used in human genetic research of disease (e.g., in studies of the HLA region, and in the identification of causes of Mendelian disorders such as cystic fibrosis).

The International HapMap Project was formally initiated in October 2002 as a means of systematically describing patterns of linkage disequilibrium in the human genome in order to catalyze medical genetic research into the heritable basis of common disease. It also represented the beginning of a paradigm shift in both the amount of data and types of questions that could be answered by human population geneticists. The stated goal of the project was to "determine common patterns of DNA sequence variation in the human genome" [21], with the goal of typing over one million SNPs in 270 individuals including 60 *trios* (samples of two parents and one of their biological children). The project has far surpassed that goal, as seen in Box 20.1.

The HapMap data provide insight into LD patterns across three populations. Chief among the concepts developed around the project is the notion of *tag SNPs* or representative SNPs in a region that can serve as "proxies" for other SNPs. That is, using tag SNPs means genetic variation can be efficiently queried for association with disease without genotyping every SNP in a given chromosomal region (therefore drastically reducing the cost of carrying out a genome-wide association study).

Tag selection methods exploit redundancy among SNPs; however, since the HapMap initially sampled only three populations, an issue for association studies is whether tag SNPs chosen from the HapMap dataset

Fig. 20.6 Inferred population structure based on 1,048 individuals and 993 markers. Each individual is represented by a thin line partitioned into *K* colored segments that represent the individuals estimated membership fractions in *K* clusters. Black lines separate populations, whose names are to the left of the figure, with continent listed on the right of the figure. The value of *K* indicates how many clusters STRUCTURE was assuming existed in the dataset for a particular set of runs for the method. From [52]



Box 20.1 Examples of publicly available human population genetic datasets

Description of three major datasets used by the human population genetics community. These are not the only large datasets available for research, but illustrate how much data are being generated to better understand human genetic variation, genetic signatures of human history, and the genetic underpinnings of disease.

Dataset Name	Initial reports of data	Amount of data generated
Human Genome Diversity Project	[4] (2002)	lymphoblastoid cell lines from 1,064 individuals in 51 populations
	[53] (2002)	377 autosomal microsatellites
	[52] (2005)	993 markers (microsatellites and insertion/deletion polymorphisms)
	[31] (2008)	650,000 SNPs in 938 of these individuals
International HapMap Project	[21] (2003 paper)	270 people across three populations (30 trios from Yoruba people of Ibadan, Nigeria; 45 unrelated individuals from Tokyo, Japan; 45 unrelated individuals from Beijing, China; 30 United States trios with northern and western European ancestry)
	[22] (2005 paper)	1 million SNPs in these individuals (1 SNP per 5 kilobases)
	[23] (2007 paper)	An additional 2.1 million SNPS (1 SNP per kilobase)
	HapMap Phase III, draft 2 reported online in January 2009	an additional 1.5 million SNPs and an increase to 1184 individuals (populations added: Chinese from Denver; Gujurati from Houston; Luhya from Webuye, Kenya; Mexican ancestry from Los Angeles; Maasai from Kinyawa, Kenya; Toscans from Italy; African ancestry from Southwest USA)
1,000 Genomes Project	www.1000genomes.org	Sequencing the genomes of approximately 2,000 people from around the world

adequately capture patterns of variation in other populations. Conrad et al. [10] showed that the portability of tag SNPs from HapMap to HGDP populations was quite good within large geographic regions such as continents. These dense genotype data reveal other important patterns resulting from continental population structure, such as an increase in LD with distance from Africa, reflecting that African lineages have smaller preserved blocks of LD due to increased time for recombination events to break up correlations (also seen in the HGDP by Conrad et al. [10]).

Data from the initial HapMap project do not enable much inference about evolutionary relationships between populations, so the genotyping of individuals from additional populations has become a priority in human population genetics. As the cost of SNP genotyping lowers, studies allow for across- and within-continental pictures of population structure to emerge. Dense genotype data from multiple populations allow the inference of both continental differentiation and the fine-scale study of within-region relationships among individuals. It is these finer-scale patterns that we will explore in the next section.

20.4 The Genetic Structure of Human Populations Within Continents and Countries

Large-scale human population genetic studies like the Human Genome Diversity Panel and International HapMap Project discussed in Sect. 20.3 initially had to choose between sampling densely geographically and sampling densely genomically. In just the last 2–3 years, improvements in genotyping technologies have allowed studies to report analyses of hundreds of thousands of SNPs genotyped in individuals from many populations. These datasets reveal the genetic signatures of historical events like migrations and conquests in more detail than geneticists could have hoped for when the field began. Here we explore how the history of Eurasia, the Americas, and Africa has shaped patterns of genetic variation of its inhabitants. (Note: the reason we have chosen to start with a discussion of Eurasia is simply that these are the populations that, to date, have been studied most intensively genetically. We believe the next few years will bring fine-scale studies of population structure across global human populations and strongly advocate these studies be undertaken, particularly in parts of the world currently understudied.)

20.4.1 Genetic Differentiation in Eurasia

Instead of grouping individuals into populations a priori (as early population genetic analyses necessitated), today we can let the data speak for themselves and tell us which individuals naturally cluster together based on genetic distance. A convenient means of accomplishing this is undertaking PCA on individual genotype scores (i.e., the “0,” “1,” “2” matrices mentioned above). Often when this is done, individuals from the same population tend to cluster together in PCA space. In fact, many PCA plots of globally distributed population structure seem to resemble geographical maps of the world with individuals from contiguous geographic regions clustering near each other in PCA space and revealing a close relationship between geographic distance and genetic differentiation (see Figs. 6.4 and 20.7).

Specifically, multicontinental studies of genomic diversity often find a clustering of populations according to their respective continents in the first few principal components, followed by differentiation between regions within continents. When sampling is dense, principal components can often serve as proxies for geographic axes [41, 42], separating Northern from Southern populations or Eastern from Western. For example, multiple studies observe North-to-South clines in European genetic differentiation, as seen using haplotype diversity in Fig. 20.8. Principal components also reveal evidence of genetic admixture between populations that can often be interpreted based on historical events such as colonization or slave trade; these signatures of admixture are discussed at length in Sect. 20.6.

As Fig. 6.4 shows, the European geographic map is an efficient summary of the first two principal components – or, put another way, dimensions – of European genetic variation. Novembre et al. [42] and Heath et al. [16] also showed that individual genotypes, despite low differentiation among populations in Europe as measured by F_{ST} (see Table 20.1), can be used to predict an individual’s geographic origin within a few hundred kilometers (when that individual’s geographic origin is representative of their ancestry). Likewise, several recent studies using high-density genotyping arrays have demonstrated the ability to reliably distinguish individuals of Ashkenazi Jewish ancestry from those without Ashkenazi Jewish ancestry in both European and European-American populations [36, 47, 64]. The ability to detect fine-scale geographic structure will only improve as whole-genome sequencing data become available; the studies discussed here are based on SNPs whose minor allele frequency is usually greater than 5%. Newer sequencing technologies will call lower frequency alleles more accurately, and low frequency alleles likely reflect recent mutations and may account for much differentiation between neighboring populations.

The larger mean heterozygosity and smaller mean linkage disequilibrium observed in Southern Europe compared to Northern Europen might be explained by an expansion in Europe from the South to the North [28]. South-to-North movement in Europe occurred during the first Paleolithic settlement of the continent by anatomically modern humans, and during the Neolothic expansion [2]. Thus we might expect to see a genetic signature of such movement, although another important controversy in historical anthropology is whether technologies such as agriculture traveled via demic diffusion (the movement of people and their genes) or cultural diffusion (the spread of technologies without a concomitant genetic signature) (see, for example, [46]).

Genetic variation in specific countries has been studied as well, such as that of Finland [24]. Studying a specific population may give insights into inbreeding or homozygosity patterns, as well as the genetic signatures of founder effects or multiple waves of migration

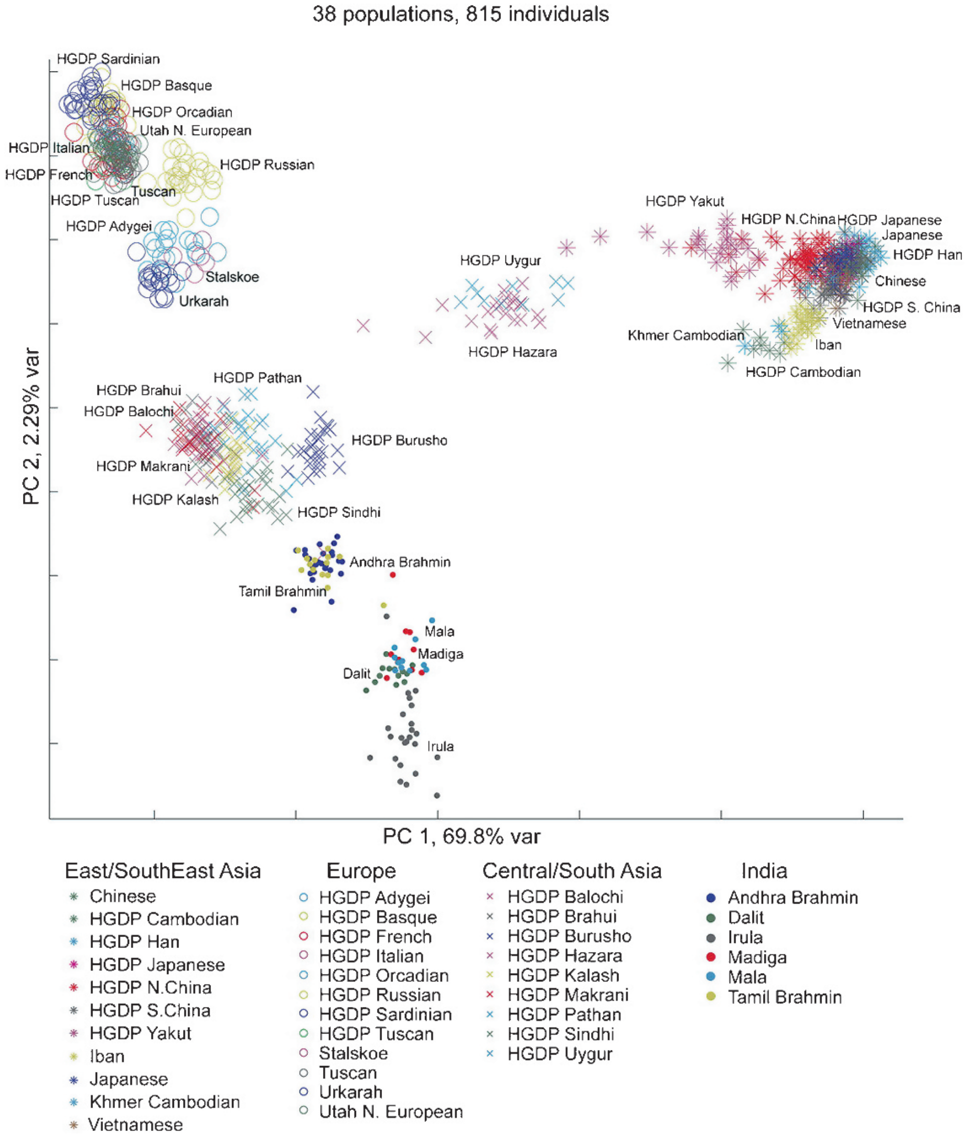


Fig. 20.7 PCA for 815 Eurasian individuals using nearly 50,000 SNPs. Individuals group closely with others in their self-identified population of origin, and the populations are differentiated in a way that mirrors a geographic map of Eurasia, much like the close relationship seen in Fig. 6.4 between genes and geography in Europe. HGDP denotes populations from the Human Genome Diversity Panel [4]. From [71]

in, for example, linkage disequilibrium patterns or admixture blocks. Jakkula et al. [24] found genetic signatures supporting multiple historical bottlenecks resulting from consecutive founder effects, in keeping with Finland's history of two major migration waves (a western one from 4,000 years ago, and a southern and western one from 2,000 years ago). A study of 7,003 Japanese individuals also shows that local regions in Honshu Island, the largest island of Japan, are genetically differentiated despite frequent migration within Japan during the last century [73].

Single-population studies are of great interest, as populations that experienced bottlenecks and subsequent low levels of immigrations (like the Finnish, Askenazi Jewish, or Icelandic peoples) may see a rise in Mendelian disease frequencies, and it has been proposed that gene mapping for complex traits may be easier in these populations than others. The Finns, for

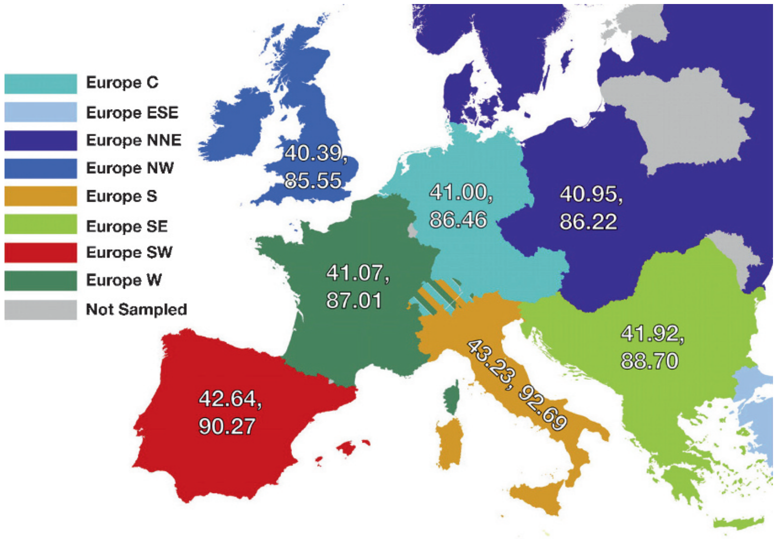


Fig. 20.8 Haplotype diversity within Europe. Two numbers are shown in each region; the first shows the mean number of distinct haplotypes in a region in a genomic window containing 10 SNPs, the second reflects haplotype diversity in a 25-SNP window. The authors find that haplotype diversity, as reflected by the numbers displayed, is higher in southern Europeans countries than in northern European countries, indicating that southern populations have larger effective sizes than northern ones and that the original peopling of Europe happened with migrations from the South to the North. From [1]

example, exhibit a substantial degree of homozygosity due to their population history, although not the amount of homozygosity seen in cultures with consanguineous marriages [24]. In such a population, the tagging of recessive variants in complex disorders may be done with common SNPs; indeed, the use of homozygous segments to identify rare alleles associated with Mendelian mutations has been successful in Finland (Meckel syndrome) [61]. Interestingly, Ashkenazi Jewish populations exhibit very similar patterns of linkage disequilibrium (and, in fact, *less* LD) than the CEPH European populations genotyped as part of HapMap, but approximately 20% higher levels of homozygosity [43]. Studies such as these address the power of genome-wide association datasets to demonstrate history-related stratification even within apparently homogeneous genetic populations, and shed light on the importance of rare variants in fine-scale genomic studies.

20.4.2 Genetic Variation in Native American Populations

During an expansion from a parent population via serial bottlenecks, sometimes called a "serial founder effect," linkage disequilibrium will increase and heterozygosity will decrease with distance from the origin of the expansion in the absence of selection [50]. Large linkage disequilibrium blocks were observed in the five Native American populations genotyped in the HGDP [10]. However, additional population samples from the Americas are important to help us understand the peopling of the Americas via the Bering Strait and what signature colonization, in this case by Europe, might have on genetic data. Wang et al. [66] studied 24 newly sampled populations of Native Americans from Canada, Meso- and South America.

The study found lower heterozygosity at microsatellite loci in indigenous Americans than in the non-American HGDP populations, and also observed a greater variance in heterozygosity among American populations. This could be a signature of a quick initial peopling of the Americas, followed by subsequent isolation of populations in the continent. A rapid coastal migration followed by a slower inland migration was supported by a higher level of genetic diversity in western South America compared to eastern South America. Wang et al. [66] also tested for correlations in differences between linguistic stocks or families and genetic distance between populations, finding that genetic distance and linguistic distance are more highly correlated within linguistic families than between families.

The study found support for an East Asian origin for Native American genetic variation, with relatively higher similarity to East Asian genetic variation in North Americans than South Americans, and also observed a private allele in the Native American samples. Wang et al. [66] showcase how a variety of hypotheses regarding demographic history can be tested with genetic data, when aligned with linguistic and archaeological data.

20.4.3 The Genetic Structure of African Populations

Africa and African populations play an important role in human evolutionary history given the African origin for anatomically modern humans and the amount of our genomic variation shaped by the out-of-Africa bottleneck [11]. However, it is important to recognize that African populations have been evolving since the human diaspora. Tishkoff et al. [65] sampled 121 African populations at over 1,000 microsatellite loci to study the demographic history of Africans as inferred from genetic data.

The investigators identified 14 ancestral clusters in Africa; these clusters approximately correspond to linguistic families, self-identified ethnicities, and/or cultural practices such as hunting-and-gathering. There was also a great deal of mixed ancestry in most populations, a signature of recent migrations in the African continent.

Three hunter-gatherer populations in the study were among the five most genetically diverse populations in the African sample, and African and Middle Eastern populations were found to share a number of alleles not observed elsewhere. Within Africa, the most private alleles were seen in click-speaking populations.

The spatial distribution of heterozygosity was used to pinpoint the origin of the modern human migration within the African continent in the same manner as by Ramachandran et al. [50]. Tishkoff et al.'s [65] analysis places this origin in southwestern Africa near the border of Namibia and Angola, which corresponds to the current San homeland. This lends support to the San being a genetically ancient population, although perhaps their current geographic origin does not reflect their ancestors' geographic location 100,000 years ago. As geographic analyses become more refined, genetic variation appears to be more clinal than clustered [50]. This is because, at within-continental geographic distances, migration levels may be high and levels of admixture across populations will increase. We cannot study population genetics within continents without understanding recent genetic admixture, the subject of the next section.

20.5 Recent Genetic Admixture

The ultimate cause for population structure is nonrandom mating. For example, if individuals from geographically distant populations are less likely to mate than individuals from the same population, over time, discernible differences in allele frequencies will accumulate (as explained in Sect. 20.1). Compounding these differences across the genome provides power for reliably differentiating individuals from different populations even if, overall, the degree of population differentiation is low [31, 42]. On the other hand, migration facilitates gene flow. Recent global exploration and colonization have led to a rapid increase in gene flow among individuals from different continents. Their offspring are referred to as admixed and we can mathematically model chromosomes from admixed individuals as mosaics of segments derived from different ancestral populations. This section summarizes variation in several admixed populations, with the goal of illustrating the power genetic data have to shed light on a population's recent history.

20.5.1 Populations of the Americas

The population history and genetic structure among the Native American groups was surveyed in Sect. 20.4.2. The first significant wave of European influence arrived in the New World with Christopher Columbus' voyages of 1492–1504. During the sixteenth to nineteenth century, between 9.4 and 12 million Africans (mostly from West and Central Africa) were transported to the New World through the transatlantic slave trade. The arrival of Europeans and Africans in the New World gave rise to numerous admixed populations. In this section, we focus on two such groups: African Americans and Hispanics.

According to the 2007 U.S. Census, 41 million U.S. residents self-identify as having some degree of direct African ancestry (i.e., identify as "black" or African-American). Studies of genetic variation among African-Americans suggest that, on average, 80% of their genetic ancestry is West African, although individual ancestry proportions vary substantially as do the average ancestry proportions for different sampling localities within the United States [44]. Based on chromosomal block lengths, the admixing time between Europeans and Africans is estimated to have occurred 7–14 generations ago [14, 74]. At 25 years per generation, this places admixture as occurring between 175 and 350 years ago. Historical records indicate that the largest sources of the African slaves were the coastline in West and West Central Africa. However, locating the precise African ancestral populations for the African Americans has been challenging, in particular due to the lack of genetic data in geographically and ethnically diverse African populations. The recent study by Tishkoff et al. [65], discussed in Sect. 20.4.3, fills in this gap by genotyping over 2,000 Africans from 113 populations; in the near future, genetic data will likely be used to characterize admixture patterns within the African component of the African Americans.

Hispanics derive their ancestry from European, African, and Native American individuals. The term Hispanic describes populations that share a common language and cultural heritage, including Mexicans, Puerto Ricans, and Cubans, to name a few. However, these groups do not constitute a uniform ethnicity with a similar genetic background. At present, Hispanics represent the largest and fastest-growing minority population in the United States. Although genetic studies characterizing the population structure in the Hispanic population have been limited both in the marker density and in subgroup representation, evidence is mounting that individual ancestry proportions vary tremendously among subgroups that are identified as Hispanic. At a population level, Puerto Ricans have higher African ancestry compared to the Mexicans [56]; even within Mexico, the Native American ancestry proportions vary among States, ranging from 35% in Sonora in the North to 65% in Guerrero in center-Pacific [59].

The history of admixed populations is clearly discernible in patterns of genetic variation as summarized by PCA. Consider, for example, the GlaxoSmithKline POPRES sample [1, 37, 42] consisting of 3,875 individuals of varying ethnic backgrounds from over 80 countries genotyped on the Affymetrix 500 K platform. In Fig. 20.9, we reproduce key results from Nelson et al. [37] on PCA analysis of PopRes and the core HapMap populations (i.e., Yoruba from Ibadan, Nigeria, CEPH with ancestry from Northern Europe, Japanese from Tokyo, and Han Chinese from Beijing). This sample contains representation from several major continental

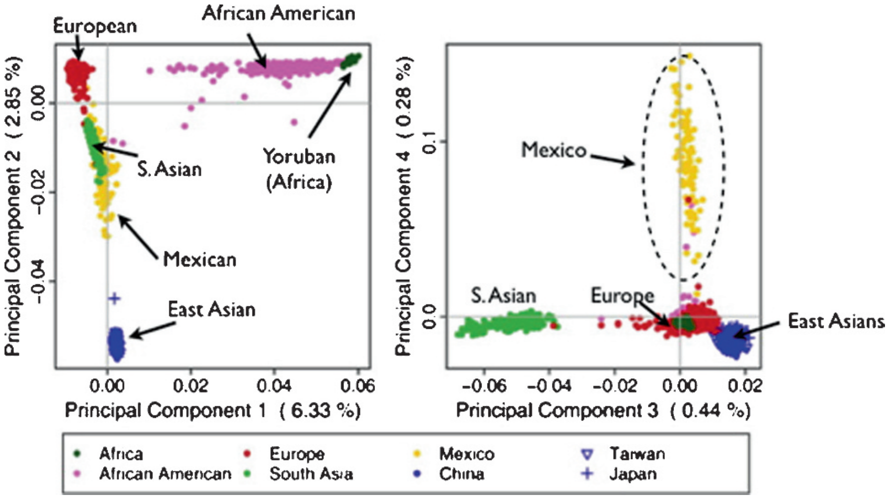


Fig. 20.9 Genetic structure in the PopRes data. Subject scores are colored by continental and/or ethnic origin (see legend). Percent of variation explained by each component is given in parentheses on each axis label. Reprinted from [37], with permission from Elsevier

populations as well as a large sample of African-Americans from the United States and Hispanics from Mexico. We note that the first principal component can be interpreted as an "African-American admixture" principal component (or, equivalently, Africa vs. Europe + Asia); principal component two corresponds to "East Asia vs. Europe"; principal component three corresponds to an "East Asia vs. South Asia" axis of variation; and principal component 4 (PC4) to a "Mexican admixture" axis. Importantly, individuals of admixed ancestry appear on the PCA map as in between the centroids of their putative ancestral populations.

An important feature of this analysis is that along the "Mexican admixture" PC, individuals show varying degrees of admixture between Europeans and a presumably "unsampled" population which likely corresponds to Native Americans. Analyzing just the European, East Asian, and Mexican samples (Fig. 20.10), we find that PC1 is an East Asia vs. Europe principal component and PC2 separates the East Asian sample from the (unsampled) Native American sample (so that the least admixed individuals are furthest away from the East Asian samples along PC2). This suggests that there is substantial genetic differentiation between East Asian and Native American populations so that the former is likely a poor proxy for the later (and vice versa).

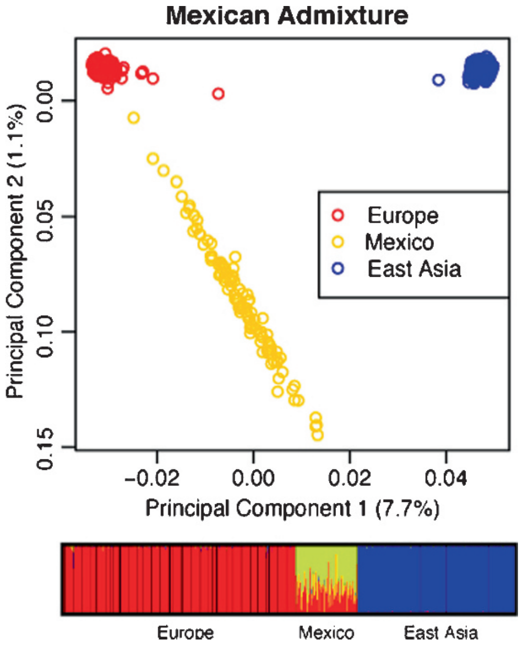


Fig. 20.10 Principal component and STRUCTURE analysis of the PopRes Mexicans from Guadalajara, Europeans from throughout Europe, and East Asians from Japan and China. From [1]

20.5.2 Admixture Around the World

Genetic admixture is a worldwide phenomenon and is not limited to the North America. An example of an admixed population in the Eurasian continent is the Uyghur population living in the Xinjiang province in western China. Because of its proximity to the Silk Road – the historically important trade route connecting East Asia with the West, Central Asia, and the Mediterranean world – the Uyghur population derives ancestry from East Asian, European and the Middle East ancestral populations [31]. Using high-density SNP markers, a recent genetic study estimated approximately equal ancestral contributions from the European and East Asian populations to the Uyghur population [72]. In central Algeria in Northern Africa, the Mozabites originated from a Berber ethnic group in the Middle East that had close cultural contact with diverse African and European populations. Not surprisingly, SNP analysis of the Mozabite individuals in HGDP detects substantial European, African, and Middle Eastern ancestry (see Fig. 20.6).

Owing to the availability of high-throughput genotyping technologies, our ability to detect finer-scale population structure and more ancient admixture has dramatically increased during the past few years. The 600,000 SNP markers typed in the HGDP samples revealed genetic structure that was not detected previously using 300 microsatellites: most prominently, the detection of the Middle Eastern populations as a separate cluster [31, 54]. Moreover, the SNP data suggest that the impact of genetic mixture is more profound than has been previously implicated. Many Middle Eastern individuals appear admixed, perhaps because of the continuous migration in this area. Finally, in the analysis of the European populations, while the genetic structure matches geography, there is undeniable continuity between populations. Thus, it is more appropriate to consider the structure within the European continent as continuous clines rather than as a discrete cluster. In summary, the increasing amount of the genetic data will allow us to characterize population structure at a higher resolution within continents.

20.6 Quantiative Modeling of Human Genomic Diversity

The primarily qualitative studies described above have given great insight into both the global and local patterns of human genetic history. Quantitative models can offer additional insights; for example, we can use quantitative models to ask how severe particular bottlenecks were, or exactly when populations diverged.

Much quantitative modeling relies on resequencing data. SNP genotyping chips provide a genome-wide picture of variation at low cost, but they can be difficult or impossible to use for quantitative inference. Primarily, this is because the sites assayed on a chip are not a random sample of the genome; they are typically chosen because they are known to be polymorphic in some smaller "discovery" population. This ascertainment process biases the resulting data [9], particularly the allele frequency spectrum. Although this bias can, in some cases, be controlled for (e.g., [25]), it is unfeasible in general [38]. In Fig. 20.11, for example, we report the joint and marginal allele frequency spectra (AFS) for the PopRes and HapMap populations. We note that the joint allele frequency spectra reproduce qualitative patterns of the PCA analysis in Fig. 20.9, such as a stronger correlation in allele frequency and lower F_{ST} for closely related populations (e.g., East Asia from PopRes and JPT + CHB from HapMap or Europe from PopRes and CEPH from HapMap). Nonetheless, the marginal (or one dimensional) spectra are quite skewed toward intermediate frequency alleles as a result of the ascertainment bias in the Affymetrix 500K chip, which favored middle frequency variants for use in GWAS. The goal of this section is to describe how (unbiased) AFS data can be used for quantitative demographic inference of both demographic history and selection.

20.6.1 Demographic History

The demographic history of a set of populations encompasses the order and timing of any divergence or admixture events, as well as changes in population sizes and rates of gene flow over time. In principle, the greatest statistical power for inferring such a model from genetic data would arise from calculating the full likelihood of the data given the model [19]. However, at present such calculations are very difficult at the genomic scale. Thus many methods for inferring demographic events rely on modeling summaries of the data. The allele frequency spectrum is a particularly popular summary. As seen in Fig. 20.1, the frequency spectrum encodes substantial information about demographic history. (Although Myers et al. [35] have shown that it does not alone uniquely determine demographic history.) For example, the center column of part D of Fig. 20.1 shows that the one-dimensional (i.e., single population) allele frequency spectrum is skewed toward rare alleles in situations of population growth, while the right two columns show that asymmetric population sizes yield an asymmetric AFS.

An early study by Marth et al. [33] introduced an analytic method for calculating the allele frequency spectrum for a single population with piecewise constant population size. Using this method to fit models for several global populations revealed signatures of ancient population growth in African-Americans (presumably occurring in their African ancestors), and bottlenecks in the history of both European-American and East-Asian populations. These historical events have been well supported by subsequent genetic studies.

Considering the joint history of multiple populations substantially complicates the models, as divergence and gene flow must be incorporated. Consequently, the computational methods become more demanding. In a ground-breaking study, Schaffner et al. [58] used extensive coalescent simulations to replicate both summaries of the allele frequency spectrum and patterns of LD for West African, European, and East Asian populations, developing the first quantitative model for their joint genetic history. The computationally intensive nature of their analysis, however, precluded them from statistically assessing the confidence of their inferences or testing multiple models.

Recent theoretical and computational advances in the simulation of the frequency spectrum with diffusion theory have enabled more comprehensive statistical characterization of such models (Gutenkunst et al., in press). Figure 20.12 shows an illustrative model of human history, and the resulting expected frequency spectra. Within this model, parameters such as divergence times, migration rates, admixture proportions, and bottleneck sizes have been quantitatively inferred.

Models have also reached further back in time, before the emergence of modern humans. In particular, a recent analysis by Fagundes et al. [13] compared several models of early human history, including the possibility of interbreeding with other hominids. The analysis showed

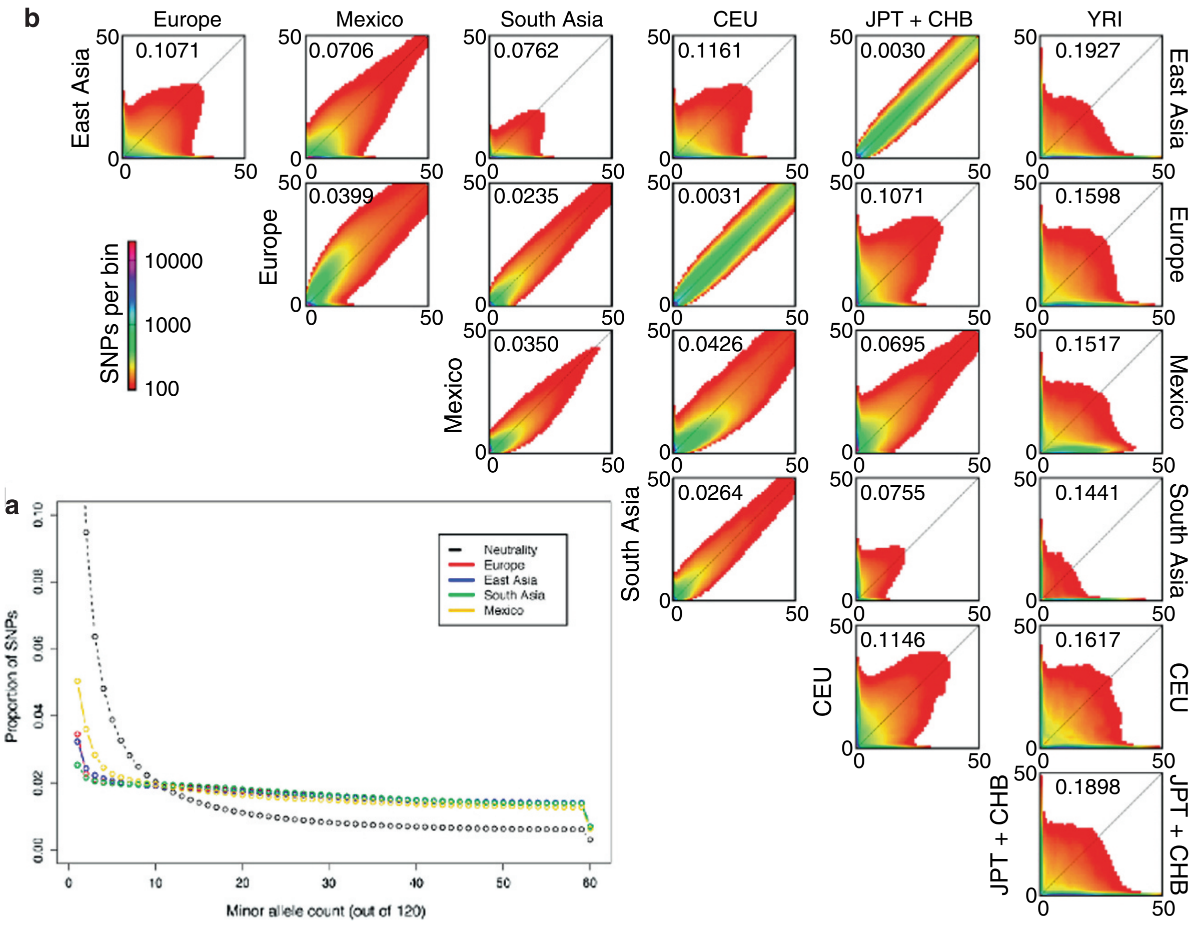


Fig. 20.11 Frequency spectra of the POPRES populations and HapMap samples (*CEU*: CEPH Utah residents with ancestry from northern and western Europe; *CHB* Han Chinese in Beijing, China; *JPT* Japanese in Tokyo, Japan; *YRI* Yoruba in Ibadan, Nigeria). (**a**) Minor Allele Frequency Spectra for the four sub-continental populations. The spectrum expected under neutrality is also shown in black. (**b**) Two-dimensional joint frequency spectra for each pairwise sub-continental population comparison. Colors represent the number of SNPs within each bin. Entries in the spectra containing less than 100 SNPs are shown in white. Autosomal estimates of F_{ST} for each comparison are shown in the upper left hand corner of each figure. From [1]

that a model in which modern humans simply replaced other hominids was best supported by the data.

20.6.2 Quantitative Models of Selection

Quantitative demographic models also play an important role in the search for evidence of selection acting on the genome. In particular, scans for selection seek genomic regions with unusual patterns of genetic variation, and demographic models define the null expectation of how unusual a region must be to be statistically significant when testing the hypothesis that it is under selection [40].

Beyond the search for unusual patterns of genetic variation, quantitative modeling has also given insight into the general signatures left by selection on the human genome. For example, an early analysis of the allele frequency spectrum for different classes of polymorphism revealed strong negative selection on mutations that change amino acid sequence. Furthermore, within those mutations computational algorithms such as Polyphen can predict which changes are most damaging [68].

More recently, the distribution of the selective effects of new mutations has been inferred from the allele frequency spectrum [3]. The selection coefficient *s* of a mutation is defined as the relative reproductive advantage conferred upon carriers of the mutation. As seen in

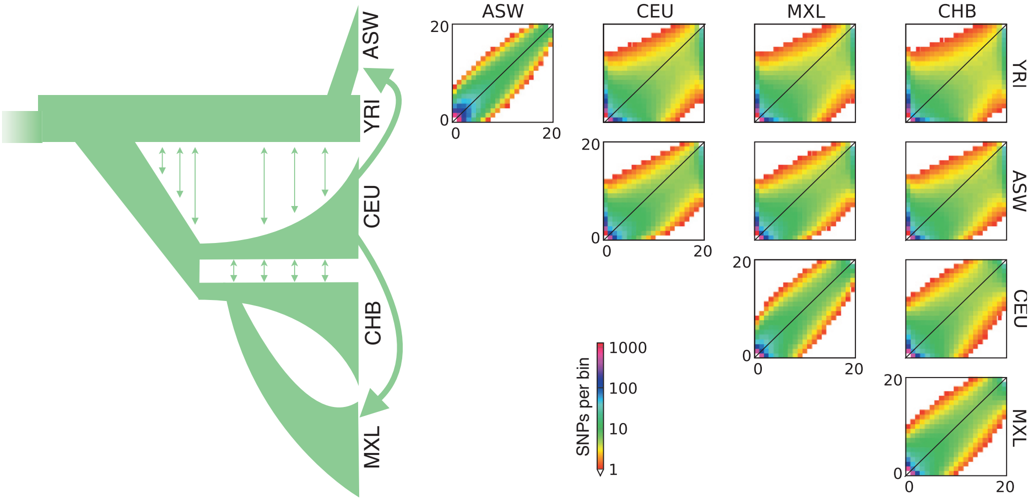


Fig. 20.12 Illustrative model of human expansion out of Africa and across the globe. The model includes African-American (*ASW*), West African (*YRI*), European (*CEU*), East Asian (*CHB*), and Mexican (*MXL*) populations. Using quantitative estimates for divergence times, population sizes, migration rates, and admixture proportions, the expected frequency spectrum under the model can be calculated using either diffusion or coalescent theory. Similar to Fig. 20.1.d., the resulting marginal spectra are shown for each pair of populations. Each spectrum shows distinct signatures of genetic history. For example, the recent European admixture into African-American and Mexican populations results in very highly correlated allele frequencies between populations pairs CEU-ASW (2nd row, 1st column) and CEU-MXL (3rd row, 1st column). Further, the Out-of-Africa bottleneck means that 2D spectra between African and non-African populations are asymmetric. When observed in real data, it is these sorts of signatures that guide quantitative modeling of human history

Fig. 20.13, the frequency spectra of synonymous and nonsynonymous variants differ dramatically. After correcting for demographic history using the synonymous mutations, it was found that the distribution of negative selection coefficients on newly arising amino-acid changing mutations possesses a very long tail. Roughly a third of amino acid substitutions are nearly-neutral ($|s|<0.01\%$), another third are moderately deleterious ($0.01\%<|s|<0.1\%$), and nearly all the remainder are highly deleterious or lethal ($|s|>1\%$). Knowledge of this distribution lets one calculate that very few of the fixed differences between human and chimp are selectively deleterious so that most are neutral or nearly, and that 10–20% of them result from positive selection. As the flood of data from the next generation of sequencing endeavors becomes available (e.g., the 1,000 Genomes Project and associated enterprises), we expect these preliminary estimates to be further refined, along with a quantitative understanding of human demographic history.

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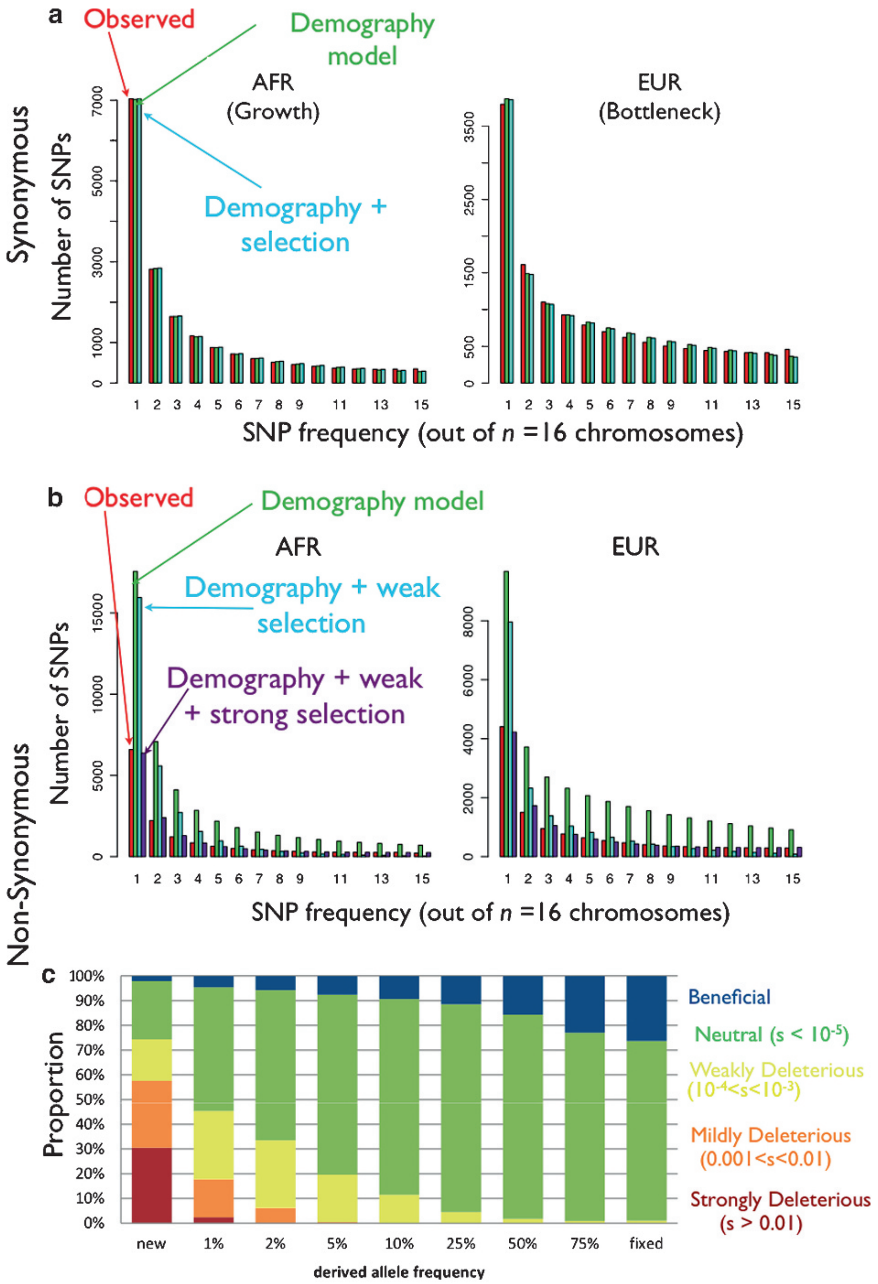

Fig. 20.13 Analysis of site-frequency spectra from over 30,000 coding SNPs found by resequencing of 11,000 genes in 20 European-Americans (*EUR*) and 15 African-Americans (*AFR*) yields estimates of demographic model and distribution of fitness effects of newly arising mutations and SNPs [3]. (**a**) Comparison of observed and predicted SFS for synonymous sites. Predictions are from two different types of models: a demographic model with growth in the AFR and a bottleneck in EUR (*green*) and for a model with weak negative selection on silent sites (*blue*). (**b**) Analogous comparison for nonsynonymous SNPs (nsSNPs) demonstrates that strong purifying selection, weak negative selection, and demographic history are all needed to accurately model the observed distribution of nsSNPs. (**c**) Estimated distribution of fitness effects for newly arising mutations in the human genome as well as SNPs at different population frequencies

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Genetic Epidemiology

21

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Abstract In this chapter, we describe both the historical and contemporary terminologies that reflect the evolving field of genetic epidemiology. We discuss the conduct of family-based studies to identify high-penetrance disease genes, along with traditional genetic analyses of human pedigrees to assess Mendelian transmission (segregation analysis) or to locate causal genes (linkage analysis/gene mapping). We also describe epidemiologic approaches used to study gene-disease associations (including genome-wide association studies) plus gene-gene and gene-environment interactions. We review analytic and methodologic issues applicable to each of these studies and emerging, nontraditional epidemiologic methods that can be used as an adjunct to traditional approaches, particularly for the simultaneous study of hundreds of thousands of data points per person. We further discuss the challenging nature of analysis, synthesis, and dissemination of these genetic data, and the value of systematic reviews, meta-analyses and consortia in evaluating large bodies of scientific evidence. Finally, we describe the need for follow-up of these results to identify causal variants and the need for translational research efforts to apply these gene discoveries to personalized medicine, such as evaluation of genetic testing in clinical practice (in terms of analytic validity, clinical validity, clinical utility), and to population health including determining the disease risk and burden in populations (e.g., absolute and attributable risks). We also consider the ethical, legal, and social implications of these discoveries.

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Contents

21.1 Introduction

Historically, the term "genetic epidemiology" has been used to denote the study of genetic factors in the occurrence of disease in populations [40]. A primary focus of genetic epidemiology has been on studying familial aggregation of disease and on statistical methods for gene discovery in family-based studies. Developments in genetic epidemiology and statistical genetics have largely centered on methods for discovering disease susceptibility genes [8]. These approaches have been successful in identifying mutations in more than 3,000 genes associated with Mendelian disorders (e.g., cystic fibrosis), as well as high-penetrance genetic variants associated with certain common diseases (e.g., hereditary breast and ovarian cancer) in families with multiple affected members [55]. We now know, however, that variants typically identified in such high-risk families explain only a small proportion of all cases; for example, *BRCA1* and *BRCA2* mutations account for an estimated 1–2% of all breast cancer cases in the general population [52].

In the last decade, the definition of genetic epidemiology has broadened, particularly with completion of the human genome project [14, 30] and with rapid developments in testing for numerous genetic variants with increased efficiency (e.g., reduced time and cost) [74]. These advances have accelerated discovery efforts particularly with genome-wide association studies (GWAS) and now make possible the evaluation of numerous gene associations and interactions. (both gene-gene and gene-environment). In an effort to capture these developments, the term "human genomics" was introduced to define "the study of the functions and interactions of all the genes in the genome" [29], and the term "human genome epidemiology" was creatd to further define epidemiological approaches used from gene discovery to applications in medicine and public health [44]. Finally, the term "public health genomics" was coined to reflect "a multidisciplinary field concerned with the effective and responsible translation of genome-based knowledge and technologies to improve population health" [6].

In this chapter, we describe both the historical and contemporary terminologies that reflect the evolving field of genetic epidemiology. We discuss traditional genetic analyses of human pedigrees to assess Mendelian transmission (segregation analysis) or to locate human genes (linkage analysis/gene mapping). We describe traditional epidemiologic approaches used to study gene-disease associations plus gene-gene and gene-environment interactions. We review analytic and methodologic issues applicable to these studies as well as emerging, nontraditional epidemiologic methods that can be used as an adjunct to traditional approaches. As the field becomes increasingly complex with the simultaneous study of hundreds of thousands of data points per person, the importance of rigorous approaches to study design, analysis and interpretation will be greatly magnified [42]. We therefore discuss the challenging nature of analysis, synthesis, and dissemination of this rapidly accumulating evidence, and the value of systematic reviews, meta-analyses and consortia. Finally, we discuss research translation efforts to apply these discoveries in personalized medicine and population health, including ethical, legal, and social implications.

21.2 Scope and Strategies of Genetic Epidemiology in the Twenty-First Century

Genetic epidemiology will continue to broaden its scope in response to continued scientific and technological advances. Monogenic diseases provide a basic understanding of biologic mechanisms in disease causation and traditional family-based studies will therefore remain critical for identifying high-penetrance genes in diseases and disorders for which there is a strong genetic component. For polygenic diseases, genome-wide association studies will continue to play an important role in identifying common genetic variants that contribute to disease. However, we believe that this, too, will evolve as newer technologies such as genome sequencing will supplant current methods. Regardless of the technology, however, most of these discovery efforts will be conducted using traditional epidemiologic studies (either cohort or case-control studies). Though studies are largely based on principles of linkage disequilibrium (LD) mapping, sequencing of the human genome will become feasible in the coming years. Critical to these efforts will be the follow-up and translation of these discoveries to identify the actual causal variants and to determine the disease risk and burden in populations. Whether intervention or prevention is feasible based on these data has yet to be demonstrated. Epidemiologic efforts to identify common variants causing disease are now largely led by multi-institutional consortia and networks intended to maximize statistical power to identify modest but true associations. Consortia will also be critical in genetic epidemiology for identifying and replicating causal associations (Table 21.1).

Although the current focus of gene discovery is on single nucleotide polymorphisms, expansion to other types of genetic variants and to other genetic changes will occur as technologies make broader efforts possible. In this century, the greatest challenge will be to translate these scientific discoveries. To this end, expansion of the field to human genome epidemiology will be critical for full understanding and characterization of the effects of genetic factors in populations [44]. Only by knowing the prevalence of genetic polymorphisms in well-defined populations, by characterizing genotype-phenotype associations, investigating gene-environment interactions, and evaluating the clinical validity and utility of genetic tests can we know how on-going discovery efforts will be useful and potentially utilized in medical practice and public health programs.

21.3 Fundamentals of Gene Discovery: Family Studies

Since human genetics was founded on observational studies of families, it is not surprising that methods of gene discovery first exploited classic linkage analysis to map causal genes for Mendelian diseases. Linkage analysis tests for co-segregation between an observed marker and an unobserved causal locus and evidence of reduced recombination between a genetic marker and a genetic disease constitute compelling evidence that a gene does control the phenotype, while simultaneously locating the causal gene within the genome. In fact, evidence of linkage used to be considered the highest level of statistical evidence that a gene does control a phenotype, since nongenetic factors can easily mimic segregation patterns of a single gene in families but it seems very unlikely that they would co-segregate with a genetic marker [20].

Although the principles of linkage analysis were developed in the 1950s, their application to families was quite limited and did not expand to a genome wide scale until the number of available genetic markers expanded with discovery of anonymous

Table 21.1 Continuum of human genome epidemiology from gene discovery to applications (adapted from [41])

Field	Application	Types of studies
Genetic epidemiology	Gene discovery	Linkage analysis, family-based association studies
Genetic/molecular epidemiology	Gene characterization	Population studies to characterize gene prevalence, gene–disease associations, and gene-gene and gene–environment interaction
Applied epidemiology and health services research	Determining health impact	Studies to evaluate validity and utility of genetic information in clinical trials or observational clinical settings

DNA markers in the 1980s. The very first genetic markers included blood groups and protein variants which were quite limited in their numbers, and their true location in the genome remained unknown until a comprehensive map of the genome could be generated. Markers in DNA itself were first discovered in the 1980s using bacterial restriction enzymes that cut DNA at specific sequences scattered throughout the genome, generating biallelic markers called restriction fragment length polymorphisms (RFLP). Later, single tandem repeat polymorphisms (STRP) or microsatellite markers were discovered to be common in regions outside of genes, and these multi-allelic markers were very informative for linkage analysis. Later still, single nucleotide polymorphisms (SNP) were found to be even more common in the genome (averaging 1 per 1,000 base-pairs), and as technology for mass genotyping improved genome wide linkage studies became more affordable; fixed panels of informative SNPs are now used to provide adequate coverage of the entire genome.

Linkage analysis is most effective for diseases with a known mode of inheritance. Parametric linkage (also called model-based linkage analysis) is a maximum likelihood approach that estimates the recombination fraction (θ), a measure of genetic distance, and uses the log-odds or LOD score to compare the hypothesis of no linkage (independent assortment of markers at two loci) with the hypothesis that linkage does exist at some recombination fraction less than the expected 50%. This parametric or model-based approach estimates the strength of linkage and tests for its statistical significance simultaneously. Statistical power to detect linkage reflects the ability to reconstruct meiotic events in informative matings (i.e., those matings involving a double-heterozygote parent who is heterozygous at both the trait locus and the marker locus). This becomes a function of the information content for meiosis in the families at hand. Marker allele frequencies are also critical in determining information content, and therefore highly polymorphic multi-allelic markers are more informative for linkage because more parents will be heterozygous, so that STRP markers (also called microsatellite markers) became popular for linkage analysis. A minimum of ~400 highly polymorphic STRPs can provide adequate coverage of the entire genome at a resolution of ≤10 centiMorgans (cM) between markers, which means on average there would be no more than 5 cM (equivalent to 5% recombination) between an unknown causal gene and a marker. This relatively loose-scale genome wide linkage array can identify chromosomal regions of interest, but would require follow-up using a separate round of genotyping with more closely spaced markers (typically more SNPs spaced 1–2 cM apart) for fine mapping. Currently SNP panels of 5,000–6,000 markers (with an average spacing of 1 cM between markers) are used for genome-wide linkage studies, although the high correlation among nearby SNPs (which tend to be in tight linkage disequilibrium or LD) must be considered in the statistical analysis. For Mendelian diseases (even those with some incomplete penetrance or possible locus heterogeneity), this strategy is effective in providing evidence sufficient to map a gene down to a small region of a chromosome. These larger SNP panels for linkage can be also used for fine mapping, since there is an average of 1 cM between markers, although even large collections of multiplex families rarely contain enough informative meiotic events to give resolution down to this level. Furthermore, even small chromosomal regions of interest can contain many genes, and the resolution of linkage signals is often limited, so that further sequencing studies will be required to identify specific mutations in genes thought to control a given phenotype.

The parametric or model-based LOD score approach has been used to detect genes exerting a "major effect" on risk (i.e., the disorder may not be strictly Mendelian, but it has limited locus heterogeneity and consistent levels of incomplete penetrance). When there is evidence of locus heterogeneity, i.e., some families show evidence of linkage to a single marker or chromosomal region and others do not, statistics such as the heterogeneity LOD score (HLOD) can be used to consider locus (or linkage) heterogeneity (see Table 21.2 for definition). This HLOD is based on the admixture heterogeneity test [71] and allows maximum-likelihood estimations of both the recombination fraction (θ) and another parameter for the proportion of linked families (α) simultaneously. Thus, parametric tests for linkage can be applied to complex diseases if one is willing to assume a specific model of inheritance. However, errors in the specified model of inheritance will diminish statistical power to detect linkage and will inflate the estimated θ, making fine mapping even less precise and less reliable.

Table 21.2 Definition of terms for linkage analysis

Term	Definition
Recombination fraction (θ)	Proportion of recombinant (nonparental) gametes formed during meiosis. For 2 unlinked loci, alleles will recombine 50% of the time ($\theta = 0.5$), and this becomes the null hypothesis of no linkage. For 2 linked loci, alleles will show <50% recombination and will tend to "co-segregate" in informative matings. Conventionally, tight linkage is considered to be $\theta = 0.05$, and $\theta = 0.0$ would imply complete linkage (no recombination) where alleles at the 2 loci are always inherited together
Informative matings	Matings involving 1 or 2 double heterozygotes which allow meiotic events to be reconstructed by examining genotypes of offspring. The simplest informative mating is between a double heterozygote and a double homozygote where phase is known, because each offspring can be directly classified as having received a "parental" or "nonparental" haplotype from the informative parent and these represent a direct count of nonrecombinant and recombinant gametes formed by meiosis in that parent
Gametic phase	Haplotype combinations of alleles at different loci, which can be inferred from observed genotypes (sometimes with certainty, but not always). In particular, the double-heterozygous individuals who are informative for linkage are ambiguous regarding gametic phase, i.e., the "AaBb" genotype could represent two possible gametic phases: AB‖ab or Ab‖aB, arbitrarily termed "coupling" and "repulsion" in the genetics literature. In family studies, phase can often be established by genotyping parents of such individuals; thus, three generational families can provide "phase-known matings," which provide more statistical power for parametric or model-based linkage analysis. "Phase-unknown matings," on the other hand, must consider the two possible phases and result in reduced power
Parametric linkage or model-based linkage analysis	A statistical approach testing the null hypothesis of no linkage versus linkage at some estimated θ based on a prespecified model of inheritance. This approach used maximum likelihood methods to both estimate θ and compute a log-odds statistic (or LOD score) to summarize the evidence for or against linkage. By convention, a LOD score ≥ 3.0 is taken as evidence for linkage (at that θ), and a LOD score < -2.0 is taken as evidence of no linkage for any single marker. Widely considered the most powerful statistical method for testing for linkage. If, however, the model of inheritance is wrong, the statistical power to detect linkage will be diminished and the estimated θ will be inflated
Nonparametric linkage or model-free linkage analysis	A statistical approach testing for excess allele sharing between affected relatives, which would reflect linkage between an observed marker locus and an unobserved causal locus. In general, the most informative type of affected relative pair is affected sib pairs, and this approach tests for excess sharing of marker alleles that are identical by descent (IBD) by comparing the null hypothesis values of 25% sharing 0 alleles IBD, 50% sharing 1 alleles IBD and 25% sharing 2 alleles between two affected sibs with the observed or estimated proportion of IBD sharing. This approach is nonparametric or model free in the sense it does not assume any particular model of inheritance (recessive, dominant, etc.) for the trait locus, although evaluating significance from the various test statistics often implies specific distributions
Identical by descent	Two alleles are considered identical by descent (IBD) if they were inherited from a common ancestor. Since humans are diploid, it is possible to share 0, 1, or 2 alleles IBD at any autosomal locus, and the prior probabilities of these sharing states are specified solely by the biological relationship (full sibs, parent-offspring, etc.). In affected sib-pair analysis testing for linkage, prior probabilities of IBD sharing are 0.25:0.50:0.25 represent the null hypothesis of no linkage and the observed probabilities are computed for each marker locus or based on a prespecified framework map of multiple markers. The genotypes of the parents are critical in specifying observed patterns of IBD sharing and the most informative type of mating is between two double-heterozygous parents carrying four different alleles, i.e., an AB × CD mating would allow all possible combinations of IBD sharing between pairs of full sibs to be classified unambiguously
Locus or linkage heterogeneity	Heterogeneity between families in evidence for linkage where some families give evidence of linkage to a given marker and other families appear to be unlinked to this same marker. In parametric or model-based linkage analysis, this introduces a second parameter to be estimated (α), which represents the proportion of linked families. In terms of testing the null hypothesis of no linkage, this second parameter requires a sequential testing strategy and an alternative test statistic, the heterogeneity LOD score or HLOD, which summarizes the evidence for linkage in the a proportion of linked families. Thus, this HLOD statistic cannot give evidence against linkage (i.e., it cannot be negative) because there it becomes impossible to distinguish between the (1-α) unlinked families and no linkage to the marker

In the absence of knowledge of any specific model of inheritance, however, more robust nonparametric or model-free methods can be used to test for linkage (although these methods do not estimate the genetic distance between a marker and the unknown trait locus). Nonparametric linkage methods are based on sharing of alleles that are "identical by descent" (IBD), i.e., marker alleles inherited from a common ancestor. Since humans are diploid, any two individuals can share 0, 1, or 2 alleles IBD at a given marker. The prior probabilities of these three sharing states are determined by the relationship between two individuals. For example, parents and offspring share exactly 1 allele IBD, but cannot share 0 or 2 alleles IBD, and therefore will not be informative for linkage. On the other hand, full siblings have a prior probability of sharing 0, 1, or 2 alleles IBD equal to 25, 50, and 25, respectively, and are therefore potentially informative for linkage. Penrose pointed out that full sibs with the same phenotype (e.g., affected sib pairs) should share marker alleles IBD more than these expected prior probabilities *if* the marker is tightly linked to a gene controlling the phenotype [60]. Testing for excess IBD sharing between pairs of affected sibs therefore provides a nonparametric test for linkage, where no model of inheritance need be specified. Other types of relative pairs can also be informative about excess IBD sharing, but to a lesser degree. For example, second-degree relatives (including avuncular pairs, grandparent-grandchild pairs, and half sibs) can share 1 allele IBD with a prior probability of 50% (and a corresponding prior probability of 50% for sharing 0 alleles IBD). If pairs of affected second-degree relatives show estimated IBD sharing above this expected value, this too constitutes evidence for linkage.

This nonparametric or model-free approach allows smaller families (i.e., those with only an affected sib pair or other relative pair) to be used to test for linkage in a robust fashion that makes sense for complex diseases (where the model of inheritance is rarely known). Several statistical methods for nonparametric linkage have been developed [21, 34], but all involve estimating IBD allele sharing between relatives for either individual markers or for multiple markers in a fixed framework map. Generalized methods for estimating IBD sharing based on multiple markers are available where hidden Markov chain algorithms are used to estimate IBD sharing [46]. These can be used to estimate IBD sharing for multiple markers in a fixed-framework map using the Lander–Green algorithm (see [73], and Chap. 7 for a discussion). Given these estimated IBD sharing probabilities for a family, then either parametric or nonparametric methods of linkage analysis can be used for multipoint linkage analysis.

For quantitative traits, parametric linkage models would require complete specification of genotypic means (and their variances) at the trait locus and allele frequencies at both the trait and marker loci, and these could be used to estimate the recombination fraction between the trait locus and the marker (and test its significance). However, good estimates of these parameters are rarely available; therefore, more robust methods are preferred. Again, estimated IBD sharing can also be used to test for linkage by using regression models to relate phenotypic differences between relatives to the observed (or estimated) IBD sharing. For individual markers, simple regression models were proposed where the squared trait difference between two sibs was regressed on the estimated probabilities of sharing alleles IBD at a marker locus [4, 34]. Later extensions showed more information could be obtained by using the squared sum of a mean corrected phenotype, as long as the sample of sib pairs is representative of the general population [21]. Sham et al. proposed regressing the estimated IBD sharing on the measures of phenotype differences between sibs and suggested this was more appropriate for ascertained samples of sib pairs [69]. These various regression approaches for quantitative phenotypes are reviewed by Schaid et al. [65] who point out opportunities for incorporating covariates, and testing for interaction.

Techniques for nonparametric linkage analysis for quantitative phenotypes are complemented by extensions to general linear models, which underlie variance components approaches. Variance components models are the foundation of the original biometrical models for estimating heritability of quantitative phenotypes, and can be extended to families of arbitrary structure [22]. By adding a term that represents an unobserved quantitative trait locus (QTL) completely linked to a marker, it becomes possible to estimate the variance attributable to sharing marker alleles IBD [1]. Using the patterns of IBD sharing among all family members, it is possible to partition the total phenotypic variance into a component attributable to a QTL, to background "polygenes," and residual environmental factors. Thus, evidence of linkage can be obtained as a proportion of the phenotypic variance attributable to IBD sharing among relatives a given marker or to a region of chromosome in a multipoint analysis. Once again, however, when this approach is applied to a quantitative phenotype where both genetic and nongenetic factors contribute to the etiology (the quantitative equivalent of a complex disease) there are limits in resolution (i.e., the peak regions of evidence for linkage are very broad) and evidence across studies is typically inconsistent (suggesting locus heterogeneity).

During the last two decades, considerable efforts have been invested in genome-wide linkage studies of complex diseases using both classic parametric and nonparametric linkage methods in both sib-pair studies and extended pedigrees. While these studies have met with some success, they were often plagued by weak and sometimes contradictory evidence of linkage, even when multiple studies are combined into a meta-analysis of LOD scores (e.g., see Marazita et al. for a meta-analysis of families ascertained through cleft lip with/without cleft palate [50]). More importantly, however, it has proven extremely difficult to narrow regions yielding evidence of linkage, because even large families cannot provide resolution much below 10 cM of genetic distance.

To summarize, linkage studies can be conducted on a genome-wide level to provide an unbiased search for causal genes using families of almost any size. However, linkage analysis always requires families informative enough to either reconstitute meiotic events within families (as part of parametric linkage analysis) or at least informative enough to document excess sharing of marker alleles between relatives in nonparametric linkage analysis. Moreover, all linkage studies of a qualitative disease phenotype (i.e., affected vs. nonaffected) require multiplex families with more than two affected members (at a minimum affected sib-pairs), and these may be difficult to recruit, so that the question arises of whether multiplex families can adequately represent all cases of a given disease and thus all of the genetic causes of that disease. Furthermore, the track record of genome-wide linkage searches is mixed at best. Many studies have failed to identify regions yielding clear evidence of linkage at the genome-wide level, and following the standard linkage approach of a course genome-wide search followed by fine mapping regions of interest does not always narrow regions of linkage for complex diseases. These failures may represent intrinsic limitations of the linkage approach (i.e., linkage alone cannot achieve resolution on a fine scale) or the presence of locus heterogeneity (multiple genes causing disease, plus some non-genetic causes) simply diminishes both power and resolution.

21.4 Fundamentals of Gene Discovery: Population Studies and GWAS

Genome-wide association studies (GWAS) have recently become a technically feasible approach for searching the human genome for genes influencing risk for complex disorders [59]. These studies typically rely on conventional epidemiologic study designs, especially the case-control design or its variations, and utilize high-throughput marker assays where 100,000–1,000,000 SNP markers are typed on each study subject (from population samples or family members). The principles underlying association studies differ from those underlying linkage studies, but both can be used to identify genes for complex disorders [51]. While linkage analysis is designed to test for co-segregation (or co-inheritance) between an unobserved causal gene and a marker within a family, association analysis relies on detecting differences in marker allele frequencies between groups of unrelated individuals such as cases and controls [15]. Close linkage can easily lead to such differences in frequencies because it creates "linkage disequilibrium" (LD), which manifests as significant correlation between alleles at two different loci (e.g., here a marker locus and a high-risk allele at an unobserved causal gene) at the population level. However, while statistical evidence of linkage results in a definitive interpretation that the marker is inherited with a marker, statistical evidence of association is more ambiguous because LD is not the only possible explanation for a difference in frequencies between unrelated cases and controls. For example, a statistically significant association could be due to an artifact created by heterogeneity in the population from which cases and controls were sampled. This phenomenon is termed "confounding" by epidemiologists and "population stratification" by geneticists. It can occur when two subpopulations which differ in both their marker allele frequencies and their disease risk are combined and treated as samples from a single, homogeneous population, creating a completely spurious statistical association between the marker allele and case/control status [58]. Although the statistical evidence from association studies is less definitive than evidence from linkage analysis, the case-control design has the advantage of being able to identify genes with more modest effects on risk that would be missed completely by linkage analysis [63]. Furthermore, because the underlying LD between markers and a potential causal gene spans a much smaller physical distance than can be detected in standard linkage analysis, finding convincing evidence of association gives greater resolution about where a causal gene lies in the genome, i.e., the resolution of association studies is greater.

The conventional case-control design requires a sound epidemiologic study design that avoids introducing various forms of bias (see Table 21.3). Ideally, cases should be a representative sample of affected individuals, preferably incident cases to minimize selection bias caused by survival. Similarly, controls should be a representative sample of at-risk but unaffected individuals. For genetic association studies, extreme caution should be exerted to ensure that the cases and controls are derived from the same base population. Since no human population can be considered completely homogeneous, there is considerable controversy about how to match cases and controls or how to adjust for possible confounding between disease status (case vs. control) and genetic background. Several options are available: (1) careful matching for genetic background (e.g., racial or ethnic background); (2) adjusted analysis using genomic control markers which would reflect effects of confounding [16]; (3) extensive quantification of the genetic makeup of cases and controls through data reduction techniques such as principal components analyzes and possibly incorporating these into the analysis as a covariate [61]; and (4) use of case-family study designs (see below). The matching of cases and controls can be as simple as recording racial/ethnic background and stratifying by these broadly defined social norms. There are also several methods to estimate population membership for individuals [62] and using these to identify and possibly exclude outlier individuals. These Bayesian methods for estimating admixture at the individual level can help identify when significant population heterogeneity or “cryptic substructure” exists in a sample of unrelated individuals [23]. Both the substructure approach and the genomic control approach require typing a substantial number of unlinked markers that are not associated with the phenotypic definition of cases and controls. Genomic control markers are used to adjust the case-control analysis for average confounding. Another adjustment approach is to use a large number of markers to quantify the genetic variance in samples of cases and controls and to condense the genetic diversity to a small number of summary measures. This usually takes the form of principal components analysis, where linear functions are fit to the data on many markers and condensed into orthogonal summary measures that account for successively smaller fractions of the total genetic variance. These linear functions can then be computed for each individual case and control and can serve either to

Table 21.3 Types of biases that can affect epidemiologic studies of gene–disease associations (adapted from [49])

Biases	
Survival bias	Case selection based on those currently available for study may miss fatal and/or quick cases or mild cases
Participation bias	Differential rates of refusal or nonresponse for study participation between cases and controls
Diagnosis bias	Knowledge of a subject’s exposure to a putative cause of disease can influence both intensity and outcome of the diagnostic process
Referral bias	Factors related to the probability of referral. Cases who are more likely to receive advanced care or to be hospitalized—such as those with greater access to health care or with co-existing illnesses—can distort associations with other risk factors
Surveillance bias	Differential detection of cases for those under frequent medical surveillance
Recall bias	Cases might recall more intensively potential causative exposures
Family information bias	Information about exposures or illnesses can be stimulated by, or directed to, a new case within a family

graphically document population substructure of the entire sample or can be treated as covariate in the conventional case-control analysis. One option for conventional case-control designs is to use "population" or "universal" controls where the controls are carefully documented to represent the population of reference, but are not necessarily free of a particular phenotype. This strategy was used in a well-recognized English GWAS of seven different complex diseases, and the control group for each case group was the same group of controls selected to carefully represent the English general population but without phenotype information [19]. This can be viewed as an incomplete case-control design, where genotype and phenotype information was available on cases but only genotype information was available on controls. In the United States, such a universal control group can potentially be derived from a sample of the population through carefully conducted representative surveys. One example of such a survey is the National Health Examination and Nutrition Survey (NHANES), which is a stratified weighted random sample of the population with overrepresentation of ethnic and racial minorities. Recently, prevalence estimates of over 90 markers were published [11]. Work is ongoing to measure genome-wide profiles from the same sample population. Nelson et al. recently suggested this type of incomplete design might be very useful for situations where the number of cases is small but the number of controls can be much larger (e.g., 10 controls matched to 1 case through multiple principal components) [57].

Finally, a variation on the conventional case-control study design is the case-family design, where an affected case and his/her relatives are sampled. One version of the case-family design is the case-sib control, where an unaffected sib is used as a control. Case-sib control designs tend to be overmatched for genetic background and thus provide less statistical power to detect effects of genes (G) than are case-unrelated control designs, but they circumvent the problem of confounding, because both cases and their sibs share the same genetic background [80]. However, given the small sibship sizes common in modern populations, case-sib control studies becomes less efficient in a sampling sense also, because not all cases will have an available sib.

Another version of case-family designs is the case-parent trio or "triad" design, where parents are genotyped and used to contrast alleles transmitted to cases to those not transmitted. In more general terms, this design tests the null hypothesis that marker genotypes are completely independent of phenotypes. Case-parent trios are particularly attractive for studies of birth defects or childhood disorders, because parents are generally available when the disorder is detected. Conversely, this design is not practical for late-onset disorders, because the parents will typically not be available. Furthermore, this type of family-based study design has the advantage of using all available cases, and not just those from multiplex families. Since there may be etiologic heterogeneity between cases from simplex versus multiplex families, it will be important to evaluate family history in GWAS, as it may be an indicator of severity. The case-parent trio design uses genotypes of the parents to contrast marker alleles in affected cases to those expected under strict Mendelian transmission. Therefore, rejecting the null hypothesis of independence gives evidence that the marker is both linked to a causal gene and in LD with a high-risk allele at that gene. Because case-family designs focus on distribution of alleles or genotypes within a family, these designs are more robust to confounding or population stratification than the original case-control design. Trio or triad designs can also be adapted to test for interaction between genes and environmental exposures (gene-environment or G×E interaction), which may be very important for many complex diseases where environmental risk factors are already recognized.

In addition, the trio or triad design provides the opportunity to test for "parent-of-origin" effects where marker alleles can give different levels of statistical evidence depending on whether they were transmitted from the mother or the father to the affected child. Evidence of parent-of-origin effects could reflect the effects of maternal genotypes on risk, presumably through control of the in utero environment, or they could reflect "genomic imprinting," where genes are turned on or off depending on if they are inherited through mothers or fathers. This represents a major advantage of the case-parent trio design, because conventional case-control designs (or even case-sib controls without parental information) cannot address the issue of parent-of-origin at any level. However, case-control designs can still address gene effects, G×E interaction and even gene-gene interaction (G×G) with potentially greater power, so that either is a viable choice when designing a GWAS.

To summarize, there are several methods for searching for genes influencing the risk for complex diseases and they should be viewed as complementary not competitive. Neither linkage nor association approaches are perfect for all circumstances. The challenge is to recognize their different strengths and use them appropriately for the question at hand.

21.5 Beyond Gene Discovery: Epidemiologic Assessment of Genes in Population Health

Numerous genome-wide association studies have been conducted to date, and there is little doubt that new susceptibility loci will be identified in the years to come. In cancer epidemiology alone, nearly 50 susceptibility loci have been identified with genome-wide significant (e.g., $P<10^{-7}$) across five major cancers (breast, prostate, lung, colorectal, melanoma) in the last 3 years [18]. ~20 have been identified for prostate cancer alone through GWAS, including SNPs in the 8q24 region. GWAS efforts include both disease associations and associations with various phenotypes and exposures/behavior. For example, GWAS in lung cancer and melanoma have identified SNPs associated with relevant environmental exposures (e.g., *CHRNA3/5* with smoking). GWAS have also identified SNPs associated with other phenotypes such as height, hair color, and skin pigmentation.

To assess the impact of genes in population health, further understanding of the distribution of genetic variants in the population and the joint effect of multiple genes or between genes and the environment (also known as gene–gene and gene–environment interactions) will be critical to explain the occurrence of most human diseases. As genetic and environmental risk factors are identified, determining whether they act independently of one another to alter disease risk, or jointly in a biological (or multiplicative) way, will determine their overall affect on disease risk. Evaluation of gene–environment interactions at the simplest level requires only displaying raw data in a 2-by-4 table (Table 21.4). In a 2-by-4 table, both exposure and the underlying susceptibility genotype are dichotomized as present or absent. The genotype could reflect a dominant model (e.g., heterozygote or homozygote variant) or a combination of alleles at multiple loci if multiple genotypes are considered. Odds ratios are computed for each stratum, using subjects who are unexposed and have no susceptibility genotype as the reference group. Though a simplistic way to present the data, there are several advantages to this approach [5]. Foremost, the role of each factor (e.g., exposure without genotype, genotype without exposure) is independently assessed both in terms of the association and of the potential attributable fraction. The presentation also underscores sample size issues because cell sizes are delineated. This approach also favors effect estimation over model

Table 21.4 Gene–environment interaction analysis in a case-control study (+ present, - absent)

Exposure	Genotype	Cases	Controls	Odds ratio[a] (OR)
–	–	A_{00}	B_{00}	$OR_{00}=1.0$
–	+	A_{01}	B_{01}	$OR_{01}=A_{01}B_{00}/A_{00}B_{01}$
+	–	A_{10}	B_{10}	$OR_{10}=A_{10}B_{00}/A_{00}B_{10}$
+	+	A_{11}	B_{11}	$OR_{11}=A_{11}B_{00}/A_{00}B_{11}$

[a] Case-only $OR=A_{11}A_{00}/A_{10}A_{01}=(OR_{11}/OR_{10}OR_{01})OR_{co}$, where $OR_{co}=B_{11}B_{00}/B_{10}B_{01}$ (control-only odds ratio)

Example: Analysis of oral contraceptive (OC) use, presence of Factor V Leiden mutation (a risk for venous thromboembolism)*. Case-only odds ratio: 25*36/10*84 = 1.1; control-only odds ratio: 2 *100/4*63 = 0.8 (*AF – Pop (%)* attributable fraction (percent) in the population, *AF – Exp(%)* attributable fraction (percent) among exposed). (From [6]; data from [77])

Factor V Leiden	OC	Cases	Controls	Odds ratio		95% CI			AF-Exp (%)
–	–	36	100	Ref	Ref				
–	+	84	63	ORe	3.7	2.18	–	6.32	73.0
+	–	10	4	ORg	6.9	1.83	–	31.80	85.6
+	+	25	2	ORge	34.7	7.83	–	310.0	97.1
Total		155	169						

testing where logistic regression is used to evaluate departure from multiplicative effects. The odds ratios themselves can be used to determine whether departure from multiplicative or additive models of interactions exists. Finally, the 2–by-4 table is advantageous in that it provides the distribution of the exposures among controls.

An alternative method for evaluating gene-environment interaction is to use a case-only study [27, 41]. This study design, where case series are used and for which there are no controls, is limited in its ability to evaluate independent effects of the genotype or exposure. However, among cases, multiplicative interaction can be detected. Specifically, assuming a multiplicative model of interaction where the genotype and exposure are independent, a departure of the case-only odds ratio from 1.0 indicates the presence of gene-environment interaction (Table 21.5).

In the context of GWAS, however, 2-by-4 tables, the use of stratified analyses and even logistic regression analysis will be limited [36]. For complex datasets, novel statistical strategies are emerging, such as hierarchical regression and Bayesian methods [17, 28]. Bayesian methods are attractive to epidemiologists because they allow scientists to integrate a priori expectations and hypotheses. A novel multilocus test of genetic association based on Tukey's 1-*df* model of interaction has been proposed [12]. This method exploits LD patterns among SNPs within a gene and simultaneously accounts for gene-gene and gene-environment interactions. Alternatively, the combinatorial partitioning method (CPM), which represents an extension of analysis of variance between and within genotypes at one locus, can be used to conduct joint analysis of multiple genes for quantitative traits [56]. An association between the gene(s) and a trait is represented in excess variability between the genotypes, relative to that within genotypes. The multifactor dimensionality reduction (MDR) method is an extension of the CPM where genotypes from multiple loci are grouped as high- and low-risk groups [33, 64]. The MDR would potentially allow persons to be classified into two or more distinct groups based on their phenotype or based on the underlying biology of the loci under consideration. Statistical methods continue to evolve, and further research and application are needed.

Ultimately, to assess the role of genes and their interactions with other genes and the environment in population health will require large representative

Table 21.5 Example of a case-only epidemiologic study design (adapted from [27])

I. Scanning for complex genotypes that could have a significant attributable fraction

Gene variants at N loci			Cases (T)
1	2	3…..N	
–	–	–……–	A
+	–	–…….–	B
.	.	………	..
+	+	+……+	X

For complex genotypes at N loci, the expected proportion of the population with such a combination will decrease markedly with increasing number of loci, even if each variant is common in the population. For example, if we have genetic variants at 10 loci each with 50% prevalence in the population, about 1 in 1,000 or fewer people are expected to be positive for all 10. Therefore, we can use the ratio of X to T to derive an upper bound of population attributable fraction for complex genotypes even in the absence of controls (for more details, consult [5]

II. Assessing etiologic heterogeneity and genotype–phenotype correlation among cases

	Cases	
Risk factor (exposure/genotype)	Phenotype 1	Phenotype 2
Yes	A	C
No	C	D

Odds Ratio = AD/BC = 1 if homogeneous subgroups

III. Screening for multiplicative gene–environment or gene–gene interaction

	Cases	
Risk factor (exposure/genotype)	Genotype 1	Genotype 2
Yes	A	B
No	C	D

Odds Ratio = AD/BC = 1 if joint effects are multiplicative and > 1 if supramultiplicative samples of populations. For published data, systematic reviews and meta-analyses provide a valuable tool for summarizing genetic effects and for identifying and explaining the underlying differences and observed discrepancies between studies [47]. Meta-analyses of gene-disease association studies are accepted as an important method for establishing the genetic components of complex diseases [37, 48]. A set of criteria (the Venice criteria) have been published as one way to assess the credibility of cumulative evidence on genetic associations derived from multiple sources [35]. These criteria take into consideration the amount of evidence, replication, and methodologic issues surrounding the quality of the evidence and potential effects from various biases.

Understanding how gene discoveries may impact population health further requires assessing various epidemiologic measures of disease outcomes, and in particular, absolute and attributable risks. *Absolute risk* is defined as the probability that persons with a particular characteristic, such as a specific genotype, will in fact develop disease (a concept similar to *penetrance* in genetics). To calculate direct estimates of absolute risk requires cohort studies, as it is estimated from the cumulative incidence of disease in a well-defined population. In case-control studies, absolute risk can be imputed if the representative population can be reconstructed and appropriate weights applied to recreate the sampling fraction.

The proportion of cases that would not have occurred within a certain time period had the risk factor (e.g., genotype) been absent is calculated with the *population attributable fraction.* It is the overall contribution of a particular risk factor (e.g., genotype) to the occurrence of disease in a given population. The formula for calculating attributable fraction as proposed by Miettinen is:

Population Attributable Fraction = $f_c(R-1)/R$ where f_c is the fraction of cases with the risk factor and *R* is the measure of relative risk [54]. Interpreting attributable fraction in the context of potential genetic and environmental interactions is currently poorly understood [78]. The concept of attributable fraction in environmental epidemiology is intuitive since it denotes a reduction in disease occurrence by removal of putative exposures from the population. Though genes cannot be removed, the concept of attributable fraction in gene-disease associations is still valid, as it approximates the influence of the genetic variant on disease occurrence in the population. We note that the genetic concept of heritability is not directly useful for determining the genetic or environmental contributions to disease or for estimating attributable fraction [8]. Heritability is population specific and depends on specific assumptions about additive effects of genetic components and lack of correlation with environmental exposures among family members.

Finally, for any gene discovery to be translated to population health for clinical application, such as genetic testing, parameters for tests in clinical practice (analytic validity, clinical validity, clinical utility) need to be applied (defined in detail in Tables 21.6, and 21.7), as first recommended by the Secretary's Advisory Committee on Genetic Testing [31, 68]. Briefly, such parameters include *analytic validity* which refers to the accuracy of a genetic test. This criterion evaluates the ability of a test to accurately "measure or detect the analyte it is intended to measure or detect" [68]. It is evaluated by calculating the test sensitivity, which provides the probability that a positive test is truly positive (e.g., will detect the analyte when it is in fact present), and test specificity, which provides the probability that the test will not detect the analyte when it is not present. *Clinical validity* com-

Table 21.6 Evaluating the analytic and clinical validity and utility of genetic markers

Type of evaluation[a]	Terms/variables	Types of studies
Analytic validity	Analytic sensitivity, specificity and predictive values	Laboratory studies "Transitional" studies
Clinical validity	Risk of current or future clinical outcomes with and without markers	Traditional epidemiologic study designs
Clinical utility	Risk of disease with and without using biomarker and accompanying interventions	Controlled clinical trials, observational clinical epidemiologic studies

[a] Adapted from the Task Force on Genetic Testing (1997) and the Secretary's Advisory Committee on Genetic Testing (2000)

Table 21.7 Defining analytic and clinical validity and utility of genetic markers*

I. Analytic validity

	Genetic marker	
Test	Present	Absent
+	A	B
-	C	D

Analytic sensitivity: A/A + C
Analytic specificity: D/B + D

II. Clinical validity of biomarkers as risk factors and as clinical tests

	Clinical outcome	
Genetic marker	Present	Absent
+	A	B
-	C	D

Odds ratio = AD/BC (for case-control study)
For cohort study:
Risk ratio = (A/A + B) / (C /C + D)
Clinical sensitivity: A/A + C
Clinical specificity: D/B + D
Positive predictive value (PPV)[a] = A/A + B
Negative predictive value (NPV)* = D/C + D
[a]Assuming total population tested

III. Clinical utility (an example) among persons with a genetic marker; comparing two hypothetical interventions in context of controlled clinical trial: risk ratio = (A/A + B) / (C/C + D)

	Outcome	
Intervention	Disease	No disease
1	A	B
2	C	D

[a]Tables are for illustrative purposes only and apply to dichotomous biomarkers (present/absent). Additional analyses may involve stratification, person-time analysis in cohort studies, adjustment for confounding and assessing for effect modification

*Adapted from the Task Force on Genetic Testing (1997) and the Secretary's Advisory Committee on Genetic Testing (2000)

prises the sensitivity, specificity, and predictive values of a test to measure its intended clinical (or subclinical) endpoint. Specifically, clinical validity is defined as the "probability that a person with disease, or who will get a disease, will have a positive test result" [68]. Measures of sensitivity and specificity are now evaluated in a representative sample of the population for whom the test is intended. Further, positive predictive value, the "probability that a person with a positive test result has, or will get, the disease for which the analyte is used as a predictor" [68] is determined. Epidemiologic study designs are critical in establishing clinical validity. Finally, *clinical utility* is ideally evaluated in clinical trials to determine the positive and negative predictive values of a test and "to demonstrate the benefits and risks from both positive and negative results" [68]. All values for analytic and clinical validity and utility should ideally be evaluated prior to implementing testing into practice.

21.6 Beyond Gene Discovery: Epidemiologic Assessment of Genetic Information in Medicine and Public Health

Translating genetic information to medicine and public health remains challenging. As in other areas of medicine and public health, translation can be delineated into four overlapping phases as described by Khoury et al. [45]. Briefly, phase 1 translational research is defined by research that seeks to move a basic genome-based discovery into a candidate health application (e.g., genetic test/intervention). Examples of phase 1 translational research are epidemiologic studies aimed at identifying and confirming gene-disease associations, such as ongoing GWAS efforts, and also characterizing gene-disease biology and gene-environment interactions. Phase 2 translational research assesses the value of a genomic application for health practice, leading to the development of evidence-based guidelines. An example of phase 2 translational research is determining the positive predictive value of confirmed gene-disease associations, such as the positive predictive value of 8q24 SNPs in prostate cancer or of *BRCA* mutations among women at high risk for breast cancer. Phase 3 translational research attempts to move evidence-based guidelines into health practice, through delivery, dissemination, and diffusion research. Though phase 3 translational research is premature for GWAS results at the current time of writing, there are successful models for phase 3 translational research, such as studies determining the proportion of women with a family history of breast or ovarian cancer who are tested for *BRCA,* and determining barriers to testing and best approaches to implementation in practice and dissemination of evidence guidelines. Finally, phase 4 translational research seeks to evaluate the "real world" health outcomes of a genomic application in practice. Identifying whether *BRCA* testing in asymptomatic women reduces breast cancer incidence or improves survival outcomes is an example of phase 4 translational research. At present, the vast majority of genomics research can be considered phase 1, as we are still in the early stages of applying genomic information to public health applications. Epidemiologic methods and approaches are critical to all phases. In phases 2 and beyond, these methods are often considered in the domains of clinical epidemiology, applied epidemiology, or health services research (Table 21.1).

"Personalized medicine" has been promised as an end-result of genomics research. However, to attain this goal will require multidisciplinary translational research efforts as described above. To date, the utility of testing for common polymorphism in predicting disease outcomes has yet to be demonstrated. Further, risk estimates and subsequent predictability resulting from joint effects of polymorphisms with other risk factors (e.g., personal and family history, environmental exposures, and behavioral risk factors) remain unmeasured [3, 32, 43, 70]. There have been varying claims regarding the utility and predictability of genetic markers as risk factors. Although the combination of each newly discovered SNP association provides high absolute risk and population attributable fraction, a recent systematic evaluation of seven common SNPs identified in breast cancer showed that the discriminatory value of all seven SNPs had less accuracy than currently known risk factors for breast cancer (e.g., based on ages at menarche and first live birth, family history of breast cancer, and history of breast biopsy examinations). Even the addition of the seven SNPs to the current risk assessment module contributed less to the discriminatory accuracy than did additional results from mammographic density, thus suggesting much larger numbers of SNPs will be required to achieve high discriminatory accuracy [26]. Similar evaluations must be taken for other diseases to determine the true predictability and contribution of SNPs to disease. Importantly, to accurately calculate the predictive values of genetic information will require consideration of gene-gene and gene-environment interactions. Therefore, additional well-conducted epidemiologic studies will be needed, both case-control and cohort studies with long-term follow-up. Only with meticulous analysis from well-conducted studies can the complex relationships between genes, exposures and other population characteristics truly be understood and used to improve population health and to achieve the goals of personalized medicine.

Several on-going initiatives reflect such efforts. The largest effort being conducted is the UK Biobank, which aims to collect information on the health and lifestyle of 500,000 volunteers aged between 40 and 69. The framework for enrolling and following up the Biobank cohort is the UK National Health Service, which provides health care to the entire UK population. Planned follow-up is for 20 or more years, upon which DNA samples and population information will be available for scientifically and ethically approved research [75]. Though no comparable infrastructure exists in the United States, with its highly decentralized health system and mobile population, the Northern California Kaiser Permanente Health Maintenance Organization has recently launched the Research Program on Genes, Environment and Health, which similarly aims to enroll 500,000 volunteers in Northern California with a questionnaire and DNA samples for future research on etiologic and pharmacogenetic outcomes [39]. The American Cancer Society is similarly aiming to enroll 500,000 men and women in their Cancer Prevention Study-3 (CPS-3) to allow evaluation of environmental, lifestyle, and genetic factors as related to cancer. Though unquestionably valuable, these population-based biobanks will require long-term follow-up to ascertain sufficient numbers of persons with selected

Table 21.8 Examples of large-scale population-based genomics studies

Study	Sample size	Population	Study objectives
Decode genetics	>100,000	Iceland	"To identify genetic causes of common diseases and develop new drugs and diagnostic tools" Measures genes, health outcomes, and link with genealogical database
UK Biobank	500,000	Population sample of persons 45-69 years	"To study the role of genes, environment, and lifestyle" Link with medical records
CartaGene[a] (Quebec)	>60,000 persons 25–74 years	Population sample	"To study genetic variation in a modern population." Link with health care records, and genealogical databases
Estonia Genome Project[a]	>1,000,000	Estonian population	"To find genes that cause and influence common diseases" Link with medical records
GenomeEUtwin[a]	~800,000 twin pairs	Twin cohorts from seven European countries and Australia	"To characterize genetic, environmental and lifestyle components in the background of health problems"
Kaiser Permanente Research Program on Genes, Environment and Health (RPGEH)	500,000	Population sample	"To identify genetic and environmental factors that can lead to disease or affect how a person reacts to medications."

[a] Part of the global P3G collaboration (Public Population Project in Genomics); complete list of P3G participants found in http://www.p3gconsortium.org/memb.cfm

health outcomes for scientific analysis [7]. In the meantime, complementary approaches have gained traction. he Wellcome Trust Case Control Consortium and the Genetic Association Information Network (GAIN) are poised to mine genetic association data within previously conducted, large-scale epidemiologic studies [25, 79]. Some on-going efforts are summarized in Table 21.8.

21.7 Policy, Ethical and Practice Considerations: the Emergence of Public Health Genomics

The potential application of genetic information in public health practice raises a number of ethical, legal, and social issues, also referred to commonly with the acronym ELSI [53, 66, 67]. As the field of genetic epidemiology moves towards big science and biospecimen-intensive collection in large population samples, ethical issues arise concerning enrollment of study participants and collection of biological specimens used for evaluation of genetic data. The specific ethical, legal, and social issues around generating genetic information will vary with time, as they are directly tied to the clinical utility of the genetic data. For example, present specific issues of concern include language on informed consent documents, recruitment of subjects, sending study results to patients who request it, and the potential for discrimination or stigmatization of individuals or groups. The creation of biobanks and large population-based studies relies on language in informed consent documents that specify the risks and benefits of participating in the study are directly related to the meaning of the results. Because we are in the early stages of understanding the role of genetic information in health and disease, the impact and potential risks remain relatively small. However, this may change during the duration of a study, as new results are published regarding potential clinical applications for medicine and public health. In consultation with a multidisciplinary group, the Centers for Disease Control and Prevention (CDC) has published an online consent form template and supplemental information that can be adapted by researchers for population-based genetic epidemiologic studies [2, 9, 10]. Informed consent will surely evolve as additional information is known about the public health significance of genes or panels of genes and gene-environment interactions.

In the meantime, there is an alarming trend of direct-to-consumer advertising of whole-genome analysis by several companies [38]. These companies offer "genetic profile" tests directly to practitioners and the public, accompanied by personalized lifestyle recommendations, such as advice on dietary supplements. This phenomenon can contribute to misunderstanding of the relationship between cause and effect at the individual and population levels [78]. Ethical, legal, and social issues would demand that these issues be explained explicitly and that transparency and rigor in genetic epidemiologic research be promoted. Unfortunately, as these tests are not currently regulated (in the United States), there is no forum for addressing ethical, legal, and social issues in such instances. However, this practice has been reviewed by Congress, and further scrutiny is likely, given their recognized potential for misleading consumers [24, 76].

Before translation of genomic information is truly integrated into clinical and public health practice, it is clear that appropriate ethical, legal, and social issues will need to be addressed. The most common concerns regarding genetic information pertain to consumer access to health insurance, employment, education, and loans [13]. Combating these concerns will require informing health providers as well as patients and the general public of the risks and benefits of genetic data and addressing current misconceptions about genetic information, such as the notion of genetic determinism. Importantly, clarifying complex issues of confidentiality and privacy will be required. As the field of genetic epidemiology and the translational aspects of research evolve, additional legislative and regulatory responses will certainly be needed.

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Pharmacogenetics

22

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Abstract The goal of pharmacogenetics is to identify genetic differences among patients that influence treatment response. Pharmacogenetics also represents a tractable target for systematic studies, because key treatment responses are both clinically important and amenable to genetic investigation. Despite a number of paradigmatic studies, pharmacogenetics has to date only had a minimal impact in medicine. Here we outline several methodological considerations for implementation in future studies and also detail how we can best apply the lessons learned from extensive genetic studies on disease predisposition in order to advance the state of pharmacogenetic knowledge and to expedite the translation of pharmacogenetic findings into clinical practice. We conclude that while the study of common variation may provide some insight, like the study of disease predisposition itself, pharmacogenetics will ultimately require characterization of rare human gene variants.

Contents

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22.1 Introduction

22.1.1 The Goal of Pharmacogenetics

Variation amongst patients in their responses to medication is a consistent clinical experience in most if not all therapeutic areas. Some of this variation is due to environmental factors, such as drug–drug interactions or co-morbid disease, and can be anticipated and at least partially avoided. A large proportion of variable drug-response, however, remains unexplained by environmental factors, suggesting that underlying genetic factors may cause or contribute to a patient's response to a particular medication. The goal of pharmacogenetics is to identify genetic differences among patients that influence treatment response.

22.1.2 Why Pharmacogenetic Studies Are Necessary

Pharmacogenetics also represents a tractable target for systematic studies, because key treatment responses are both clinically important and amenable to genetic investigation. The need for better understanding of the genetic determinants of treatment response is particularly essential with respect to three major response phenotypes: efficacy, safety, and optimum dose.

22.1.1.1 Efficacy

Despite the plethora of pharmacological treatments that exist for any given disorder, drug resistance/non-response remains a serious clinical issue. It is estimated, for example, that 30% of patients with epilepsy, and 30–50% of patients with schizophrenia do not respond to any of the currently available drug treatments. For these treatment-resistant patients there are frequently few, if any, treatment options, which results in a significant proportion of patients with a definitive diagnosis of disease but no possibility of medical intervention.

Efficacy issues also become apparent when the current crisis in drug development is considered: starting in 1998, despite an increase in the productivity of drug discovery, the number of new molecular entities submitted to the FDA and EMEA decreased by nearly 50%. At the same time, clinical trial budgets have increased by 58%, while the failure rate of phase III clinical trials is nearly 50% [26]. The incorporation of pharmacogenetic information into trials could be of financial benefit, however, if preclinical studies began to include pharmacogenetic components. The increased ability to predict the success – or failure – of a drug based on pharmacogenetic knowledge prior to large-scale, expensive trials will simplify phase III trials, reduce costs, and increase the chance of success [26]. In addition, pharmacogenetic discoveries that elucidate the underlying molecular bases of treatment failure stand to facilitate the development of new compounds to treat patients that are resistant to current medications.

22.1.1.2 Safety

Drug treatment is frequently associated with adverse drug reactions (ADRs), which can vary in severity from mild to life threatening. Indeed, many medications fail clinical trials because of the incidence of ADRs, and multiple medications have been withdrawn from the market post approval following the emergence of severe ADRs in patients. Torsade de pointes (TdP/prolonged QT interval) is one severe ADR that has led to the withdrawal of several drugs, including astemizole (an allergy medication), grepafloxacin (an antibiotic), and sertindole (an antipsychotic), in the past 10 years. Currently, an estimated 5–13% of hospital admissions are due to adverse reactions even to FDA-approved drugs [2, 8].

In addition to severe ADRs, there are also adverse reactions that, while not immediately life threatening, are nonetheless treatment limiting. For example, ADRs such as hypersomnolence, diplopia, tremor, or dizziness may prohibit the administration of therapeutic doses. Furthermore, marked weight gain is often associated with several drugs, such as the tricyclic antidepressants amitriptyline and nortriptyline, the anticonvulsants valproate and pregabalin, and the atypical antipsychotics clozapine and olanzapine, among others. The increase in body mass index (BMI) as a result of these treatments can be considerable and can lead to such obesity-related comorbidities as type II diabetes, hypertension, and cardiovascular disease.

The frequency of mild ADRs is difficult to estimate, as they commonly go unreported; however, it is not uncommon for these types of ADRs to precipitate treatment discontinuation, regardless of efficacy outcomes, representing a significant limitation to effective treatment.

22.1.1.3 Dose

Optimal drug dosing represents a third clinical area that stands to be improved by pharmacogenetic investigation. Many clinically used drugs have a narrow therapeutic window, so that doses that are too low are not effective, while doses that are too high quickly lead to drug toxicity and dose-related ADRs. For many of these same drugs, different patients also require dramatically different therapeutic doses. For example, this represents a particular clinical challenge with respect to antiepileptic drugs, where patients must be started on low doses and these slowly titrated until seizure control is achieved or until unacceptable ADRs necessitate medication withdrawal. Unfortunately, dose titration can take months, during which time seizures persist. Pharmacogenetic predictors of dose, however, could allow clinicians to safely expedite the determination of optimal dose and, particularly for patients who require extremely high doses, minimize the amount of time a patient remains in the subtherapeutic range.

22.2 Important Pharmacogenetic Discoveries to Date

The concept that treatment outcome may be affected by genetic variants was first posed by Motulsky in 1957 [32], and the term "pharmacogenetics" was coined by Vogel in 1959. In the 1970s twin studies demonstrated that monozygotic (identical) twins demonstrate highly concordant plasma levels of multiple drugs, whereas the plasma levels in pairs of dizygotic (fraternal) twins vary widely [1, 43], indicating a strong genetic influence. The subsequent identification of debrisoquine polymorphism (cytochrome P450 2D6; CYP2D6) motivated many of the early pharmacogenetic studies.

22.2.1 CYP2D6 Polymorphism and Pharmacogenetics

Debrisoquine polymorphism was identified on the basis of drug clearance studies of the antihypertensive drug debrisoquine, in which two distinct phenotypes were identified: extensive metabolizers (EM) who excrete significant amounts of debrisoquine metabolite, and poor metabolizers (PM) who excrete very little metabolite [7, 24]. This phenotype was later found to be caused by defects in a cytochrome P450 gene [13, 14] now known as *CYP2D6*.

Early studies in pharmacogenetics focused considerable attention on *CYP2D6*, because the gene is highly polymorphic with over 70 identified allelic variants (http://www.cypalleles.ki.se/cyp2d6.htm) and the CYP 2D6 enzyme metabolizes approximately 25% of all medications, including a large number of antidepressants and neuroleptics (Table 22.1). Several studies have found a relationship between the PM phenotype and serum concentrations of a number of drugs, including amitriptyline, nortriptyline, mirtazapine, and aripiprazole [14, 15, 18]. Although there is a clear relationship between *CYP2D6* status and serum concentrations, the correlation does not appear to extend predictably to other important clinical outcomes, such as dose (as determined in empirical medication trials) or efficacy. Therefore, the extent of the clinical utility of *CYP2D6* genotype has not been determined, and there

Table 22.1 Some drugs metabolized by CYP2D6

Drug	Drug class
Sparteine	Antiarrhythmic
Amitriptyline	Antidepressant
Desipramine	Antidepressant
Fluoxetine	Antidepressant
Imipramine	Antidepressant
Nortriptyline	Antidepressant
Paroxetine	Antidepressant
Dextromethorphan	Antitussive
Codeine	Analgesic
Tramadol	Analgesic
Metroprolol	b-Adrenoreceptor blocker
Propanolol	b-Adrenoreceptor blocker
Halperidol	Neuroleptic
Perphenazine	Neuroleptic
Risperidone	Neuroleptic

are currently no guidelines for the incorporation of *CYP2D6* genetic testing into treatment practice.

22.2.2 Classic/Paradigmatic Studies

22.2.2.1 Isoniazid, Azathioprine, Mercaptopurine and Other Poor Metabolizer Phenotypes

As previously discussed, ADRs are a major cause for concern in clinical use of medications. Indeed, some of the early pharmacogenetic studies concerned ADRs occurring in patients exposed to the drugs isoniazid (used to prevent/treat tuberculosis) and the immunosuppressants azathioprine and mercaptopurine. Each of these drugs is associated with significant ADRs (hepatotoxicity and thiopurine toxicity, respectively), and initial candidate gene studies assessed functional genetic variation in the major drug-metabolizing enzymes (DMEs) of these drugs. These studies revealed that genetic changes resulting in reduced/absent activity of each drug's major DME (*N*-acetyltransferase 2 (*NAT2*) and thiopurine *S*-methyltransferase (*TPMT*), respectively), were associated with the incidence of these ADRs [9, 21, 34]. As a result of these important DME studies, one of the few clinical pharmacogenetic applications became standard: testing of *TPMT* genotype or phenotype (TPMT enzyme level) is used clinically to identify those at risk for thiopurine toxicity upon exposure to azathioprine and mercaptopurine, and dosage adjustments are made before treatment to prevent the occurrence of severe ADRs.

22.2.2.2 Efficacy of Asthma Treatment

Another early pharmacogenetic discovery assessed the efficacy of inhaled albuterol for the treatment of asthma. Albuterol is an agonist of the β-2 adrenergic receptor (*ADRB2*) and provides relief of asthma symptoms. The initial study in 1997 [30] showed a significant association between improved response with a coding polymorphism (Arg-16-Gly) in the *ADRB2* gene with analyzes indicating that homozygotes of the Arg-16 allele were about five times as likely to respond to treatment as homozygotes of the Gly-16 allele. Heterozygotes are 2.3 times as likely to respond. These results may be particularly important because the Arg-16-Gly is a common allele [minor allele frequency (G, Gly) is 0.47], but the clinical relevance of this polymorphism is still under investigation.

22.2.2.3 Abacavir Hypersensitivity

Abacavir is a transcriptase inhibitor used to treat infections with human immunodeficiency virus (HIV). After abacavir was released, it emerged that 4.3% of patients exposed to the drug developed a hypersensitivity reaction that presents as fever, rash, and/or GI upset and can ultimately result in death [19]. This idiosyncratic syndrome usually regresses after treatment withdrawal and has been noted to be much more severe upon rechallenge with the drug. Genetic studies were undertaken focusing on the HLA region of the genome, and initial results indicated that the HLA-B*5701 allele is a significant predictor of the occurrence of the hypersensitivity reaction [20, 29]. Prospective clinical use of this pharmacogenetic association appears to be effective, with no cases of the hypersensitivity reaction reported in a study of 260 patients [35], and systematic genetic testing has been posited as a cost-effective method of avoiding the development of this severe ADR [22].

22.2.2.4 Tranilast and Clinical Pharmacogenetic Investigations

Tranilast is a drug that entered clinical trials as an antirestenosis drug, but during the course of the phase III trials 12% of patients had a rise in their levels of bilirubin. Pharmacogenetic tests were carried out during the trial and identified a polymorphism in the glucuronyltransferase 1A1 gene (*UGT1A1*) that was significantly associated with the incidence of hyperbilirubinemia [6]. This TA-repeat polymorphism is also known to predispose some individuals to Gilbert's syndrome, an inherited variant that causes hyperbilirubinemia [31].

Tranilast was subsequently abandoned as an antirestenosis drug owing to lack of efficacy; however, the study itself demonstrates the value of including pharmacogenetics in clinical studies and the importance of collecting DNA samples and detailed phenotype information on efficacy and safety during the course of preclinical and clinical trials.

22.2.2.5 Cancer Pharmacogenetics and Somatic/Acquired Polymorphisms

Though pharmacogenetic studies of many medications focus on germline polymorphisms and the roles that they play in treatment response, cancer pharmacogenetics often seeks rather to discover somatic or acquired polymorphisms in tumor cells that affect treatment outcome. Furthermore, pharmacogenetics of cancer therapy is particularly important, as anticancer treatments have some of the lowest efficacy rates among clinical treatments, at 20–80% for first-line treatments and 5–30% for subsequent treatment regimens [25].

Cancer pharmacogenetics already has a few paradigmatic studies in its relatively short research history. For example, trastuzumab (Herceptin) is an anticancer agent and monoclonal antibody that acts on a particular epidermal growth factor receptor *HER2/ERBB2*. The gene for this particular receptor is amplified in 25–30% of human breast cancer tumors, and the resulting receptor overexpression is associated with tumor aggressivity and an increased incidence of relapse and death (for review see [37]). The drug trastuzumab binds to the overexpressed receptor and signals cancer cells for destruction by the immune system. As a result, the use of trastuzumab is specifically indicated as a treatment option for tumors with *HER2/ERBB2* gene amplification and overexpression [44].

The efficacy of another drug, imatinib (Gleevec), which is used to treat chronic myeloid leukemia (CML), has also been found to depend on the genetic make-up of cancer cells. As a first-line treatment, imatinib is a very effective treatment for CML, effectively binding and inactivating the BCR-ABL protein, a tyrosine kinase that activates downstream proteins that drive white blood cell proliferation (the underlying cause of CML). However, imatinib resistance can be caused by acquired mutations in the BCR-ABL protein complex that inhibit drug binding, and among the patients in whom this occurs there is a significant rate of relapse and a much poorer prognosis. Several such somatic mutations have been characterized, though only six mutations are common and these, taken together, account for 60–70% of all mutations [45].

22.3 Pharmacogenetic Methodology: Clinical Practice

While the above studies and many others have begun to identify variants that affect treatment response to an array of medications, the field of pharmacogenetic research as a whole has been severely limited by a number of methodological issues, including phenotypic characterization, limited sample sizes, and subpar genomic methods.

22.3.1 Phenotype

One of the most difficult components of designing pharmacogenetic studies is the definition of phenotypes, which must be done in a way that is amenable to genetic study but also reflects clinical endpoints. For the study of ADRs, the definition of phenotype is relatively straightforward as it is frequently easy to classify a patient as having experienced or not experienced a particular adverse reaction. ADRs are not always unambiguous, however, and in order to accurately ascertain their presence/absence or classify the severity of an adverse event it is sometimes necessary to conduct specific tests prospectively. For example, cognitive side effects on exposure to several neuropsychiatric medications, including antipsychotic, antiepileptic, and antidepressant medications, are a clinical concern, but are not easy to discern in a purely clinical setting because patients are being treated for diseases that affect brain function, and many of them are concomitantly also being treated with other neuropsychiatric drugs. In such a scenario, a complaint of a cognitive adverse event cannot easily be attributed to exposure to a single medication. To assess these types of effects as accurately as possible, it is necessary to set up a prospective study design that includes testing the cognitive function of the patient before and during the administration of a drug and close monitoring for any other changes in treatment, disease state, or patient behavior that could also have effects on the outcome. Without detailed information and the ability to compare the change in cognitive ability, the presence and the severity of cognitive adverse events cannot be accurately quantified.

Efficacy studies also present a challenge to pharmacogenetics in terms of defining phenotype. To an extent it is possible to classify patients as responsive or nonresponsive to a particular drug, but there are many other issues that complicate the matter. For example, in epilepsy treatment with antiepileptic drugs (AEDs) it is quite common for patients to be treated with more than one AED, as no AED used in monotherapy has proved sufficient to control seizures. The patients are "responsive," but not to any single drug, so that they are phenotypically different from patients who respond well to the first AED to which they are exposed. In addition, it is not uncommon for patients to have a partial response, where symptoms are significantly affected by a drug but not completely controlled.

Efficacy phenotypes are further complicated by the heterogeneity of certain diseases. Continuing with epilepsy as an example, the disease etiology itself is poorly understood, but it is commonly accepted that patients with epilepsy suffer from a number of different pathophysiological causes. The underlying cause of epilepsy may have a significant impact on treatment outcomes, but this cannot always easily be accounted for in pharmacogenetic studies.

In all, there is no obvious way to assign phenotypes for efficacy; however, it is essential that the designation of phenotype is thorough and unambiguous, with careful consideration of disease etiology and past and present medication use.

22.3.2 Sample and Patient Recruitment

Clinical cooperation is, of course, necessary for pharmacogenetic studies, and study design can be manipulated to accommodate the clinical advantages and limitations at every site. The quality of a genetic study reflects the quality of study design and clinical information that is captured and included. In general, clinics can approach genetic studies in three manners: clinical trials, single-hospital studies, and multiple-hospital studies, each having their own advantages and limitations.

Clinical trials by far surpass other settings in their ability to collect rich information and to selectively enroll patients who meet very particular inclusion criteria. However, clinical trials, by necessity, focus only on the most relevant efficacy and adverse reaction phenotypes, and particularly on those that might prevent a medication from being approved and reaching the market. In such a setting, although the clinical data collected are detailed and accurate, it is not statistically feasible to run analyzes on all clinical measures. Such open-ended studies are limited in statistical power owing to the multiple-comparison corrections that must be made with respect to the assessment of statistical significance. Perhaps most importantly, clinical trials have strict inclusion and exclusion criteria, which mean that the medicines are not always used in a way that reflects real clinical practice.

Single-hospital studies involve the recruitment of patients from a single medical center, which helps to be more certain about the homogeneity of clinical assessments, but also makes it possible to study only common phenotypes. Recruitment from a single medical center is likely to result in patient numbers only in the hundreds, and although these cohorts may outnumber many past pharmacogenetic studies, genome-wide association (GWA) studies require still larger cohorts for statistical power. Therefore, for the study of some of the more severe, and rarer, adverse reactions, single-hospital settings are not likely to provide sufficiently large cohorts.

Studies that draw patients from multiple hospitals are far more likely to involve the large numbers of patients necessary for GWA studies. In particular, genetic assessment of rare adverse reactions will likely be possible only through the collaboration of multiple medical centers. Unfortunately, although study size will be increased in this type of study, patient cohorts are also at risk of inconsistent phenotypic classification. Phenotypic heterogeneity is one of the commonly accepted causes of ambiguous results in genetic studies, and therefore great care must be taken to minimize this factor.

While building up large cohorts is a priority for genome-wide association studies aimed at identifying common variation that contributes to treatment response, some pharmacogenetic phenotypes may be due to rare, highly penetrant variants. Such variants can be identified by whole-genome sequencing in small, carefully collected and phenotyped cohorts (see below). Obviously, the design of the genetic study will dictate which method of sample collection is appropriate.

22.4 Pharmacogenetic Methodology: Genomics

22.4.1 Candidate Gene Studies

While it is likely that genetic variation throughout the genome plays a role in influencing treatment outcomes, a priori hypotheses based on the pharmacokinetic and pharmacodynamic properties of a drug implicate a much smaller number of genes in pharmacogenetic processes. Pharmacokinetic processes are those that are involved in the absorption, distribution, metabolism, and excretion of a drug (the action of the body on a drug), and pharmacokinetic candidate genes include genes that encode drug-metabolizing enzymes and drug transporters. Pharmacodynamic processes describe the action of the drug on the body, and pharmacodynamic candidates include genes that encode drug targets and genes within the pathways of drug targets (Fig. 22.1). This philosophy may narrow the genomic space to which many researchers confine their pharmacogenetic investigations; however, the results obtained by such methods are mixed: candidate gene studies on the widely used anticoagulant warfarin have found that dose depends largely on genetic variation in its drug-metabolizing enzyme (CYP2C9) and its drug target (VKORC1, see below), but candidate gene studies on most other drugs have had negative or inconclusive results.

In general, a candidate gene approach has been the method of choice, but candidate gene studies have several important limitations. Even though the effects

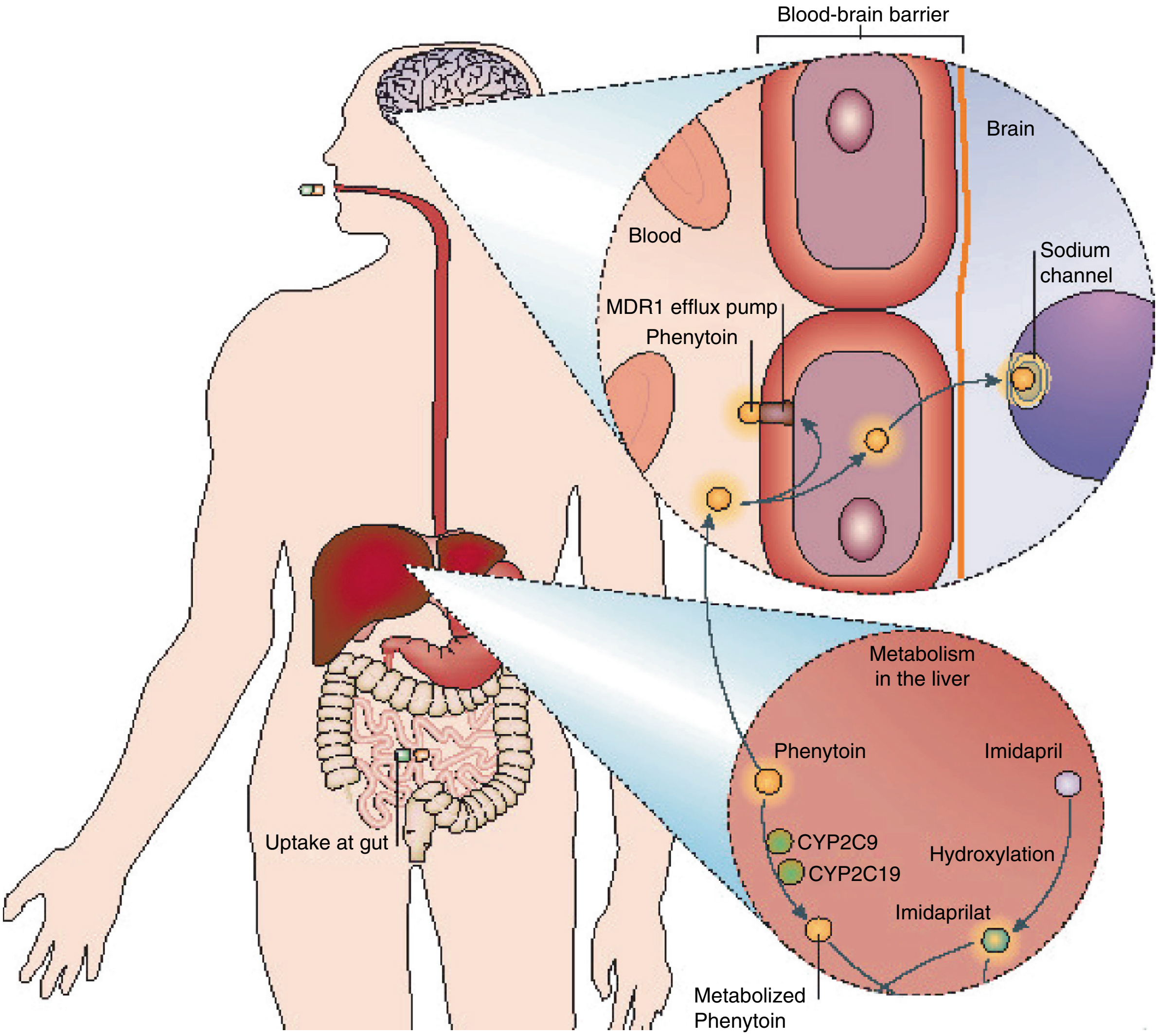

Fig. 22.1 The pharmacokinetic and pharmocodynamic properties of a drug can be used to select candidate genes. Phenytoin (PHT) is illustrated here as an example, PHT is absorbed in the gut, metabolized by two enzymes (CYP2C9 and CYP2C19) in the liver, and transported across the blood-brain barrier. Once in the brain, PHT can act on its drug target, the sodium channel (SCN1A), but is also removed by the MRD1 transporter. All of these genes represent good candidates for genetic studies on PHT

of a drug on the body are thoroughly researched prior to drug approval, it seldom happens that *all* aspects of drug pharmacokinetics and pharmacodynamics are clearly understood. As a result, candidate gene approaches to pharmacogenetics can easily overlook relevant genes. In addition, many studies combine a candidate gene approach with a candidate variant approach, focusing only on single nucleotide polymorphisms (SNPs) or variants that are known to have a specific function. This approach, which fails to consider uncharacterized variation in genes, severely limits the scope of a study.

22.4.2 Whole-Genome SNP Analyzes

It is now routine to carry out genome-wide association studies that effectively represent most of the common variation in the human genome. To understand whole-genome technology, it is first necessary to understand linkage disequilibrium and gene "tagging," the principles on which the technology is based. First, although there are about 10 million common SNPs in the human genome, there are correlations between and among some of these SNPs. The strength of such correlation is measured by the property of r^2, with an r^2 value of 1 indicating a perfect correlation (e.g., when adenosine (A) is found at one SNP, cytosine (C) is always found at a second SNP). Owing to this property, in a genetic study it is unnecessary to type both of these SNPs, because it is known that if SNP 1 is A then SNP 2 is C. In such a scenario, SNP 1 can be used as a "tag" for SNP 2, decreasing the number of SNPs that need to be genotyped (Fig. 22.2).

Recently, the HapMap project [11] has characterized 3.1 million SNPs in 270 individuals from four populations and is estimated to have captured all common variation with an $r^2 \geq 0.9$ (90% certainty). The tagging strategy has been applied to these data, and subsets of tagging SNPs have been identified that essentially represent all common variation across the genome. Technologies have been simultaneously created to genotype up to 1 million tagging SNPs in a single multiplex genotyping array [17]. Although overall cost remains nonnegligible, the cost per genotype is much lower than for single-SNP genotyping. As these platforms continue to become more affordable, and because they represent nearly all common variation across the human genome, whole-genome genotyping

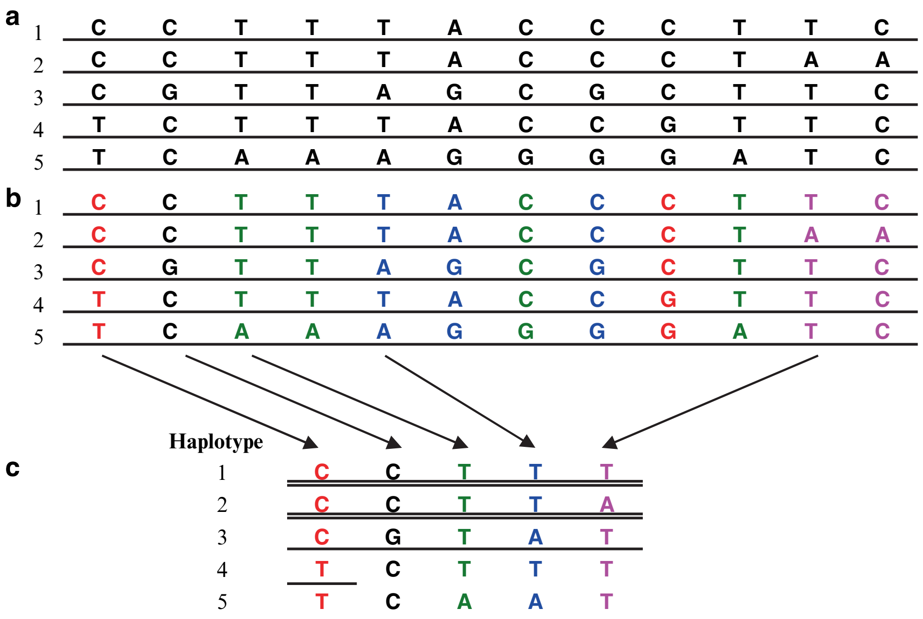
a
1 C C T T T A C C C T T C
2 C C T T T A C C C T A A
3 C G T T A G C G C T T C
4 T C T T T A C C G T T C
5 T C A A A G G G G A T C
b
1 C C T T T A C C C T T C
2 C C T T T A C C C T A A
3 C G T T A G C G C T T C
4 T C T T T A C C G T T C
5 T C A A A G G G G A T C
c
Haplotype
1 C C T T T
2 C C T T A
3 C G T A T
4 T C T T T
5 T C A A T

Fig. 22.2 (**a–c**) SNP tagging. This diagram represents five different haplotypes defined by 12 SNPs, laid out here in the order in which they occur in the genome (**a**). In this example, the selection of just one SNP from each color-coded group (**b**) will be sufficient to represent all genetic diversity in this area of the genome. Therefore, genotyping only of these five SNPs (**c**) is necessary is currently the most appropriate technology for pharmacogenetics studies.

Although genome-wide association (GWA) studies currently represent the most comprehensive way to carry out association studies of common variation, they also come with their own suite of problems. Specifically, GWA analyzes require a large number of statistical tests, which significantly decreases the power to detect significant associations unless the cohort and the genetic effect are sufficiently large. In theory, large sample sizes are not a prohibitive issue, but in practice sample size is frequently limited by cost. Sample sizes will almost certainly be inadequate when genetic studies on rare phenotypes are carried out, and the homogeneity of clinical data is often difficult to maintain in large studies.

22.4.3 Rare Variants and Whole-Genome Sequencing

A more fundamental constraint is that after whole-genome scans were completed for most common diseases it became clear that, with a few exceptions such as macular degeneration and Alzheimer's disease, common variants have only a modest impact on disease risk [12]. While there have been few whole-genome studies for drug-response phenotypes, it seems likely that GWA in pharmacogenetics will follow a similar pattern, where a few traits show marked dependence on common variation but many, or perhaps most, do not. These traits will need to be approached by way of whole-genome sequencing.

Rapid advances in sequencing technologies now make it feasible to sequence the entire genome to identify all variants. While the cost per genome remains high, in order to minimize cost an extreme phenotype study design is appropriate. This approach features the selection of patients with only the most severe or definite phenotypes to be whole-genome sequenced. Rare variants that are identified as potential risk factors in the small discovery cohort can then be genotyped in large cohorts to assess their contribution to the response. For example, patients who develop Stevens-Johnson syndrome (SJS) on carbamazepine exposure and patients who have never had allergic reactions or rashes to any medications can be considered extreme phenotype cases and controls. Then, rare variants that are suggestive of being functional can be genotyped in a large cohort of patients exposed to carbamazepine, (a) to corroborate that these rare variants only occur in patients who develop SJS and (b) to assess the contribution of these variants to the development of rash on exposure to carbamazepine. Such a study design is a cost-effective way to include large cohorts of patients in the analysis while only carrying out whole-genome resequencing in a small number of cases and controls.

22.5 Challenges in Pharmacogenetics

22.5.1 Polygenic Inheritance

As with the study of human disease, it remains unclear whether most of the genetic control of drug-response is due to rare variants of relatively large effects or to common variants across multiple genes, with each gene variant having only a modest effect. In the latter case, large GWA studies are necessary, while the former case will require a whole-genome sequencing approach.

Pharmacogenetic studies of warfarin dose illustrate that common variants sometimes have major impacts on complex traits. Warfarin is an anticoagulant with a narrow therapeutic window and with serious consequences (bleeding) in the event of an overdose. The variation in maintenance dose of warfarin is largely attributable to genetic variants in the genes that encode the drug target, vitamin K epoxide reductase complex, subunit 1 (VKORC1), and the major metabolizing enzyme, CYP2C9 [5, 36, 38, 41]. When considered together, these genetic polymorphisms explain 30–40% of the total variation in dose. This not only represents one of the largest genetic predictors of a complex phenotype discovered to date, but also is likely to be implemented in clinical practice on completion of prospective studies.

22.5.2 Pharmacogenetics in the Clinic

Although the goal of pharmacogenetics is to provide guidance to physicians so as to help them know which drugs can be safely used based on a patient's genotype, realization of that goal is proving to be more difficult than initially expected. As previously discussed, there is a lack of well-designed, comprehensive studies in the field, which has resulted in only a modest number of definitive discoveries. In addition, the translation of pharmacogenetic discoveries into clinical practice will only occur after prospective clinical trials have assessed their accuracy and utility and have provided evidence-based guidelines for the clinical use of genetic information. To date, few such studies have been carried out.

Despite these shortcomings in pharmacogenetics, there are a small number of drugs that have had genetic information added to their labeling by the US Food and Drug Administration (Table 22.2). However, the inclusion of genetic warnings on drug labels currently neither requires the incorporation of genetic tests for the clinical use of these drugs nor outlines guidelines for the use of genetic information in clinical practice.

22.5.3 Pharmacogenetics Across Ethnic Groups

Origin of ancestry and ethnicity is an important consideration in genetic studies. In the most general terms, ethnicity was first divided by HapMap into populations of European (CEU), African (YRI), and Asian (CHB + JPT) ancestry. More recently, additional populations have been added to the HapMap databases, which now describe 11 different populations (www.hapmap.org). The inclusion of multiple populations in HapMap has made it quite clear that, across populations, LD patterns and allele frequencies are highly variable (see Table 22.2 for allele frequency differences of pharmacogenetic variants), a fact which has consequences not only for study design, but also for the clinical use of drugs.

With respect to genetic association studies, particularly GWA studies or any study that employs a tagging-based approach, an association relies on LD between a genotyped SNP and a causal genetic variant. When the LD patterns between two populations are different, an association that is observed in one population will not be observed in the other. However, a failure to replicate in a population of different ancestry could be due to several different scenarios that are indistinguishable when the causal variant is not specifically known:

1. The association is not observed in population #2 because of a breakdown in LD
2. The association is not observed in population #2

Table 22.2 Drugs that include pharmacogenetic information in FDA labeling

Drug	Clinical phenotype observed/studied	Associated gene variant	Frequency of risk alleles CEU	YRI	CHB	JPT
Azathiopurine	Thiopurine toxicity	*TPMT* PM	0.036	0.06	0.023	0.003
Mercaptopurine	Thiopurine toxicity	*TPMT* PM	0.036	0.06	0.023	0.003
Irinoetcan	Neutropenia	UGT1A1*28	0.32	0.43	0.13	0.113
Erlontinib	Efficacy	*EGFR+*	n/a	n/a	n/a	n/a
Trastuzumab	Efficacy	*HER2* overexpression	n/a	n/a	n/a	n/a
Abacavir	Hypersensitivity	*HLA*-B*5701	0.061	0.0	0.011	0.0
Maraviroc	Efficacy	*CCR5*	0.951	1.00	0.99	1.00
Warfarin	Dose	*VKORC1*	0.432	0.086[b]	0.924	0.891
		CYP2C9*2, *3	0.1 (*2)	0.0 (*2)	0.0 (*2)	0.0 (*2)
			0.058 (*3)	0.0 (*3)	0.04 (*3)	0.03 (*3)
Atomoxetine	Dose	*CYP2D6* PM[a]	0.07	0.023	0.01	0.01
Thioridazine		*CYP2D6* PM[a]	0.07	0.023	0.01	0.01
Carbamazepine	Stevens-Johnson syndrome	HLA-B*1502	0.0	0.0	0.011	0.0
Codeine		*CYP2D6* PM[a]	0.07	0.023	0.01	0.01

Populations: *CEU* Utah residents with Northern and Western European ancestry, *YRI* Yoruba in Ibadan, Nigeria, *CHB* Han Chinese in Beijing, China, *JPT* Japanese in Tokyo, Japan; *PM* poor metabolizer phenotype

[a] Allele frequency is from an African-American population

because the first association is a false positive, or

3. The association is not observed in population #2 because the variant that is causal in the genetic background of population #1 is not causal in the genetic background of population #2.

In fact, of the pharmacogenetic variants that are recognized by the FDA (Table 22.2), at least three are present in only one population (CYP2C9, HLA-B*5701, HLA-B*1502), while the phenotypes that they affect (variation in warfarin dosing, abacavir hypersensitivity, and carbamazepine-related SJS) are relevant concerns in all populations. Therefore, it must be emphasized that pharmacogenetic results are not necessarily applicable across populations and that population-specific studies are necessary to assess the presence and effect of pharmacogenetic variants worldwide.

In addition to population-specific genetic variants, drug administration has also been affected by the observation of population-specific treatment outcomes. For example, it has been noted that ACE inhibitors and beta blockers are not as effective for the treatment of heart failure in African Americans as they are in Caucasians [10]. The FDA has included ethnicity guidelines on ACE inhibitors and at least five other drugs that are currently used (Table 22.3). The disparate effects of these drugs may well be due to pharmacogenetic factors; however these have yet to be determined. Until genetic causes for the inter-ethnic effects of these drugs are elucidated, the administration of some medications will continue to be loosely guided by ethnic considerations.

Another issue of ethnicity that it is important to consider in large-scale population-based studies is the effect of population stratification. Population stratification is caused by differences in allele frequencies across populations, and it is well-established that population stratification can cause both type I and type II error in genetic association studies. While limiting study samples to one ethnic population is good practice for minimizing intercontinental population structure, more subtle allele frequency differences may remain within a continental or ethnic population. Such cryptic population stratification is less easily controlled for methodologically (i.e., by case-control matching), and several statistical methods that identify and correct for population stratification have been developed and validated (reviewed in [42] and in Chap. 20).

Table 22.3 Drugs with FDA warnings concerning the ethnicity of the patient

Drug	Ethnicity information/ indication
ACE Inhibitors	Smaller effects in blacks
Isosorbide dinitrate-hydralazine	Indicated for self-identified blacks
Rosuvastatin	Lower dose for Asians
Tacrolimus	Higher dose for blacks
Oseltamivir	Neuropsychiatric events reported mostly in Japan
Warfarin	Lower dose for Asians

The ability to accurately quantify and adjust for population stratification now makes it possible to use large, common population control sample sets in GWA studies that do not have to be carefully matched for ethnicity or geography. This method can enhance the power of a study by increasing the size of the control population and also reduce cost, as it will no longer be necessary for every study to genotype its own large sets of controls. Large, common population control samples will be especially important in cases where the causal variant is rare and large control samples are necessary to demonstrate a statistically significant allele frequency difference between cases and controls. This method has already had some success in disease predisposition studies, resulting in the recent identification of rare copy number variants that cause autism and schizophrenia [33, 39, 46] and is likely to be important in future pharmacogenetics studies when large-effect, but rare, variants influence a drug-response phenotype.

22.5.4 *Drug Classes of Urgent Interest*

Among all drug classes, a pharmacogenetic focus is specifically indicated where adverse events are the most common and severe and where efficacy rates are low. One drug class that is in urgent need of pharmacogenetic study is that of neuropsychiatric drugs, specifically antipsychotics/antidepressants and antiepileptics.

Antipsychotics and antidepressants have been the most studied of all drugs in pharmacogenetics. As a class, they have efficacy rates of 5–70%, and they are associated with significant ADRs. In general, it has been found that serum levels of both antipsychotics and antidepressants correlate strongly with genotype at the relevant drug-metabolizing enzymes, and there have been multiple reports of an effect of functional variation in dopamine and serotonin receptors on efficacy outcomes (reviewed in [3, 4]). However, these associations have not yet proved to have any clinical relevance, as serum levels do not strictly correlate with efficacy. A recent retrospective study of the CATIE trial, a large-scale trial designed to estimate the efficacy and incidence of ADRs of antipsychotics showed no significant effect of functional variation in drug-metabolizing enzymes on clinical measures of dose, ADRs, or efficacy [16].

Antiepileptic drugs represent a class of medicines with similarly low efficacy rates (less than 70%), and significant ADR profiles, but very little attention has been devoted to pharmacogenetic studies in antiepileptic drugs. Despite the lack of focus, pharmacogenetic studies have discovered an *HLA-B* allele (*HLA-B*1502*) that is associated with carbamazepine-induced SJS in Asians (Table 22.2) [23, 27]. Unfortunately, aside from the clinical implications of HLA testing for Asian patients beginning treatment with carbamazepine, the field of pharmacogenetics with respect to neuropsychiatric drugs is devoid of discoveries that affect important clinical phenotypes and have important clinical implications.

22.6 Conclusions

As discussed, the next step for pharmacogenetic association studies is to incorporate genome-wide analyzes into study designs that include carefully phenotyped patient cohorts. While this has already become common practice in disease predisposition studies, its application has been limited in pharmacogenetics and there are very few whole-genome association studies of treatment response. This anomaly needs to be corrected, and the examples of warfarin dosing, abacavir hypersensitivity, and statin-induced myopathy [28] illustrate that systematic GWA studies for variable drug responses are likely to generate new findings. What is needed for future success in this research area is a commitment to patient collection, cataloging of common and rare adverse events, exploration of appropriate efficacy and ADR phenotypes, and a transition from candidate gene-based studies to GWA studies. There is, however, every reason to believe that treatment response genetics will follow a similar course to disease genetics, and that even after systematic GWA studies have been performed, much of the variation in treatment response will remain to be explained, even where a strong genetic component is likely. In such cases, discovery genetics will depend on whole-genome sequencing approaches.

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Behavioral Genetics

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Introductory Note by Michael R. Speicher

The investigation of behavioral genetics is a complicated task, as behavior is caused by combinations of genetic and environmental factors. Some behavioral phenotypes have a strong genetic component. There is, for example, compelling epidemiological evidence indicating that over 50% of the risk for developing alcohol dependence stems from genetic susceptibility, and genetic studies have already identified several risk alleles. However, for many other behavioral traits the degree of genetic contribution is unclear and often a matter of debate. It seems that – in addition to environmental factors – social behavior and social cognition are not the result of variation in a single gene, but are instead modulated by a number of genetic variants, each of which has only a modest effect on behavior. Furthermore, classification and description of a certain behavior is often difficult and standardization is frequently lacking. As a consequence, the linking of specific genetic or environmental risk factors to typical or atypical behaviors represents a challenge and new conceptual tools are being developed in addition to the currently accepted approaches. These tools include classic, traditional approaches, such as twin studies, family-based studies, and chromosome analysis using biochemical and molecular techniques. Furthermore, animal models involving cross-species trait genetics have made a significant contribution. More recently, genome-wide association studies have elucidated positional candidate genes, which will help to illuminate the often complex etiology of behavioral disorders.

For clinical purposes and for the social aspects of behavioral traits it is very important to understand the neurobiology and neurogenetics of social cognition and behavior. At the same time, behavioral genetics involves highly complex and fluid ethical considerations. Therefore, data on actual risks and benefits of research on behavioral genetics and potential clinical applications has to be monitored carefully and appropriate ethical safeguards need to be developed in parallel.

These aforementioned aspects of behavioral genetics will be addressed for a variety of different behavioral phenotypes in the following chapters (Chaps. 23.1–23.7).

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The Genetics of Personality

23.1

Jonathan Flint and Saffron Willis-Owen

Abstract The genetic analysis of human personality, like many other complex traits, has undergone a metamorphosis over the last 10 years. The historically predominant techniques of candidate gene association and linkage are now being replaced by genome-wide association approaches aimed at identifying the small genetic effects that contribute towards individual differences in personality. Like their predecessors, however, these approaches suffer from their own limitations, and as yet there has been no definitive identification of a human gene or variant that robustly contributes to a personality trait (such as "Neuroticism"). In this chapter we discuss the evidence that personality is heritable, consider the main approaches used to identify individual contributory factors, and outline the barriers to success in this rapidly changing field.

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Personality, so frequently assessed by self-administered questionnaires, does not at first glance appear to be a favorable target for molecular genetic investigation. Two criticisms have been leveled against the enterprise: that the assessments are state dependent, and therefore meaningless for genetic studies, and second that the answers do not reflect the activity of any meaningful biological process (studying the genetics of personality would be similar to studying the genetics of fever, for instance). Before reviewing the literature on genetic studies of personality, we shall deal with both these points.

One of the most contentious issues in the literature of personality assessment is the degree of state dependence of personality assessment: people may answer the same question in very different ways depending on the circumstances in which they find themselves. Surely, the argument runs, a state-dependent measure can be of little value in genetic studies, since the latter require traits that are, at least in part, free from environmental malleability? One simple refutation of this concern is the observation that questionnaire assessments are remarkably consistent. For example, correlations

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in excess of 0.9 are found when the same questionnaire is administered even after an interval of more than 2 years [82]. In other words, personality assessments can be considered as stable as many measures of physiological variation (such as blood pressure) during middle adulthood. They do indeed appear to measure traits, not states.

However, response consistency does not guarantee that a personality assessment means very much, if anything at all. Ideally we would like to show that variation in personality reflects the activity of a neurobiological system, as has been claimed by a number of psychologists. To appreciate these arguments it is important to understand how personality factors are generated from questionnaires. It might seem that the value of a completed personality questionnaire depends on how truthfully it has been answered. Hans Eysenck, creator of one the most widely used assessments, gives an example that illustrates the fallacy of this interpretation: "One might see some unfortunate individual sitting down with the questionnaire, his hands trembling and sweating with excitement, his face getting pale and flushed alternately, and his tongue licking his lips, his whole body in a tremor of nervousness; on going over to reassure him, one would find after the question, "Are you generally a nervous sort of person?" he had boldly put the answer, 'No'." [23].

In fact psychologists do not use the individual items: they use factors derived from the pattern of responses. Consider these two questions: "Are your feelings easily hurt" and: "Do ideas run through your head so that you cannot sleep." Although there is no logical reason why the same response should be given to both questions, in fact people who answer yes to the first tend to answer yes to the second, and vice versa. Since this is true for other questions, it is possible to pull out a set of correlated answers. The correlations allow us to recover a relatively small number of correlated responses, or factors.

Over the many years that have passed since the notion of personality was first conceived, evidence has accumulated in favor of a small number of factors. The five-factor model (FFM) divides personality into the five dimensions of Neuroticism, Extraversion, Openness to Experience, Agreeableness, and Conscientiousness [17] (easily remembered by the acronym OCEAN). The FFM is hierarchical; that is to say each broad trait dimension may contain further, more specific, features of personality. For example, Neuroticism contains personality features such as anxiety, angry hostility, depression, self-consciousness, impulsiveness, and vulnerability.

The fact that personality factors are heritable shows that they have, at least in part, a biological basis. Twin studies have been instrumental in demonstrating that about 40% of variation in personality is due to genetic variation (this figure varies between factors, though not substantially) [20, 25, 49, 52].

Heritability does not imply that personality has a biological function. In fact, as Tooby and Cosmides argue, it is consistent with personality being evolutionary noise: "[S]election, interacting with sexual recombination, tends to impose relative uniformity at the functional level in complex adaptive designs, suggesting that most heritable psychological differences are not themselves likely to be complex psychological adaptations. Instead, they are mostly evolutionary by-products, such as concomitants of parasite-driven selection for biochemical individuality" [76]. There are problems with using heritability to validate personality constructs; many valid psychological processes do not appear to be heritable (e.g., the language you speak), while many trivial processes are (such as the sort of car you own).

Does this mean that personality factors have no value in their own right? This has struck a number of investigators as unlikely, since, assuming that the concordance between personality dimensions is meaningful, "[T]he probability is that they are based on the way in which our biology has evolved to cope with the extraordinary range of social structures and physical environments on this planet" [88].

Evidence that other species also have heritable variation in personality traits, homologous to human factors, supports the idea that personality traits have a biological function. Gosling's review of animal personality literature up to 2001 concluded that a number of dimensions appeared repeatedly across multiple species, including a dimension reflecting an individual's reaction to novel stimuli or situations (termed Reactivity, Emotionality, or Fearfulness and possibly homologous to neuroticism) [29]. According to Gosling, "The FFM dimensions of Extraversion, Neuroticism, and Agreeableness showed considerable generality across the 12 species included in their review … The way these personality dimensions are manifested, however, depends on the species. For example, whereas a human scoring low on Extraversion stays at home on Saturday night or tries to blend into a corner at a large party, the octopus scoring low on Boldness stays in its protective den during feedings and attempts to hide itself by changing color or releasing ink into the water" [29].

The combination of heritability, a concordance between personality dimensions measured by different questionnaires, and evidence that other species have similar, if not identical, personality dimensions has been instrumental in promoting a biological interpretation of personality [18]. Gray, arguing for congruence between animal models of trait anxiety and human neuroticism [31-33], put forward the view that there are two interrelated but separable brain systems that subserve both human and rodent traits. The first system, which he terms the fight/flight system, subserves flight, defensive aggression, freezing, and associated autonomic activity. The second system, termed the behavioral inhibition system, subserves the cognitive and information processing aspects of anxiety. Within Gray's theory, neuroticism or emotional stability was seen as a measure of sensitivity to reinforcing events.

Cloninger has argued strongly that personality factors reflect the action of neurobiological systems [14]. He identifies three independent and heritable dimensions of personality: (a) novelty seeking: frequent exploratory activity and excitement in response to novel stimuli; (b) harm avoidance: the tendency to respond intensely to aversive stimuli and to learn to avoid punishment and novelty; (c) reward dependence: the tendency to respond intensely to reward. Cloninger associates each with a different neurobiological system: novelty seeking with low basal dopaminergic activity, harm avoidance with high serotonergic activity, and reward dependence with low basal noradrenergic activity [14]. These views have been important in setting the scene for many of the genetic studies reviewed below because they can be used to justify the choice of candidate genes.

23.1.1 Neuroticism

Neuroticism (N) is a longitudinally and culturally robust measure of emotional stability. Although there are still some divergences in trait designation between measurement instruments, the various manifestations of N have been shown to exhibit considerable overlap in terms of both concept [1, 54, 89] and etiology. A recent longitudinal study of N, for example, revealed large phenotypic, genetic, and environmental correlations (0.57, 0.91, and 0.42, respectively) between N dimensions derived from two independent measurement instruments over a period of 22 years [83]. Likewise, other authors have also shown that N can be detected within a variety of different social strata and cultures [20] and may even be recognized in the behavior of other, less complex, organisms [29, 30].

Neuroticism represents an important construct in the study of human psychiatric disease. Both prospective and cross-sectional studies reveal a close relationship between N and psychiatric phenotypes, including perhaps most notably, major depression. High N scores are robustly associated with an increased risk for depression [2, 36, 42, 44, 79], and experience of a depressive episode yields an elevation in N which persists after recovery (i.e., a scar effect) [62]. Critically, these epidemiological data are also supported by biological evidence of shared causality, which indicate that approximately half of the genetic determinants of these phenotypes are shared (yielding a genetic correlation of 0.49–0.68) with no discernable sex-difference in the magnitude of this correlation [24, 43]. Since N accounts for a substantial proportion of comorbidity between psychiatric disorders – 20–45% of internalizing disorder comorbidity and 19–88% of comorbidity between internalizing and externalizing disorders [47] – it may be hypothesized that N represents a common liability factor for stress and anxiety related disorders.

23.1.1.1 Genetic Association Studies

Historical attempts to identify the genetic determinants of N can primarily be divided into two camps; genome-wide linkage and candidate gene association. While candidate gene studies have been prolific in their production, these studies have focused on relatively few theoretically plausible candidate genes and have been plagued by issues of nonreplication.

The serotoninergic system has been the principal focus in molecular genetic investigations of N. Genetic studies have focused in large part on the role of the serotonin transporter gene (5-HTT), which regulates the re-uptake of 5-HT at synapses. 5-HTTLPR is a polymorphism in the promoter of this gene, which carries two main alleles: a long allele with 16 repeats (*L*) and a short allele with 14 repeats (*S*). The genetically dominant *S* allele results in lower transcriptional activity than the *L* allele, leading to a relative reduction in mRNA levels, serotonin binding, and re-uptake.

In 1996 Lesch et al. demonstrated that the 5-HTTLPR associates significantly with N in a total of 505 individuals [51]. Although a number of subsequent studies have observed a similar association, based both on an N phenotype [19, 51] and on a range of other conceptually related diseases and traits (including Harm Avoidance [40], generalized anxiety disorder [87], and depression [15, 37]), several large investigations have failed to identify any significant effect [26, 82]. Furthermore, where a positive association has been identified its effect size has typically been small, occasionally reversed in direction [8], and sex-specific (although not consistently for the same sex [19, 34]. Similar observations have been made in other complex phenotypes, with the likelihood of successful replication limited by design parameters such as low sample size in the originating study [38].

One method of resolving discrepancies is to apply meta-analytic techniques. Meta-analysis is a method of combining results from independent studies to acquire a much larger data set from which more robust conclusions can be drawn than are obtainable from each of the smaller component studies. The fact that there have been a number of meta-analyzes [55, 57, 66, 67] indicates difficulties in arriving at conclusive decisions about the role of the 5-HTTLPR in personality, and it should be recognized that meta-analyzes are not substitutes for well-powered individual studies [56]. However, it now seems unarguable that if the 5-HTTLPR *does* underlie variation in neuroticism, its impact is likely to be extremely small, contributing much less than 1% of the phenotypic variation.

A number of additional candidate genes have been tested for association to N. However, like the serotonin transporter, most of these genes have been selected using pre-existing evidence of involvement in mood-related neurological systems and/or structures, thereby limiting the potential to detect novel mechanisms of trait causation. Brain-derived neurotrophic factor (BDNF), for example, is known to be expressed in the hippocampus (part of the limbic system), where it moderates neuronal growth, differentiation, and survival, as well as synaptic plasticity. The gene exhibits a transcriptional response to both stressful [69] and antidepressant [28] events and carries a functional mutation in the 5¢ prodomain that influences intracellular BDNF trafficking and activity-dependent secretion [21]. An equivalent mutation has recently been generated in the mouse, and behavioral analysis of these animals suggests that altered BDNF secretion may translate behaviorally to heightened emotionality in anxiogenic contexts [11]. Together, these and other similar data have led researchers to consider the 5¢ functional BDNF mutation (termed val66met) as a candidate for a variety of mood-related phenotypes, including N [50] However, as in the case of the serotonin transporter, evidence of association has been mixed [81].

More recently, a novel class of candidates has emerged; that of the semaphorin axon guidance molecules and their associated co-receptors, the plexins. This family of secreted and transmembrane proteins has been implicated in the etiology of N through several convergent lines of investigation; including both theory- and position-based analyzes. Epidemiologically N is known to predict several clinical phenotypes, including both major depression and schizophrenia. One of the semaphorin receptors, *Plexin A2* (*PLXNA2*) has recently been shown to associate with schizophrenia through genome-wide association [53], and this association appears to replicate successfully between cohorts. Although the mechanism of action is as yet uncharacterized, current evidence indicates that semaphorin-plexin signaling may influence cell migration via an effect on centrosome positioning [63], with a particular emphasis on hippocampal mossy fiber projections [73]. These data fit with current hypotheses regarding mood disorder pathogenesis, which postulate an abnormality in neurogenesis within the adult hippocampus. Consequently, *PLXNA2* has emerged as a potential candidate for N, which may serve as a common susceptibility factor for both clinical phenotypes. Recent analyzes corroborate this hypothesis, revealing significant associations between *PLXNA2* mutations and N, as well as a range of other related phenotypes [84].

23.1.1.2 Gene by Environment Effects

The importance of gene by environment interaction in personality studies is producing a literature no less contentious than that dealing with the main effects of genetic variation on phenotype. We all know that different people, faced with the same stressful situation, react differently, and it is scarcely controversial to assert that this variation has, in part, a genetic origin. Nonetheless, while few doubt that gene by environment interaction exists, its importance has been difficult to assess. The older literature was not optimistic: Jinks and Fulker [39], using the correlation between identical twin intrapair differences and pair sums, found little evidence for genotype environment interaction for cognitive and personality traits.

More recently, the tide has turned. In an influential article published in 1994 Bronfenbrenner and Ceci argue strongly that interaction needs to be taken into account in behavioral genetic studies: "The mechanisms by which genotypes actualize into phenotypes vary as a function of environmental context. When proximal processes are weak, that is when the environment is not conducive to expression of that genotype, heritability is low, as genetic potential is not realized" [7]. A study of cognitive ability in 7-year-old children taking part in the National Perinatal Collaborative Project found that for disadvantaged children, environmental influences accounted for nearly 60% of the variance in IQ, while genetic factors accounted for negligible variance. However, in advantaged children the pattern was almost reversed, good evidence therefore of an interaction [77], a finding that has been replicated in an independent study [35].

In a cross-fostering analysis [13], crime rates in male Swedish adoptees were found to be greatest when both heritable and environmental influences were present, with the interaction accounting for twice as much crime as genetic and environmental influences alone. Cadoret et al. [9] studied adoptees whose parents had either antisocial personality and found that an adverse adoptive home environment interacted with adult antisocial personality in predicting increased aggression in the offspring.

Given that the interactions are there and are important, could it be that molecular variants will not be found unless gene by environment interaction is taken into account? Perhaps modeling the joint effects of genes and environment is necessary to obtain sufficient statistical power to detect the effect. Empirical evidence in favor of this view comes, yet again, from a study of the 5-HTTLPR. Caspi et al. report, that carriers of the 5-HTTLPR short variant are twice as likely to become depressed after stressful events such as bereavement, romantic disasters, illnesses or job loss, and childhood maltreatment significantly increases this probability [10].

Studies of a variant of 5-HTTLPR in nonhuman primates supports this finding. In rhesus monkeys, maternal separation during the first months of life adversely affects later social interaction behavior. As in humans, there is a repeat length variation in the promoter of the serotonin transporter gene (the variant is called rh5-HTTLPR). The rh5-HTTLPR genotype interacts with deleterious early rearing experience to influence attentional and emotional behavior, stress reactivity, and alcohol preference and dependence [5].

The trouble with this argument is that the environmental causes of personality variation are frequently as mysterious as the genetic. Where the environmental effect is known, then increased power might be obtainable, but what happens if we are not so sure? This problem is well known to epidemiologists, who have been struggling for some years to detect subtle environmental effects [74].

Clayton and McKeigue, in a discussion of the value of gene by environment studies, argue as follows: "If we could specify in advance that the effect of the environmental factor on disease risk would be restricted to a subgroup of individuals with a particular genotype, there would, of course, be a gain in power from testing only this subgroup for the effect of the environmental factor. In practice, such an extreme situation is unlikely to be frequently encountered in the study of complex diseases, and entails a level of knowledge of underlying biology which would probably render epidemiological studies redundant. In less extreme situations, and where previous knowledge is more limited, a combined test would need to be done for the main effect of environmental exposure and its interaction with genotype. Since such tests have multiple degrees of freedom, the gain in power is much reduced; indeed, power might even be lost" [12].

As an example, consider the problem of defining environmental effects on depression, a phenotype that is genetically closely related to neuroticism. Here we know that stressful life events (SLE) have an important role in the onset of depression [46], but the temporal relationship between the two is less well characterized. The largest effect is seen in the month succeeding the SLE, but this depends on the type of event [45], raising the possibility that a gene by environment effect will depend on the type of SLE. Some people might be genetically predisposed to weather a marriage break-up better than others, but not to deal so well with the death of a spouse. As important subgroups of each SLE are found we may be faced with an exponentially increasing list of environmental effects to investigate, with consequent disastrous consequences for our power to detect anything at all.

The literature in support of the detection of gene by environment effects at individual loci looks qualitatively very similar to the initial reports of genetic association studies of the main effect of 5HTT [55]: there have been a small number of high profile findings, followed by a mixture of replications and nonreplications [78]. The pattern appears to be pervasive and indicates that the current studies of gene by environment effects are underpowered. It is worth noting that the largest studies of putative gene by environment effects to date have largely produced null results [72], suggesting that findings in smaller studies may represent false positives. In short, we do not yet know whether gene by environment studies will fare any better than other, genetic association studies with simpler designs.

23.1.1.3 Linkage Studies

Linkage studies, on the other hand, while substantially fewer in number, have provided arguably more robust evidence of region involvement. To date five linkage studies have been published describing the genetic basis of N. The first, published in 2003, identified five chromosomal regions. All of these regions exceeded an empirically determined significance threshold [27], and most spanned a region exceeding 30 cM in length, which supports their status as true effects rather than false positives [75]. These loci were positioned on chromosomes 1q, 4q, 7p, 12q, and 13q. In addition to these sites, a number of additional sex-specific loci were also identified, an observation consistent with the elevated phenotype concordance that can be detected in same-sex siblings as opposed to opposite-sex pairs and indicative of partially divergent genetic causality between the sexes.

Two studies included N as a secondary measure, with a primary focus on alcohol [48] and nicotine dependence [60]; two other studies [59, 85] genotyped only extreme-scoring siblings (an extremes design). The joint Australian/Dutch study [85] was particularly impressive in that it combined large sample sets from two populations, providing an opportunity for the investigators to replicate findings. Overall, however, the findings are disappointing. No one locus is consistently found in all studies, although, across all studies, replication can be observed on chromosomes 1, 7, 12, and 18. Additionally, if one considers binary disease traits that are conceptually related to N (e.g., major depression or generalized anxiety disorder), further evidence of genetic overlap can be obtained [80].

Although these data implicate a small number of discrete chromosomal regions in the etiology of N, these regions are extensive, thereby precluding identification of an experimentally tractable number of candidate genes and/or regulatory sequences for further investigation. The N locus originally mapped to chromosome 1 by Fullerton et al. [27] extends over 191 Mb from D1S2667-D1S249, encapsulating over 2,000 known/predicted genes and a multitude of intuitively plausible candidate genes (including two serotonin receptors, a cannabinoid receptor, and a glutamate receptor amongst many others). Since these mapping data were derived from a large, highly selected, sample (consisting of the phenotypic extremes of the N distribution selected from a population in excess of 88,000 individuals) and assessed using a well-validated quantitative measure of liability, it becomes apparent that alternative approaches are likely to be required for the fine-scale dissection of the N phenotype.

23.1.1.4 Genome-Wide Association Studies

The advent of micro-array methods of genotyping, in which hundreds of thousands of genetic variants can be assayed at one time, has made it possible to test all genes in the genome by genetic association. A whole genome association has been used to investigate the genetic basis of N [68]. The results confirm that the genetic effects contributing to heritability of Neuroticism are small. More than 600,000 genetic variants were analyzed and just one, rs702543, replicated in a separate sample. This is hardly robust evidence of genetic association. But the negative evidence is important. Failure to find any genetic variants accounting for more than 1% of the variance means the 40% heritability of N is likely to arise from a large number of loci, each explaining much less than 1% of variance, and/or that N may be determined by rare variants or singleton polymorphisms [41] that are not adequately tagged by current haplotype-based SNP chips in the absence of enrichment (pedigree-based) sampling.

23.1.2 Extraversion

To date no genome-wide positional cloning studies of Extraversion (E) or its close relative novelty seeking conducted either through linkage or association have been reported in the literature. Instead, there has been a predominant focus on candidate gene studies, and as in the case of N, a specific emphasis on a single biochemical pathway and its cognate genes. The dopaminergic system is involved in appetitive and motivational behaviors [16], and pharmacological challenge studies have suggested a relationship between dopaminergic hyperactivity and reward seeking, as well as motivational factors associated with both extraversion and novelty seeking (Netter 2006); all this suggests that these traits may share a common neurobiological basis. On these grounds, dopaminergic genes, in particular the dopamine D4 receptor (*DRD4*) have emerged as plausible candidates for interindividual variation in novelty seeking and extraversion.

Although *DRD4* is highly polymorphic, research evaluating its role in behavioral and psychiatric phenotypes has largely focused on a single variant: a variable number tandem repeat (VNTR) located in exon III, and specifically the presence or absence of a 7-repeat ("long") allele. This variant has been reported to display several functional characteristics, including decreased ligand binding [3], decreased gene expression in vitro, and attenuation of cyclic AMP formation when dopamine is bound to the receptor [4] relative to 6-repeat or shorter allele.s There is, however, some disagreement regarding the optimal grouping of VNTR alleles. The *DRD4* gene also includes a single nucleotide polymorphism in the promoter region (C-521T), which has been reported to be in linkage disequilibrium with the exon III VNTR [22, 71] and is also associated with variation in expression of the D4 receptor, with the *T* allele yielding a ~40% reduction in transcription compared with the *C* allele [61, 64].

By 2008 almost 50 studies had been published reporting data on the association between *DRD4* and extraversion. Many of these studies are assimilated in a meta-analysis published in 2002, which provides support for the association between *DRD4* and trait novelty seeking, both based on the VNTR and C-521T polymorphisms [65]. A further meta-analysis published in 2008 also finds evidence for an effect and shows that the effect size might actually be relatively large by the standards of genetic association studies of behavioral traits (although nevertheless small in absolute terms) [58]. The pooled effect size estimate suggests that the C-521T SNP may account for 2% of the phenotypic variance. Whether this finding will hold up in large-scale individual studies remains to be determined.

Extraversion also associates significantly with drug dependence, with a change of one standard deviation yielding a 24% increase in risk for drug dependence [47]. However, a combination of low E and high N scores also appears to predict several anxiety disorders, including both social phobia and agoraphobia [6]. In keeping with these observations, *Rgs2*, a gene previously shown to underlie murine variation in emotionality by positional cloning [86], has recently been shown to associate with low E scores (introversion) and increased limbic activation during emotion processing in humans [70]. This association cannot be explained by variation in N. These data therefore suggest that genetic variation in *Rgs2* may predispose towards anxiety disorders through the generation of a hyperreactive state in the limbic circuitry, manifesting behaviorally as introversion. These data therefore highlight the potential utility of investigations focused on quantitative personality traits in the genetic dissection of qualitative, diagnosis-based psychiatric phenotypes, and also indicate that cross-species comparisons may be informative in the genetic dissection of human behavioral traits.

23.1.3 Implications for Future Research

The genetic analysis of human complex traits has undergone a metamorphosis over the last 10 years. The predominant techniques of candidate gene association and linkage are now being replaced by genome-wide association approaches aimed at identifying the small genetic effects that contribute to individual differences in the personality factor neuroticism (N). All these approaches, however, suffer from their own limitations, and as yet there has been no definitive identification of a human gene that robustly contributes to N across multiple populations. Alternative approaches are likely to be required, not to replace existing methodologies, but rather to supplement and guide ongoing investigations. For example, the contribution of model organisms, specifically considering the ways in which synteny between human and mouse QTL may be used to guide human association studies, is likely to become increasingly important. In order to progress our knowledge of N and other complex behavioral traits, it may therefore be necessary to adopt a more inclusive, interdisciplinary approach to complex trait analysis, incorporating several convergent sources of information for the identification of clinically relevant genes.

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Mental Retardation and Intellectual Disability

23.2

David L. Nelson

Abstract Cognitive or intellectual disability (ID) in humans is a common trait that has a genetic etiology in as many as one-half of cases. Genetic causes range from single-gene defects to trisomies. The discovery of mutations that lead to ID has led to improved diagnosis and the opportunity for reproductive decisions based on prenatal diagnosis. In addition, understanding the gene defects in ID has elucidated molecules and pathways important for normal cognition, assisting with efforts to understand brain function. This chapter touches on the definition and history of ID, then considers in detail several classifications of gene defects that lead to ID. These include unbalanced gene dosage, with attention to Down syndrome and Prader-Willi and Angelman syndromes, and single gene disorders, particularly X-linked disorders Fragile X and Rett syndromes. Each disorder illustrates important principles in human genetics. Future directions in gene discovery, mutation detection and treatment are discussed.

Contents

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23.2.1 Introduction

Interest in the genetics of human cognition has spanned the period since the rediscovery of Mendel's work. It has been a controversial area, particularly the study of cognitive abilities within the typical (or normal) range, but also in its treatment of patients. The study of the genetics of intelligence is beyond the scope of this chapter. Here, we consider the genetic conditions that can lead to cognitive disabilities.

In recent years, the term "mental retardation" is no longer favored. "Intellectual," and "cognitive," disability

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have been proposed as preferred terms. Throughout the history of studies of intellectual disability, numerous terms for description of the condition have fallen out of favor as they are adopted by the general public and are viewed as insensitive to those who are disabled. A noteworthy example of this is the use of the terms "idiot," "imbecile," and "moron" to define individuals with varying degrees of mental retardation based on scores on tests that measure intelligence. These terms were once used in the medical literature to classify individuals with intellectual disability, but this is no longer the case, since they became pejorative in the general language. A similar shift away from "mental retardation" has occurred in recent years. In this chapter, the term intellectual disability (ID) will be utilized to conform with current terminology. Nonetheless, the term mental retardation remains widespread, and it is important to note that ID is meant to be synonymous with mental retardation. A useful discussion of issues surrounding this nomenclature can be found elsewhere [100].

23.2.2 Definition of ID

The most widely accepted definition of ID is that it has an age of onset below 18 and is characterized by significant limitations in both intellectual function and adaptive behavior. In this case, "adaptive behavior" refers to conceptual, social, and practical adaptive skills. For intellectual function, disability is typically measured by intelligence test instruments that generate an intelligence quotient, or IQ score. IQ tests are designed to generate a normal distribution in the general population, and scores are distributed around a mean of 100. ID means an IQ score that is less than two standard deviations beneath the mean. Generally, IQ scores below 70 are considered as an indicator of disability, but this is not universally accepted, and it is useful to keep in mind that adaptive aspects of a particular individual's ability can contribute to his or her classification [1].

23.2.3 History of ID

The presence of individuals with significant ID has been noted in the written records of many ancient cultures. It is only in recent centuries that these individuals have received special societal attention, initially in the form of housing in group institutions. Much more recently, a trend toward "mainstreaming" for all children with disabilities (physical and cognitive, as well as those involving senses such as hearing and sight) has taken hold in the United States and elsewhere. This began in the second half of the twentieth century, giving children with ID access to educational opportunities alongside other children and encouraging parents to raise their children at home. The practice has been mandated through federal legislation in the United States that began in 1975, allowing many children who would otherwise have been committed to state institutions to receive appropriate education in the ordinary schools.

A greater appreciation of genetic forms of ID began in the mid-nineteenth century and accelerated with the Darwinian revolution. Francis Galton took a keen interest in understanding variation in the human population, attempting to implicate heritable components with genius [34]. He established the concept of a "normal distribution" for achievement using, for example, academic scores in medical school and appreciated that such a distribution must contain individuals at the low end of the scale. The normal distribution could be applied to many characteristics, and a quantitative measure of "intelligence" was introduced by Binet and Simon in France in 1907 [16], with the first tests designed to evaluate learning potential. Binet's motivation was principally to classify students for appropriate educational levels during schooling, and the score was defined as a "mental age" to help with placing students. Students whose mental age lagged behind their chronological age were classified as mentally retarded. Later, and in contrast to Binet's wishes, these tests were adapted to measure general intelligence, and a scale was developed with a normal distribution of scores with the average set at 100 and a standard deviation of 15, establishing the currently defined IQ score.

23.2.4 Frequency of ID

Today, IQ testing is carried out using a variety of instruments. Among the most common are the Wechsler Intelligence Scales, a series of tests that can be used for individuals within different age groups. These provide subscores in both "verbal" and "performance" in addition to a "full-scale" or combined score. Some forms of genetic ID can result in differences between verbal and performance scores, and the ability to score both can be instructive for some patients. Based on a normal distribution of IQ scores, it is expected that the frequency of ID would be slightly more than 2% in the general population. It has been difficult to define precise frequencies, however a recent study from the US Centers for Disease Control estimated the number of affected individuals among school age children in metropolitan Atlanta, Georgia at 1.2% [75].

Males exceed females with cognitive disability by approximately 50% [110]. This has led many to propose that the increased male vulnerability stems from X-linked mutations or gene variants that result in cognitive problems in hemizygous males, while sparing heterozygous females. We will consider X-linked genetics and intellectual disability in more detail below. In brief, a large fraction of X-linked genes can result in ID when mutated, but these have not explained the enhanced rate of ID in males. ID can result from many causes, including both environmental and genetic [2]. Environmental causes are numerous, and include exposure in utero to many substances such as alcohol, poor prenatal care, birth trauma, an inadequately enriched environment, malnutrition (including iodine deficiency), and infectious disease. It is estimated that the fraction of cases that result from genetic causes approaches 50% and is higher (perhaps 60%) [74] among more severely affected patients (IQ below 50).

The evidence for a genetic contribution to ID is abundant, with many identified causes in both sporadic and familial forms. The best-characterized forms of genetic ID are syndromic; patients exhibit additional symptoms beyond the cognitive disability that allow their disorder to be categorized. Attention has turned more recently to ID without additional symptoms, referred to as nonsyndromic ID, particularly in the case of families with an X-linked inheritance pattern, but also among families without X-linkage and even in sporadic cases. This chapter will discuss some causes in depth. It is not possible to be comprehensive, however, since there are several hundred known genetic lesions that confer cognitive disability. Indeed, based on data from study of X-linked families [114], it is now estimated that mutations in about 10% of all genes can result in ID, emphasizing that normal cognition is dependent upon a very complex interplay of gene products.

23.2.5 Gene Dosage and ID

In his preface to the 1972 edition of Lionel Penrose's classic monograph "The Biology of Mental Defect," J.B.S. Haldane wrote the following [83]:

> The most sensational discovery about mental defect since the last edition [1963] is that not only mongolism, but several other conditions combining mental defect and physical abnormality, are due to cytological aberrations, the commonest being an extra chromosome. All told, they only account for a small fraction of mental defect, but they lead to a conclusion of great importance. A mongoloid is not an imbecile or idiot because he or she possesses abnormal genes in the cell nuclei, like an epiloiac or an amaurotic idiot, but because he or she has too many of certain normal genes. Penrose believes that many unclassified defectives are defective because, though cytologically normal, they have too many genes of a kind which are found in most normal people, and are on the whole desirable in the heterozygous condition.

Haldane's passage and Penrose's insight are remarkable today as the appreciation of gene copy number changes in genetic forms of ID continues to grow as more sensitive means of detection are deployed. The increasing detection of deletions and duplications in patients with developmental delay, particularly de novo events, is one of the more significant advances in the last 5 years of research into the genetics of ID [94].

Changes in gene dosage resulting from deletion or duplication are a common cause of ID and have long been recognized through cytogenetic methods. It has been estimated that as much as 15% of severe ID is accounted for by cytogenetic abnormalities visible in the light microscope with chromosome banding methods [95]. These include whole-chromosome alterations, principally trisomies, and also many deletions and duplications, some involving apparently balanced translocations. Each of the viable trisomies results in ID. Numerous examples of recurrent deletions that can be detected in a karyotype have been described to result in ID.

Down syndrome remains the most frequent form of genetic ID, with an incidence of approximately 1/700. Described as a form of ID that exhibited distinctive physical features in 1866 by J. Langdown Down in England [28] (and by others earlier), Down syndrome was discovered to result in the majority of cases from trisomy of the smallest human chromosome, chromosome 21, in 1959 by multiple groups [17, 31, 49, 63] shortly after the consensus developed regarding the human chromosome number. The other viable trisomies of autosomes also result in ID. Individuals carrying additional copies of the X chromosome are not usually cognitively disabled, but learning disabilities have been described in up to one half of boys with a 47, XYY karyotype [120]. Turner syndrome, or monosomy X, is the only viable human monosomy and can also result in learning disability, but does not typically cause ID [97]. A variety of smaller deletions resulting in partial monosomy and well-defined syndromes including ID are discussed in more detail below. Many partial trisomies can also lead to developmental delay and ID, and a Down phenotype in partial trisomy 21 is perhaps the most common of these. As methods are refined for the detection of smaller trisomies, it is likely that additional syndromes will be described involving recurrent duplications that lead to cognitive disability.

23.2.6 Down Syndrome

Down syndrome is characterized by developmental delay and a constellation of physical features. The epicanthic fold present in these patients' eyelids led to the use of the term "mongolism" to describe individuals with Down syndrome. This term has been discouraged since 1961, but is still encountered [3]. Other prominent features include upslanting palpebral fissures, a flat nasal bridge, a single transverse palmar crease, short stature, short limbs, and poor muscle tone. Individuals are often born with cardiac defects, often ventricular septal defects, requiring surgical correction. Life expectancy has improved with better care, but there is an increased rate of an Alzheimer-like dementia in older individuals, as well as an increased likelihood of leukemia.

In addition to simple trisomy 21, patients with Down syndrome may also exhibit partial trisomies and Robertsonian translocations that lead to triplication of regions or the entire chromosome 21. Approximately 95% of patients have a spontaneous acquisition of an extra chromosome 21 due to nondisjunction. A small fraction of these patients are mosaic for trisomic and normal cells. Robertsonian translocations are commonly inherited as a 14;21 translocation. There is a maternal excess of spontaneous nondisjunction resulting in Down syndrome. The increased incidence of Down syndrome with maternal age was described by Penrose in 1933 [84]. Current data suggest that more than 90% of nondisjunctions are maternal, with three quarters of those occurring in meiosis I. Paternal nondisjunction accounts for some 4% of cases, while another 2% occur in early embryogenesis as a mitotic event [32]. Advanced maternal age is a risk factor for both meiosis I and meiosis II nondisjunction events. Factors that lead to increased nondisjunction in older eggs remain a subject of much research. The long period of time (from fetal development) that eggs remain in stasis after meiosis I is commonly thought to play a role, but other factors, such as patterns of chromosome recombination, are also likely involved [107].

In contrast to ID caused by single-gene disorders, treatment in Down syndrome is particularly daunting owing to the large number of gene aberrations. However, the completion of the DNA sequence of human chromosome 21 provided an important milestone in Down syndrome research, as it made available to investigators the entire catalog of chromosome 21-encoded genes for analysis, particularly for testing hypotheses regarding the gene or genes that might be critical for aspects of the trisomy 21 phenotype [36, 45]. Numerous individual candidates have been considered, and most recently these have been tested in mouse models, where increased copy number of individual genes can be achieved through transgenesis. An example is the DYRK1 gene, which was found (through creation of transgenic mice with additional copies) to alter cognitive function and later shown to induce developmental delay and motor abnormalities [4, 108]. The conservation of gene order between human and mouse chromosomes along with more detailed maps and then sequences from both species, has facilitated the development of trisomy models in the mouse designed to closely approximate the human trisomy. While the mouse chromosome 16 carries many of the human chromosome 21-encoded genes, it is not completely syntenic, with others carried on chromosomes 17 and 10. The Ts65Dn mouse [92] is trisomic for nearly half of the human chromosome 21 genes, and demonstrates a number of phenotypes that resemble those in Down patients. Additional mouse lines with smaller regions of trisomy that overlap the Ts65Dn region have provided the ability to ascribe certain phenotypes in the mouse to subsets of genes [79, 80]. This approach is beginning to provide candidate sets of genes for phenotypes, allowing consideration of specific therapies for patients. Ts65Dn mice, for example, show alterations in neuronal function that suggest the possibility of treatment with specific neurotransmitter antagonists. These have shown efficacy in modifying neuronal phenotypes in the mouse models and led to the possibility of improved medications for Down syndrome patients.

Prenatal screening for trisomy 21 has been indicated for more than 25 years after advances in amniocentesis and chorionic villus sampling. The guidelines have focused on screening pregnancies in mothers of advanced age (mid-30s), since the incidence of Down syndrome rises dramatically from ~0.1% in younger mothers to more than 3% in women aged 45. It is important to note, however, that the majority of Down syndrome children are born to younger women, since so many more children are born to women below age 30 [85]. Advances in screening for Down syndrome in fetuses have continued in recent years, reducing costs and allowing screening in younger populations at reduced risk to the patient. Screening of maternal serum for levels alphafetoprotein, estriol, human chorionic gonadotropin, and inhibin alpha (the Quad Screen) [13] can be utilized as a first-pass screen for the possibility of a Down (or trisomy 18) fetus. Combined with ultrasound detection of nuchal translucency, it is possible to detect up to 95% of trisomies without invasive testing of the fetus. Efforts continue to develop detection of fetal cells in the maternal circulation for improved identification of trisomies and other cytogenetic abnormalities [57].

23.2.7 Recurrent Deletions and Duplications and ID

Numerous syndromes where ID is a feature have been discovered that result from recurrent deletion of chromosomal regions. Many of these were first described using light microscopy and chromosome banding techniques. These include Prader-Willi [60] and Angelman [66] syndromes, Miller-Dieker [27], Williams [38], DiGeorge [23, 39], and Smith-Magenis [40, 72] syndromes, along with 1p [103] and Xp22 [9] deletions. The recognition that deletion of the terminal ends of chromosomes is relatively common among individuals with ID led to the development of "multi-telomere" fluorescence in situ hybridization, using probes that detect subtelomeric regions of each of the chromosomes [55, 59, 90, 102, 125], with a detection rate of more than 2% in all samples studied. More recently, the adoption of DNA-based comparative genome hybridization methods has exposed additional regions that could not be detected with standard cytogenetic methods [6].

Beginning with the completion of a reference chromosome map and subsequently a reference DNA sequence for the human genome, it became possible to contemplate interrogating human samples for copy number changes at increasing fine scale. These analyses began with the efforts of Pinkel and Gray and their coworkers [53], who recognized the capability to carry out comparative hybridization of two fluorescently labeled DNA samples against chromosomes, and later clones immobilized on a surface [87]. Relative hybridization levels allow determination of regions that are under- or overrepresented in one sample relative to the other. These started with large insert-cloned DNAs (e.g., bacterial artificial chromosomes) representing regions of the genome spotted on glass slides. In the initial version, the aim of these studies was to improve understanding of changes in tumor DNAs relative to DNA from normal cells taken from the same individual. However, this became a general method for comparing any sequence to a reference, and the methods have improved to allow use of oligonucleotide probes spotted on slides to vastly increase the resolution of the method. Such arrays that allow comparative genome hybridization (CGH) analysis have quickly became an important adjunct to standard cytogenetics for characterizing patients with a variety of maladies, including developmental delay and ID [5].

The role of repeated DNA sequences in mediating the rearrangements that lead to recurrent deletion or duplication disorders is now appreciated. In particular, low copy repeats have been found to flank regions commonly deleted in a number of well-described common deletion disorders. These repeated sequences are typically uncommon in the genome, usually repeated locally at the site of the deletion, and often polymorphic in the human population. The repeats are often conserved in recent evolution. Lee and Lupski recently reviewed the contribution of repeats of this type to a variety of neurological disorders that involve recurrent deletions or duplications including ID [61]. The principal mechanisms that can explain the generation of rearrangements that involve repeats are nonallelic homologous recombination and nonhomologous end joining [104]. However, some events that appear more complex can be explained by a replication-based mechanism, rather than by recombination [62].

23.2.8 Prader-Willi and Angelman Syndromes

A recurrent deletion in chromosome 15 (15q11.2--q12) was identified in the early 1980s as a cause of Prader-Willi syndrome, a rare, usually sporadic form of mild ID that has other physical features and unusual behaviors, such as hyperphagia [60]. It was noteworthy when it became clear that Angelman syndrome patients were found with the same cytogenetic lesion [66], since the clinical phenotypes are quite distinct, with Angelman patients showing more severe ID along with characteristic language delays, movement disorders, and a happy demeanor with hand flapping. The recognition that deletions from paternally contributed chromosome 15 led to Prader-Willi, while maternal deletions resulted in Angelman syndrome [56], supported the notion that imprinting of the genes in this region was responsible for the differences in phenotype. The recognition that uniparental disomy can cause both Prader-Willi and Angelman syndromes brought significant confirmation of the imprinting hypothesis [76, 77].

In the intervening years since the recognition that imprinted genes in the 15q11-q12 region can result in different forms of ID, intensive efforts by many groups have led to the recognition of individual genes responsible for the bulk of each disorder. Smaller mutations have been found that support the candidates. For Angelman syndrome, a ubiquitin ligase (UBE3A) is silenced on the paternal chromosome and the maternal allele is expressed in brain regions (hippocampus and cerebellum) that support a role in the phenotypic features of the disease [50]. Mice lacking the gene are found to have learning impairments, further supporting the role of UBE3A in the disorder [115]. For Prader-Willi syndrome, the principal candidate is a cluster of small nucleolar RNA genes that are specifically expressed from the paternal chromosome [26, 98].

23.2.9 Future Directions in Genome Rearrangements and ID

The 15q11.2 locus underscores the complexity of genomic rearrangements that lead to ID. It is 20 years since the initial description of the paired deletions in Prader-Willi and Angelman syndromes, and the specific gene lesions have only recently been uncovered. The development of DNA sequence data and reference genomes has allowed such loci to be exhaustively studied. However, regions with recurrent rearrangements tend to have a complex repeat architecture that is often polymorphic in the human population and can confound genome sequence assemblies [8]. Indeed, variation in copy number is a very common feature of the human genome and appears not to be confined to rearrangement-prone regions [54]. The ongoing efforts to characterize these regions in multiple individuals will begin to address the variation in gene and sequence content in the general population, which should in turn provide better assessment of these regions in individuals with ID. Very recent studies using array CGH have pointed to regions with deletions that can result in multiple phenotypic outcomes, reminiscent of the early Prader-Willi and Angelman findings. An example can be found for a region of chromosome 15, which has been found to have copy number variants in individuals with intellectual disability, autism, epilepsy, or psychiatric disorders such as schizophrenia [14, 48, 109]. Whether imprinting, the fine details of the rearrangements (or of the individual's genomic architecture prior to the rearrangement), or effects of variation in the remaining alleles are responsible will likely be different for each locus. Happily, the tools for distinguishing between these possibilities have been developed.

23.2.10 Single-Gene Mutations and ID

23.2.10.1 Autosomal Recessive

A large number of single gene mutations can result in ID, although each individually is quite rare. Many of these also result in inborn errors of metabolism and have specific features of these syndromes in addition to the problems with cognition, but it is likely that many more genes have the potential to contribute to

ID. Examples include lysosomal enzyme storage diseases such as Tay-Sachs, Sandhoff, Niemann-Pick, and forms of Gaucher diseases, which are recessive, typically progressive, and cause ID through neurodegeneration. As most of these are autosomal recessive conditions, their frequency is typically quite rare outside of populations that carry founder mutations or cases with consanguinity.

Diseases that result from defects in amino acid metabolism can also result in cognitive disabilities. The example of phenylketonuria (PKU), most frequently caused by mutation in the phenylalanine hydroxylase gene (PAH), is of particular note. Early detection of newborns with PKU provides the ability to place the patient on a special diet with restricted pheylalanine, reducing or eliminating the likelihood of brain damage and ID. The adoption of newborn screening for elevated phenylalanine levels in the blood of newborns has allowed many individuals who would otherwise have been cognitively damaged to escape this consequence. It is estimated that some 250 of cases of ID have been prevented each year in the US through newborn screening and early treatment with diet [2]. This represents a significant success story for research into genetic causes of ID.

Additional genes involved in autosomal recessive ID are being sought. A very useful approach has made use of families with consanguinity, identifying regions of homozygosity, and screening genes within the region(s) for mutations. Syndromic forms with symptoms beyond ID have been more amenable to this approach, but even nonsyndromic forms have been identified [95]. As the cost of DNA sequencing continues to fall, it may be possible to employ mutation detection on a much larger scale for identification of variants in such families, and possibly in sporadic patients. Efforts to identify causative variants in X-linked forms of ID provide insight into the difficulties involved in embarking on such an approach (see below).

23.2.10.2 Autosomal Dominant

Since genetic alterations that lead to moderate or severe ID rarely lead to successful reproduction in individuals who carry them, most autosomal dominant forms of ID result from new mutations. As described above, spontaneous deletions and other chromosome alterations are common forms of new mutation in ID. However, there are some well-described autosomal dominant single gene disorders that feature milder ID with variable expression. These are often described as learning disabilities. It is likely that many more genes will be uncovered and shown to result in ID or learning disability when haploinsufficient, once the genes that underlie the regions with deletions found by array CGH are characterized and tested in additional sets of patients. An example of such a single-gene disorder is neurofibromatosis type I, which is a relatively common (1/4,000) dominant disorder that is inherited in roughly one half of cases, with the remainder appearing de novo. Some 50% of patients are cognitively impaired. In addition to its role as a tumor suppressor and regulator of myelin, the NF1 gene is involved in early brain development through its regulation of the Ras signal transduction pathway [46], and in other aspects of brain function, which may explain the learning issues in patients.

Another dominant disorder involving disturbed signal transduction is tuberous sclerosis. It is caused by dominant loss of function mutations in either TSC1 or TSC2, and patients with this relatively common (1/6,000) disorder can have severe to mild ID or be cognitively unaffected. They typically develop benign tumors in the brain and other organs, and many exhibit seizures. A number of investigators have determined that signaling through the mTOR pathway is altered in TSC and have successfully demonstrated the use of mTOR inhibitors (rapamycin) for modifying phenotypes in cellular and mouse models of the disease. Application of these results to patients in clinical trials is under way, demonstrating the value of understanding basic mechanisms of gene function for potential treatment regimens in disorders involving ID [96, 99].

Myotonic muscular dystrophy (~1/8,000) is typically caused by a large expansion of a trinucleotide repeat sequence in the DMPK gene [18]. This CTG repeat, located in the 3’ untranslated region of the gene, can expand to sizes into the thousands of triplets. The repeat has a tendency to expand in subsequent generations and disease severity correlates with repeat length. Very large expansions, inherited almost exclusively from female carriers, can result in a congenital form of the disorder where ID is very common. In typical, adult-onset forms, cognitive difficulties are more often found to be mild, but there can be progressive cognitive difficulties that manifest as dementia, which would not qualify as ID [70, 71]. However, it is clear that the variable and pleiotrophic effects of the DM mutation can lead to cognitive involvement even in individuals without the congenital form. Significant effort in recent years has pointed to a toxic gain of function by mutant mRNA carrying the expanded CUG triplets as a major cause of the disorder. This has the consequence of reducing availability of proteins that normally interact with RNAs bearing this sequence, altering RNA metabolism. One consequence is that changes occur in alternative splicing patterns in a number of genes [81, 82, 89].

23.2.11 X-Linked ID

Our understanding of single-gene forms of ID has benefited significantly from the study of X chromosome-linked forms. The ability to identify families with X-linked inheritance patterns segregating ID allowed linkage mapping to identify regions associated with the disorder in the family. The likelihood of X-linked forms of ID was suggested as early as in the 1930s, and families with an X-linked pattern of inheritance were described shortly thereafter [68]. Numerous groups have collected families of this type and have described both X-linked syndromic forms (those with other phenotypes beyond the ID) and families with ID and no other symptoms, termed "nonsyndromic." A recent tabulation of X-linked conditions with ID discloses a total of 215 defined conditions, with 66 of these classified as nonsyndromic. More than 80 specific genes have been implicated in X-linked ID (XLID), and nearly 100 more have been assigned to chromosomal regions by linkage mapping (Fig. 23.2.1) [21, 93].

A large-scale DNA sequence-based study of some 75% of the known coding exons present on the X chromosome was recently completed in some 200 families with XLID and no known mutation [114]. Mutations could be definitively identified in one quarter of the families, using criteria of multiple mutations in the gene, clear loss of function mutations, segregation of the mutation with disease in the families, and absence of mutation in unaffected individuals. This study added nine genes involved in XLID and provides novel additional mutations and genes for further diagnostic efforts and research into functional contribution to cognition. There were other findings of note, however, which serve as cautions against scaling these types of studies to the whole genome. Approximately 10% of genes studied were found to have clear loss of function mutations in males that did not confer a phenotype and were present in the general population. This suggests that a similar, or higher, frequency of such events can be expected for autosomes, with consequent difficulties in sorting out their relationship to phenotypic effect. In addition, among the families where a clear causative mutation could not be identified numerous unique variants were found (an average of six per individual) that might have a role in disease. These could not be definitively ascribed, even with the benefit of X-linked inheritance patterns and additional affected family members. The variants discovered in this study were confined to the coding sequences, and the complications of analysis can be predicted to be much greater as we begin to consider noncoding variants. This study underscores the difficulties that will be faced as fewer and smaller families, along with singleton cases of ID are considered for mutation analysis.

The large number of genes involved in XLID, and the diverse functions disrupted, have been surprising. These findings have resulted in a reassessment of the role of X-linked genes in the excess of males with ID, since few X-linked loci with a large contribution to ID have been discovered. Instead, many loci with small numbers of patients and families appear to be the pattern, and many males in families with an X-linked pattern of inheritance remain unexplained by mutation. The excess of males with ID may represent a liability conferred by other factors, perhaps hormonal [20, 67]. The families with an X-linked pattern without detectable mutation may represent chance occurrence of multiple members with ID, which is common in the population in general.

Most families examined for potential XLID exhibit the typical X-linked pattern of inheritance, with affected males, no male-to-male transmission, and carrier females. However, some of the X-linked forms of ID manifest in females (e.g., fragile X syndrome), while others are lethal in males in the prenatal period (e.g., Rett syndrome), leaving only affected females. These exceptions to the usual inheritance pattern have been of great interest, and studies of X-linked forms of ID have led to some interesting new principles in human genetics. These studies have also yielded a bountiful number of genes that have impact on cognition.

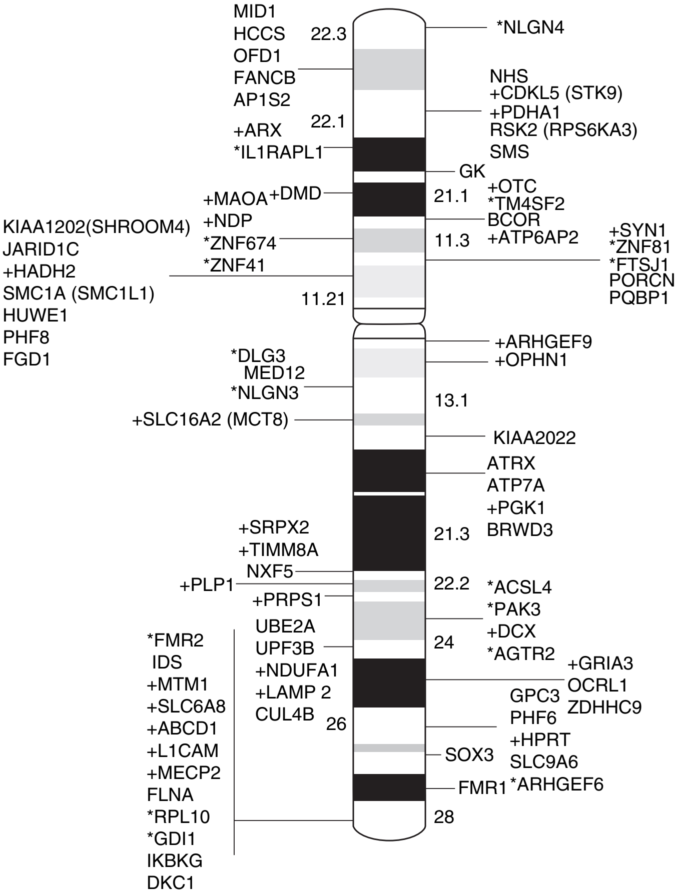

Fig. 23.2.1 Ideogram of the X chromosome with the positions of the 82 known XLMR genes as of 2007. Genes written in *black* cause syndromes, those in *gray* preceded by a *plus sign* cause neuromuscular disorders, and those written in *gray* preceded by an *asterisk* are involved in nonspecific conditions. (Adapted by permission from Macmillan Publishers Ltd: European Journal of Human Genetics, copyright 2008 [21])

23.2.11.1 Fragile X Syndrome

Fragile X syndrome is the most common inherited form of ID, and is second to Down syndrome as a genetic cause of ID. It is estimated to have a prevalence of ~1/3,500 males, and is found in most populations at similar rates [122]. The disorder was first described by Martin and Bell in 1943, who proposed it as an XLID on the basis of a single family with affected males [68]. They described many of the hallmark physical characteristics of the disease, which include a high forehead, large and often protruding ears, large head, and, in postpubescent males, enlarged testicular volume (Fig. 23.2.2). Developmental delay is common, often with speech and language delays, but slow acquisition of motor skills is also typical, with delayed milestones for sitting, standing, and walking. Behavioral characteristics in males are shyness, gaze avoidance, hand flapping, and hypersensitivity to novel environments and intense stimuli. A significant fraction, perhaps as high as 50%, of males with fragile X syndrome can be classified as autistic [35]. The degree of cognitive involvement is highly variable, but ranges from mild to severe. Girls can also be affected, but their cognitive abilities range from normal to moderate levels of ID (Table 23.2.1).

Fragile X syndrome received its name from the discovery in 1969 by Lubs et al. of an unusual chromosomal abnormality present on the X chromosomes of patients with the Martin-Bell phenotype [65]. The "fragile site" was present at the distal end of the long arm, in band q27.3, and manifested as a secondary

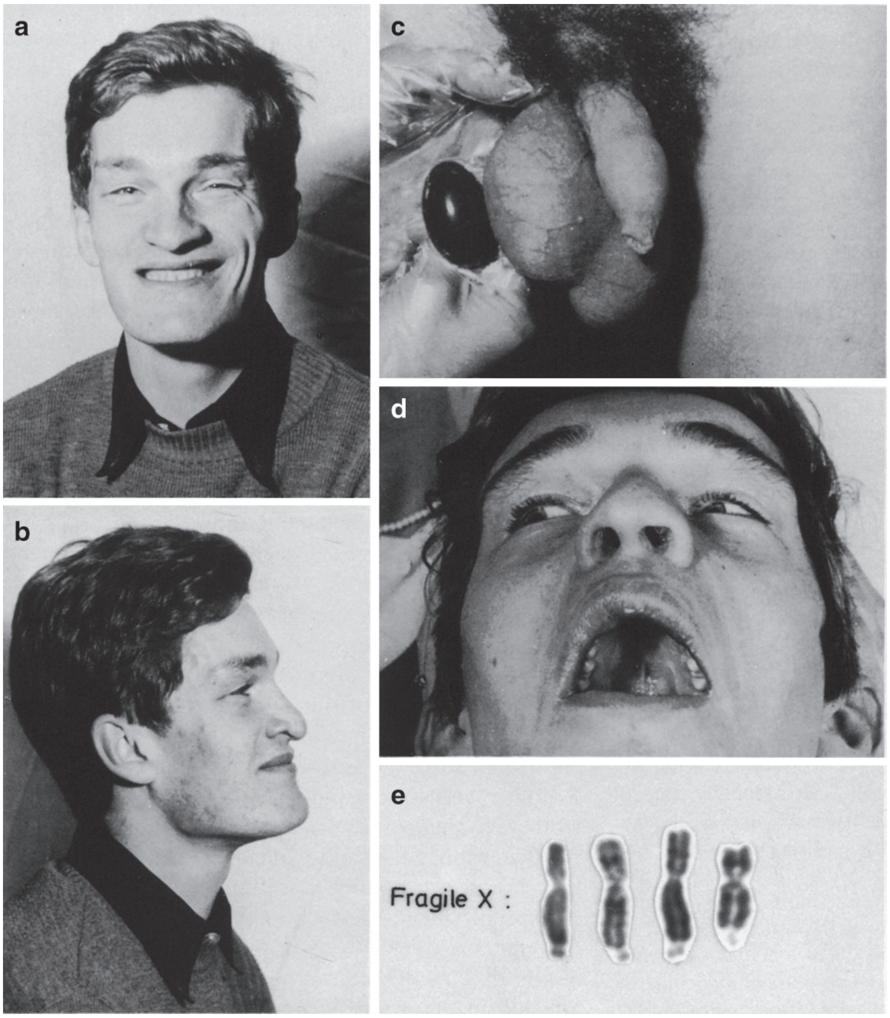

Fig. 23.2.2 Patient with X-linked mental retardation (Martin-Bell or fragile X syndrome): (**a**, **b**) typical face; **c** macro-orchidism; (**d**) high-arched palate; and **e** X chromosomes indicating the fragile X site. From [113]

Table 23.2.1 Clinical features of fragile X syndrome from [119]

Intelligence	IQ range 30–65, sometimes borderline normal or even normal. Occasional hyperactivity or autism in childhood; generally friendly, shy, nonaggressive as teenagers; speech anomaly
Growth	Birth weight normal; usually heavier and taller than normal sibs; head circumference above 50th, sometimes above 97th percentile
Facies	Prominent forehead and jaws, long face, and big ears
Testicles	May be 3–4 cm^3 in childhood (normal 2 cm^3); postpubertal boys 30–60 cm^3 (normal < 25cm^3)
Occasional features	Epilepsy; increased reflexes in lower extremities; gynecomastia, striae, fine skin; thickening of scrotal sac

constriction. The region distal to the fragile site was occasionally broken off in cytogenetic preparations, leading to the constriction being termed a fragile site [111]. A number of reports in the 1970s described this and many more fragile sites in the human genome that could be elicited by a variety of drugs added to cells in culture. The Xq27.3 was found to be induced by low folate media, or by the addition of high levels of folate, and is one of the "folate-sensitive" fragile sites [112]. Interestingly, only a moderate fraction of cells from a patient with fragile X syndrome would exhibit fragile site induction, and rates were never above 50%. The presence of the fragile site was utilized as a diagnostic test for fragile X syndrome for more than a decade.

With a reasonable diagnostic tool, many groups began to collect families with fragile X syndrome and to carry out genetic analyses. These led to a very significant finding: pedigrees segregating fragile X syndrome had unusual transmission characteristics. There were unaffected males in the families, typically grandfathers, who contributed X chromosomes to their grandsons with the disorder. These men were termed normal transmitting males (NTM). The daughters of NTMs were found to have no risk for cognitive problems, but their sons (in aggregate) had a risk that was less than that predicted by Mendelian genetics – just 40%. In some pedigrees, however, it was possible to find generations with typical Mendelian segregation of the disease from carrier females. These typically were found to be in the more recent sibships. These observations were quite puzzling- and were referred to as the Sherman paradox after two papers published by Stephanie Sherman and Patricia Jacobs and their coauthors in the mid 1980s provided the data demonstrating the peculiar pattern of inheritance in the disorder (Fig. 23.2.3) [105, 106].

Linkage mapping using X chromosome markers demonstrated that the fragile X syndrome mutation was in the same region as the cytogenetic abnormality. Using somatic cell genetics, Stephen Warren was able to generate a panel of hybrid cell lines that retained chromosomes with breakpoints apparently at the fragile site, and these were used to determine the locations of fragments of DNA from the X chromosome that were used as markers [123]. Abnormal DNA methylation was detected in the vicinity of the fragile site in male samples using Southern blotting of large restriction fragments separated by pulsed-field electrophoresis, suggesting an unusual mutation [12, 118]. Yeast artificial chromosomes containing the region were identified by several groups and used to characterize the site and identify coding sequences [47]. An expanded, methylated sequence was found to be present in individuals with fragile X syndrome by Southern blotting, but not in controls, or in unaffected individuals carrying the same X chromosome in the pedigree [78, 117, 127]. The expansion was found to arise from a normally polymorphic CGG trinucleotide repeat present in the 5′ untranslated region of the FMR1 gene [117]. The repeats were found to be very unstable within families, and the instability explained the unusual genetics of the disease. NTMs were found to have small expansions, termed premutations. Study of the available families revealed that repeat lengths in individuals with fragile X syndrome exceeded ~200 triplets and were typically several hundred to thousands. Patients showed variation in repeat length and indicated mosaicism, which can include premutation alleles [33, 86].

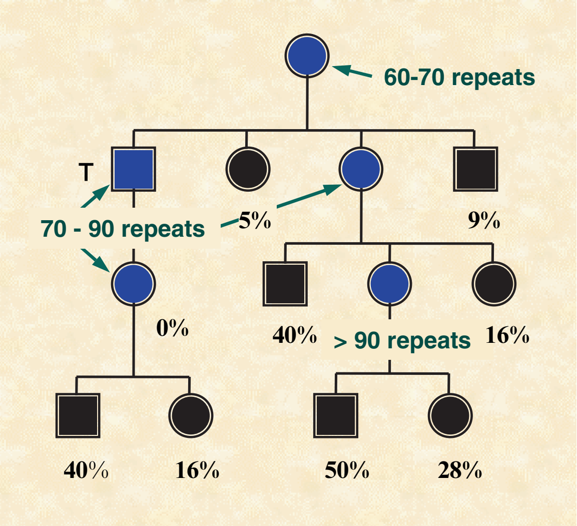

Fig. 23.2.3 Sherman paradox of fragile X inheritance. In this theoretical pedigree, the *black* percentages represent the risk of intellectual disability in a pedigree segregating fragile X syndrome. Unaffected carriers are in *blue*. Note that the risk increases with each generation, and can reach Mendelian expectations for males (*right side*, final generation). The male in the second generation designated by a *T* is a 'normal transmitting male,' who passes the disorder to grandchildren without being cognitively disabled. The *repeat numbers* in *green* indicate the likely CGG repeat lengths that correspond to the risks observed by Sherman et al. The increasing length of repeats in subsequent generations explains the finding that risk is dependent on position in the pedigree

Discovery of the CGG triplet repeat in fragile X syndrome was followed by that of a large number of unstable triplet repeats that give rise to human genetic disorders. Many of these mutations result in degeneration of neuronal subsets and are present in coding sequences of genes in the form of CAG codons, which encode a stretch of polyglutamines. The first of these to be described was in spinal bulbar muscular atrophy, and was followed by others found in Huntington disease and many spinocerebellar ataxias. Myotonic muscular dystrophy, described above, was also found to result from abnormal expansions of a CTG triplet in the 3′ untranslated region of a gene termed DM protein kinase. The discovery of trinucleotide repeat expansions as mutant alleles in more than one dozen human genetic disorders has been one of the more significant advances in the past two decades [91].

The three allele classes of the CGG repeat in the FMR1 gene are defined by their lengths. Individuals in the general population carry alleles that range from as few as 5 to as many as 54 repeats, with the most common alleles being 29 and 30 triplets with AGG triplet interruptions at the 10th and 20th positions. These are transmitted without change to offspring. The moderately expanded premutation alleles are found to range from ~55 to ~230 repeats and are characterized by instability on transmission to the next generation. Premutations alleles typically exhibit long pure CGG sequences, and the development of a premutation from typical alleles appears to often involve loss of one or both of the interrupting AGG triplets. Instability of CGG repeats is found once the length of uninterrupted CGG exceeds approximately 35 triplets [30, 58]. There is a bias toward expansion, with increases outweighing decreases several fold. Interestingly, only female transmissions support the expansion of the premutation to sizes above ~230 repeats, which lead to fragile X syndrome. These larger, full, mutations show methylation of the C residues in the repeat, and this methylation, along with other alterations in chromatin, reflects the loss of FMR1 transcription. While there can be considerable variation among patients owing to mosaicism in methylation and repeat length, the principal result of the full mutation is to eliminate expression of the FMR1 gene.

Premutation alleles are found in all pedigrees with fragile X syndrome; there are no documented examples of a full mutation developing from an allele that would be found in the general population. Moreover, there is no example of a premutation developing from a normal allele. It is estimated that the growth of normal alleles to premutation length takes many generations [73], and the existence of predisposed haplotypes in the general population with unusual patterns of interruption supports the notion of a lengthy process for the development of premutations. The high frequency of fragile X syndrome coupled with the observation of apparently de novo cases led to the prediction that the mutation rate at the locus involved would be extremely high. While this is true in the sense that the premutation to full mutation transition has a high frequency, it is also the case that the high mutation rate is the result of pre-existing premutations, which are primed for additional mutation.

A late-onset neurodegenerative disorder has been described among up to one half of male carriers of premutations [43]. This disorder has been termed fragile X tremor ataxia syndrome (FXTAS) and is characterized by a Parkinsonian tremor, ataxia, cognitive decline, neuronal loss, and the presence of ubiquitin-staining nuclear inclusions in neurons and other cells. Interestingly, patients with fragile X syndrome do not exhibit this disorder, and accumulating evidence points to the expression of CGG-containing RNAs as the toxic agent [44, 51, 52]. This RNA is not expressed in fragile X patients, but is found in premutation carriers, possibly at elevated levels. Some female carriers of premutations have been found with FXTAS, but it appears to be rare in this group.

Counseling in families with fragile X syndrome has benefited from the knowledge of patients' repeat lengths, but it has also introduced considerable complications. The risk of bearing a full-mutation fetus for women carrying a premutation is dependent on the length of the premutation. Risk of an expansion to the full mutation reaches 100% for alleles above ~100 repeats (leading to the expected 50% Mendelian risk), but is very low for those below 60 repeats. Between these two thresholds, the risk escalates with length. An additional complication is presented by the ability to detect female fetuses carrying the full mutation. Since these girls are affected (i.e., have ID defined by IQ testing) in about one half of cases, counseling is difficult, since there is typically no additional predictive information. The realization that premutation carriers are also at risk for a late-onset disorder has also led to concern about how this information should be utilized, particularly in the setting of widespread screening of newborns, which is currently being considered for fragile X syndrome [7].

23.2.11.1.1 FMR1 Function and Potential Therapy in Fragile X Syndrome

Considerable effort from many groups has provided a picture of the function of the FMR1 gene product, although it is far from complete [10]. The protein (FMRP) is a member of a small family (three members) of RNA-binding proteins that have roles in translation of target RNAs. One of the important sites of action for FMRP is in neuronal dendrites, where it is part of a signal transduction cascade that responds to synaptic signaling, FMRP may be involved in transport of specific RNAs to dendrites, as it is found in RNA:protein particles. The protein can be found on polyribosomes, suggesting a role in translational control, and its primary role may be suppression of translation. The absence of FMRP in animal models supports this idea, since overproduction of protein is observed in these models [88].

A number of observations in animal models led to the development of the "mGluR theory of fragile X syndrome" [11]. This theory postulates that glutamate signaling through type I metabotropic receptors (mGluR1 and mGluR5, which are G protein-coupled receptors sensitive to glutamate) is modulated in part by FMRP through translational suppression, and that in FMRP's absence, this system is overstimulated, with a number of consequences, including difficulty for synapses to mature [10]. A prediction of this theory is that reduction of signaling through the mGluRs would ameliorate these effects and might prove therapeutic. Tests of specific inhibitors of mGluR5 proved to modify phenotypes in mouse and fly models [25, 69, 126], and these results have encouraged clinical applications of similar compounds in patients with fragile X syndrome [15]. If these therapies prove effective, this will represent a significant triumph for functional analysis of gene function leading to rational therapy in a common form of genetic ID.

23.2.11.2 Rett Syndrome

Rett syndrome is caused by mutations in the *MECP2* gene in Xq28. The disorder affects girls almost exclusively and is characterized by a progressive loss of function beginning in the 2nd year of life and resulting in severe cognitive disability along with a constellation of physical and behavioral abnormalities (Fig. 23.2.4) [19]. Severity can be highly variable, likely owing to patterns of X-inactivation that differ among patients. The incidence of disease has been estimated to be between 1/10,000 and 1/15,000, and it is found in all populations. Leading up to the description of mutations in the *MECP2* gene, the evidence in favor of a genetic etiology for Rett syndrome was sparse. Most cases appeared to be sporadic, with only a small number of recurrent cases now known to have resulted from mothers who were unaffected owing to favorable lyonization or germline mosaicism. Males carrying typical loss of function mutations in *MECP2* are not viable as embryos, and affected females do not typically bear children, so that, as expected, most cases (~95%) result from de novo mutation. With the focus on the *MECP2* gene, it has become apparent that there are additional disorders that result from *MECP2* mutation, including a relatively common duplication that confers ID in males [24, 116].

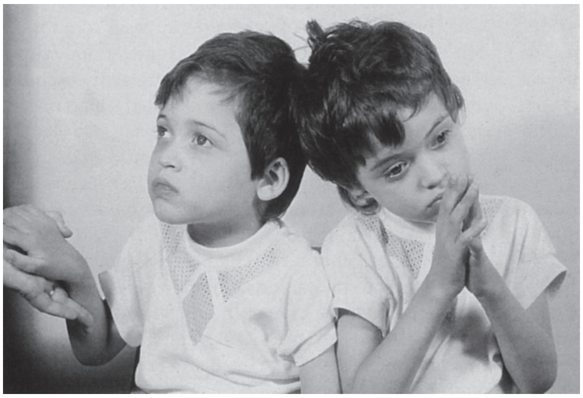

Fig. 23.2.4 Monozygotic twins with Rett syndrome, at the age of 9 years. (Courtesy of Dr. G. Tariverdian)

The *MECP2* gene product is a nuclear protein that recognizes methylated DNA and, together with other transcription factors, is involved in control of gene transcription. In affected girls, loss of protein function in a portion of cells leads to dysfunction, while overexpression in males is also problematic for normal cognition. A male with a triplication at the locus and a more severe phenotype suggests significant dosage sensitivity for *MECP2* levels [24]. Mice with mutations in *Mecp2* mirror human phenotypes [22, 41, 101]. Of particular note is that adult restoration of *Mecp2* expression can rescue phenotypes [37, 42], offering the possibility that the disorder is dependent on acute levels of *MECP2*, rather than a result of developmental damage. Similar data have been developed in other mouse models for genetic forms of ID, with rescue by gene replacement of drug treatment [29]. These exciting results portend well for potential therapy in Rett syndrome and in other ID disorders.

These findings in Rett sydrome and related disorders underscore the likelihood that the ongoing efforts to identify genomic regions with deletions and duplications will likely be fruitful for further delineation of dosage-sensitive genes involved in ID, and predict that even more subtle alterations in expression levels could prove important for behavioral and cognitive phenotypes, both pathogenic and within the typical range.

Among the other common X-linked forms of ID are mutations in ARX and SLC6A8, which each account for a few percent of XLID [93]. Each of the genes with mutations in XLID has an important story to tell about gene function, mutational mechanism, and genotype/phenotype correlation. Unfortunately, it is beyond the limitations of this chapter to discuss them in detail. A general principle, however, is that the classification of XLID into syndromic and nonsyndromic forms broke down as gene mutations were identified. There are now several examples of mutations in the same gene that can give rise to both syndromic and nonsyndromic forms of ID with X-linked inheritance patterns [93].

23.2.12 Future Directions

The large abundance of genes that can give rise to XLID suggests that a similar fraction of autosomal genes might contribute to cognitive disability. This is likely to be 10% or more of genes and suggests that study of inbred populations will uncover causative homozygous mutations that may be limited to a small number of families. Of possibly more interest will be uncovering the heterozygous mutations that might confer a milder effect, yet could be of appreciable frequency in the general population. The study of autosomal ID in inbred families may point to some of these.

With the large-scale XLID sequencing study [114] and the prior efforts to sequence individual diploid genomes [64, 121, 124], we have come to appreciate that the level of variation between individuals' genomes is large. It will require large numbers of genome sequences from well-characterized individuals to begin to associate sequence variation with phenotype, as many variants will have no effect, while others will be essentially private, making them very difficult to associate with phenotype without family studies or development of models. It is an exciting time, but the accumulation of large data sets of sequence will have to be accompanied by equivalent efforts in determining phenotypes at high precision for the correlations to be worthwhile.

The use of models, principally mouse models, for understanding consequences of mutations on cognition has been very effective. Examples from Rett and fragile X syndromes are noted above. These models have been essential for understanding the functions of the affected proteins, and are being used to devise and test therapies. Nonetheless, the laboratory mouse has significant limitations for study of human cognition. Defects in learning and memory are typically mild if present. This is due in part to the nature of the laboratory mouse, which through inbreeding has been selected for docility. At present, however, there is no viable alternative mammalian model from a cost or convenience viewpoint. The fruit fly *Drosophila* has also been of particular value; the rapid ability to identify modifying genes and even to carry out drug screens has had a very favorable impact for study of mutations of the orthologous fly gene products.

As therapies are developed for disorders that include ID, there will be more interest in developing newborn screening approaches for identifying candidates for treatment. This will be particularly important for disorders such as ID, where early intervention may be of considerable value. The situation in PKU provides an important example, where early diet therapy can prevent a devastating cognitive disability. Testing for mutations in most of the newly discovered genetic forms of ID will require more sophisticated, likely DNA-based, testing, however, which will involve a much greater cost than the current newborn screening for blood analytes. As DNA sequencing costs continue their precipitous decline, it appears likely that complete genome sequencing of newborns may be within reach in the not-too-distant future, providing a comprehensive approach to mutation detection for ID and charting a course of treatment for affected individuals that will preserve or even improve their cognitive capacities. The past 50 years have seen remarkable advances in identification of causes of ID, and the next half century should yield an equivalent series of discoveries that lead to effective therapies for cognitive disability.

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Genetic Factors in Alzheimer Disease and Dementia 23.3

Thomas D. Bird

Abstract Alzheimer disease (AD) is a common and complex disorder affecting several million people world-wide. It is defined clinically as a progressive dementing illness associated with Aβ-amyloid neuritic plaques and neurofibrillary tangles in the brain. From a genetic standpoint it is a heterogeneous disorder. Three separate genes (APP, PSEN1, PSEN2) each cause an autosomal dominant, highly penetrant, early-onset, familial form of the disease. The three proteins encoded by these genes all influence the production of the toxic $A\beta_{1-42}$ form of amyloid. Commercial genetic testing is available for these rare forms of AD. However, mutations in these genes represent less than 2% of all cases of AD. The more common late-onset form of AD is thought to be polygenic and multifactorial. The ε4 allele of apolipoprotein E (ApoE) is a known genetic risk factor for late-onset AD, lowering the average age of onset by unknown mechanisms. Numerous other candidate risk genes are being identified through genome wide association studies, but have been difficult to confirm. Other familial forms of dementia, such as frontotemporal dementia (FTD), prion-associated diseases, and CADASIL, may be caused by autosomal dominant mutations occurring in their respective genes (MAPT, GRN, PRNP, Notch-3).

Contents

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23.3.1 Clinical Manifestations of Alzheimer Disease

The major clinical manifestation of Alzheimer disease (AD) is dementia that typically begins with subtle and poorly recognized failure of memory and slowly becomes more severe and, eventually, incapacitating. Other

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common findings include confusion, poor judgment, language disturbance, agitation, withdrawal, and hallucinations. Occasionally, seizures, Parkinsonian features, increased muscle tone, myoclonus, incontinence, and mutism occur [8]. Because the first symptoms of AD are subtle it is difficult to know when the disease begins. Presumably there is brain deposition of Aβ peptide and tau for years before the onset of clinical symptoms. Mild memory loss is common with advancing age. A syndrome called mild cognitive impairment (MCI) refers to a condition where the memory loss is noticeable but not disabling and not associated with other symptoms. Persons with MCI slowly convert to a diagnosis of AD at a rate of approximately 10% per year. However, in some persons MCI does not progress and they do not develop AD. At the present time there is no definitive method of distinguishing between these two groups.

Death usually results from general inanition, malnutrition, and pneumonia. The typical clinical duration of the disease is 8–10 years, with a range of 1–25 years.

23.3.1.1 Establishing the Diagnosis

Establishing the definitive diagnosis of AD relies upon clinical-neuropathologic assessment [104]. Neuropathologic findings on autopsy examination remain the gold standard for diagnosis of AD (Fig. 23.3.1). The clinical diagnosis of AD (prior to autopsy confirmation) is correct about 80–90% of the time [74].

Clinical signs: slowly progressive dementia.

Neuroimaging: CAT and MRI imaging studies will show diffuse cerebral cortical atrophy, often severe in the hippocampal region of the medial temporal lobe [51]. SPECT and PET radionuclide scans may show diffuse decreased cerebral metabolic rates, often beginning in the parietal region. A special PET scan (PIB) using a radioactive ligand for Aβ-peptide shows early increased deposits of material throughout the cortex and basal ganglia [108].

Neuropathologic findings: microscopic extracellular Aβ-amyloid neuritic plaques, intraneuronal neurofibrillary tangles, and amyloid angiopathy at postmortem examination. The plaques should stain positive with Ab-amyloid antibodies and be negative for prion antibodies, which are diagnostic of prion diseases. The numbers of plaques and tangles must exceed those found in age-matched controls without dementia (Fig. 23.3.2). Guidelines for the quantitative assessment of these changes exist [11, 80]. Aggregation of alpha synuclein in the form of Lewy bodies may also be found in neurons in the amygdala [89]. Cerebrovascular disease with microscopic infarcts may also contribute to the pathology [124].

Cerebrospinal fluid (CSF): Analysis shows reduced levels of Aβ-peptide (the metabolic product of the amyloid precursor protein that accumulates in the brains of persons with the disease) and increased levels of tau protein (found in neurofibrillary tangles).

23.3.1.2 Prevalence

AD is the most common cause of dementia in North America and Europe, with an estimated 4 million affected individuals in the US.

The prevalence of AD increases with age. Mild memory loss is often called mild cognitive impairment

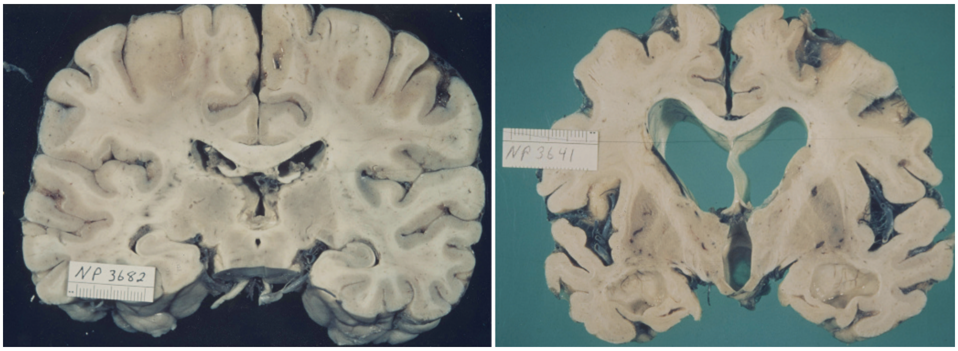

Fig. 23.3.1 Normal adult brain (*top*) compared with Alzheimer brain (*bottom*), showing marked diffuse cortical atrophy and ventricular enlargement. (From [7], with permission)

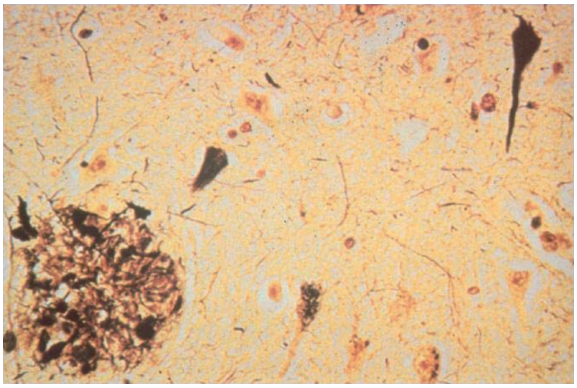

Fig. 23.3.2 Microscopic neuropathology of Alzheimer disease showing an extracellular neuritic plaque (*lower left-hand corner*) and a neurofibrillary tangle containing neuron (*upper right-hand corner*). (From [6], with permission)

(MCI). In many persons MCI is considered an early stage of AD.

The incidence of AD rises from 2.8 per 1,000 person-years (one person living for 1 year = 1 person-year) in the 65- to 69-year age group to 56.1 per 1,000 person-years in the age group older than 90 years [57]. Approximately 10% of persons over age 70 years have significant memory loss, and more than half of these individuals have AD. An estimated 25–45% of persons over age 85 years have dementia.

23.3.2 Causes

About 1–6% of all AD is early onset (before age 60–65 years), and about 60% of early-onset AD is familial, with 13% appearing to be inherited in an autosomal dominant manner [7, 16, 102] (Table 23.3.1).

The distinction between early-onset familial AD (onset before age 60–65 years; EOFAD) and late-onset familial AD (onset after age 60–65 years) is somewhat arbitrary. Early-onset cases can occur in families with generally late-onset disease [14].

Table 23.3.1 Causes of Alzheimer disease

Cause	% of cases
Chromosomal (Down syndrome)	< 1%
Familial	~25%
• Late-onset familial (AD2)	• 15–25%
• Early-onset familial AD (AD1, AD3, AD4)	• < 2%
Unknown (includes genetic/environment interactions)	~75%

23.3.2.1 Environmental

No environmental agents have been proved to be directly involved in the pathogenesis of AD. It is often speculated that late-onset AD (LOAD) is the result of unknown environmental factors acting on a predisposing genetic background [9]. Twin studies have implicated both genes and environment [30]. The Gatz et al. study found concordance for AD in MZ vs DZ twins of 45% vs. 19% and 61% vs. 41% in males and females, respectively. It was noted that age of onset can vary considerably with twin pair, and heritability in this twin study was estimated at 58–79% [30]. Potential environmental factors include head trauma, viruses, toxins, and low education level. Several investigators have suggested that persons with AD had subtle cognitive or linguistic signs in childhood or early adulthood [120, 132].

23.3.2.2 Heritable Causes [6, 7]

23.3.2.2.1 Chromosomal

Down Syndrome. Essentially all persons with Down syndrome (DS; trisomy 21) develop the neuropathologic hallmarks of AD after age 40 years. More than half of individuals with DS, if carefully observed or tested, also show clinical evidence of cognitive decline [15]. The presumed reason for this association is the lifelong over-expression of the *APP* gene on chromosome 21 encoding the amyloid precursor protein and the resultant overproduction of Aβ-amyloid in the brains of persons who are trisomic for this gene.

The amyloid-β (Aβ) deposition in the brain may begin in the first decade of life in persons with DS [63]. AD was not noted clinically or pathologically in a 78-year-old woman with partial trisomy 21 who did not have an extra copy of the *APP* gene [90]. Two studies have found no association of ApoE genotype with age of onset of dementia in DS [59, 71], but one study did find an association of onset age with a polymorphism in the *APP* gene [71]. Schupf et al. [111] found an unexplained increased risk for AD in mothers who gave birth to children with DS prior to age 35 years (rate ratio = 4.8, 95% CI 2.1–11.2). This study has not been confirmed.

23.3.2.2.2 Single Gene

About 25% of AD is familial (i.e., two or more family members have AD). Familial cases appear to have the same clinical and pathologic phenotypes as non-familial cases (i.e., an individual with AD and no known family history of AD [42, 81]) and are thus distinguished only by family history or by molecular genetic testing. A large volume of research on the molecular and genetic basis of AD has been summarized elsewhere [6, 7, 36, 64, 82, 106, 107, 118].

Late-Onset Familial AD (AD2)

Many families have multiple affected members, most or all of whom have onset of dementia after age 60 or 65 years (Table 23.3.2). Disease duration is typically 8–10 years, but ranges from 2 to 25 years. Investigations have supported the concept that LOAD is a complex disorder that may involve multiple susceptibility genes (reviewed and summarized in [49, 108, 114]. Bertram et al. [5] have performed a meta-analysis on these data. The following information is currently available about genes or loci actually or potentially altering risk for late-onset AD:

There is a very well-documented association of LOAD with the *APOE* e4 allele (19q13). The *APOE* e4 allele, by unclear mechanisms, appears to affect age of onset by shifting the onset toward an earlier age [52, 75, 109].

Apolipoprotein E is a cholesterol transport protein that circulates in plasma and is the major apolipoprotein in brain, where it is synthesized by glia, macrophages and neurons [2]. In humans the three common isoforms differ by a single amino acid: e3 has cysteine at position 112 and arginine at 158, e2 has cysteine at both positions, and e4 has arginine at both positions. Frequencies of ApoE genotpyes in the general population are shown in the first column of Table 23.3.3. Note that 3/3 is most common and 4/4 is the least common. It is not known exactly how ApoE influences the biology of AD [45]. Apo e4 differs from e2 and e3 in lacking a cysteine, having a greater tendency to form a molten globule state, and exhibiting domain interactions [136]. These domain interactions lead to cleavage fragments that may be toxic. An important finding has been that a transgenic mouse model of AD with a mutation in the *APP* gene has a greatly reduced deposition of amyloid plaques when produced on an ApoE knockout background [1]. This demonstrates an important role of ApoE in amyloid plaque formation. The use of APoE genotyping in diagnosis and risk assessment of AD is discussed in Sect. 23.3.3.

– Several other potential genes are under investigation:
– *SORL1* on chromosome 11q23, a protein involved with APP protein trafficking [103].
– *A2M* on chromosome 12 [23]
– *GST01* and *GST02* on chromosome 10 [69]
– *GAB2* on chromosome 11q14 interacting with the *ApoE4* allele [98]
– *PCDHIIX* on the X chromosome [17]

Several other potential loci are under investigation on the following chromosomes:

- 12 [24]
- 10 [40, 78, 100]
- 2q, 9p, and 15q [68, 112]
- 19p13 [133]
- 7q36 [93]
- 9q22 *(UBQLN1)* [4, 50, 119]
- Studies of LOAD in a genetically isolated Dutch population have suggested linkage of AD to markers on chromosome 1q22, 3q23, 10q22 and 11q25 [70]
- A genome-wide association study (GWAS) of more than 1,300 cases identified 2 potentially important loci (14q, 6p) in addition to APoE [3].
- A linkage study of >300 families with LOFAD has found several loci of interest [62].

Table 23.3.2 Late-onset familial Alzheimer disease: molecular genetics

Locus name	Gene symbol	Chromosomal locus	Protein name	Test availability
AD2	*APOE*	19q13.2	Apolipoprotein E	Yes

Early-Onset Familial AD (EoFAD)

- *Clinical Features.* Early-onset familial AD (EOFAD) refers to families in which multiple cases of AD occur with the mean age of onset usually before age 65 years, although some studies have used age 60 years or 70 years. Age of onset is usually in the 40s or early 50s, although onset in the 30s and early 60s has been reported. Campion et al. [16] found a prevalence of EOAD in the general population of 41.2 per 100,000 persons at risk (ages 40–59 years). Sixty-one percent of these individuals with EOAD had a positive family history, and 13% met stringent criteria for autosomal dominant inheritance (i.e., affected individuals in three generations). EOFAD cannot be clinically distinguished from nonfamilial AD except on the basis of family history and age of onset. The dementia phenotype is similar to that of LOAD, sometimes with a long prodrome [35, 60, 61].
- *Molecular Genetics.* At least three subtypes of EOFAD (AD1, AD3, AD4) have been identified based on the causative gene. All are autosomal dominant. The relative proportion of each subtype and the causative genes are summarized in Table 23.3.4 [16, 46, 96, 115]. All three types are related to amyloid precursor protein (APP) metabolism. The APP is cleaved by beta and gamma secretases to form the A beta peptide, which is the primary component of the extracellular amyloid plaque deposited in AD (Fig. 23.3.3). (APP may also be cleaved inside the A-beta peptide domain by alpha secretase.) Cleavage at the gamma secretase site may produce Aβ peptide with 40 or 42 amino acids. The Aβ 42 peptide has been demonstrated to be the 'toxic' amyloid plaque-forming peptide. Presenilin 1 (PS1) is part of the gamma secretase complex (and PS2 is a close homolog of PS1). Thus, the three primary genes associated with EOFAD are all related to APP and Aβ amyloid molecular biology. It remains unclear exactly how mutations in these three genes alter APP metabolism and cause AD, whether by increasing production of Aβ 42 or changing the Aβ 40/42 ratio, or some related mechanism [10, 123]. There are transgenic mouse models containing one or more mutations in APP and/or PS1, and even a triple transgenic mouse with mutations in APP, PS1, and MAPT (tau) [83]. It is likely that other genes will be identified as a cause of EOFAD, because kindreds with autosomal dominant FAD with no known mutations in *PSEN1*, *PSEN2*, or *APP* have been described [20, 95].
- Of these three genes, mutations in PS1 are most common. More than 170 mutations have been described in PS1 [61]. Age of onset ranges from 25 to 65 years, usually in the forties. Penetrance is >95%. Very early onset before age 30 has been reported (e.g., mutations I143T, L166P, P436Q). Myoclonus, seizures, aphasia, and cerebellar plaques may occur. Deletions in exon 9 have been associated with spasticity and giant amyloid 'cotton wool' plaques.
- Several different missense mutations have been reported in APP and tend to occur near the secretase cleavage sites (see Fig. 23.3.4). V717I is one of the most common and occurs in the g-secretase site. Mutations near the alpha secretase site may be associated with severe amyloid angiopathy.
- Mutations in PS2 are the least common cause of EOFAD [65]. Only about 12 such mutations have been described. Asn141Ile is a founder mutation that has been found in families with the same Volga German background [65]. Age of onset in PS2 mutations ranges from 40 to 75 years with about 95% penetrance.

Table 23.3.3 Percent of *APOE* genotypes in caucasian controls and individuals with AD (modified from [47])

APOE Genotype	Normal Controls ($n=304$)	All individuals with AD ($n=233$)	Individuals with AD and positive family history of dementia[a] ($n=85$)
e2/e2	1.3%	0%	0%
e2/e3	12.5%	3.4%	3.5%
e2/e4	4.9%	4.3%	8.2%
e3/e3	59.9%	38.2%	23.5%
e3/e4	20.7%	41.2%	45.9%
e4/e4	0.7%	12.9%	18.8%

[a] Most families would be considered to have late-onset familial AD

23.3.2.3 Unknown

Individuals with nonfamilial AD meet the diagnostic criteria for AD and have a negative family history. Onset can be any time in adulthood. The exact pathogenesis of

APP

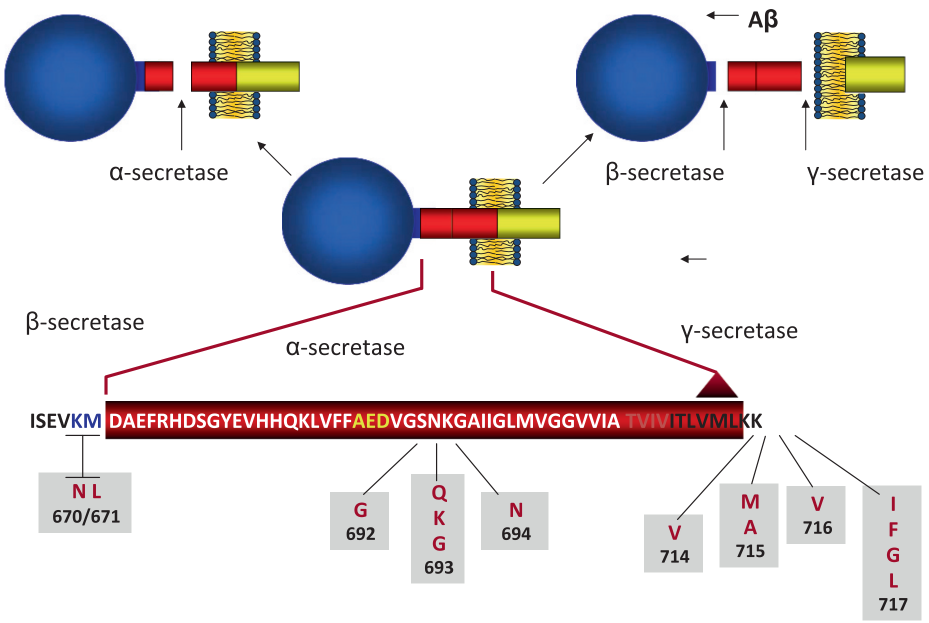

Fig. 23.3.3 Molecular aspects of the APP gene and protein, showing the sites of cleavage by a-, b-, and g-secretases, production of the Ab-peptide (*upper right*) and sites of several disease causing mutations (From [6], with permission)

the disease is unknown. A common hypothesis is that nonfamilial AD is multifactorial and results from a combination of aging, genetic predisposition, and exposure to one or more environmental agents, such as head trauma, viruses, and/or toxins, although no environmental agents have been proved to be directly involved in the pathogenesis of AD [21].

23.3.2.4 Molecular Genetic Testing

23.3.2.4.1 Late-Onset Familial AD

The association of one or two copies of the *APOE* allele e4 (i.e., genotypes e2/e4, e3/e4, e4/e4) with LO AD is well documented (Table 23.3.3) [47, 52, 72].

Table 23.3.4 Early-onset familial Alzheimer disease (EOFAD): molecular genetics

Locus name	Proportion of EOFAD	Gene symbol	Chromosomal locus	Protein N	
name	Test availability				
AD3	20–70%	*PSEN1*	14q24.3	Presenilin-1	Clinical
AD1	10–15%	*APP*	21q21	Amyloid precursor protein	Clinical
AD4	Rare	*PSEN2*	1q31-q42	Presenilin-2	Clinical

- The association between *APOE* e4 and AD is closest when the individual has a positive family history of dementia. The last column of Table 23.3.3 largely represents late-onset familial AD.
- The strongest association between the *APOE* e4 allele and AD, relative to the normal control population, is with the e4/e4 genotype. That genotype occurs in about 1–2% of the normal control population and in nearly 19% of the familial AD population.
- In individuals who have the clinical diagnosis of AD, the probability that AD is the correct diagnosis is increased to about 97% in the presence of the *APOE* e4/e4 genotype [109]. However, note that e4 homozygotes are relatively uncommon in the general population, and the clinical diagnosis of AD by experienced physicians is correct 85–90% of the time without ApoE genotyping.
- The increased risk of AD associated with one *APOE* e4 allele or two *APOE* e4 alleles is also found in African Americans [38] and Caribbean Hispanics [105]. In African Americans the 3/4 genotype is associated with an odds ratio of 2.32 and the 4/4 genotype with an odds ratio of 7.19 for developing AD compared with the 3/3 genotype [77].
- Approximately 42% of persons with AD do *not* have an *APOE* e4 allele. Thus, *APOE* genotyping is not highly sensitive for AD. The absence of an *APOE* e4 allele does not rule out the diagnosis of AD [74]. Thus genotyping is not highly specific [79].
- Breitner et al. [13] have estimated lifetime risks for developing AD based on gender and *APOE* genotype (see Sect. 23.3.4.4.2). This group emphasizes that ApoE genotype primarily affects age at onset of AD, rather than lifetime susceptibility [52]. Figure 23.3.4 shows lifetime risks for AD for various ApoE genotypes.

The usefulness of *APOE* genotyping in clinical diagnosis and risk assessment remains unclear. (See list of "Statements and Policies Regarding Genetic Testing.")

- Although the presence of one *APOE* e4 allele or two *APOE* e4 alleles is neither necessary nor sufficient to establish a diagnosis of AD, *APOE* genotyping may have an adjunct role in the diagnosis of AD because a large proportion of individuals with one *APOE* e4 allele or two *APOE* e4 alleles who are demented have been found to have neuropathologic confirmation of AD at autopsy [47, 73, 78, 131].
- In contrast, *APOE* genotyping was not found to be of significant diagnostic use in identifying AD in a community-based sample with late-onset dementia [125].
- There is some evidence that the *APOE* e2 allele may have a protective effect in regard to risk for AD (Table 23.3.4).

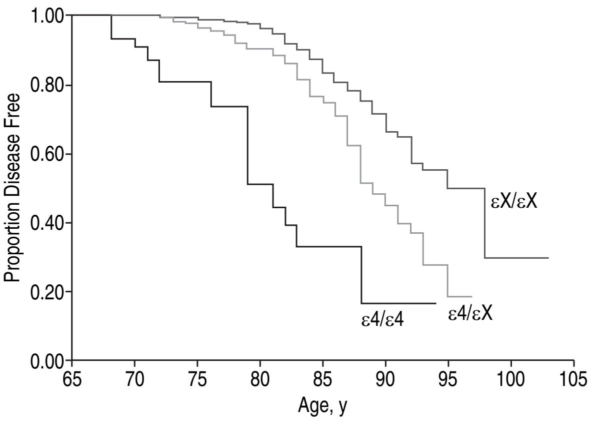

Fig. 23.3.4 Unadjusted disease-free survival by age, stratified by 0, 1, and 2 apolipoprotein E (APOE) e4 alleles, among 3,308 residents of Cache County, Utah, 1995–1997 and 1998–2000. The graph shows three unadjusted product-limit estimates for the three strata of 0 (*hatched line*), 1 (*gray line*), and two (*black line*) APOE e4 alleles (*X* allele 2 or 3). (From 51, with permission)

Another way to look at this association between AD and an *APOE* e4 allele is with *APOE* e4 allele frequencies (Table 23.3.5).

Table 23.3.5 *APOE* allele frequencies in controls and individuals with AD (modified from [47])

APOE allele	Normal controls ($n=304$)	All individuals with AD ($n=233$)	Individuals with AD and positive family history of dementia[a] ($n=85$)
e2	9.0%	3.9%	5.9%
e3	76.5%	60.5%	48.2%
e4	13.7%	35.6%	45.9%

[a] Most families would be considered to have late-onset familial AD

23.3.2.5 Early-Onset Familial AD

The three known subtypes of EOFAD, called AD3, AD1, and AD4 [46], can only be distinguished by molecular genetic testing (Table 23.3.4). Genetic testing of individuals who are simplex cases (i.e., a single occurrence of EOAD in a family) is controversial and should be undertaken in the context of formal genetic counseling [127]. A small proportion (<5%) of such cases will have a mutation in PS1.

23.3.3 Genetic Counseling

23.3.3.1 Mode of Inheritance

Because AAD is genetically heterogeneous, genetic counseling of persons with AD and their family members must be tailored to the information available for that family. AD is usually considered polygenic and multifactorial. EOFAD is inherited in an autosomal dominant manner.

23.3.3.1.1 Risk to Family Members: Late-Onset Nonfamilial AD

Genetic counseling for people with nonfamilial AD and their family members must be empiric and relatively nonspecific. It should be pointed out that AD is common and that the overall lifetime risk to any individual of developing dementia is approximately 10–12%.

First-degree relatives of a person with AD have a cumulative lifetime risk of developing AD of about 15–30%, which is typically reported as a 20–25% risk [25, 116]. This risk is about 2.5 times that of the background risk (~27% vs 10.4%) [22, 37].

There is some disagreement as to whether the age of onset of the affected person changes the risk to first-degree relatives. One study found that EOAD increased the risk [116], while another study did not [37].

The number of additional affected family members probably increases the risk to close relatives, but the magnitude of that increase is unclear unless the pattern in the family is characteristic of autosomal dominant inheritance. Having two, three, or more affected family members probably raises the risk to other first-degree relatives in excess of that noted above for nonfamilial cases, although the exact magnitude of the risk is not clear. Heston et al. [43] found a 35–45% risk of dementia in individuals who had a parent with AD and a sib with onset of AD before age 70 years. Jayadev et al. [48] also report data suggesting that offspring of parents with conjugal AD (i.e., both parents affected) had an increased risk of dementia.

23.3.3.2 Risk to Family Members: EOAD

Many individuals diagnosed as having EOAD have another affected family member, although family history is negative 40% of the time [16]. Family history may be ‘negative’ because of early death of a parent, failure to recognize the disorder in family members, or, rarely, a de novo mutation. The risk to sibs depends upon the genetic status of the affected proband’s parent. If one of the proband’s parents has a mutant allele, then the risk to the sibs of inheriting the mutant allele is 50%. Individuals with EOFAD (and a mutation in APP, PS1 or PS2) have a 50% chance of transmitting the mutant allele to each child. The risk to other family members depends upon the status of the proband’s parents. If a parent is found to be affected, his or her family members are at risk.

23.3.3.3 Related Genetic Counseling Issues

23.3.3.3.1 Use of *APOE* Genotyping for Predictive Testing

In contrast to the utility of *APOE* testing as an adjunct diagnostic test in individuals with dementia, there is general agreement that *APOE* testing has limited value when used for predictive testing for AD in asymptomatic persons. Data suggest that a young asymptomatic person with the *APOE* e4/e4 genotype may have an approximately 30% lifetime risk of developing AD [12]. Further refinement of this risk reveals that females with an *APOE* e4/e4 genotype have a 45% probability of developing AD by age 73 years, whereas males have a 25% risk [13]. These risks are lower – and the likely age of onset later – for persons with only one *APOE* e4 allele (peak age 87 years) or no *APOE* e4 allele (peak age 95 years). These estimates are not generally considered clinically useful; however, a research study to assess the potential utility of *APOE* testing in relatives of individuals with LOAD is under way [37, 101]. The relatively high risk in ε4/ε4 homozygotes is notable, although this is relevant to the smallest number of persons in the general population.

*Down Syndrome***.** Family members of persons with Down syndrome are not at increased risk for AD.

23.3.3.3.2 Testing of At-Risk Asymptomatic EOAD Family Members

- *Testing of At-Risk Asymptomatic Adults*. Testing of asymptomatic adults at risk for EOFAD caused by mutations in the *PSEN1*, *PSEN2*, or *APP* gene is available clinically. Testing results for at-risk asymptomatic adults can only be interpreted after an affected family member's disease-causing mutation has been identified. It should be remembered that testing of asymptomatic at-risk individuals with nonspecific or equivocal symptoms is predictive testing, not diagnostic testing.Preliminary results have shown that while relatively few family members choose such testing, they usually cope well with the results, which can affect personal relationships and emotional well-being. [122]. However, significant depression has been reported following such testing [92].
- *Preimplantation Genetic Diagnosis.* Preimplantation genetic diagnosis (PGD) and embryo transfer have been successfully used to achieve pregnancy in a 30-year-old asymptomatic woman with an *APP* disease-causing mutation, resulting in the birth of a healthy child who does not have the *APP* disease-causing mutation identified in the mother and her family [129].

23.3.4 Management

23.3.4.1 Treatment of Manifestations

The mainstay of treatment for AD is necessarily supportive, and each symptom is managed on an individual basis [8]. In general, affected individuals eventually require assisted living arrangements or care in a nursing home.

Although the exact biochemical basis of AD is not well understood, it is known that deficiencies of the brain cholinergic system and of other neurotransmitters are present. Drugs that increase cholinergic activity by inhibiting acetylcholinesterase produce a modest but useful behavioral or cognitive benefit in some affected individuals. The first such drug was tacrine; however, this agent is also hepatotoxic. Newer such drugs with similar pharmacologic action, such as Aricept® (donepezil) [86, 113] (rivastigmine) [26], and galantamine [95], are not hepatotoxic.

Memantine, an NMDA receptor antagonist, has shown some effectiveness in the treatment of moderate to severe AD [99]. Memantine is often added to a cholinesterase inhibitor.

Antidepressant medication may improve associated depression.

23.3.4.2 Therapies Under Investigation

Treatment trials evaluating use of anti-inflammatory agents (NSAIDs), estrogens, nerve growth factors, ginkgo biloba, statins, BACE inhibitors, and antioxidants are under way or have recently been reviewed [54, 66, 67, 73, 85, 134, 135]. Attempts to ameliorate the tau pathology are also underway [41].

23.3.4.3 Other

Vitamins and other over-the-counter medications have been used in the treatment of AD [54, 73, 135].

Some, but not all, reports suggest that affected individuals taking HMG-coenzyme A reductase inhibitors for hypercholesterolemia have a reduced incidence of dementia [66, 67, 134].

Immunization of an AD mouse model with Aβ-amyloid has attenuated the AD pathology and stimulated the search for a possible vaccination approach to the treatment of human AD [110]. A human trial of this approach was halted because of encephalitis in a few subjects [18, 27, 33, 44]. Alternative approaches to immunization therapy have been proposed, including the use of antibodies to A beta peptide [91].

Thus far, treatment of symptomatic AD with estrogens has not proved beneficial [72, 76, 130].

23.3.5 Other Causes of Dementia

Differential diagnosis of AD includes other causes of dementia, especially treatable forms of cognitive decline, such as depression, chronic drug intoxication, chronic CNS infection, thyroid disease, vitamin deficiencies (especially B12 and thiamine), CNS vasculitis, and normal-pressure hydrocephalus [8]. Of these conditions, depression and chronic drug effects are the most common and are treatable with antidepressants and elimination of the drug, respectively.

Other degenerative disorders associated with dementia, such as frontotemporal dementia (FTD), Picks disease, Parkinson disease, diffuse Lewy body disease (LBD), Creutzfeldt–Jakob disease (CJD), and CADASIL, may also be confused with AD [104].CT and MRI imaging are valuable for identifying some of these other causes of dementia, including neoplasms, normal-pressure hydrocephalus, frontotemporal dementia, and cerebral vascular disease.

23.3.5.1 Frontotemporal Dementia

Frontotemporal dementia (FTD or Pick's disease) is an important cause of dementia that is less common than AD, but typically has an earlier age of onset, such that it is more common than AD in the 5–7th decades of life [28, 39]. The early symptoms of FTD are usually behavioral with a personality change. There may be apathy, loss of social inhibitions, agitation, or inappropriate behavior. Language difficulties are also common. Memory is relatively intact early in the disease, and this helps distinguish it from AD. Brain imaging studies and neuropathological findings demonstrate focal lobar atrophy of the frontal and/or temporal lobes. There is no specific treatment for FTD.

About 30% of FTD cases are familial. Two genes have been discovered that cause familial FTD. One form is produced by a variety of missense or splice mutations in MAPT (microtubule-associated protein tau) [29, 88, 97] (see Fig. 23.3.5). These mutations are autosomal dominant with high penetrance and often associated with tau aggregation, such as neurofibrillary tangles in the brain. Exon 10 may be included or spliced out of the tau mRNA, resulting in 4-repeat or

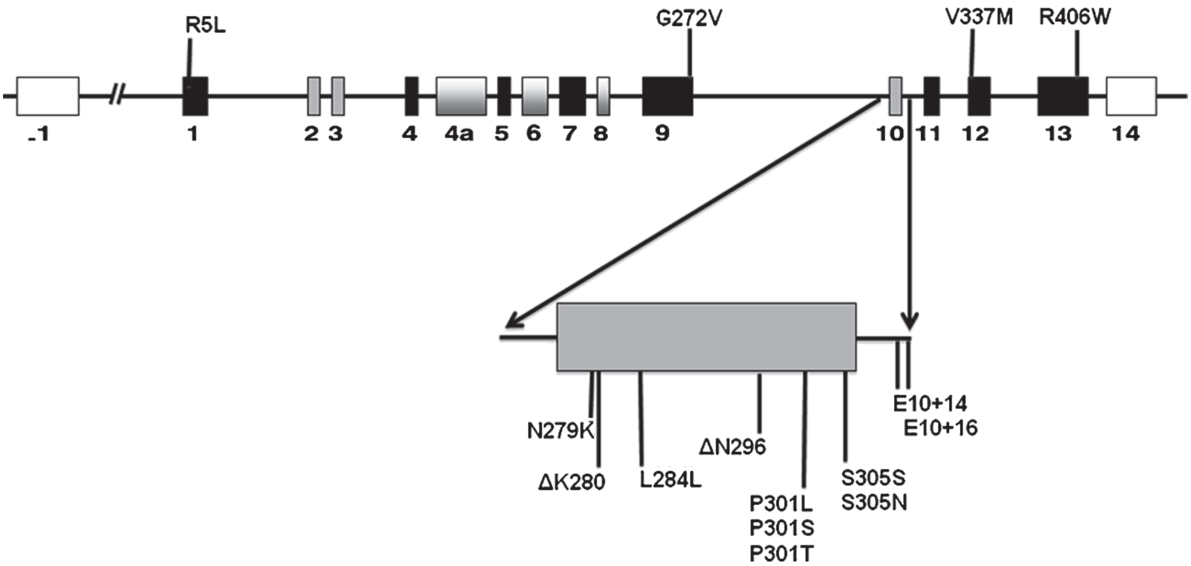

Fig. 23.3.5 MAPT (microtubule-associated protein tau) gene showing sites of several mutations causing hereditary frontotemporal dementia. Exon 10 is a hotspot for mutations and P301L and E10+16 are two of the most common. Splicing of exon 10 determines whether the resulting protein is 3-repeat or 4-repeat tau. *Numbers* below *boxes* in the *top line* refer to exons. Exon 10 is exlarged between the *arrows*. *Numbers* pointing within exons or introns indicate mutations causing FTD designated by codon and amino acid change (e.g., <P301L) or position within the intron (e.g., E10+14). (Courtesy of P. Poorkaj-Navas)

3-repeat tau protein. Many disease-causing mutations are in exon 10 or its adjacent splice site. The mutation may disrupt the function of microtubules or alter the normal 50/50 ratio of the 3-repeat and 4-repeat splicing variants of tau. The most common mutations in MAPT are P301L and E10+16.

The second gene causing FTD is progranulin (GRN) [29, 128]. This variety of FTD is associated with neuronal inclusions of TDP-43 (TARDBP43, a DNA-binding protein). Almost all of the mutations in GRN are prematurely truncating mutations that result in haploinsufficiency of the protein because of nonsense-mediated decay of mRNA [32]. The most common mutation is R493X [94]. Penetrance is reduced with some mutations in GRN, and mutations have been found in occasional sporadic cases. Because of nonsense-mediated decay there are reduced plasma levels of GRN in persons with mutations [31]. Interestingly, mutations in the TARDBP43 gene cause a familial form of ALS (amyotrophic lateral sclerosis), and not FTD [121, 126]. Other rare genetic causes of FTD have occurred with mutations in the valosin-containing protein (VCP, associated with inclusion body myositis and Paget's disease of bone) [53] and CHMP2B genes [117].

23.3.5.2 Familial Prion Disorders

Creutzfeldt–Jakob disease (CJD) is a rare progressive neurodegenerative disease caused by a conformational change in the prion protein with a prevalance of about 1 per million. There are rare familial forms of CJD, representing about 15% of all cases [34]. These familial cases are caused by a variety of missense mutations or insertions in the prion (PrP) gene [55, 58]. These mutations are autosomal dominant but may have reduced penetrance. The clinical syndrome varies considerably including typical CJD (rapidly progressive dementia with rigidity and myoclonus), a more slowly progressive dementia easily confused with AD and the rare but unusual familial fatal insomnia (FFI) [19]. The two most common mutations in PrP are E200K and V210I [56]. Homozygosity for methionine at codon 129 is a risk factor for CJD and also may affect the phenotype in familial cases. When the mutation is D178N and valine is encoded at codon 129 on the same chromosome the phenotype is typical CJD. With the same mutation (D178N) in *cis* with methionine at 129, the phenotype is FFI.

23.3.5.3 CADASIL

CADASIL (*c*erebral *a*utosomal *d*ominant *a*rteriopathy with *s*ubcortical *i*nfarcts and *l*eukoencephalopathy) is caused by missense mutations in the Notch3 gene [87]. A large number of mutations have been found to be spread throughout the 33 exons and cause an autosomal dominant disorder. The clinical characteristics are early migraine headache, followed by recurrent strokes and/or slowly progressive dementia, typically in the 5–7th decades [84]. Brain MRI shows progressive diffuse hyperintensity in cerebral white matter on T2 imaging that may be apparent years prior to any symptoms. Cerebral arterioles show abnormal histopathology of the media with osmophilic granules on electron microscopy. There is no specific treatment.

Published Statements and Policies Regarding Genetic Testing

- American College of Medical Genetics/American Society of Human Genetics Working Group on ApoE and Alzheimer's disease (1995) Statement on use of apolipoprotein E testing for Alzheimer's disease
- American Society of Human Genetics and American College of Medical Genetics (1995) Points to consider: ethical, legal, and psychosocial implications of genetic testing in children and adolescents
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Genetics of Autism

23.4

Brett S. Abrahams and Daniel H. Geschwind

Abstract We have learned more about the molecular genetics of autism in the last 3 years than in the previous 30. This includes both a new appreciation for the role of rare genetic variation and the identification of the first contributory common variants by genome-wide association. These data show that although the population attributable risk of common variation may be moderate to large, the genotype risk of common variants at the individual level are small. In contrast, a large number of diverse rare mutations of large effect have been identified, but none appear specific to autism. All of these findings point to extreme genetic heterogeneity suggesting complex gene–gene or gene–environment interactions in autism etiology. Available knowledge, reviewed below, also suggests that phenotypic presentation is the result of complex interactions, and that implicated genetic risk factors in many cases cross the boundaries of established clinical diagnostic categories. Acceptance of this complexity and efforts to understand genetic variation in terms of intermediate phenotypes represent important directions for future research.

Contents

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23.4.1 Background

Autistic disorder is characterized by specific deficits in three core domains, language, social behavior, and cognitive flexibility prior to 3 years of age although presentation varies substantially between cases with variable impairment in additional sensory, motor, and medical domains ([56], Table 23.4.1). Current practice does not typically view autistic disorder in isolation, but rather as one of several entities collectively referred to as the autism spectrum disorders (ASDs). Asperger syndrome, where language capability is relatively sparred, would be included here, as would individuals diagnosed with pervasive developmental disorder-not otherwise specified (PDD-NOS), a catch-all used to capture cases showing a subset of relevant behavioral abnormalities but not meeting criteria for autistic disorder as defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV). Childhood disintegrative disorder

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and Rett syndrome, distinguished by regression (loss of learned skills) and deterioration, respectively, represent a smaller fraction of cases but are likewise included under the ASD umbrella. The development of standardized diagnostic tools including the Autism Diagnostic Interview (ADI-R) and the Autism Diagnostic Observation Schedule (ADOS) have proved critical in characterization of cases and development of uniformity between clinical centers. Recent estimates of frequency for conditions on the spectrum have been discussed in detail elsewhere [54], but are as high as 1/150 when broadest definitions are employed [36].

As highlighted in Fig. 23.4.1, work towards an understanding of the ASDs has covered much ground over the last quarter century. Central to this progress was the shift away from attributing etiology to aspects of parenting, particularly insufficient attention or affection from mothers, a notion popularized (by the prominence of) psychodynamic theories in the 1960s. Subsequent recognition of rare chromosomal aberrations in patients together with the identification of rare syndromes in which autistic symptomatology is elevated was important here and was central for both acceptance of a medical model and the recognition of genetic factors in the ASDs.

Twin studies provide additional important support for a major role for genetic factors in autism susceptibility [11, 123]. Separate studies documenting an increase in the "broader autism phenotype" or subclinical ASD-like behavioral abnormalities amongst first-degree relatives of cases also lend support for genetic factors underlying components of the autistic spectrum [18, 21]. The importance of genetic factors was further highlighted by reports that an autism diagnosis in a first child was associated with a ~10% recurrence rate in subsequent progeny, a significant increase over population levels [108]. This said, early genetic studies were quick to point to significant genetic heterogeneity [72] and question whether genetic factors may govern subcomponents of disease, rather than inheritance of the clinical entity as a whole [53]. Although much has been learned in recent years, such questions remain topical and will be revisited below.

Although the overall focus here is to review current knowledge regarding genetics, consideration of how environmental contributions may likewise impact

Table 23.4.1 Domains of impairment in the autism spectrum disorders (ASD)[a] (reprinted, with permission, from the Annual Review of Medicine, Volume 60 2009, by Annual Reviews, www.annualreviews.org) [56]

Domain	Autism	Asperger	PDD-NOS[b]	ASD
Social communication	Required	Required	Required	
Language	Required	–	Variable	
Repetitive and/or restrictive behaviors	Required	Required	Variable	
Sensory abnormalities[c]	>90%	80%	Variable	94%
Developmental regression[d]	15–40%	?	?	15–40%
Motor signs[e]	60–80%	60%	60%	60–80%
Gross motor delay	10%	?	?	5–10%
Sleep disturbance	55%	5–10%	40%	50%
Gastrointestinal disturbance[f]	45%	4%	50%	4–50%
Epilepsy[g]	10–60%	0–5%	5–40%	6–60%
Comorbid psychiatric diagnosis[h]	70%	60%	>25%	25–70%

[a] Diagnostic features are denoted as required, while those that are not observed are denoted with a dashed line. There have been few large-scale, epidemiologic studies of features associated with the ASDs, so that the frequencies presented above are conservative estimates based on an amalgamation of information from various references [12, 59, 63, 71, 77, 84, 88, 94, 119, 131, 138, 146]. The ASD column at the far right provides estimates for the broad group of related conditions, which includes autism, Asperger syndrome, and PDD-NOS

[b] Pervasive developmental disorder, Not otherwise specified (PDD-NOS) is a defined as a condition in which some, but not all, features of autism or another defined pervasive developmental disorder are present

[c] Responses to sensory stimuli (typically auditory or tactile) different to those observed in typically developing children

[d] Loss of function in either language and social skills (or both)

[e] Motor signs include hypotonia, gait problems, toe walking and apraxia

[f] Six months or more of diarrhea, constipation, reflux, or bloating

[g] The presence of epilepsy varies as a function of other co-morbid features resulting in a relatively large range

[h] Includes mood disorders, conduct disorders, aggression, and attention deficit/hyperactivity disorder (ADHD)

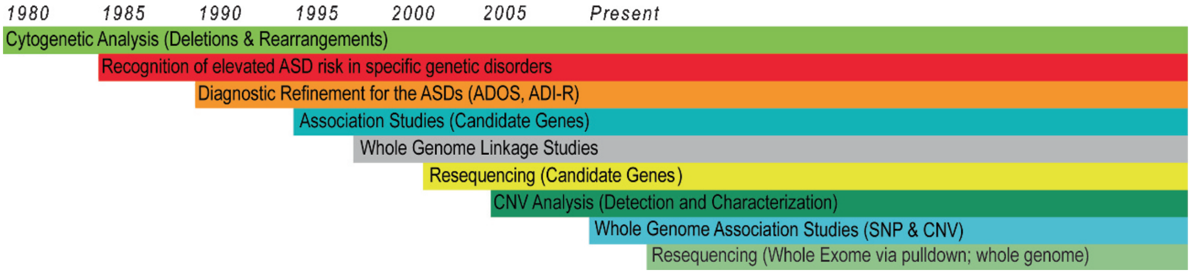

Fig. 23.4.1 Methodological changes have accelerated progress in ASD genetics. Collection of large cohorts via international collaboration, together with array-based technologies enabling genome-wide interrogation of variation, has resulted in major advances. Similar progress will come from massively parallel sequencing of partial and whole genomes. Although such experiments are soon likely to become routine, interpretation of results, particular in the context of diverse phenotype data, will require substantial computational infrastructure. (Reproduced from *Archives of Neurology*, with permission [1])

risk is warranted. Early evidence for a viral link, particularly rubella, suggests that maternal infection in early child development may be contributory [46]. Later work indicates that an array of pre- and perinatal complications is likewise increased in cases [60], but does not highlight specific complications that are obviously increased in cases versus controls. Increased maternal and paternal age have also been implicated [34, 107], although the manner by which having an older parent may come to influence risk remains unclear. It is tempting to speculate that older parents may generate offspring who as a group harbor *de novo* copy number variations (CNVs) (for explanation, see 3, Sect. 3.4.4.4) at an increased frequency but the contribution of maternally derived chromosomal anomalies and paternally derived point mutations must also be considered. Such data are important in the context of an apparent increased prevalence in the ASDs over recent years [54]. Although attributable at least in part to cultural factors – including a broadening of diagnostic criteria and reduced stigma – such effects are unlikely to account for the full extent of the observed increase [66]. Moving forward, our understanding of how risk is modulated and presentation shaped will no doubt benefit from joint consideration of genetics and environment.

Here we review progress in identification of genetic contributions to ASD, ranging from genetic syndromes resulting from rare point mutations and/or copy number variation (CNV) to common genetic variants established to be contributory. One key theme is that although Mendelian mutations clearly play a larger part than previously suspected, some highly associated rare or recurrent mutations show patterns of inheritance more consistent with complex inheritance. Stated otherwise, although major effect alleles exist and play an important part in modulating risk, most if not all of these appear to operate in concert with ancillary factors, environmental or genetic, to shape ultimate presentation.

23.4.2 Cytogenetic Findings

For many years, cytogenetic identification of rare chromosomal abnormalities in cases served as the principal means by which disease-related regions could be isolated [140]. Although typically spanning many megabases, encompassing 10 or more genes, and in many cases complex, such anomalies are estimated to be present in 6–7% of ASD children [90]. This rate increases if ascertainment is limited to individuals with dysmorphic features and intellectual disability but none of these variants or other "syndromic" causes of autism account for more than 1% of ASD, and most are rarer (Table 23.4.2). Although it is well recognized that large maternally derived duplications at 15q11-13 explain the etiology in an estimated 1–2% of cases [41], establishing such relationships for less frequently observed structural variants is challenging.

An additional issue is that because cytogenetically visible events encompass a large number of genes, clear involvement of a particular genomic region does not typically reveal individual molecules which may be contributory. This is important because identification of individual genes that underlie the effects of such variation is necessary to provide insights regarding mechanisms. Within the core 15q11-13 interval, involvement of each of *UBE3A* (a ubiquitin ligase) and

Table 23.4.2 ASD-related syndromes[a],* (modified from [3])

ASD-related syndrome	Associated gene(s)	Proportion with ASD	Proportion ASD with syndrome	References
1q21 Duplication	Many	50%	~1%?	[91, 128]
3p Deletion / duplication	*CNTN4*	<50%	~1%	[51, 61, 110]
15q Duplication (maternal)	Many (including *UBE3A, GABRB3, SNRPN,* and *SNURF*)	High	~1%	[41]
15q13 Deletion	Many (including *CHRNA7)*	<50%	Unknown	[15, 118]
16p11 Deletion	Many (including *SEZ6L2*)	High	~1%	[78, 79, 90, 144]
22q11 Deletion (aka VCFS / DiGeorge)	Many (including *TBX1* and *COMT*)	15–50%	<1%	[52, 139]
22q13 Deletion	*SHANK3*	High	~1%	[48, 89, 95]
Angelman (15q11-13)	Maternal *UBE3A*	40–80%	<1%	[22, 102]
Beckwith Weidemann (11p15)	*IGF2* and *CDKN1C*	~7%	Unknown	[73]
Cortical dysplasia focal epilepsy (7q35-36)	*CNTNAP2*	70%	Negligible	[68, 125]
Cowden/BRRS (10q23)	PTEN	20%	>10% with macrocephaly	[101, 135]
Down (trisomy chr.21)	Many	6–15%	Unknown	[86]
Fragile X (Xq27)	*FMR1*	25% of males 6% of females	1–2%	[64]
Potocki-Lupski (17p11)	Many (including *RAI1*)	~90%	Unknown	[106]
Smith–Lemli–Optiz (11q13)	*DHCR7*	50%	Negligible	[129]
Prader–Willi (15q11-13)	Paternal deletions	20–25%	Unknown	[45]
Rett (Xq26)	*MECP2*	N/A	~0.5%	[5]
Timothy (12p13)	*CACNA1C*	60–80%	Negligible	[120]
Tuberous sclerosis (9q34 and 16p13)	*TSC1, TSC2*	20%	~1%	[10]

**BRRS* Bannayan-Riley-Ruvalcaba syndrome, *CACNA1C* calcium channel voltage-dependent L type alpha 1C subunit, *CDKN1C* cyclin-dependent kinase inhibitor 1C, *CNTN4* contactin 4, *CNTNAP2* contactin-associated protein-like 2, *DHCR7* 7-dehydrocholesterol reductase, *FMR1* fragile X mental retardation 1, *GABRB3* GABA A Receptor, beta 3 subunit, *IGF2*, insulin-like growth factor 2, *MECP2* methyl CpG-binding protein 2, *PTEN* Phosphotase and tenoin homolog deleted on chromosome 10; *RAI1*, retinoic acid-induced 1, *SEZL6* seizure-related 6 homolog (mouse)-like 2; *SHANK3* SH3 and multiple ankyrin repeat domains 3; *SNURF,* SNRPN upstream reading frame; *SNRPN* small nuclear ribonucleoprotein polypeptide N, *TSC1* tuberous sclerosis 1, *TSC2* tuberous sclerosis 2, *UBE3A* ubiquitin protein ligase E3A, *VCFS* velocardiofacial syndrome

[a] The reader should compare values listed above to ASD prevalence in the general public (0.2–0.7%) and among individuals with nonsyndromic MR (~15%) [36]. Other etiologically heterogeneous clinical entities that show unexpectedly high overlap with the ASDs include: bipolar disorder, epilepsy, Joubert syndrome, schizophrenia, specific language impairment, and Tourette syndrome

GABRB3 (an inhibitory neurotransmitter receptor subunit) is well established. Additional GABA receptors (A5 and G3 subunits) and the imprinted *SNURF-SNRPN* transcripts receive less attention but are also likely to be contributory. This is further complicated by independent structural variants on either side of this core region, which likewise appear to modulate risk. Amongst particularly intriguing candidates here are (*CYFIP*) the cytoplasmic FMR1 interacting protein 1 [19, 47, 98, 100, 122], *MAGEL2* involved in regulation of circadian rhythms [29, 76], the neurexin adaptor molecule *APBA2 / MINT2* [16, 75, 99], the nicotinic acetylcholine receptor subunit *CHRNA7* [118], and a related hybrid gene *CHRFAM7A* [44]. Dissection of how individual molecules contribute independently and together to clinical variation in cases represents an important set of problems for the future.

As discussed below, additional variation within such intervals or modifiers elsewhere in the genome are likely to further regulate risk and presentation even in the face of major effect alleles. Along these lines recent work has complicated the relatively simple interpretation that deletions involving the distal portion of 22q are attributable to the postsynaptic scaffolding molecule *SHANK3* [48]. Despite the fact that losses encompassing *SHANK3* variants are present in an estimated 1% of ASD cases [95], subsequent identification of comparable deletions in typically developing children [61] suggests that additional, as yet unknown, factors must be present to give rise to disease. Similarly, although cytogenetic variation at 7q first identified a possible involvement of the cell adhesion molecule *CNTNAP2* in the modulation of neuropsychiatric phenotypes [136], subsequent work suggests that heterozygous disruption of this gene can be observed in phenotypically normal individuals [14]. As reviewed elsewhere [140], other important regions including 2q37, 5p15, 17p11, and Xp22 shown to harbor cytogenetic lesions in multiple ASD cases represent important targets for further evaluation.

23.4.3 Linkage

Linkage studies have yield mixed results with regards to the identification of ASD loci [3], but the approach was invaluable in the localization of genes underlying ASD-related syndromes (see Table 23.4.2 and below). Genetic heterogeneity likely underlies the observation that increased sample size appears to confer only modest gains in autism linkage studies (Fig. 23.4.2). For example, no locus met criteria for genome-wide significance in the largest linkage study published to date [128]. And yet regions highlighted by this study, including 11p12-p13, may prove to be interesting in prioritization of emerging candidate genes. Other loci have been identified and replicated in multiple studies (e.g., 7q and 17q11–17q21) [9, 33, 130], and although contributory variants have been observed at each of 7q [4, 7, 13, 23, 30, 58, 87, 114] and 17q [126, 127, 143], no single common variant in either region can account for the observed linkage signals. These results suggest that numerous alleles, potentially in multiple different genes, are likely contributory. Strategies designed to enrich for homogeneity amongst cases [28, 96, 124] and employ ASD-related endophenotypes such as cognitive flexibility, social behavior, or language performance [4, 43, 49, 145] are likely to prove useful in future work employing a range of approaches.

23.4.4 Syndromic ASDs

Multiple rare genetic syndromes have been associated with the ASDs [148]. An important distinction here, however, is that in contrast to the cytogenetically recovered loci discussed above, the availability of relatively larger numbers of unrelated probands has permitted the identification of the underlying molecular deficit in a growing number of such entities (Table 23.4.2). Best known is fragile X syndrome, with mutations in the gene for the RNA-binding protein *FMR1* observed in 1–2% of individuals with an ASD. Because only ~25% of boys with *FMR1* mutations meet diagnostic criteria for an ASD, results again suggest that multiple factors are at play. Results are similar for individuals with tuberous sclerosis, which is attributable to autosomal dominant mutations in either *TSC1* or *TSC2*. Although *TSC1/2* mutations are present in an estimated 1% of ASD cases, amongst individuals

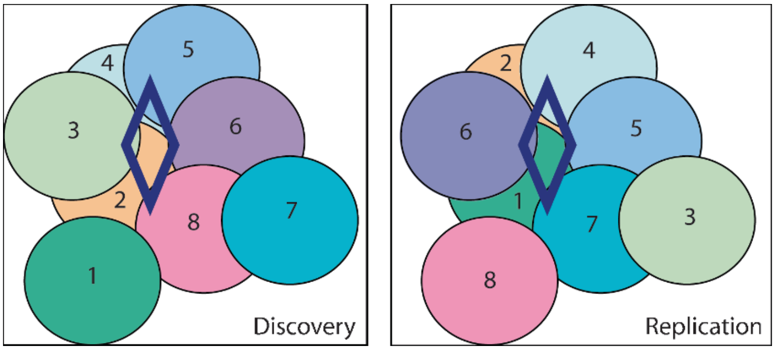

Fig. 23.4.2 Extreme heterogeneity is likely to impede replication of true risk alleles. Known and yet to be identified ASD risk alleles (*colored circles*) each appear to contribute to only a subset of cases. Although allele frequencies are stable across populations (*outer squares*), enormous variation is observed when sampling is incomplete (*blue diamonds*). Thus, alleles that appear to be over-represented amongst cases in a discovery cohort (*orange; left*) may fail to replicate on follow-up (*orange; right*). Larger cohorts and statistical methods designed to address this heterogeneity will be required. (Concept and execution by Veronia J. Vieland, PhD, Battelle Center for Mathematical Medicine, The Research Institute at Nationwide Children's Hospital)

with tuberous sclerosis only ~20% present with an ASD. Angelman syndrome, most often resulting from the *de novo* loss of the maternal allele at 15q11-13, is likewise observed in ~1% of ASD cases. In contrast to the near 1:1 complete correspondence between maternal *duplications* at this locus and an ASD diagnosis, however, maternal *deletions* appear to give rise to autism in only an estimated 40% of carriers. Additional syndromes, including Down syndrome and neurofibromatosis, show a lower incidence of autistic disorder (~6 and 4%, respectively) but are significantly elevated over baseline levels, which are estimated at ~0.2%. Increased penetrance with regard to a spectrum diagnosis has been reported for less frequently encountered clinical entities, but additional cases will be required here to obtain stable estimates. All told, known syndromic conditions such as those referenced in Table 23.4.2 some of are likely to account for ~15–20% of ASD cases. Identification of the molecules underlying some of these conditions has enabled functional investigation of defined signaling pathways. Discussed below are separate findings obtained through the use of reverse genetics in which genotypes are used to define patient groups with subsequent effort directed towards understanding the associated phenotype.

23.4.5 Re-sequencing

Sequencing of candidate loci, based on linkage or cytogenetic findings [70], along with genome-wide characterization of structural variation [see below] has likewise identified rare variants at multiple loci enriched in cases versus controls. The emerging "genotype first paradigm" is an approach likely to gain even further prominence as sequencing throughput is increased. Also key and reviewed below is the growing recognition that individual variants map only imprecisely onto individual clinical entities, necessitating the collection and analysis of more detailed phenotypic data in cases and controls.

Identification of coding mutations in Neuroligin 3 (*NLGN3*) and Neuroligin 4 (*NLGN4X*) was an important advance that served to focus attention towards the synapse [149]. It was in this context, for example, in which rare missense mutations in the neuroligin interactor Neurexin 1 (*NRXN1)* [25] were identified in cases [50]. Subsequent recognition of de novo deletions at *NRXN1* reinforced the importance of variation at this locus [128] and serve to support an important role for Neuroligin-Neurexin signaling in the ASDs. Such data also helped to make sense of frameshift mutations [68, 125] in the neurexin family member *CNTNAP2* [105]. Recessive inheritance of such mutations in the Amish present with a congenital epilepsy characterized by developmental brain abnormalities, language regression, and an ASD diagnosis in a majority of cases. Likewise, the identification of *SHANK3* as an important modulator of risk [48] was facilitated by the biological plausibility of this candidate given the demonstrated role of other molecules involved in synaptic function. Although these and other rare variants have been important clues in ASD pathophysiology, they are infrequent causes of the ASDs [13, 147]. Given that events in these molecules can also be associated with a diverse number of outcomes including: intellectual disability without features of autism [80], schizophrenia [75, 111], Tourette syndrome [81, 136] and typical development [61, 74], variation at individual loci is unlikely to provide sufficient information for accurate prediction of clinical presentation. The converse – that joint consideration of multiple loci along with relevant environmental risk factors will increase information content – is also true and will be discussed below.

As is hopefully clear from the above discussion, the identification of *NLGN3/4* mutations was an important advance in ASD. At the same time, this discovery has come to so dominate the field that appropriate consideration of molecules operating in distinct signaling pathways has arguably suffered as a result. Support for PI3K-AKT related molecules, for example, merits increased attention. Central here is the finding that an estimated 20% of heterozygous carriers of germline mutations in the phosphatase and tensin homologue deleted on chromosome 10 (*PTEN*) present with an ASD and macrocephaly [27]. Strikingly, and in contrast to other rare variants discussed thus far, available estimates indicate that heterozygous mutations in *PTEN* may be present in as many as 10% of individuals with an ASD diagnosis and macrocephaly [101, 135]. That the receptor tyrosine kinase *MET* (discussed below) and each of *TSC1* and *TSC2* (discussed above) likewise act on PI3K signaling is notable [82]. Recent evidence tying *CACNA1C*, the L-type calcium channel subunit mutated in Timothy syndrome [120], to this same pathway [35] provides additional potential coherence at the molecular level. Whether or not additional calcium channels implicated in the ASDs (e.g., *CACNA1G* [126], and *CACNA1H* [121]) modulate risk by acting on this same pathway remains to be seen. Current notions of these pathways are likely to be primitive but may nevertheless prove useful in organizing emerging results.

MECP2, the gene mutated in Rett syndrome, likewise appears to provide important links between key molecular entities, a somewhat unexpected finding given the phenotypic differences between girls with this disorder and those with autism. That males harboring *MECP2* duplications [134] present with MR suggests that subtle dysregulation of gene expression in either direction can interfere with normal brain development. A relationship to the 15q11-13 locus is supported by the observation that a subset of *MECP2* mutation carriers present clinically with an Angelman-like phenotype [93]. Additional convergence comes from the fact that genes within the Angelman region are dysregulated not only in Rett Syndrome but also in idiopathic autism [115]. Finally, *MECP2* can also be connected to *CADPS2,* a Ca2+ dependent modulator of *BDNF* release, in which rare missense variants are overrepresented in cases relative to controls [114]. The finding that *BDNF* is a direct target of *MECP2* [39], and observed to be an important modulator of disease progression in a mouse model of Rett syndrome [37], provides important additional support for the notion that these independently identified molecules may operate together at a functional level.

Examination of familial segregation of mutations at loci identified by re-sequencing suggests that individual variants are largely insufficient to independently give rise to an ASD. For example, although mutations in axonal initial segment localized sodium channel subunits *SCN1A* and *SCN2A* were seen to be significantly overrepresented in cases relative to controls [142], such variants were observed both in affected children and in their parents. A similar overrepresentation of rare missense variants in the T-type calcium channel subunit *CACNA1H* has likewise been observed in cases compared with controls [121]. Despite the demonstration that individual variants altered channel activity, mutations were again seen both in cases and unaffected relatives. The point here is not to argue that these rare variants are unrelated to disease, but rather underscore the point that many (if not all) risk variants show an imprecise mapping onto affection status. As elegantly noted elsewhere, "[O]nly when penetrances are well above 50% does one approach a familial concentration that begins to look like a standard Mendelian segregation" [20].

23.4.6 Copy Number Variation

Most of the points raised above in the context of re-sequencing are also applicable to analyses of structural variation. Although resolution is much reduced relative to sequencing, the entire genome can be interrogated in parallel using array-based technologies, obviating the need for candidate-driven strategies. Considerable excitement was recently garnered by the identification of recurrent de novo variation at 16p11, observed in an estimated 1% of cases [78, 90, 144]. Subsequent identification of duplication and deletion events in healthy controls [17, 61], however, points again to oligogenic mechanisms, incomplete penetrance, and variable expressivity, consistent with genetic complexity.

Another relatively new finding is that of individuals harboring characteristic 1.5-Mb deletions at 15q13.3 involving *CHRNA7* and the subsequent elucidation of a relationship to intellectual ability and epileptiform abnormalities [118]. Again, however, diverse clinical manifestations of this single variant have been reported, with carriers also showing either schizophrenia [67, 122], autism [15], or typical development [133]. These results underscore the need to understand individual variants in the context of modifiers which may shape presentation.

Other recently published work [26, 29, 61] points to additional loci of interest. Cai et al. [29] determined that duplications encompassing the *ASMT* gene, the last enzyme required for melatonin synthesis, were elevated in cases versus ethnically matched controls ($p=0.003$ by Fisher's exact test). These results are intriguing in the face of a related study highlighting the relationship between common variation at this locus and each of autism and melatonin levels [92]. Glessner et al. [61] report enrichment of CNVs proximal to each of neuronal cell-adhesion and ubiquitin-degradation molecules. Novel ASD loci were also identified, including a region at 2p24.3 near the uncharacterized cDNA AK123120, which was observed to harbor variants in cases more frequently than in controls ($p=3.6\times10^{-6}$; $OR=5.5$). Our own investigations [26] prioritized exonic CNVs in an attempt to identify variants most likely to interfere with gene function. These analyses led to case-specific in both *BZRAP1*, an adaptor molecule known to regulate synaptic transmission ($p=2.3\times10^{-5}$) and *MDGA2* ($p=1.3\times10^{-4}$), a cell adhesion molecule with striking structural similarity to Contactin 4 [51, 110]. Each of these results is intriguing and should be explored in additional cohorts. That hundreds of distinct rare variants were each seen only in a single case, however, suggests that massive cohorts will be required to identify the subset of rare alleles relevant to disease.

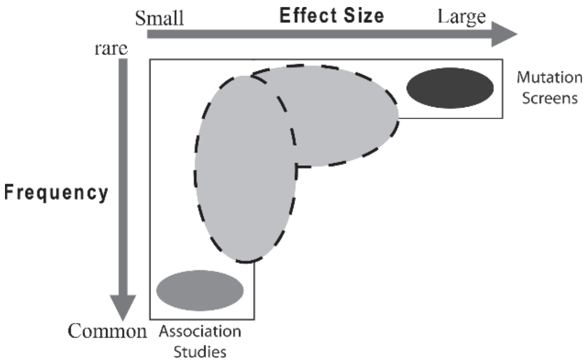

Fig. 23.4.3 Current studies typically attend to two extremes on the allelic spectrum**.** SNPs with modest effects sizes (*small gray oval*) have been identified through association studies. Rare events that segregate with disease have also been identified through re-sequencing efforts and CNV studies (*small black oval*). Less attention has been directed at events not at either extreme, but also likely to modulate risk. Larger studies will permit consideration of intermediate effect alleles (*ovals with dotted lines*). Although available data do not support the presence of common alleles of large effect for affection status (*empty bottom right corner*) it is possible that such associations may be observed in the future when disease-related endophenotypes, measuring aspects of social behavior or language performance are employed as quantitative endpoints

Unlike re-sequencing studies discussed above, it can be challenging in CNV analyses to define individual genes within regions of interest that may be contributory. This is made all the more complicated by the fact that clinical outcomes may in some cases be attributable to multiple genes within a region. Moreover, multiple distinct variants – likely to differ in effect size and function – can be observed at particular loci of interest (Figs. 23.4.3 and 23.4.4). Results obtained from sequencing candidate genes at 16p11 [79] are consistent with independent contributions from multiple genes including *SEZ6L2*.

International consortia established to make such data available to investigators online will facilitate interpretation of how rare variants come to shape presentation. Such work will likewise benefit from the ongoing collection and characterization of large patient cohorts, particularly when associated data and biomaterials are made available to the entire research community, as is the case with the Autism Genetics Resource Exchange (AGRE) [57].

To round out this discussion of rare variations it should be emphasized again that most individuals harbor multiple rare variants and the presence of individual events in a single patient with a common disease need not be meaningful. That de novo events are observed in ~1% of controls [116] is important here and demands caution in the attribution of observed effects to individual variants. Nevertheless, that such events are increased in frequency in simplex cases (7–10%) suggests that as a group such variants are indeed contributory [90, 116]. The same concern arises in consideration of rare point mutations. For example, it is well established that premature truncation in the

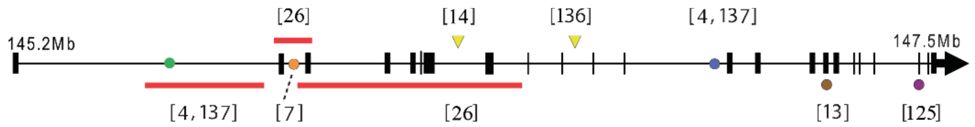

Fig. 23.4.4 Single genes are likely to harbor a variety of functionally distinct alleles. Individuals homozygous for a truncating mutation in *CNTNAP2* (*purple circle*) present with intellectual disability, cortical dysplasia, focal epilepsy, and behavioral abnormalities [125]. Chromosomal translocations at this locus [14, 136] have also been observed (*yellow arrowheads*) as have CNVs (*red lines*) which eliminate exons [26] or an apparently functional *FOXP2*-binding site (*green circle*) [4, 137]. Although potentially contributory, none of these variants are sufficient in the heterozygous state to cause disease, as each has been observed in unaffected parents. Less clear, but of substantial interest, are rare amino acids substitutions (e.g., I869T; *brown circle*) observed in cases [13]. In support of still further complexity, separate SNPs within *CNTNAP2* have been associated with different endpoints. rs7794745 (*orange circle*) is associated with affection status [7], whereas rs2710102 (*blue circle*) is associated with language-related measures [4, 137]. Given that each of these risk alleles is common it may be that the two interact in at least some individuals. That similar effects have been described at other loci suggests that the relationship between genetic variation and outcome is complex even when considering only single genes. Numbers in square brackets identify studies listed in the reference section in which variants were identified or characterized

X-linked calcium channel subunit *CACNA1F* results in congenital night blindness. Less straightforward, however, is whether a gain of function allele at this locus contributes to intellectual disability and autism in a subset of males in from a large family New Zealand [65]. Involvement is plausible, given evidence implicating other related calcium channel subunits, [e.g. 120] but the question is one that requires additional attention. Clear to us is that an integration of results from risk loci across the genome will be required for accurate risk assessment within individuals.

23.4.7 Common Variation

Many candidate genes have been studied, but few replicated. In addition to loci reviewed elsewhere [3], linkage and association at *PRKCB1* support potential involvement in disease risk [103]. Results are particularly compelling given replication in an independent cohort [85]. Additional regionally directed screens point to common variation within *CACNA1G* [126], a T-type voltage gated calcium channel subunit, as well as *DOCK4* [87] observed to regulate dendritic morphogenesis in the hippocampus [132]. As emphasized in these reports and discussed above, however, observed associations are insufficient to account for linkage.

Exciting additional clues are also emerging from initial analyses of recently published GWA data [141]. Wang et al. employed Illumina HumanHap550 data for 2,000 Caucasian cases and contrasted allele frequencies against almost 6,500 unrelated controls of European origin. Not only was a SNP at 5p14.1 found to meet genome-wide significance (rs4307059, $p=3.4\times10^{-8}$), but additional SNPs within a 100 Kb linkage block also showed P-values less than 1×10^{-4}. Importantly, association at this intergenic locus between cadherin 9 and cadherin 10 was confirmed in an additional two independent sets with combined p-values ranging from 7.4×10^{-8} to 2.1×10^{-10}. As is the case with all common variants associated with disease, much work will be required to understand how variation within this intergenic region may act to impact presentation. Of potential utility, however, is the observation that *CDH10* is present at high levels in the developing frontal cortex, a result similar to that observed previously for *CNTNAP2* [2, 4].

Qualitatively similar results at a nonoverlapping locus were obtained from analysis of 1,000 partially overlapping multiplex families genotyped on Affymetrix 5.0 arrays [6]. Although no associations in this initial cohort met genome-wide significance (rs10513025, $p=7.3\times10^{-7}$), focused follow-up in additional families resulted in a genome-wide significant effect ($P=6\times10^{-9}$) at an intergenic region between *TAS2R1* and *SEMA5A*. Although the identity of the underlying causal variant again remains unclear, differential expression of *SEMA5A* in lymphoblasts from cases versus controls supports the hypothesis that the GWAS-identified variant modulates risk by altering transcript levels. This result is of particular interest together with an established interplay between *SEMA5A* and *PLXNB3* [8], in which common variation has been associated with language performance and a reduction in white matter volume [112]. That PLXNB3 likewise interacts with ASD-associated MET [31, 32, 40] suggests that these results may serve to further PBK-AKT refine the discussed above.

Availability of these genome-wide data should also obviate the need for exploratory studies at individual loci. Because different results were obtained from each study despite partially overlapping cohorts, it remains debatable, however, the extent to which these initial results will predict future observations. Differences between the arrays employed and SNPs interrogated are certainly important contributory factors here. Incomplete sampling is also likely to be an important issue (Fig. 23.4.2), and calls for the assembly and analysis of larger cohorts. Although these and other SNPs yet to be identified may only modulate overall risk in subtle ways, the impact on population prevalence is likely to be substantial. For each, identification of the underlying causal variant(s) tagged by the anonymous markers interrogated represents an important next step. If results at *CNTNAP2* (Fig. 23.4.4) generalize to other parts of the genome, individual genes may harbor multiple variants, common and rare, with strongest associations to distinct aspects of presentation [4, 7, 137].

23.4.8 Towards Convergence

It is critical to consider, as we have emphasized above, scenarios in which individual genetic variants are not strongly associated with an ASD diagnosis, but rather to intermediate phenotypes that collectively define ultimate presentation. This paradigm is strongly supported by the observation that first-degree relatives of autistic probands are often enriched for features of the "broader autism phenotype" [21, 83, 104, 145] characterized by subclinical language dysfunction, autistic-like social abnormalities, or increased behavioral rigidity relative to unrelated controls. That study of such intermediate phenotypes [62, 109] has proven useful in the identification of ASD risk loci [38, 117] provides further support for such models. In addition, this model predicts that such variants will contribute both to normal variation and risk for neurodevelopmental conditions that are considered clinically distinct [55]. This is certainly supported by work at the *CNTNAP2* locus which appears modulates language function across in both the ASDS and SLI [4, 137].

From this perspective, the ASDs may best be conceptualized as the end result of multiple rare and common alleles that act in combination to shape different aspects of cognition and behavior (Fig. 23.4.5). Available data are further consistent with the notion that current diagnostic classifications do not adequately capture the underlying etiologies [24, 42, 137]. Careful examination of patient records supports a similar interpretation – that individual genetic risk factors may ultimately predispose to a range of related clinical conditions [113]. As discussed elsewhere [1], this is particularly important in the context of the developing brain, whose circuits should not be expected a priori to show good correspondence to clinically defined disease boundaries.

Thus, genes for which a relationship to ASD is established should be considered as potential candidates for related disorders of cognition including specific language impairment, schizophrenia, bipolar, and mental retardation (and vice versa). Evaluation of how individual variants linked to different disorders are related to underlying endophenotypes will be of criti-

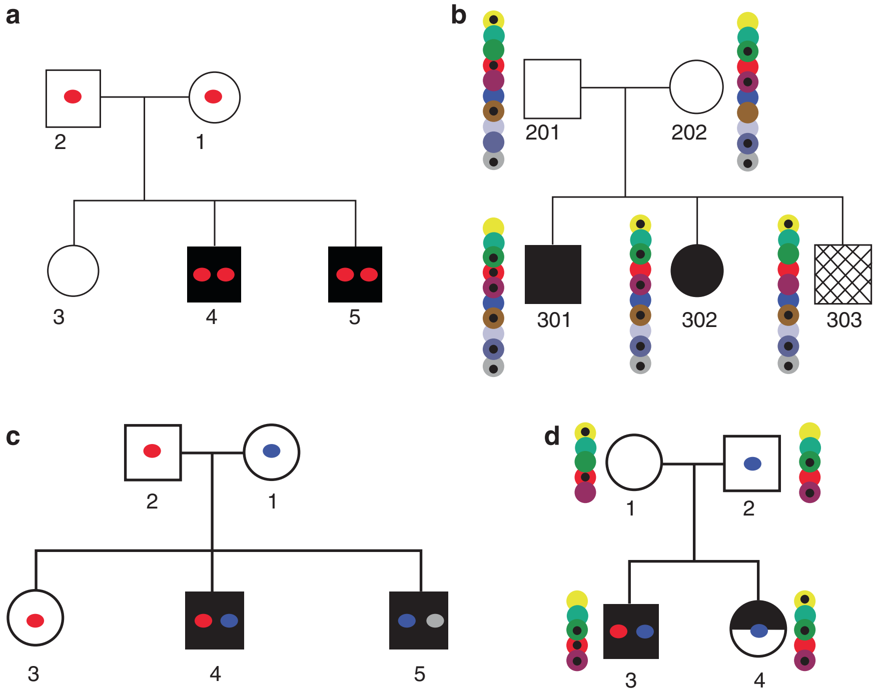

Fig. 23.4.5 Genetic models should conform to available data. Examples of rare alleles which segregate with disease in a Mendelian fashion (**a**) and common variants associated with smaller effects (**b**) are well established. Less widely considered, however, are scenarios in which rare variants of major effect show incomplete penetrance (**c**). Deletions at *NRXN1* and 16p11 appear to fall in this category with at least a subset of carriers showing normal development. Illustrated in **C** is a family in which no rare variant of major effect (*red*, *blue*, or *gray circle*) appears sufficient to cause disease; instead, all affected individuals carry multiple risk alleles. More complicated still, and most likely with regards to the ASDs, is a scenario in which alleles of varying effect interact to shape presentation and determine affection status (**d**). *Circles* and *squares* correspond to females and males, respectively. *Filled* or *hatched* shapes highlight affected individuals. *Colored circles* alongside or within individuals represent alleles of minor and major effect, respectively

cal importance for explaining the relationships between such conditions. It should be emphasized also that the ever-growing number of genes (Table 23.4.3) makes it increasingly difficult to keep track of the manner by which different kinds of variation contribute to risk. Fortunately, however, it seems that at least a subset of molecules now implicated in pathogenesis can be connected at the level of molecular signaling. Emerging

Table 23.4.3 Evidence scores for *promising* and for *probable* ASD genes[a] (modified from [3], with permission)

Gene	Total score	Syndrome/ mutations	Replicated association	Analysis of variant	Mouse model	Other evidence
Promising						
BZRAP1	1	0	1	0	0	
CACNA1G	1	0	1	0	0	No
DISC1	1	0	0	0	1	No
DOCK4	1	0	1	0	0	Involved in regulation of hippocampal dendrite morphology
ITGB3	1	0	1	0	0	No
MDGA2	1	0	1	0	0	No
PRKCB1	1	0	1	0	0	No
AHI1	2	2	0	0	0	No
ASMT	2	0	1	0	0	ASD-associated variant also associated with melatonin levels
AVPR1A	2	0	0	0	1	Dysregulation in plasma of cases versus controls
CACNA1H	2	0	1	1	0	No
CNTN4	2	2	0	0	0	No
GRIK2	2	0	1	0	0	Homozygous mutation results in nonsyndromic MR
SCN2A	2	0	1	1	0	No
SLC25A12	2	0	1	0	0	Associated with neurite outgrowth; up-regulated ASD brain
Probable						
EN2	3	0	1	1	1	No
MET	3	0	1	1	0	Expression reduced in brains of cases versus controls
NRXN1	3	2	0	0	0	Interacts functionally with neuroligins
OXTR	3	0	1	0	1	Expression reduced in blood of cases versus controls
SHANK3	3	2	0	0	0	Modulates glutamate-dependent reconfiguration of dendritic spines
SLC6A4	3	0	1	1	0	Clinical benefit from inhibitors; variation linked to gray matter volume
CACNA1C	4	2	0	1	0	Linked to PI3K signaling along with *PTEN / TSC1 / TSC2*
CADPS2	4	2	0	1	1	No
CNTNAP2	4	2	1	0	0	Downstream target of *FOXP2*
DHCR7	4	2	0	1	0	Hypocholesterolemia in a proportion of probands
FMR1	4	2	0	1	1	No
NLGN3	4	2	0	1	1	No
NLGN4X	4	2	0	1	1	No
GABRB3	5	2	1	0	1	Expression is dysregulated in PDDs
MECP2	5	2	0	1	1	*MECP2* deficiency causes reduced expression of *UBE3A* and *GABRB3*
PTEN	5	2	0	1	1	Linked to PI3K signaling along with *TSC1 / TSC2 / CACNA1C*
TSC1	5	2	0	1	1	Regulates dendrite morphology and function of glutamatergic synapses
TSC2	5	2	0	1	1	Regulates dendrite morphology and function of glutamatergic synapses
UBE3A	5	2	0	1	1	Expression is dysregulated in PDDs
RELN	6	2	1	1	1	Levels reduced in brains of cases versus controls

(continued)

Table 23.4.3 (continued)

AHI1 Jouberin, ASD autism spectrum disorder, *ASMT* acetylserotonin *O*-methyltransferase, *AVPR1A* vasopressin V1a receptor, *BZRAP1* benzodiazapine receptor (peripheral), associated protein 1, *CACNA1C* calcium channel, voltage-dependent, L type, alpha 1C subunit, *CACNA1G* calcium channel, voltage-dependent, T type, alpha 1G subunit, *CACNA1H* calcium channel, voltage-dependent, T type, alpha 1H subunit, *CDH9* cadherin 9, *CDH10* cadherin 10, *CADPS2* calcium-dependent secretion activator, 2, *CNTN4* contactin 4, *CNTNAP2* contactin-associated protein-like 2 precursor, *DHCR7* 7-dehydrocholesterol reductase, *DISC1* disrupted in schizophrenia 1, *DOCK4* dedicator of cytokinesis 4, *EN2* homeobox protein engrailed-2, *FMR1* fragile X mental retardation 1 protein, *GABRB3* gamma aminobutyric acid receptor subunit beta-3 precursor, *GRIK2* glutamate receptor, ionotropic kainate 2 precursor, *ITGB3* integrin beta-3 precursor, *MDGA2* MAM domain containing glycosylphosphatidylinositol anchor 2, *MECP2* methyl-CpG-binding protein 2, *MET* met proto-oncogene, *MR* mental retardation, *NLGN3* Neuroligin 3, *NLGN4X* Neuroligin-4, X-linked precursor, *NRXN1* Neurexin-1, *OXTR* oxytocin receptor, PDDs pervasive developmental disorders, *PRKCB1* protein kinase C, beta 1, *PTEN* phosphatase and tensin homolog, *RELN* Reelin precursor, *SCN2A* sodium channel, voltage-gated, type II, alpha subunit, *SEMA5A* sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A, *SHANK3* SH3 and multiple ankyrin repeat domains protein 3, *SLC6A4* sodium-dependent serotonin transporter, *SLC25A12* calcium-binding mitochondrial carrier protein Aralar1, *TSC1* hamartin, *TSC2* tuberin, *UBE3A* ubiquitin-protein ligase E3A.

[a] Because the relationship to disease is most clear for rare variants, we biased our scoring accordingly. Genes associated with an ASD-linked syndrome or mutation resulted in 2 points, whereas other lines of evidence resulted in 1 point. Observation of a rare variant at a particular locus (CNV or bp) was insufficient for inclusion here; the additional requirement of statistical enrichment relative to controls was also necessary. We also excluded regions of clear interest (*e.g.* 1q21) for which the role of individual genes remains ambiguous. Likewise, although compelling evidence supports involvement of regions proximal to each of *AK123120* [61], *CDH9/10* [141], *NHE9* [97] and *SEMA5A* [6] it remains to be determined whether these nearby genes are involved in modulation of risk. To qualify as a mouse model, two out of three "core features" were required to be present. Mean score for the 35 genes included here was 2.97, with a standard deviation of 1.50. We assigned genes with scores 3 or greater ($n = 20$) as probable ASD genes and those with scores less than 3 ($n = 15$) as possible ASD genes. Although we have done our best to systematically and comprehensively evaluate available data for each of the different molecules discussed, we recognize that these evidence scores are largely arbitrary. Emerging genome-wide data suggests that a large number of molecules are likely to modulate risk in a variety of different ways. Despite unintended omissions of genes likely to be important, we hope this updated table might continue to serve as a starting point for discussion.

data are consistent with a variety of models, although it is increasingly difficult to defend single-gene explanations, even in rare situations. Finally, although the concept of genetic complexity is widely accepted, the logical consequences of this disease architecture, integrating both rare and common variation, must be more widely embraced for necessary progress to occur.

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The Genetics of Alcoholism and Other Addictive Disorders

23.5

David Goldman and Francesca Ducci

Abstract Addictions are common, complex disorders that are to some extent tied together by shared genetic and environmental etiological factors. They are frequently chronic, with a relapsing/remitting course. Addictive disorders, which are in part volitional, in part inborn, and in part determined by environmental experiences, pose the full range of medical, genetic, policy, and moral challenges. Genetic factors account for 40–70% of the variance in addiction liability. There is little evidence for large influences on overall population vulnerability from any single gene. Instead, multiple genetic loci are likely to be involved, each with a small attributable risk. Gene discovery is being facilitated by a variety of powerful approaches and tools, but is in its infancy. Susceptibility loci for addictions include both drug-specific genes (e.g., alcohol-metabolizing genes) and loci moderating neuronal pathways, such as reward, behavioral control, and stress resiliency, that are involved in several psychiatric diseases (e.g., *MAOA* and *COMT)*. In recent years, major progress has been made in identification of genes using intermediate phenotypes such as task-related brain activation that confer the opportunity of exploring the neuronal mechanisms through which genetic variation is translated into behavior. Fundamental to the detection of gene effects are also the understanding of the interplay between genes and of genes/environment interactions. The identification of genes altering the liability to addiction and treatment response (e.g., *OPRM1*) could provide new therapeutic targets and an ability to individualize treatment. Although the genetic bases of addiction remain largely unknown, there are reasons to think that more genes will be

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discovered in the future. Multiple and complementary approaches will be required to piece together the mosaic of causation.

23.5.1 Introduction

Addictions are multistep pathologies associated with maladaptive and destructive behaviors. They share the persistent, compulsive and uncontrolled use of a drug or, more generally, an agent or activity, for example gambling, shopping, or use of the internet.

Once exposure to the addictive agent has occurred, repetitive use induces neuroadaptive changes which promote further agent-seeking behaviors and ultimately lead to an uncontrolled pattern of use. These adaptive changes are the bases for the establishment of tolerance, craving, withdrawal, and affective disturbance that persist long after consumption of the addictive agent ceases. These changes serve as a basis for the cue- and stress-induced triggering of relapse and rapid reinstatement of use. Owing to these progressive and self-maintaining neurobiological mechanisms, addictive disorders are chronic and relapsing in nature [88].

Addictions by definition require exposure to an environmental agent. Both the probability of initiation and the probability of developing a pathologic pattern of use are influenced by individual characteristics (e.g., genetic vulnerability, sex, age, cohort, and psychopathology), environment (e.g., drug availability, social support, exposure to stressful events), and the nature of the addictive agent (e.g., rewarding properties, mode of administration, physiological response, secondary pathology). As genetically influenced diseases, addictions thus fall within the group of diseases that are thought of as complex. Yet, as will be seen, much has been learned about the inheritance of addictions and the influence of specific genes. Gene discovery is clarifying the neurobiological mechanisms of addiction and is being informed and aided by advances in our understanding of those mechanisms.

23.5.2 Definition of Substance Use Disorders and Other Addictions

Addictive disorders are clinically defined by two main systems: the Diagnostic and Statistical Manual of Mental Disorders (DSM) of the American Psychiatric Association, and the International Classification of Disease (ICD) of the World Health Organization. The same diagnostic criteria apply to the variety of addictive agents to which people are exposed. Two categories of addiction are recognized: Abuse (DSM-IV)/harmful use (ICD-10) and dependence. Abuse and dependence are both maladaptive patterns of behavior leading to clinically significant impairment or distress. Diagnostic criteria for substance abuse and dependence are shown in Table 23.5.1.

Table 23.5.1 Diagnostic criteria for substance-use disorders (SUDs) according to the fourth edition of the Diagnostic and Statistical Manual of Mental Disorders (DSM-IV, issued by the American Psychiatric Association)

Substance-use disorders, including abuse and dependence, are maladaptive patterns of substance use that lead to clinically significant impairment or distress. The diagnosis of substance dependence requires at least three of seven criteria and the diagnosis of substance abuse requires one of four criteria.

The seven criteria for substance dependence:

1. The need for markedly increased amounts of the substance to achieve intoxication or desired effect, or diminished effect with continued use of the same amount (tolerance).
2. Withdrawal syndrome or use of the substance to relieve or avoid withdrawal symptoms.
3. One or more unsuccessful efforts to cut down or control use.
4. Use in larger amounts or over a longer period than intended.
5. Important social, occupational or recreational activities are given up or reduced because of substance use.
6. A large amount of time is spent in activities that are necessary to obtain, to use or to recover from the effects of the substance.
7. Continued use despite knowledge of having persistent or recurrent physical or psychological problems that are caused or exacerbated by the substance.

The four criteria for substance abuse:

1. Recurrent use resulting in a failure to fulfill the main obligations at work, school or home.
2. Recurrent use in physically hazardous situations.
3. Recurrent substance-related legal problems.
4. Continued use despite persistent or recurrent social or interpersonal problems that are caused or exacerbated by the substance.

For both disorders, symptoms must occur within the same 12-month period. The abuse diagnosis is excluded in patients who have ever been dependent.

The development of valid and reliable criteria for addictive disorders provided a unifying framework for epidemiologic, treatment, and genetic studies worldwide [51]. However, the nosology of addictions has important limitations. The diagnostic categories are syndromic (based on clusters of symptoms and clinical course) rather than etiologic. The diagnoses are categorical, assuming a cut-off between normal and abnormal behavior, when many of the same problems are found in people who fall below the threshold for diagnosis. Pathologic use (e.g., leading to dangerous driving or problems at work) is underdiagnosed via the categorical DSM approach. Also, the diagnostic categories do not capture features of addiction that are important clinically and in research. For example, binge drinking is a pattern of alcohol use characterized by episodic bouts of intense drinking. In some American Indian Tribes, binge drinking is a common pattern of alcohol use that is generally, but not always, seen in the context of alcohol dependence. Regardless, binge drinking is a strong independent predictor of problems in all four DSM addiction major symptom areas: social, work, physical, and violence/lawlessness [123]. There is also a need to understand, and integrate, etiologic factors that act across diagnostic boundaries. As will be discussed later in this chapter, twin studies detect evidence of etiologic factors shared between addictions and other psychiatric diseases [81] and linking normal (personality) and abnormal (psychopathology) variations [88]. Thus, addiction disease categories are etiologically connected to other psychiatric diseases and to "normality." Future versions of diagnostic classifications may incorporate dimensional indices such as age at onset, and frequency, quantity, and years of use. However, it is improbable that the nosology of addiction will advance until neurobiological indicators, including genotypes, are integrated.

23.5.3 Epidemiology and Societal Impact of Addiction

Substance use and substance use disorders are common According to the World Health Organization (WHO), worldwide there are 2 billion alcohol users, 1.3 billion tobacco users, and 185 million users of illicit drugs (http://www.who.int/substance_abuse/facts/global_burden/en/). In the United States, according to the NESARC Survey (National Epidemiologic Survey on Alcohol and Related Conditions) the lifetime and 1-year point prevalences of alcohol dependence are 12.5 and 3.8%, respectively, [46]. In the same survey, prevalences of 12-month and lifetime drug dependence were 0.6 and 2.6%, respectively, [22]. Finally, the 1-year point prevalence of nicotine dependence is estimated to be 12.8% [48].

From a public health perspective, the cost of substance use and addictions in term of mortality and morbidity is enormous [147] and comparable to that of the worst chronic diseases, including diabetes and cancer (www.drugabuse.gov/about/welcome/aboutdrugabuse/magnitude).

The costs of addictions include teratogenic effects. In the United States, approximately 30% of women consume alcohol during pregnancy, leading to an incidence of fetal alcohol syndrome (FAS) of 0.2–2.0/1,000 live births, a rate comparable to that of Down syndrome. Alcohol crosses the placental barrier and can impair brain development even if the exposure occurs in the third trimester. FAS cognitive disabilities include deficits in memory, attention, behavioral inhibition, and reasoning. Later in life, the affected child is more vulnerable to psychiatric disorders and addictions, perpetuating a cycle of risk. The lifetime medical and social costs of FAS are high: as much as $ 800,000 per child.

Although genetic factors contribute to individual differences in vulnerability to addictions [43], these disorders – at least in theory – can be prevented by environmental intervention [100] and choice not to consume the addictive agent. There is data to validate this view. Alcohol consumption correlates with risk of developing organ damage such as liver cirrhosis at both individual [110, 152] and population levels [119] (Fig. 23.5.1).

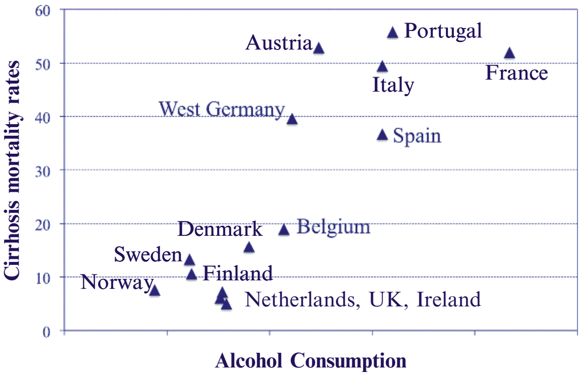

Fig. 23.5.1 Correlation between per capita alcohol consumption and rates of mortality from liver cirrhosis in 13 European countries, adapted from [119]; only data on males reported

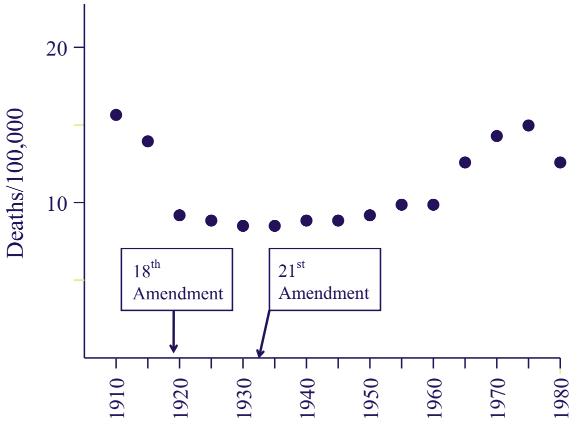

Fig. 23.5.2 Temporal variation in cirrhosis deaths in the U.S in relation to the Prohibition years between the 18th (January 29, 1920) and 21st (December 5, 1933) Amendments to the U.S. Constitution

As shown in Fig. 23.5.2, countries with higher per capita alcohol consumption tend to have higher rates of mortality from cirrhosis. Thus, social policies and customs play a crucial role in determining overall rates of addictions within populations, because they moderate the overall level of exposure. For example, the United States attempted to rid itself of the problem of alcoholism in 1920, when the manufacture, distribution, and sale of alcoholic beverages were prohibited. The "Prohibition," and the Temperance movement that preceded it, appear to have almost halved rates of cirrhosis (see Fig. 23.5.2). However, public demand led to a growth in organized crime, and ultimately the effect of the 18th Amendment to the U.S. Constitution, passed in 1919, was reversed by the 21st Amendment, passed in 1933.

It is important to note that although exposure to legal addictive agents is pervasive, there is an enormous interindividual variation in the pattern of use in terms of quantity, frequency, and duration. In the United States more than 70% of alcohol is consumed by 10% of the population [152]. Similarly, nicotine-dependent and psychiatrically ill individuals consume approximately 70% of cigarettes [48]. Since exposure to addictive agent is widespread, it is important to recognize that a substantial proportion of the exposed population will become addicted, and these vulnerable individuals will account for a large fraction of use of the agent. These statistics highlight the priority of developing targeted preventive strategies focused on individuals who are more vulnerable to developing pathologic use, and they highlight the fact that changes in public policy that are innocuous for most people represent hazards for others.

23.5.4 Genetics: Family and Twin Studies

Family studies have shown that both alcoholism and other addictions cluster in families [8, 77, 101, 104]. First-degree relatives of subjects with substance use disorders have an eightfold increased risk of developing a substance use disorder as compared to relatives of controls [101]. The increased risk among members of the same family might result from either environmental or genetic influences. Twin studies and studies on adoptees have made an enormous contribution to disentangling those contributions. In the classic twin study design the phenotypic resemblance of monozygotic (MZ) twins is compared with that of dizygotic (DZ) twins to estimate three main sources of variation:

1. Heritability (A): Proportion of variance due to additive (A) genetic factors.
2. Shared (or common) environment (C): Proportion of the variance due to factors shared by siblings. C is directly measured in the context of adoption.
3. Unique environment (E): Proportion of the variance due to environmental factors unique to the individual.

Both A and C are sometimes collectively termed familial influences because they contribute to similarities between members of the same family.

It has already been noted that the diagnostic assessment of addictions is imperfect. The usual effect of measurement error is to limit the maximum heritability (A) and to increase the proportion of variance attributable to E. In the same vein, it is important to stress that A, C, and E are latent (unmeasured) influences that should not be interpreted as absolute values. They provide broad estimates of sources of variation in the particular context in which they are evaluated, and do not, positively or negatively, predict effects of intervention to prevent or treat addictions. Furthermore, they do not inform us of sources of variation at the individual level. Nor are they informative of the specific genes and environmental factors involved.

Some of the questions that can be addressed by twin studies of addictions are: Are addictions heritable? Are genetic risk factors moderating vulnerability different in men and women? What is the developmental dependency of genes and environment in risk? What is the nature of inheritance? Are the genetic and environmental risk factors agent-specific or nonspecific? Are genetic and environmental factors independent of each other?

23.5.4.1 The Heritability of Addictions

Results from large, carefully characterized cohorts of twins, including epidemiologically ascertained twins from Virginia, USA) and Australia [15, 32, 53, 71–73, 75, 80, 83, 84, 93, 95, 116, 142], indicate that addictions are among the most heritable psychiatric disorders and most heritable complex traits [Fig. 23.5.3), with heritability estimates ranging from 0.39 (for hallucinogens) to 0.72 (for cocaine) [43].

The moderate to high heritabilities of addictions that were found are paradoxical, because addictions are by definition environmentally mediated, being contingent on exposure and choice. However, it is clear that susceptibilities to several complex diseases, including coronary artery disease, obesity, and diabetes, are genetically influenced, but also depend profoundly on lifestyle choices. Moreover, there is a genetics of choice: individuals vary in their capacity to resist impulse, exploratory behavior, cognitive resources to evaluate risk, and emotional traits that set the stage for vulnerability. In this regard, heritabilities for initiation and use of addictive agents are generally lower than for addictions, but are still significant [31, 79, 93].

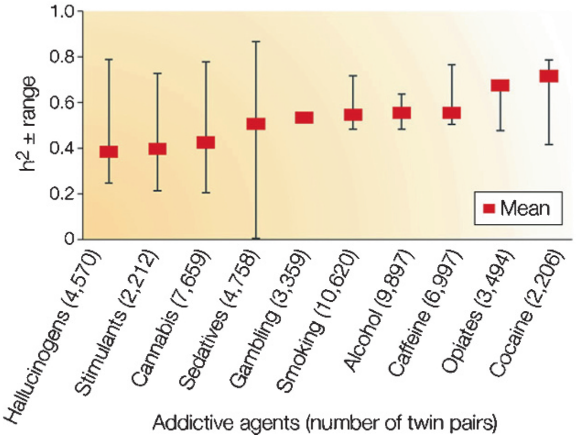

Fig. 23.5.3 The heritability (weighted means and ranges) of ten addictive disorders. These include hallucinogens, stimulants, cannabis, sedatives, gambling, smoking persistence, alcohol dependence, caffeine consumption or heavy use, cocaine dependence or abuse, and opiates. From [43], with permission

It is important to note that in several instances the twins analyzed in studies of the inheritance of addictions were collected using epidemiologic methods such that twins in a particular geographic region and place in time were evaluated systematically and fairly completely (e.g., the Virginia and Australia twin studies), and other twin datasets considered here also represented a systematic sampling within some other general framework (e.g., samples of WWII and Vietnam veterans). Therefore, these do not represent heritabilities within groups of severely affected patients who might be ascertained only in medical settings. However, most of the twin studies were conducted on Caucasian samples from the United States, Europe, and Australia, and thus the results might not be generalizable to other populations.

23.5.4.2 Do Genetic Factors Moderating Risk Differ in Men and Women?

Across populations and on a worldwide basis, addictions are more common in men. Is this because of sex-specific or sex-influenced genetic factors? Alternatively, is it a manifestation of gender-determined differences in opportunities and expectations? Indeed, changing cultural norms have led to disproportionate increases in the risks of addictions among women. However, gender disparities remain. U.S. males have more than twice the risk of alcohol dependence [OR (99%CI): 2.4 (1.75–3.16)] and drug dependence [OR (99% CI): 2.7 (1.16–6.37)] [49].

Twin studies can investigate whether different sets of genes act in males and females. As shown in Fig. 23.5.4, this is accomplished by comparing phenotypic correlations across three types of DZ twin pairs: sex-concordant (male-male and female-female) pairs and opposite-sex (female-male) pairs. If genes act similarly, correlations in these three types of twin pairs will be similar Fig. 23.5.4, scenario A). If the magnitude of genetic influence is higher in males correlations will be higher in male-male DZ pairs (Fig. 23.5.4, scenario B). Finally, if the trait is equally heritable in both sexes but the genes involved are different, there

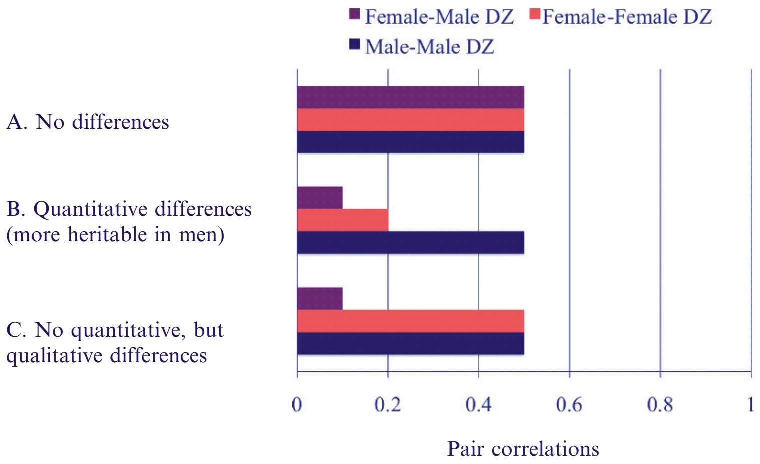

Fig. 23.5.4 (**a–c**) Phenotypic correlations in gender-concordant (male-male, female-female) and gender-discordant (female-male) dizygotic (DZ) twin pairs, assuming quantitative and/or qualitative differences between the two sexes in genes moderating a hypothetical trait. If gender differences are the same in the two genders, correlations in sex-discordant and sex-concordant twin pairs are similar (**a**) If the magnitude of genetic influence is higher in men than women, correlations will be highest in male-male DZ pairs (**b**) Finally, if the trait is equally heritable in both genders but the genes involved are different, there will be a lower correlation in sex-discordant pairs than in both kinds of sex-concordant pairs (**c**) (see text). Modified from [74], with permission

will be a lower correlation in sex-discordant pairs (Fig. 23.5.4, scenario C).

Twin studies indicate that genetic influences on alcoholism are approximately equivalent in males and females [54, 113]. However, the lesser similarity of opposite-sex pairs provides evidence for qualitatively different influences on risk in the two sexes [113]. At the gene level, gender-specific effects on addiction liability have been described for catechol-*O*-methylransferase (COMT) [35], an enzyme that metabolizes catecholamine neurotransmitters (see Sect. 23.5.6.1.2). Furthermore, animal models have shown sex differences in the regulation of ethanol-stimulated mesolimbic dopamine release by the μ-opioid receptor [67]. However, for nicotine, twin studies do not provide evidence for differences in genetic influences between men and women [96], and for illicit substance use disorders gender differences have not be fully addressed owing to low numbers of affected women in most of the twin studies conducted so far.

23.5.4.3 Developmental Dependence of Genes and Environment in Risk

Longitudinal studies reveal that the impact of genetic factors on addictions changes during development and across the lifespan. Addiction-related behaviors that are not heritable early in life become highly heritable later. Kendler et al. [84] found that the effect of shared environment (C) declines from adolescence into adulthood and disappears by age 35 for nicotine and by age 40 for alcohol (Fig. 23.5.5). In contrast, gene effects (A) are not detectable in early adolescence but gradually grow in importance.

These results can be explained by the action of some genetic factors only after repetitive exposure to the addictive agent, or by genes that act only after the brain has fully developed. This is supported by studies showing that specific genetic factors influence the likelihood of developing a maladaptive pattern of use after initial exposure [1, 78, 111]. Another possible explanation is that during the progression to adulthood, the individual has increasing latitude to shape her own choices and social environment, thus increasing the relative role of genotype [82] (see also active rGE in Sect. 23.5.4.6].

23.5.4.4 What Is the Nature of the Inheritance of Addictions?

Studies of families with addictions do not reveal a Mendelian pattern of inheritance. Factors complicating the inheritance of addictions include:

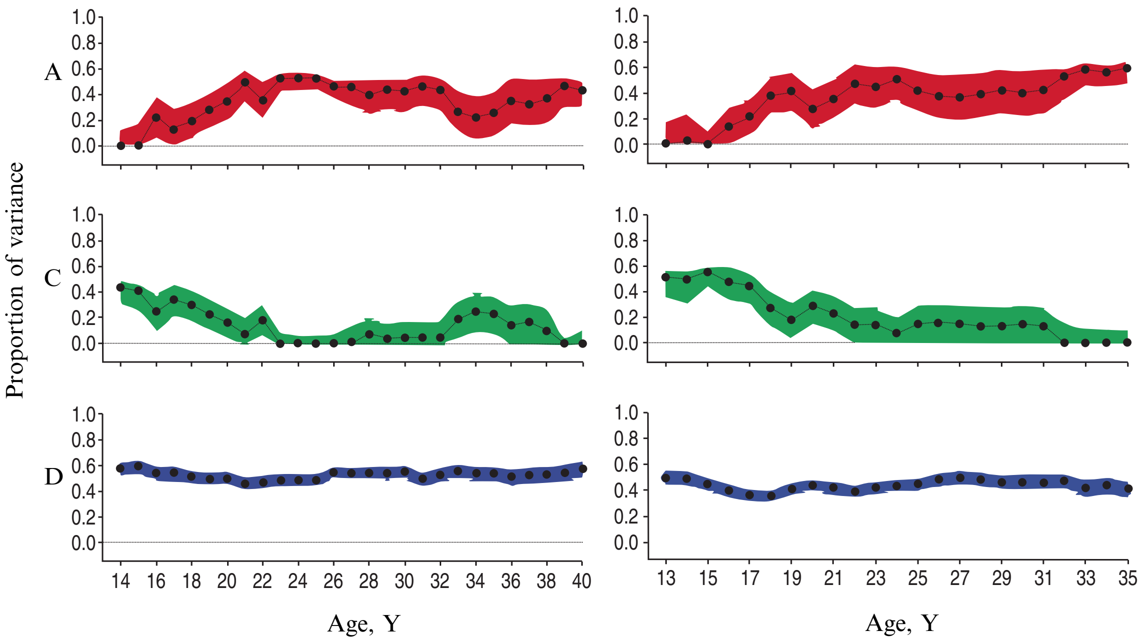

Fig. 23.5.5 Age-dependency of genetic and environmental contributions to variation in liability to alcohol (*left*) and nicotine (*right*) use. Additive genetic effects (*A*), familial environmental factors (*C*) and individual-specific environment (*E*) are represented by age in years (*Y*). The actual parameter estimates for A, C, and E are depicted by the *black lines*. *Colored regions* represent the possible range of estimates ±1 standard error. For both nicotine and alcohol use, genetic influences increase and shared family environmental influences decrease, moving from adolescence to adulthood (see text). Adapted from [84], with permission

Phenocopies: an individual who is not at genetic risk may become at risk because of a severe environment.

Incomplete penetrance: an individual at genetic risk does not become addicted, for example because of a decision to abstain or the intercession of a vigilant spouse.

Assortative mating and bilineal transmission: addicted individuals have a strong tendency to mate [59], enhancing the likelihood of bilineal transmission from both the maternal and paternal sides of the family.

For the addictions and other complex diseases, there is little evidence for large influences on overall population vulnerability from any single gene. Instead, multiple genetic loci are likely to be involved, each with a small attributable risk (for review see [140]). However, there are divergent models involving the action of multiple loci in overall population risk. Two of the most important divergent models, genetic heterogeneity and polygenicity, are contrasted in Fig. 23.5.6.

In the polygenic model, multiple functional alleles act in combination to lead to the phenotype in a fashion that is not merely addictive. Therefore, the effect of individual loci would not be independent, a phenomenon also known as gene-by-gene interaction (G×G). In classic American movie terms, the polygenicity model resembles the strategy employed by "The Joker" (Batman's nemesis) to terrorize the citizens of Gotham City, an imaginary American metropolis. The criminal mastermind distributed cosmetics that were harmless when used individually but fatal when used in some combinations. On the left side of Fig. 23.5.6, addicted individuals have different polygenic combinations of alleles that have pushed them over the threshold of vulnerability. In contrast, on the right side of the figure causation via genetic heterogeneity is illustrated. Multiple alleles are predisposing or protective, but the effects of any one can suffice. Genetic heterogeneity can occur at the same gene (within-locus heterogeneity) or at different genes (between-locus heterogeneity) (leading to genocopies of the illness).

Twin concordance ratios make it possible to distinguish between the genetic heterogeneity and polygenicity models to some degree (see Fig. 23.5.7).

As illustrated, polygenic inheritance tends to produce high MZ:DZ concordance ratios, because the

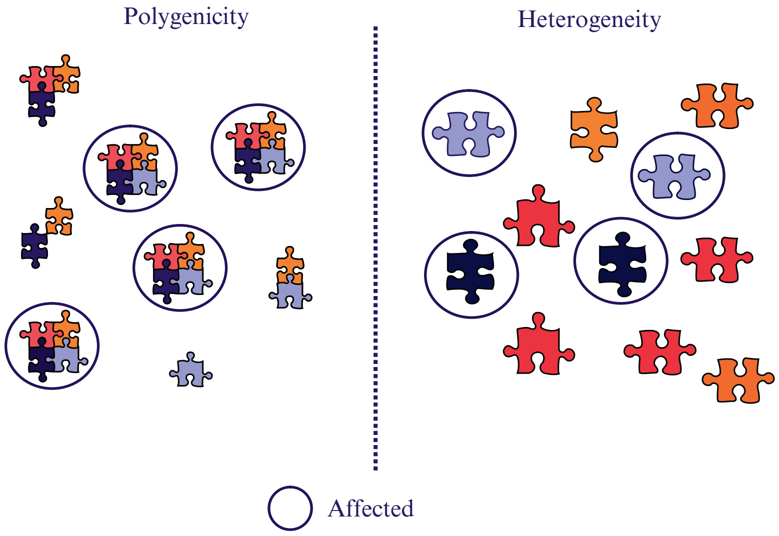

Fig. 23.5.6 Comparison of the polygenicity and heterogeneity models for a hypothetical complex disease in a sample of unrelated individuals. Affected individuals are indicated with a *circle*. Under the polygenicity model (*left*) a combination of different alleles (represented as different *jigsaw puzzle pieces*) is required to determine the disease. Under the heterogeneity model a single allele is sufficient to cause the disease, but different alleles (represented as *dark-blue* and *light-blue pieces of jigsaw puzzle*) are involved in different individuals. In other words, different alleles can cause the same disease (see text). Modified from [43], with permission

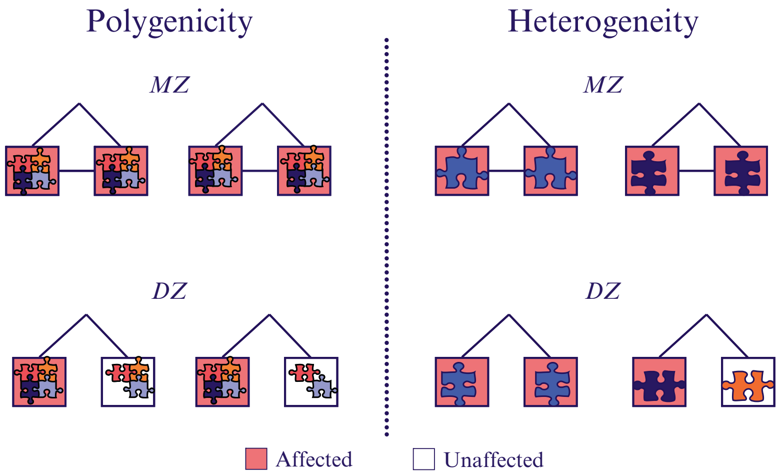

Fig. 23.5.7 Monozygotic (MZ) and dyzygotic (DZ) twin concordances for a hypothetical trait under the polygenicity and heterogeneity models. Under the polygenicity model (*left*) concordances tend to be much higher in MZ pairs than in DZ pairs, because DZ pairs are unlikely to inherit all the alleles (represented by *four pieces of puzzle*) necessary to develop the disease. As a consequence, MZ/DZ phenotypic concordance ratios will be >2:1 (see text). Under the heterogeneity model even just one allele is sufficient to cause the disease (either the *light-blue* or *dark-blue piece of puzzle*). Therefore, concordance ratios between MZ and DZ will tend to be 2:1 (dominant model) or 4:1 (single recessive locus) (see text). Modified from [43], with permission

odds of the DZ twins sharing a large number of alleles is not high. In fact it is $(1/2)^z$ multiplied by the number of such combinations that are available in the twins, where z is the number of alleles necessary to produce vulnerability. On the other hand, monogenic inheritance tends to produce 2:1 MZ: DZ concordance ratios for dominant allele effects and 4:1 ratios for recessive allele effects. As shown in Fig. 23.5.8, the MZ:DZ concordance ratios for addictions approximate the 2:1 ratio expected for monogenic inheritance, with the exception of a 3.7:1 ratio for cocaine. Also arguing against the polygenicity model, in families the degree of phenotypic similarity for addictions tends to fall off in proportion to the decrease in identity by descent, again supporting the heterogeneity model [14].

In line with the heterogeneity model, it has recently been shown that several neuropsychiatric diseases, including schizophrenia and autism [134, 145], are

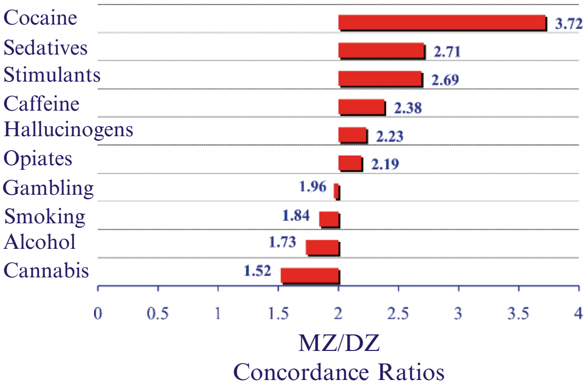

Fig. 23.5.8 MMZDZ twin concordance ratios for ten addictions. MZ/DZ ratios tend to converge on two, consistent with the heterogeneity model (see text). Modified from [43], with permission

sometimes caused by rare, highly penetrant mutations, including large deletions and insertions (copy number variations) that appear to be specific to single cases or families.

23.5.4.5 *Agent-Specific and Nonspecific Genetic and Environmental Factors*

Co-morbidity (the co-occurrence of different disorders in the same individual) is common amongst the addictions and between addictions and other psychiatric diseases. Both types of co-morbidity are substantially in excess of what would be expected by chance [49, 52, 85]. Such co-morbidity can indicate the existence of etiologic factors that are shared (co-causation) and thus the non-independence of the risk of the two diseases that co-occur. However, co-morbidity can also indicate that one disease tends to lead to the other (e.g., the gateway hypothesis, and self-medication explanations for addictions). Drug use can lead people to alter their environment in such a way that exposure to another drug occurs. For example, 85% of chronic alcoholics smoke cigarettes, but perhaps this is because bars are smoke-filled or because alcohol-consuming peers are more likely to smoke. Twin studies can establish the origins of co-morbidity and evaluate the extent to which genetic and environmental risk factors moderating liability to different diseases are shared or unshared. A common genetic source of co-morbidity between two disorders can be detected by identifying cross-inheritance of vulnerability.

23.5.4.5.1 Co-morbidity Among Addictions

Twin and family studies have revealed that genetic factors acting on different addictive disorders include both substance-specific genetic factors and genetic factors that are shared between different addictions (reviewed in [40, 58]). In a large sample of adult male twins who were veterans of WWII [138], a common genetic factor accounted for more than two-thirds of the total genetic variance in risk for disorders involving marijuana, stimulants, sedatives, and psychedelics, whereas 0.7 of heroin dependence heritability was found to be substance specific. Kendler et al. [83] explored the genetic overlap between several licit and illicit substances, including alcohol, caffeine, nicotine, cannabis, and cocaine, in a portion of a Virginia twin sample consisting of almost 5,000 twins. In this study genetic risk factors for dependence on different psychoactive substances could not be explained by a single factor acting across all substances. Rather, two shared genetic factors were found: one factor mainly explained vulnerability to cannabis and cocaine dependence (illicit drug genetic factor); a second factor mainly explained vulnerability to alcohol, caffeine, and nicotine (licit drug genetic factor). These two factors were not independent but highly correlated. Large substance-specific genetic factors were found mainly for nicotine and caffeine (see Fig. 23.5.9).

23.5.4.5.2 Co-morbidity Between Addiction and Other Psychiatric Disorders

Addictions frequently co-exist with other psychiatric diseases, including both internalizing disorders (disorders marked by anxiety or problems of mood) and externalizing disorders (disorders marked by problems of impulse control) [47, 85]. Twin studies consistently reveal the existence of shared genetic influences between alcoholism and externalizing disorders [59, 81, 88]. Longitudinal studies have shown that externalizing disorders of childhood, such as conduct disorder (CD) and attention deficit hyperactivity disorder (ADHD), are important risk factors for the subsequent development of alcoholism [130]. Evidence from twin

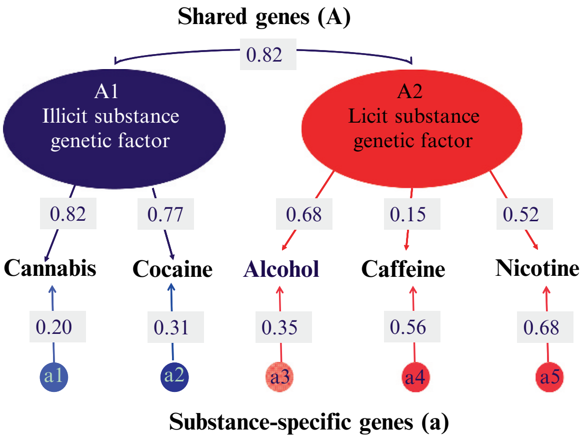

Fig. 23.5.9 Shared and substance-specific additive genetic influences acting on symptom counts for cannabis, cocaine, alcohol, caffeine, and nicotine abuse or dependence. Two common genetic factors are identified, one for illicit substances (*A1*) and one for licit substances (*A2*). Substance-specific genetic influences (*a1-a5*) are represented at the *bottom* of the figure. Path values are standardized loadings and thus need to be squared to reflect the proportion of variation in liability in the observed variable accounted for by the factor. For example, the illicit substance genetic factor (*A1*) accounts for 0.67 (0.82^2) of the total variance in cannabis symptoms in this population. The cannabis-specific genetic factor (*a1*) accounts for 0.04 (0.20^2) of the variance in this trait. Therefore, the total variance of cannabis symptoms determined by genetic factors – heritability – is 0.71 (0.67 + 0.04). The remaining variance (1–0.71 = 0.29) is explained by environmental influences that are not represented here. The *double-headed arrow* connecting the illicit and licit substance genetic factors represents the genetic correlation between these factors (see text). Adapted from [83], with permission

studies for shared genetic influences between alcoholism and internalizing disorders are more controversial [76, 81, 114]. However, longitudinal studies have shown that anxiety disorders such as panic disorder and social phobia predict subsequent alcohol problems in adolescents and young adults [154].

Overall, twin studies predict that genes involved in vulnerability to addiction include both substance-specific genes and genes that act on general (common) pathways involved in different diseases. Substance-specific genes include genes involved in pharmacokinetic or pharmacodynamic processes specific to a particular drug. For example, genetic variation in alcohol-metabolizing genes moderates risk to develop alcoholism (see Sect. 23.5.6.1.1). Nonspecific genes include genes affecting neurobiological networks involved in vulnerability to different types of addictions and also genes predisposing to addictions and other psychiatric diseases, such as genes involved in reward, stress resiliency, behavioral control, and personality. For example, the dopamine system is fundamental for the reward effects of all addictive agents [143], and genetic variations in the gene encoding the dopamine two receptor (*DRD2*) have been linked to different types of addictions [23, 148], although with some inconsistencies [42]. As will be discussed, other genes such as monoamine oxidase A (*MAOA*) (see Sect. 23.5.6.1.2), the serotonin transporter (*SLC6A4*) (see Sect. 23.5.6.1.2), and catechol-*O*-methyl transferase (*COMT*) (see Sect. 23.5.6.1.2) have been implicated in the shared genetic liability between addictions and other psychiatric diseases.

23.5.4.6 Are Genetic and Environmental Risk Factors Independent of Each Other?

The simplest, and oldest, model for the etiology of behavioral differences and psychiatric diseases regarded genetic and environmental factors as separate entities contributing to disease liability in an additive fashion. This model polarized discussion, leading to the gene vs environment debate, a false dichotomy.

At the height of the eugenics movement, which in the United States was powerfully embodied at the Cold Springs Harbor Laboratory led by Charles Davenport, Charles Darwin's Malthusian principle of survival of the fittest had been used to develop the concept of Social Darwinism. Those who were psychiatrically ill, cognitively deficient (e.g., illiterate), and poor were thought to be genetically inferior. Social effects included the compulsory sterilization of many thousands of vulnerable individuals. The effect of this movement is encapsulated by the case Buck vs Bell, heard before the U.S. Supreme Court in 1927. Carrie Buck was a ward of the Virginia State Colony for Epileptics and Feebleminded. Although later in her life she became an avid reader, she was one of many thousand proposed for sterilization because she was "feeble-minded" and incorrigibly promiscuous (actually she had given birth to a child after being raped). Carrie Buck's mother had been a prostitute. Writing for an 8–1 majority, Chief Justice Oliver Wendell Holmes, Jr. stated,

> It is better for all the world, if instead of waiting to execute degenerate offspring for crime, or to let them starve for their imbecility, society can prevent those who are manifestly unfit from continuing their kind. The principle that sustains compulsory vaccination is broad enough to cover cutting the Fallopian tubes.

At the end of his opinion, which greatly accelerated state-sponsored euthanasia for eugenic purposes, Chief Justice Holmes memorably concluded: "Three generations of imbeciles are enough." Carrie Buck, and later her daughter, were among 65,000 individuals compulsorily sterilized in the United States. There were also large eugenic programs in other nations, including Japan, Canada, and Sweden, where in that one nation 21,000 people were forcibly sterilized. Most infamously, Nazi Germany sterilized over 400,000 people and murdered more than 6 million institutionalized individuals, homosexuals, gypsies, and Jews.

Although it is valid to argue that genetic studies on the addictions and other psychiatric diseases have emerged from a different imperative and tradition – namely the need to understand behavior and to prevent and treat psychiatric diseases – the disciplines of psychiatry and psychiatric genetics encouraged and were highly intertwined with the eugenics movement, and with the genocides that were justified on that basis. Therefore, it remains imperative to be alert to the threat of misuse of genetic information, especially where it may be misused to stigmatize whole groups of individuals.

At the opposite pole is the view that addictions and other psychiatric diseases emerge only through upbringing, experience, and choice. Like the genetic view, which was adopted by the Social Darwinists and Nazis to justify policy, the environmentalist viewpoint was also co-opted for political purposes, for example by socialists, whose main point was that all people should not just be treated equally but that there should be equality of outcomes. In the Declaration of Independence, the Founders of the United States uttered the memorable words "All men are created equal"; however, this was a statement of universal rights (for white males!) and not an assertion of equality of ability or vulnerability. Paradoxically, the equal outcomes that are sometimes thought of as a desirable societal goal can only be achieved if individual differences, including the vulnerabilities of individuals, are recognized. However, socialist utopian appeals rested on an assumption of human malleability and ultimately culminated in the excesses of Lysenkoism, a dark time in the former Soviet Union when geneticists studying inheritance were repressed in favor of a Lamarckian view of genomic malleability. On the whole, the unidimensional environmental perspective has also been stigmatizing, in part because since this model of behavioral causation left society with a set of intractable problems that were in some vague sense the fault of the individual, the family, or the community. Indeed, regardless of whether behaviors have a genetic or environmental origin, behaviors will be used for the purposes of stigmatization. American Indians had to contend with the "fire-water myth," though in fact particular American Indian tribes have low rates of alcoholism and within tribes there is differential vulnerability owing to genetic variation. The Irish are frequently characterized as alcoholic although rates of alcoholism are not higher than in several other European countries. Within the Irish, differential vulnerability is again genetically transmitted, as revealed by Prescott et al. [93]. On the other hand, as we understand the genetic and neurobiologic origins of addiction, it is clear that the seeds of these problems are latent in all of us, albeit to different extents, causes are understood, removing the need for over-generalizations, and destigmatization flows from this more sophisticated understanding.

Studies that have examined the combined effect of genes and environment in addiction have revealed that the individual's genotype in probabilistic fashion shapes her relative risk for these common disorders. Genotype also determines reaction range – the range of possible responses to environment. Most people are probably at some risk for an addiction and could become addicted under certain circumstances, and thus the reaction range of most of our genotypes encompasses addiction. However, a shift in level and type of environmental exposure uncovers or covers part of the overall risk density distribution, as shown in Fig. 23.5.10. What are these factors? Several environmental influences powerfully moderate risk of developing addictions, including exposure to maltreatment during infancy [27], age at onset of drinking [45, 46], low socioeconomic status [99], adverse life events, low social support, poor parenting, religiosity [83], peer influences [61], and drug availability.

There are two main types of violations of gene-environment independence: gene by environment correlation and gene by environment interaction.

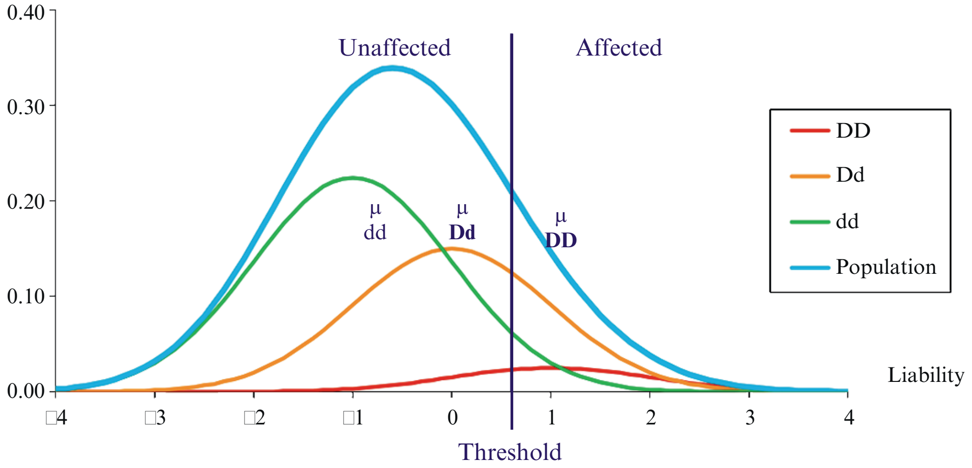

Fig. 23.5.10 Genotype-specific liability and disease threshold. Liability or predisposition to a disease is simplified here as a single continuous dimension. Once liability passes a threshold the discrete phenotype emerges (affected versus unaffected). Given a disease-linked locus with two different alleles (*D* and *d*), carriers of the three different genotypes (*DD*, *Dd*, *dd*) have three different liability curves that together compose the overall liability distribution for that population. Factors that change the threshold will therefore change the number of affected and unaffected individuals within each genotype group as well as in the overall population

23.5.4.7 Gene by Environment Correlation

Gene × environment correlation (rGE) occurs when genotype correlates (*r*) with probability of exposure to environmental stressors. There are three main mechanisms leading to rGE: *active rGE, evocative rGE,* and *passive rGE.* Active rGE occurs when an individual's genotype can shape her choice of environment. For example, children with conduct disorder (CD), a precursor to antisocial personality disorder (APD) tend to seek out antisocial peers, exposure to whom increases their risk of developing antisocial behavior and addiction. In evocative rGE, an individual can indirectly shape his/her environment. For example, a child with CD may evoke harsher discipline from her parents, in turn promoting the risk of addictions and other pathologies. In passive rGE, alleles conferring risk in a child also alter the behavior of the parent transmitting the allele. Thus, the children of an addicted parent are at enhanced risk both via transmission risk alleles and via family environment and teratogenic effects of the drug.

Twin studies can address the existence of rGE by measuring the "genetics of the environment." Although it may appear paradoxical, if behavior alters environmental exposures and if the relevant aspect of the behavior is subject to genetic influences, then the environmental measure will be heritable. Inheritance of environmental exposures has been observed. Kendler and Backer [70] reported modest to moderate heritabilities (ranging from 7 to 39%) for several categories of environmental factors that are important or potentially important in addiction vulnerability: stressful life events, parenting, family environment, social support, peer interactions, and marital quality.

Twin studies can help us understand whether the relationship between an environmental variable and an outcome is causal (e.g., directly mediated by an environmental effect) or mediated by genetic/shared environmental influences (as occurs with rGE). The discordant twin design, in which twins discordant for exposure to the environmental factor are studied, is a powerful test of causality. The discordant twin design offers the possibility of testing whether the association between the environmental factor and the disease persists after controlling for genotype and for other shared-environmental factors, such as socioeconomic status and home environment. In this design, the association between the environmental variable and the disease is evaluated in the entire sample, in DZ pairs discordant for exposure, and in MZ pairs discordant for exposure. Figure 23.5.11 shows three possible patterns. If association between the environmental variable and the outcome is entirely mediated by unique environmental factors (case A), the strength of the association (measured by the odds ratios, ORs) will be the same in the whole sample and among MZ and DZ pairs (Fig. 23.5.11a). In case B, the association of the environmental variable is partially mediated by genetic factors. Here the ORs are highest in the total sample (where the association is not controlled for shared environmental and genetic confounds), intermediate in DZ twins (where the association is controlled for shared environment and partially for genetic factors), and lowest among MZ pairs (where the association is fully controlled for both shared environment and genetic factors). Finally, in case C the association is entirely mediated by genetic factors, because it completely disappears among discordant MZ pairs (OR = 1).

This approach has been used to deconstruct the origins of an important gene × environment correlation, namely age at first use of the psychoactive substance. Early initiation of alcohol use is associated with an increased risk of developing addiction. According to the NESARC epidemiologic survey, odds of lifetime substance dependence among users are reduced by 4% for illicit drugs and 9% for alcohol for each additional year that onset of drug use is delayed [45, 46]. This association between age at initiation and risk can arise from different mechanisms. It can result from a direct-causal effect (early onset directly increases risk) or might be mediated by genetic/familial influences (early onset and addiction result from a broad shared liability). Results from the Virginia Twin Registry study are more consistent with the second hypothesis. Prescott and Kendler et al. [115] showed that twins with late onset of alcohol use had the same risk as co-twins who experienced early onset of alcohol use. This result indicates that the association between early exposure and alcoholism may result from a shared genetic liability and that early exposure does not independently influence risk of developing alcoholism. This is important, because preventive efforts at delaying drinking are likely to be useful for preventing alcohol-related accidents and injuries, but might not necessarily reduce the risk of developing alcoholism in adulthood. However, the impact of early alcohol exposure on risk of alcoholism remains a critically important issue: few would risk unnecessary early drug and alcohol exposures in children with developing brains and imperfectly developed impulse control.

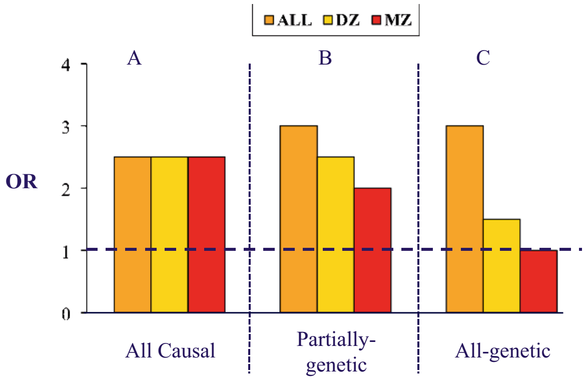

Fig. 23.5.11 (**a–c**) Causal and noncausal relationship of a hypothetical environmental variable and a hypothetical outcome using MZ and DZ twin pairs that are discordant for exposure. If association between the variable and the outcome is entirely mediated by unique environmental factors, the strength of the association [measured by odds ratio (*OR*)] will be the same in the whole sample and among MZ and DZ pairs (**a**) If the association is partially mediated by genetic factors, the ORs are highest in the total sample, intermediate in DZ twins, and lowest among MZ pairs (**b**) If the association is entirely mediated by genetic factors, it completely disappears among discordant MZ pairs (OR = 1) (**c**) (see text). (Modified from [74], with permission)

23.5.4.8 Gene by Environment Interaction

Gene × environment interaction (G × E) occurs when the effect of the environmental exposure on a certain outcome is strongly influenced or contingent upon genotype and vice versa (gene effect on the outcome is contingent on exposure)(for review see [17]).

G × E has been observed for several of the functional alleles identified so far in addictions and psychiatric genetics. By comparison with other complex diseases, including cancer, diabetes, cardiovascular, infectious diseases, and hematologic diseases, where genetic variation moderates resiliency and vulnerability to chemicals, pathogens, nutrients, caloric load, oxidants, and ionizing radiation, in psychiatric genetics much of the progress has been made through the discovery of alleles that influence stress resiliency. Severe childhood stress and neglect both increase vulnerability to addiction and multiple addiction-related psychiatric diseases, including antisocial personality disorder (APD), CD, anxiety disorders, and depression, with the risks of these common diseases being elevated severalfold in the stress-exposed [122, 144]. However, not all people who are exposed to early life stress develop addiction or other psychiatric diseases, indicating wide variation in resiliency to stress. Functional loci that have been shown to partially account for interindividual differences in stress resiliency include monoamine oxidase A (*MAOA*) (see Sect. 23.5.6.1.2) [18], the serotonin transporter *SLC6A4* [19] (see Sect. 23.5.6.1.2), *COMT* [20] (see Sect. 23.5.6.1.2), the corticotrophin-releasing hormone receptor 1 gene [11], neuropeptide Y (*NPY*) [153], and *FKBP5* [10].

We believe it is important to clarify here the distinction between addictive vs non-additive (or interactive) effects implied in either GXG or GXE. Indeed, misunderstanding of the concept of interaction between factors is frequent. In assessing effects of risk factors on ORs, the ORs attributable to additively acting risk factors are multiplied to predict their combined effect. Thus, if risk factor A confers a relative risk of 2 and risk factor B confers a relative risk of 3, the additive risk (A + B) is 2 × 3 = 6. Additivity is frequently mislabeled "interaction," when what has been observed is an additive increase in risk. Also, it is important to recognize that interactions, unless they are large, can be more difficult to detect and accurately quantitate than main effects, thus requiring larger sample sizes or more ingenious sampling frameworks (contrasting populations with different severities of exposure, accessing intermediate phenotypes, etc.).

23.5.5 Progress Through Intermediate Phenotypes

One strategy to discover gene effects in addictions and other etiologically complex diseases is the deconstruction of complex phenotypes into components that are etiologically more homogeneous. Intermediate phenotypes access mediating mechanisms of genes and environmental effects on behavior. Heritable intermediate phenotypes that are disease associated have been termed "endophenotypes" [44]. Several intermediate phenotypes have been specifically associated with addiction. These include alcohol-induced flushing, which is a protective endophenotype, and low response to the effects of alcohol, which is an endophenotype predictive of risk of alcoholism. The genetic origins of alcohol-induced flushing are discussed later (in Sect. 23.5.6.1.1). In humans, the level of response to alcohol is believed to reflect mainly pharmacodynamic variation in response [128] rather than variation in metabolism. A low response to alcohol predicts increased risk of developing alcohol use disorders [55, 124, 129] and has been associated with genetic variation in the serotonin transporter gene (*SLC6A4*) and in the gene encoding the subunit a6 of the g-aminobutyric acid receptor A (*GABRA6*) [62]. Other intermediate phenotypes assist in the exploration of genetic vulnerability to addictions (as well as other psychiatric diseases), and these addiction-relevant intermediate phenotypes include electrophysiologic, neuropsychologic, neuroendocrinologic and, more recently, neuroimaging measures.

Neuroimaging provides access to the neuronal mechanisms underlying emotion, reward, and craving and therefore represents an extraordinary tool to link genes to the neuronal pathways that produce behaviors (for reviews see [102, 149]). For example, amygdala activation after exposure to stressful stimuli predicts anxiety and captures interindividual differences in emotional response and stress resiliency [50]. On the other hand, activation of the prefrontal cortex during working memory performance is used to evaluate prefrontal cognitive function that is impaired in several psychiatric diseases, including addictions. The combination of genetic analysis with brain imaging illustrates the power of cross-disciplinary science.

23.5.6 Finding the Specific Genes Underlying Vulnerability to Addiction

Two main strategies have been used and are increasingly integrated to identify the specific genetic variations influencing addiction: the candidate gene and the genome-wide approaches. In the former, genes known to influence processes involved in the pathogenesis or treatment of addiction are selected. In the latter, the whole genome is interrogated simultaneously in a hypothesis-free fashion. A point of integration between the methods is the study of candidate genes located in chromosome regions implicated by genome-wide scans.

23.5.6.1 Candidate Genes

23.5.6.1.1 Substance-specific Genes Moderating Liability: ADH1B and ALDH2

The alcohol dehydrogenase IB (*ADH1B*) and aldehyde dehydrogenase 2 (*ALDH2*) genes encode for two enzymes catalyzing consecutive steps in alcohol metabolism. In adults, these enzymes have an important role although there are also several other enzymes that can carry out both of these metabolic steps, including catalase, cytochrome P450, and additional enzymes in the ADH and ALDH gene families. In the liver, three ADH genes are expressed at high levels and to some extent at different times of development, and these three enzymes are primarily expressed in hepatocytes. However, it will be seen that despite this complexity of enzyme action in alcohol metabolism, individual functional alleles altering the function of only one enzyme are sufficient to exert a major biochemical effect and an effect on risk. Probably this is because of the relatively greater importance of ADH1B in the adult liver and its lower Km and higher capacity for metabolism than in some of the other enzymes. The ALDH2 enzyme is an ALDH that is encoded in the nuclear genome but translocated to the mitochondrion, where it plays a critical role in the ability of hepatocytes, and other cells throughout the body, to metabolize acetaldehyde. The main roles of the enzymes that have maintained these genes through at least 80 million years of mammalian evolution are in fact somewhat obscure. In their natural environment, mice and rats are not heavy consumers of alcoholic beverages! Yet our distant mammalian cousins possess a full complement of these enzymes. Perhaps the reason for this is that although the liver metabolizes ethanol ingested in beverages it also has to utilize alcohols that are the product of bacterial fermentation in the gut.

The product of ADH is acetaldehyde, a toxic intermediate that may react with a variety of biomolecules. Indeed, acetaldehyde adducts with DNA and both it and alcohol are formally recognized as mutagens. Acetaldehyde is a potent releaser of histamine, triggering the aversive flushing reaction. Symptoms include headache, nausea, palpitations, and flushing of the skin. Ordinarily, acetaldehyde is rapidly converted to acetate, and levels of acetaldehyde remain very low – in the nanomolar range. However, if aldehyde dehydrogenase is blocked by disulfiram (which is used to help alcoholics maintain abstinence) or certain drugs used to treat protozoal infections (e.g., metronidazole) then the flushing reaction is observed after the ingestion of only small quantities of alcohol. Also, if acetaldehyde accumulates the individual is at substantially increased risk of upper gastrointestinal tract cancer, and this can occur either via to pharmacologic blockade of aldehyde dehydrogenase or as a result of natural genetic variation, a factor that physicians may wish to consider in counseling individuals who drink despite carrying the genetic variations that will next be described [12].

Nature has provided two common natural examples of genetic predisposition to alcohol-induced flushing, and it is not surprising that the enzyme variants that lead to flushing are protective against alcoholism. The most important functional loci at *ADH1B* and *ALDH2* are the *ADH1B His47Arg* missense polymorphism, in which Arg47 is a hyperactive allele acting in co-dominant fashion, and *ALDH2 Glu487Lys*, in which the Lys487 allele inactivates ALDH2 dominantly (a manifestation of the tetrameric structure of ALDH2). Higher activity of ADH1B, conferred by Arg47, or lower activity of ALDH2, conferred by Lys487, leads to accumulation of acetaldehyde following alcohol consumption and the flushing reaction. In East Asian populations (e.g., China and Japan), where both His47 and Lys487 are highly abundant, and in Jewish populations, where His47 is abundant, many individuals carry genotypes that are protective against the development of alcoholism. The protective effect seems to vary across environments [139] and shows genotype–genotype additivity [135]. Following up the connection of acetaldehyde to mutation, both polymorphisms have also been associated with enhanced risk of cancers of the oropharynx and esophagus [12, 151]. Both of these functional polymorphisms appear to be ancient in human populations, occurring on characteristic and highly diverged haplotypes. On that basis it is unlikely that either the Arg47 or Lys487 was selected to high frequencies in East Asian populations as protective alleles against alcoholism. One possibility, still speculative, is that the polymorphism alters susceptibility to protozoal infections of the gut, including amebiasis, because an action of metronidazole (an antiprotozoal drug of unknown mechanism) is to inhibit ALDH [41]. However, regardless of the forces responsible for their high frequencies, the pervasive environmental exposure to alcohol that occurs in modern societies has added other dimensions to their effects.

23.5.6.1.2 Genes Moderating Liability to Addiction and Other Diseases: *COMT*, *MAOA*, and *SLC6A4*

Monoamines, including serotonin (5-HT), norepinephrine (NE), and dopamine (DA), are fundamental modulators of emotionality, cognition, reward, and behavioral response to stimuli. Therefore, it is unsurprising that genes regulating monoamine*s* levels such as catechol-*O*-methylransferase (*COMT*), monoamine oxidase A (*MAOA*), and the serotonin transporter (*SLC6A4*) have been implicated in vulnerability to several psychiatric diseases, including addiction, antisocial personality disorder, depression, and anxiety. In line with these ideas, drugs increasing monoamines in the synaptic cleft or drugs that target receptors of monoamine neurotransmitters are used in the treatment of several psychiatric diseases.

COMT metabolizes DA, NE, and other catecholamine neurotransmitters. COMT plays an important role in the regulation of dopamine levels in the prefrontal cortex because of the paucity of the dopamine transporter in this region [92, 98]. *COMT* knockout mice have increased levels of dopamine in this brain region [37, 150]. In mammals, the COMT enzyme occurs in two distinct forms: as a soluble, cytoplasmic, protein (S-COMT) and as a membrane-bound form (MB-COMT) which – in humans – has 50 additional amino acid residues at the N-terminus. S-COMT predominates in most tissues, accounting for 95% of total COMT activity [66]. However, in brain, the amount of MB-COMT activity is much higher [121]. Val158Met is a common functional single nucleotide substitution of COMT [89], replacing methionine for valine at codon 158 of MB-COMT and at codon 108 of S-COMT. Via its effect on enzyme stability [127, 146] the Met158 allele is three- to fourfold less active than Val158 [21], and the alleles act co-dominantly [138]. Because of its higher activity and the importance of COMT for dopamine metabolism in the frontal cortex, the Val158 allele was predicted to lower dopamine level in that region. Consistent with this idea and with the role of dopamine in tuning frontal cortical function, the Val158 allele has been linked to inefficient frontal lobe function evaluated with different methodologies [29, 39, 97]. Also, in a pharmacogenetic study, the COMT inhibitor tolcapone improved executive function in val/val homozygotes, but not in individuals homozygous for the met allele, a finding consistent with the higher levels of cortical dopamine already expected in individuals with this genotype [38]. On the other hand, the Met158 allele, although associated with better cognitive performance, is associated with decreased stress resiliency and increased anxiety. This allele has been associated with increased anxiety among women populations [33], with increased pain–stress response and a lower pain threshold [26, 155], and with increased amygdala reactivity to unpleasant stimuli [133]. In certain addicted populations, e.g., polysubstance abusers [141], both the Val158 and Met158 alleles have been associated with addictions. The Val158 allele was found to excess among methamphetamine, nicotine, and polysubstance addicts [141]. On the other hand, in addicted populations with high frequencies of internalizing disorders, such as late-onset alcoholics in Finland [137] and Finnish social drinkers [69], increased risk appears to be conferred by the Met158 allele.

This account of multilevel association of a functional locus of the *COMT* gene to behavior illustrates the relative strength of allele effects on intermediate phenotypes such as brain imaging measures of metabolic activity during cognitive tasks or after a painful or emotional challenge test, and the much more modest effects on the common, complex disease. The disease is a more complex phenotype emergent from differences in internal states that are accessed via the intermediate phenotype measures, but the disease state may also emerge for other reasons, or fail to manifest at all. Frustratingly, the addictions are not etiologically defined (people become addicted for different reasons) so that it is illogical to expect gene-consistent effects across different populations of patients (e.g., addicted patients who might differ at age at onset, or whose additions varied in severity, or who had different risk exposures). In the addictions, studies identifying genes such as COMT have had the beneficial effect of focusing more attention on intermediate phenotypes and clinical subgroups, and on the history of environmental exposure, all information with which genetic markers are likely to be used in concert to improve the nosology of these common diseases.

MAOA is an X-linked gene encoding monoamine oxidase A, a mitochondrial enzyme that metabolizes monoamine neurotransmitters including norepinephrine, dopamine and serotonin. *MAOA* knockout mice have higher levels of these neurotransmitters and manifest increased aggressive behavior and stress reactivity [16]. In the human, different *MAOA* genetic variants impair MAOA activity to different degrees, and the reduction in enzyme activity appears to parallel the effect on behavior. In 1993, Brunner et al. [13] reported a Dutch family in which eight males were affected by a syndrome characterized by borderline mental retardation and impulsive behavior including impulsive aggression, arson, attempted rape, fighting, and exhibitionism. The cause was a stop-codon variant in the eighth exon of *MAOA* leading to complete and selective deficiency of MAOA activity, with an X-linked pattern transmission from unaffected mothers carrying the stop-codon (see Fig. 23.5.12).

Discovery of this mutation led to attempts to identify it in other individuals with behavioral dyscontrol, including individuals accused of serious crimes. However, and despite intensive effort, the stop-codon variant was not found in other individuals, and thus represents an example of a rare, private allele. More recently, a common *MAOA* polymorphism influencing MAOA transcription was discovered [126]. This locus, termed the MAOA-linked polymorphic region (*MAOA–LPR*), is a variable-number tandem repeat (VNTR) located approximately 1.2 kb upstream of the *MAOA* start codon and within the gene's transcriptional control region [25, 126]. Alleles at this VNTR have a different number of tandem copies of a 30-bp sequence, with the three- and four-repeat alleles being by far the most common. Alleles with four repeats are transcribed more efficiently than alleles with three copies of the repeat, and therefore lead to higher MAOA enzyme activity [126]. In a longitudinally studied cohort of boys, Caspi et al. [18] found that *MAOA–LPR* moderated the effect of childhood maltreatment on vulnerability to develop antisocial behavior. In this study maltreated boys with the low-activity genotype were more likely to develop antisocial problems later in life than boys with the high-activity genotype. Meta-analysis of several studies that represent attempts at replication revealed a significant pooled G×E effect for *MAOA* and stress. A similar *MAOA* × stress interaction appears to occur in women, although of course a much smaller percentage of women are homozygous for the low expression allele (males being hemizygous for *MAOA*). In a sample of Native American women, the effect of childhood sexual abuse (frequent among women in this and other populations) on risk of developing alcoholism and antisocial personality disorder was contingent upon *MAOA–LPR* genotype [27]. Sexually abused women homozygous for the low-activity *MAOA–LPR* allele had high rates of both disorders, and heterozygous women displayed an intermediate risk pattern. However, in the absence of childhood sexual abuse, there was no relationship between *MAOA* genotype and these disorders.

MAOA G×E has also been studied in animal models. These are useful because of the ability to control stress exposures and many other environmental variables. In the Rhesus macaque (*Macaca mulatta*) early life stress exposure, particularly early separation from the mother, leads to dyscontrolled behavior and enhanced stress response later in life. The behaviors observed in the stressed animals include increased alcohol consumption, higher impulsive aggression, incompetent social behavior and serotonin dysfunction, and increased behavioral and endocrine responsivity to

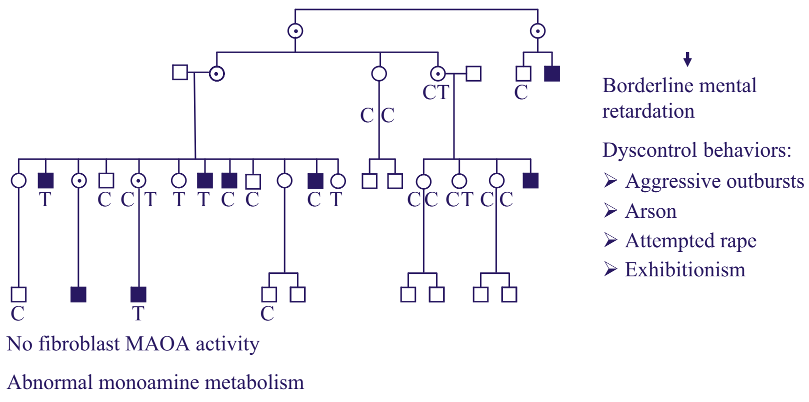

Fig. 23.5.12 Pedigree of a Dutch family with eight males affected by Brunner syndrome, X-linked behavioral dyscontrol caused by a stop codon in monoamine oxidase A. This variant (C936T) leads to complete and selective deficiency of MAOA activity. The X-linked pattern of transmission features unaffected carrier mothers, and both affected (carrier) and unaffected (noncarrier) male offspring

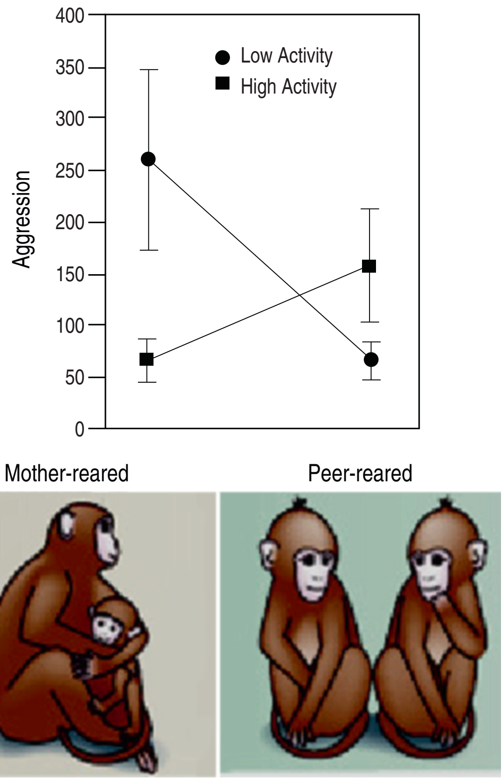

Fig. 23.5.13 Interactive effect between a genetic variant (rhMAOA-LPR) in the promoter of the rhesus monkey MAOA gene and maternal separation on aggressive behavior. The low-activity MAOA allele is associated with increased aggression only among mother-reared monkeys (*left*), and not among peer-reared monkeys (*right*). Adapted from [105], with permission

stress (for review see [5]). Remarkably, an orthologous (same evolutionary origin and same function) VNTR polymorphism is also found in the promoter region of *MAOA* in the Rhesus macaque. Also similar to the human, the lower activity allele predicts aggressive behavior in these animals, and the association is dependent on whether the monkey had been separated from its mother (see Fig. 23.5.13) [105].

Where in the brain does MAOA mediate its effects on behavioral variation? MAOA activity in the hippocampus, a brain region that processes emotional experience and memory, may be critical to the interaction between *MAOA* and childhood trauma. Carriers of low-activity *MAOA–LPR* allele hyperactivate the hippocampus and amygdala during the retrieval of negatively valenced emotional material, but not during the retrieval of neutral material [103]. Therefore, the increased sensitivity to adverse experiences of carriers of the low activity *MAOA* allele might be due to their stronger activation by negative stimuli and their converse impairment in extinguishing adverse memories and conditioned fears.

How do endocrine factors that modulate behavioral control and aggression interact with *MAOA* alleles that modulate the same behaviors? This is a complex question because of the possibility that effects of hormones and *cis*-acting genetic elements could converge on the expression of the gene (in this case *MAOA*). On the other hand, the hormonal environment of the brain could interact with the genotype-influenced availability of monoamine neurotransmitters. In fact, there is a powerful gene × endocrine interaction between *MAOA* and testosterone on the outcome of dyscontrolled and aggressive behavior. This interaction was explored because of the role of testosterone in aggressive behavior, which in part explains high male:female ratios for acts of violence and aggression resulting in criminal convictions. Within males (females have much lower testosterone levels) there is a moderate correlation between testosterone level and lifetime aggression score. However, this relationship is contingent upon the male having the low-expression *MAOA* genotype, as approximately half of males do [132] (Fig. 23.5.14).

The mechanism of the interaction is an open question, because while both low activity and high testosterone lead to behavioral dyscontrol, androgens increase *MAOA* expression through response elements located within the *MAOA* promoter [108].

The serotonin transporter (*SLC6A4)* is a key regulator of the level of serotonin in the synapse. The effects of serotonin are illustrated in part by serotonin transporter blockade, which can be accomplished with drugs such as the serotonin-specific reuptake inhibitors commonly used to treat depression, anxiety, and chronic pain. The serotonin transporter gene *SLC6A4* has a common polymorphism in its promoter region (*5-HTTLPR*). The major alleles, which affect transcription of the gene, involve 16 (*L*) or 14 (*S*) copies of a 20- to 23-bp imperfect repeated sequence [91]. Furthermore there is a relatively common, functional A>G substitution within the *L* allele [63]. The low-transcribing *s* allele has been inconsistently associated with trait anxiety, depression, and alcoholism. However, the effect of this allele on behavior appears to be stronger if stress exposure is taken into account. *5-HTTLPR* moderates the impact of stressful life

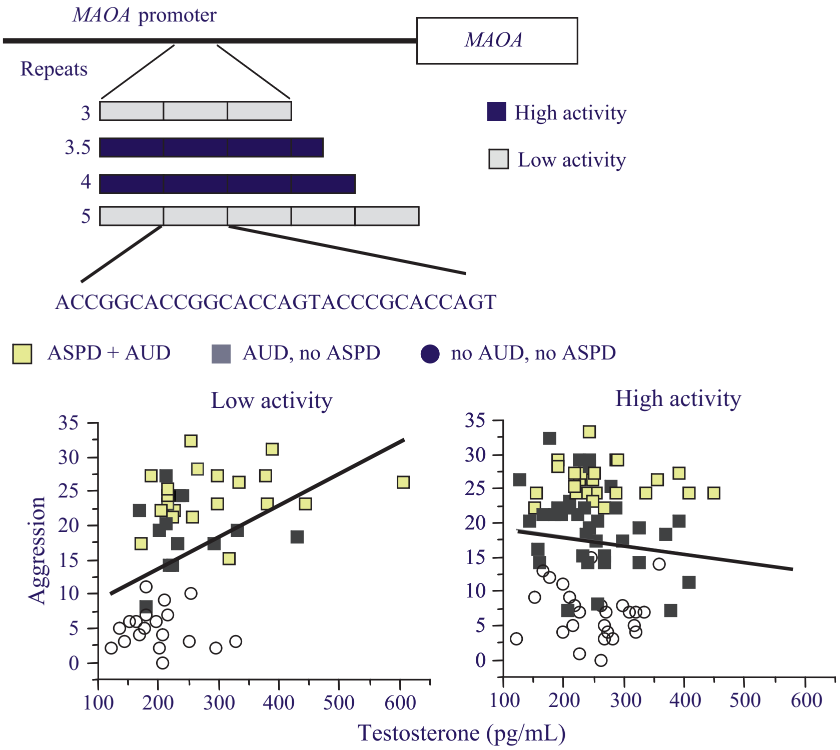

Fig. 23.5.14 Interaction between testosterone level and a genetic variant (MAOA-LPR) in the promoter of the human MAOA gene on lifetime aggression in males from a Finnish population. These include alcoholics with co-morbid antisocial personality disorder (*AUD + ASPD*), alcoholics without co-morbid antisocial personality disorder (*AUD, no ASPD*) and controls (*no AUD, no ASPD*). (**a**) Alleles at the MAOA-LPR locus differ for number of copies of a 30-bp repeated sequence. Alleles with 3.5 and 4 repeats are transcribed more efficiently than alleles with three and five copies of the repeat, and are therefore associated with increased MAOA activity. Indeed, some controversies exist for the activity associated with the five repeats allele (see text). (**b**) Elevated level of testosterone is associated with increased aggression among carriers of the low-activity allele. In contrast, no association between testosterone level and aggression is detected among carriers of the high activity allele. Adapted from [132], with permission

events on risk of depression and suicide [19, 125]. Carriers of the low-transcribing *S* allele exhibit more depression and suicidality following stressful life events than *L* individuals with two copies of the *L* allele [19] (Fig. 23.5.15).

Furthermore, *5-HTTLPR* has been shown to moderate the functions of brain regions, such as the amygdala, that are critical in emotional regulation and response to environmental changes. Carriers of the low-activity allele display increased amygdala reactivity to fearful stimuli [50], reduced amygdala volume [111], and enhanced functional coupling between the amygdala and the ventromedial prefrontal cortex [57], a brain region that ordinarily modulates the activity of the amygdala such that emotional responses are buffered. Closer to the function of the gene, an effect of *5-HTTLPR* genotype on transporter expression in brain in vivo has been reported in some studies [56] but not in others [131]. As was the case for *MAOA*, the Rhesus macaque again has an orthologous polymorphism in the promoter region of its gene. Consistent with findings in humans, the macaque rs-5HTTLPR polymorphism influenced alcohol consumption and stress response, depending on rearing conditions. Carriers of the low-expression serotonin transporter genotype that were separated from their mothers at an early age displayed higher stress reactivity and ethanol preference [6]. The combined effect of *rh-HTTLPR* and environment on stress reactivity suggests that the influence of *HTTLPR* on behavior might be traced to altered regulation of the hypothalamic–pituitary–adrenal (HPA) axis.

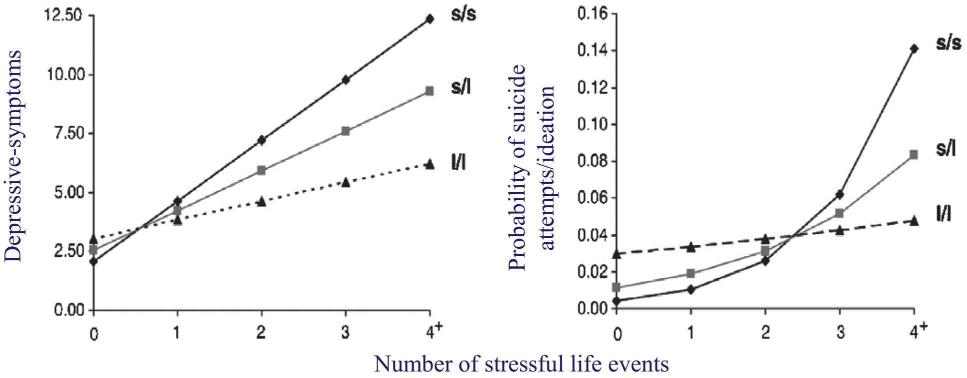

Fig. 23.5.15 Influence of life stress on depression and suicide: moderation by a polymorphism (5HTT-LPR) in the promoter of the serotonin transporter gene (SLC6A4). Individuals homozygous for the low-activity allele (*ss*) who were exposed to more than four stressful events were at higher risk for depression and suicide attempts/ideation than were those homozygous for the high activity allele (*ll*). Differences in risk between the three genotypes emerged progressively in the context of stress life exposure, consistent with a gene by environment interaction. Adapted from [19], with permission

23.5.6.2 Genetic Mapping

Genetic mapping is the localization of genes underlying a trait on the basis of correlation with DNA variation, without the need for prior hypotheses of biological function [3]. For the addictions, genome-wide scans, including whole-genome linkage and whole-genome association (WGA), have implicated several chromosome regions. Here the perspective is the progress of these efforts and the genes thus far identified in what will come to be viewed as the early days of these efforts: in particular a nicotinic receptor gene that has a role in vulnerability to nicotine addiction, as well as lung cancer.

23.5.6.2.1 Whole-Genome Linkage

In whole-genome linkage studies a panel of polymorphisms is tested for meiotic linkage to a disease in family-based samples. This is done by identifying chromosome regions that are shared more often among phenotypically concordant relatives than among phenotypically discordant family members. The implicated chromosomal regions are usually broad, e.g., greater than 10 Mb. Therefore, a more refined search for candidate genes within the disease-linked region is subsequently conducted using association or sequencing.

To perform whole-genome linkage analysis for alcoholism and other addictions, several large family-based data sets have been collected. These include the Collaborative Study on the Genetics of Alcoholism (COGA) [120], the Roscommon study of Irish families [119]; a sample of multiplex families collected in the Pittsburgh area [60]; and samples collected from relatively isolated populations, including Native Americans [28, 30, 94] and Finns [90]. Such isolated populations, and large families within them, are likely to confer the advantage of reduced genetic heterogeneity. A non-exhaustive list of convergent findings across family linkage studies includes a region on chromosome 4q that contains the alcohol dehydrogenase (*ADH*) gene cluster [30, 94, 117, 120] and a chromosome 4p region containing a γ-aminobutyric acid receptor (*GABA*$_A$) gene cluster [94, 120]. In the COGA sample there was also evidence for linkage to chromosomes 1 and 7, and to chromosome 2 at the location of an opioid receptor gene [60]. A region on chromosome 1 was linked to alcoholism and affective disorder in the COGA data set [106], providing more information in support of the existence of a genetic overlap between alcoholism and internalizing disorders. A region on chromosome 7 was linked to alcoholism and/or illicit drug disorders in a subset of COGA families with high density for childhood and adult antisocial behaviors [65]. Linkage analyses have also been conducted with intermediate phenotypes for alcoholism, including low response to alcohol [106], neurophysiological endophenotypes such as P300 [114], and reduced alpha power [28, 36], and chromosome regions identified by these studies overlap partially with those reported for alcoholism.

GABA$_A$ Receptors. γ-Aminobutyric acid (GABA) is the primary inhibitory neurotransmitter in the central nervous system. GABA$_A$ receptor-mediated chloride currents into neurons are facilitated by various drugs including ethanol, benzodiazepines, and barbiturates. Several lines of evidence suggest that GABA is involved in many effects of alcohol, including tolerance, dependence, and cross-tolerance to benzodiazepines and barbiturates. A series of mouse ethanol-related behaviors, including preference, withdrawal severity, and sedation sensitivity, map to quantitative trait loci (QTL) regions where *GABA$_A$* receptor-gene clusters are located [24, 87]. In the rat, an Arg100Gln missense variant located in the GABA$_A$ α6 subunit gene (*GABRA6*) was associated with variation in ethanol and benzodiazepine sensitivity [87]. In humans, AUD has been linked to both the chromosome 4 [28, 34] and chromosome 5 [118] GABA clusters. Linkage signals appear to derive from *GABRA6* on chromosome 5 [118] and *GABRA2* [28, 34] and *GABRG1* [33] on chromosome 4. In the COGA sample, the association between *GABRA2* and alcoholism was mainly driven by alcoholics who also abused illicit substances, indicating that this gene might contribute to the shared liability to a different class of addictive disorders [2]. A human variant of *GABRA6* (Pro385Ser), which is located in the chromosome 5 cluster, was associated with sensitivity to alcohol [62] and benzodiazepines [64].

23.5.6.2.2 Whole-Genome Association

Large-scale genotyping techniques have recently become available, and genome-wide analyses for several complex diseases, including diabetes, obesity, bipolar disorder, inflammatory, bowel disease, prostate cancer, breast cancer, colorectal cancer, and rheumatoid arthritis (for review see [3]), have been completed within large data sets of unrelated individuals using dense panels that can include up to 1 million single nucleotides (SNPs). These WGA studies have the advantage of increased power for detecting effects of relatively common alleles (>0.05) and more refined localization of signals to smaller chromosome regions than family-based linkage analyses, which have a reciprocal advantage of being powerful for detecting effects of rare and uncommon alleles that are present in only a small proportion of probands and their families.

A WGA on nicotine addiction was performed using number of cigarettes per day regularly smoked as a phenotype, in two European populations with a total of 7,500 persons. Although no SNP reached genome-wide statistical significance, a trend toward association was found for a common haplotype in the *CHRNA3-CHRNA5* nicotinic receptor subunit gene cluster on chromosome 15. This result has been replicated in a third set of 7,500 additional European individuals [7]. A, a missense mutation in the alpha5 nicotinic cholinergic receptor (*CHRNA5*), has been shown to influence vulnerability to nicotine dependence [9] and lung cancer [136].

For alcoholism, a WGA scan was conducted in a sample of unrelated alcohol-dependent ($n = 120$] and control ($n = 160$) individuals sampled from the COGA pedigrees [68]. This study identified several candidate genes that might moderate vulnerability to alcoholism and whose products are involved in cellular signaling, gene regulation, development, cell adhesion and Mendelian disorders. However, these findings are weakened by the small sample size and the consequent lack of power to identify exhaustively common alcoholism-causing alleles. WGA in large case-control data sets will soon be reported for alcoholism; there are ongoing studies on alcohol consumption including more than 20,000 individuals!

23.5.7 Treatment of Addictions

Treatment of addictive illnesses is enormously beneficial, in the same sense as it is also worthwhile to treat other diseases, such as cancer, where success rates are substantially less than perfect, and where – as in the addictions – volition and lifestyle choices also have a powerful role in etiology and outcome. The maintenance of abstinence for multiyear periods has enormous benefits to the individual, family, and community. Half or more of addicted individuals can make the transition to lifetime freedom from relapse, although this is usually only accomplished through a combination of individual will, lifestyle changes, family support, self-help groups (including Alcoholics Anonymous), and medical care. The multidimensional nature of addictions makes their etiology more difficult to comprehend, but paradoxically increases the range of opportunities for interventions that can sometimes be used in complementary fashion. Effective interventions extend from the spiritual and religious to drug therapies that ease withdrawal, block the action of an addictive drug (antagonist therapies), substitute for the addictive agent (agonist therapies), or reduce symptoms such as anxiety and depression which accompany long-term withdrawal and can trigger relapse. Also, understanding the role of neurobiology and genetic factors in vulnerability to addictions has been crucial in the destigmatization of these illnesses, thus encouraging their diagnosis and treatment. Lastly, the identification of genes altering the liability to addiction and ability to recover are a major focus for genetic studies, because these could provide new therapeutic targets and an ability to individualize treatment (so-called personalized medicine). One of the first examples of pharmacogenetic prediction of treatment response in the addictions is a common functional missense variant of the mu-opioid receptor (*OPRM1* Asn40Asp). In several studies, naltrexone, a mu-opioid receptor antagonist, was observed to augment abstinence and good therapeutic outcome in recovering alcoholics. Carriers of the Asp40 allele appear to be highly likely to show clinical improvement when treated with this drug, encouraging the idea that the treatment of this large clinical population can be better targeted [4, 107].

23.5.8 Conclusion

Addictions are common, complex disorders illustrating the interplay of gene × environment interaction. These disorders, which are in part volitional, in part inborn, and in part determined by environmental experience, pose the full range of medical, genetic, policy, and moral challenges. Gene discovery is being facilitated by a variety of powerful approaches, but is in its infancy. It is not surprising that the genes discovered so far act in a variety of ways: via altered metabolism of drug (the alcohol metabolic gene variants for alcohol), via altered function of the receptor of the drug (the nicotinic receptor for nicotine), and via general mechanisms of addiction (genes such as monoamine oxidase A and the serotonin transporter that modulate stress response, emotion, and behavioral control).

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Behavioral Aspects of Chromosomal Variants

23.6

Michael R. Speicher

Abstract Humans with chromosome aberrations often show, along with many other findings, behavioral abnormalities, which may be mild or severe and may either affect all carriers of an aberration or only some of them. Although chromosome aberrations influence embryonic development in multiple and ill-defined ways, certain genetic syndromes do result in relatively specific patterns of behavior and related attributes. Although sophisticated approaches to examine such "behavioral phenotypes" have been developed, the description and characterization of behavioral aspects is also frequently accompanied by claims and counterclaims which often complicate the subject. Relative to the general population, individuals with intellectual disabilities are at much higher risk of experiencing behavioral, emotional, and psychiatric problems. However, many mental health professionals do not appreciate the co-occurrence of psychiatric problems and intellectual disabilities. Therefore, there are several gaps in the research and treatment of mental health concerns in people with autosomal chromosomal aberrations. Nevertheless, chromosomal aberrations offer the unique opportunity to relate behavioral phenomena to independently ascertained and relatively well-defined genetic causes. In this chapter the impact of various chromosomal rearrangements, ranging from whole-chromosome copy number changes to small deletions or duplications, on behavior is described and discussed.

Contents

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23.6.1 Introduction: Human Chromosome Aberrations and Behavior, Possibilities, and Limitations

Humans with chromosome aberrations often show, along with many other findings (Sect. 23.6.4), behavioral abnormalities, which may be mild or severe and may either affect all carriers of an aberration or only some of them. Although chromosome aberrations influence embryonic development in multiple and ill-defined ways, certain genetic syndromes do result in relatively specific patterns of behavior and related attributes. Several approaches to examination of such “behavioral phenotypes” have been developed [25]. A special group that has given immense impetus to the delineation and study of behavioral phenotypes is that of parent support groups. These groups have advocated and raised awareness of major behavioral problems associated with chromosomal aberrations, some of which are often unfamiliar to the clinicians with whom the families have been dealing. However, the description and characterization of behavioral aspects is also frequently accompanied by claims and counterclaims which often complicate the subject.

Most unbalanced autosomal aberrations lead to multiple and severe malformations (Sect. 23.6.4) that also affect the brain, and may therefore cause cognitive impairment ranging from developmental delay to severe mental deficiency. Relative to the general population, individuals with intellectual disabilities are at much higher risk of experiencing behavioral, emotional, and psychiatric problems. However, many mental health professionals do not appreciate the co-occurrence of psychiatric problems and intellectual disabilities. Therefore there are several gaps in the research and treatment of mental health concerns in people with autosomal chromosomal aberrations. Nevertheless, chromosomal aberrations offer the unique opportunity to relate behavioral phenomena to independently ascertained and relatively well-defined genetic causes.

23.6.2 Numeric Autosomal Aberrations

Key findings of behavior and emotional problems in humans with Down syndrome are summarized here.

23.6.2.1 Down Syndrome

Down syndrome is the most common syndrome with a trisomy of an autosome (Sect. 23.6.4). The exact definition of a Down syndrome cognitive phenotype is a particular challenge, as it is influenced by biological development, such as the maturation of specific neural systems over time on the one hand, and experiences and training on the other hand. There are only a few studies following individuals longitudinally, and assessing them at key points in time takes very complex and difficult efforts [11, 51]. As a consequence, it is not surprising that estimates of the intelligence quotient (IQ) attributed to Down syndrome have varied over the years.

With all known shortcomings of IQ estimates it is apparent that cognitive deficits and the IQ range show considerable heterogeneity within the population with Down syndrome. Some may have IQ scores between 50 and 70, indicating moderate intellectual disabilities. However, the vast majority of humans with Down syndrome have scores from over 20 up to 50, corresponding to severe intellectual disability, while a very few have scores of 20 or below [62]. Nevertheless, there is evidence that over the life span performances may decline relatively more frequently in humans with Down syndrome than in the general population [10].

Individuals with Down syndrome may have significant delay in nonverbal cognitive development accompanied by additional specific deficits in speech, language production, and verbal working memory [11, 51]. However, many of these persons can be educated to read and write.

Compared with other groups with specific syndromes or with various causes for their respective disabilities, children with Down syndrome generally show lower rates of significant behavioral or emotional problems [13]. Although rates of psychopathology are relatively low, children with Down syndrome may show behavioral problems. These include – relative to typically developing controls – a higher frequency of externalizing behaviors such as stubbornness, oppositionality, inattention, attention-seeking, and impulsivity [12]. As a consequence, about 6–8% of children with Down syndrome are diagnosed with attention deficit/hyperactivity disorder (ADHD) [13]. In contrast to the prevalent stereotype and view of children with Down syndrome as friendly, sociable, and charming, there is a growing recognition of the co-occurrence of Down syndrome and autism spectrum disorder (ASD) in about 7–10% of the cases [30].

Children with Down syndrome go from having relatively few behavioral problems in their early years to being at markedly increased risks for depression and clinical symptoms of dementia in their adult years. The aforementioned externalizing problems often decline as early as during adolescence. At the same time, internalizing symptoms, such as withdrawal and being more secretive and quiet, increase during this time span, so that persons with Down syndrome often show an age-related withdrawal [14].

Neuropathologic signs of dementia become apparent in most individuals with Down syndrome aged 40 years and older. The full pathology of Alzheimer disease appears to be invariably present from 35 years of age onwards – 50 years earlier than in the normal population. In fact, Down syndrome is unique in conferring a 100% risk of developing early Alzheimer disease [2]. In addition to Alzheimer disease, adults with Down syndrome are particularly prone to depression. This is even true when compared with other groups with intellectual disabilities, and the prevalence rates of depression are estimated to range from 6.1 to 11.4% [34]. In the general population memory changes represent usually preclinical stages of dementia. In contrast, in adults with Down syndrome behavior and personality changes are frequently found at the beginning of dementia, suggesting that frontal lobe dysfunction and dementia of the frontal type may be characteristic of the early course or manifestation of Alzheimer disease in Down syndrome [5].

Other behavioral aspects of adults with Down syndrome include a much lower likelihood of physical aggressiveness than in their counterparts and relatively rare occurrence of bipolar disorder and schizophrenia [13]. Future research efforts will certainly have to focus on the more detailed identification of risk or protective factors for these psycho-behavioral aspects, and also on the efficacy of different interventions and treatment forms.

23.6.3 Copy Number Variations Associated with Behavioral Disorders

Genomic variability is to a large extent caused by structural alterations, such as deletions, duplications or inversions. The widespread presence of copy number variations (CNVs) even in normal individuals was first reported in 2004 [27, 48] and has subsequently been confirmed in numerous other reports (see Sect. 23.6.3). Such structural variations have now been implicated in various behavioral disorders, such as autistic spectrum disorder, schizophrenia, and bipolar disorders. The association between CNVs and these disorders will be described in greater detail in the chapters specifically devoted to them (e.g., autistic spectrum disorder: Chap. 23.4 and schizophrenia: Chap. 23.7) and the treatment of this topic is therefore kept very brief here.

23.6.3.1 *Autistic Spectrum Disorder*

Array-comparative genomic hybridization (CGH, see Sect. 3.4.3.5.3) performed with genomic DNA of individuals with autistic spectrum disorder and their parents revealed a significant association between de novo CNVs and autism [4, 49]. This observation was confirmed in another study [32]. The CNVs often involve genes critical for CNS development, such as the *SHANK3-NLGN4-NRXN1* postsynaptic density genes, *DPP6-DPP10-PCDH9* (synapse complex), *ANKRD11*, *DPYD*, or *PTCHD1* [37], supporting the notion that the observed CNVs represent a significant risk factor for autistic spectrum disorders. Based on these results, cytogenetic and array analyses should be included in the standard diagnostic program in persons with suspected autistic spectrum disorders.

23.6.3.2 *Schizophrenia*

A series of reports also described correlations between CNVs and the occurrence of schizophrenia. Some of these CNVs were also implicated in other psychiatric diseases, such as autistic spectrum disorders and mental retardation. The evolving pattern is that there are some rare CNVs, which contribute to the genetic component of schizophrenia owing to their high penetrance [67]. In particular, deletions in 1q21.1, 15q11.2 and 15q13.3 were found to be significantly associated with schizophrenia [55].

23.6.3.3 Bipolar Disorders

For bipolar disorders there is also increasing evidence for an association with rare structural variants, especially if the age of onset of mania was below 18 years [65, 68].

23.6.4 Aberrations of the X Chromosome

Numeric and structural aberrations of the X and Y chromosomes generally lead to much milder disturbances in embryonic development than autosomal aberrations do (Sect. 3.6.1). Many somatic abnormalities found in these syndromes are related to abnormal sexual development. The psychological disturbances are less overwhelmingly severe and may sometimes be specific.

23.6.4.1 Turner Syndrome

Clinical and chromosomal findings of Turner syndrome are described in Sect. 3.6.3.3.3. The standard karyotype is 45,X; however, many mosaics and structural variations are observed. Turner syndrome is associated with a number of characteristic physical features, such as short stature and absent ovaries, as well as a set of common neuropsychological deficits and social and behavioral features.

Earlier investigations more than 50 years ago reported a significant reduction in mean IQ [23]. Subsequently it has been acknowledged that the lower IQs reflected a significant reduction in Performance IQ, whereas Verbal IQ was normally distributed [17]. Areas in which Turner syndrome females typically score below population norms include Arithmetic, Picture Completion, Coding, and Object Assembly subtests [33, 45]. In fact, visuospatial problems are the cardinal cognitive deficit of women with Turner syndrome. However, selective deficits in attention, memory, and executive processing can also be seen.

Despite the fact that verbal abilities are within normal limits, individuals with Turner syndrome may show reduced fluency, poor articulation, and difficulty processing syntactic structures [61]. In tests of academic achievement individuals with Turner syndrome demonstrate considerable difficulty with arithmetic but perform adequately for age in terms of their reading and spelling and are often described as avid readers. Therefore, at school many girls with Turner syndrome are identified as having a nonverbal learning disability and may have an increased need for special education [45].

In childhood a significant proportion of Turner syndrome patients tend to be hyperactive, while up to 10% have ADHD in adolescence. However, many others are extremely inhibited and shy, especially in adolescence, which is a particularly unhappy time for teenagers with Turner syndrome [45].

Most of the observations seen in children with Turner syndrome persist into adulthood, particularly their visuospatial and visual memory deficits [42, 44]. In contrast, some abilities improve with age, such as weaknesses in perceptual judgment and motor planning skills [41]. A large number of individuals with Turner syndrome demonstrate excellent musical aptitude.

Less than 5% of Turner syndrome women achieve higher professions, although a few individuals with Turner syndrome hold graduate degrees and have become physicians or lawyers. The majority of individuals with Turner syndrome hold clerical or semiprofessional positions (i.e., teaching, nursing, early childhood education) and they tend to be overrepresented in child-care positions. There is a high incidence of dependence, with many living at home together with their parents as adults [43].

Psychosocially females with Turner syndrome show a definite female gender identity and assume a typical female gender role. Social immaturity is often described in individuals with Turner syndrome, and their social relations are often difficult [45]. The majority have few friends during adolescence, and even in adulthood difficulties with relationships and coping with Turner syndrome are commonly reported. Owing to their particular set of physical stigmata they generally have a poor body image and low self-esteem [45].

23.6.4.2 Klinefelter Syndrome

Klinefelter syndrome is a relatively common (1/500 to 1/1,000) genetic syndrome caused by an extra X chromosome in males, leading to an XXY karyotype; other karyotypes and also mosaics occur. Adult patients are an average of a few centimeters taller than their normal brothers; in particular, their legs are longer in relation to their overall stature. This growth pattern can be observed as early as in childhood; at the age of puberty the subnormal sexual development becomes obvious: the testicles are small, and there is aspermy (Sect.3.6.3.3.4).

Although Klinefelter syndrome is not rare, many men with Klinefelter syndrome are not aware of their genetic impairment. A recent epidemiologic study suggests that more than 75% of subjects with Klinefelter syndrome are not diagnosed [8]. Furthermore, in many cases diagnosis is significantly delayed, made not during childhood but in adolescence, or even later. Thus, most men with Klinefelter syndrome and their relatives are not aware of the genetic constitution that underlies their cognitive and behavioral problems. As a result, our knowledge of the social behavioral phenotype of Klinefelter syndrome is limited. In fact, cognitive and behavioral dysfunctions in Klinefelter syndrome have generally been underrated relative to the endocrinological and physical features. Previous studies have predominantly assessed global functioning (e.g., academic achievement, occupation, or marital status) rather than specific social abilities. Some studies have specifically focused on social adjustment in adolescents and men with Klinefelter syndrome, yet they primarily collected categorical data (e.g., someone can be either sociable, passive or shy) or involved small sample sizes or lacked control data recorded in individuals from the general population [64].

In most cases, the physical and neurobehavioral characteristics of Klinefelter syndrome are relatively mild, and Klinefelter syndrome is not usually associated with moderate or severe mental retardation. However, Klinefelter syndrome is often associated with significant language-based learning disabilities and executive dysfunction, and there is a general impression that men and boys with Klinefelter syndrome often struggle with social situations (e.g., at school or at work) [19]. Many of the psychological symptoms encountered in males with Klinefelter syndrome can be explained by their diminished androgen production, which is normally required for the expression of male-specific psychological development.

Several studies suggest that individuals with Klinefelter syndrome are at risk for psychosocial and emotional problems such as social withdrawal, social anxiety, shyness, impulsivity, and inappropriate social behavior [19, 52]. In early adulthood a significant portion of XXY men have few or no friends, poor relations with siblings and parents, little energy and initiative, and few or no spare time interests. Their vitality and ability in establishing social contacts is often reduced [35].

The patients show on average slightly reduced intelligence with special difficulties in learning how to read and write. However, IQ values well above average are not rare. On the other hand, Klinefelter syndrome has been found more often in series of mildly mentally subnormal subjects. The literature reports do not unanimously support a well-defined specific defect of mental abilities.

These psychosocial aspects of Klinefelter syndrome can have a significant impact on school performance and learning. School problems are more frequent than expected from intellectual ability and seem to be caused by behavioral problems. Adult patients often hold unskilled jobs; success in higher professional careers has been reported but does not seem to be very common.

A recent study described the psychosocial morbidity in a cohort of young males with hypogonadism attributable to Klinefelter syndrome [52]. One aim of this study was to document the effect of androgen replacement on behavior. Seventeen of 32 postpubertal patients with Klinefelter syndrome required testosterone therapy, while in 11 serum testosterone in the normal adult range was documented. Significant psychosocial and behavioral problems were present in 22 out of 32 of patients with Klinefelter syndrome, including seven who were testosterone replete, with an identifiable pattern of disorder, including marked lack of insight, poor judgment, and impaired ability to learn from adverse experience. Use of long-term replacement testosterone treatment reduced episodes of behavioral indiscretion. This suggests that inadequately treated hypogonadism in Klinefelter syndrome may increase recognized psychosocial morbidity [52].

Therefore, there is a need for prospectively planned and timed support for young men with Klinefelter syndrome, in order to ameliorate current poor psychosocial outcomes.

23.6.4.3 Triple-X Syndrome

This syndrome is described in Sect. 3.6.3.3.5. Many women with the karyotype XXX have developed normally and have children. However, the 47,XXX individual may be at greater risk for poor psychosocial adaptation and early adulthood. As pointed out in Sect. 3.6.3.3.5, much of the available data is based on isolated case studies of women ascertained through the presence of another condition. There are only a few studies in which patients with 47,XXX had been identified through chromosomal screening of newborns and followed up longitudinally [24, 46].

One of these few studies compared 47,XXX women and female sibling controls during adolescence and during early adulthood. The study revealed that 47,XXX woman are less well adapted during both adolescence and young adulthood and they described their lives as more stressful. Furthermore, they had more work, leisure, and relationship problems. Their IQ was lower and they showed evidence of more psychopathology than subjects in the reference group composed of female siblings. Propositi with lower IQs tended to demonstrate poorer psychological adaptation. However, psychiatric status was not determined solely by intelligence; psychological dysfunction occurred even among women with IQs in the average range. Although these women seem to have more difficulties, most of them are self-sufficient and functioning reasonably well, albeit less well than their siblings [24].

Another study reported milder impairment in 47,XXX women [46]. However, a concern of these studies is their representativeness, as all of them have small sample sizes. In summary, there is evidence that children with a supernumerary X chromosome score consistently below controls on Verbal IQ and subtests comprising the Verbal Comprehension factor, but did not differ in Performance IQ, which was in the normal range. Academic achievement is not affected in aneuploid females with higher levels of intelligence.

23.6.5 Aberrations of the Y Chromosome

23.6.5.1 XYY Syndrome

For a description of somatic symptoms of the XYY syndrome see Sect. 3.6.3.4.1. The mean stature of these men is usually taller than that in the population of their origin. Many show normal sexual development and are fertile. 47,XYY males may have delayed speech, lower cognitive function (IQ), hyperactivity, learning disabilities, and other central nervous system (CNS) abnormalities [1]. Several males with an XYY constitution and a normal phenotype have also been reported.

23.6.5.2 Higher Prevalence Among "Criminals"

The XYY syndrome has become widely known since Jacobs et al. [28] carried out a survey of patients who were mentally subnormal and under surveillance in a special institution because of "dangerous, violent, or criminal propensities." Among 196 probands 12 had an abnormal karyotype; 7 with XYY and 1 with XXYY. This frequency was much higher than expected; however, the authors stated that they could not determine whether these men had been institutionalized mainly because of mental subnormality, aggressive behavior, or some kind of combination of these factors. Their results were soon confirmed in a number of studies from institutions for mentally subnormal men with behavior problems, especially among particularly tall inmates. On the basis of such evidence it was concluded that their antisocial behavior was caused by the additional Y chromosome, and that they were genetically predisposed to criminality. The explanation seemed simple. Normal men are more aggressive than normal women; normal men have one Y chromosome, while women do not. Hence, if someone has two Y chromosomes, he should be twice as aggressive as normal men; his aggressiveness may fall outside the socially acceptable range, and he may commit acts of violence.

Gradually, however, some pertinent questions were asked: above all, how frequent is the XYY karyotype in the general population of nonconvicts? Studies on the incidence among male newborns showed a frequency of around 1:1,000, or even higher, similar to that of Klinefelter syndrome [20]. Even in the absence of reliable prevalence studies among the male adult population it was fair to conclude that the prevalence differs little from the incidence at birth, i.e., that there is no preferential mortality. This, however, could just mean that the great majority of XYY men do not come into conflict with the law.

Another question was whether the nature of their crimes revealed a certain pattern and, more specifically, whether acts of violence and sexual aggression prevailed. This was in general not the case: using a population-based sample of men with sex chromosome abnormalities by screening 34,380 infants at birth, Götz et al. [20] compared XYY men, XXY men, and controls for the frequency of antisocial personality disorder and rates of criminal convictions. This study report showed that, with adjustment for the number of years at risk of receiving a criminal conviction the difference in overall delinquency rate ratios was significant at the 1% level ($p=0.01$), revealing that the XYY men were more likely to have a criminal record than chromosomally normal controls. However, there was no evidence from the sentences imposed on the subjects that the offenses committed by the XYY men were more serious than those committed by the controls. In fact, XYY men committed significantly more offenses within categories such as "breach of the peace" ($p<0.005$) and theft ($p<0.01$), yet not in other subcategories, such as "assault," "criminal damage," "alcohol and drug related," or "sexual offenses." Furthermore, there was no evidence from the length of imprisonment or the magnitude of the fines that the delinquent acts of the cases were more extreme than those of controls. In particular, there was no significant difference in crimes of sexual nature, such as indecent assault and shameless indecency. The XYY men received their first conviction at a mean age of 17.6 years, not significantly younger than the controls, at 18.1 years [20]. Thus, while XYY men committed more offenses overall, the offenses were not more serious than those committed by the controls.

Furthermore, the image of XYY men as especially aggressive is also not supported by the behavior of XYY men when they become institutionalized. The question was asked whether they are more aggressive than other men detained in the same institutions. In fact they turned out to be more agreeable; on average they had better relationships with supervisory personnel [56]. Many more psychological and psychiatric studies were carried out. While varying in details, their overall picture seldom differed from that of chromosomally normal inmates of the same institutions with the same range of intelligence.

All these results suggest alternative explanations for the undisputedly higher frequency of XYY probands in institutions for law offenders.

23.6.5.3 Intellectual Dysfunction or Simply Stature?

Many studies have been carried out on convicted and imprisoned law offenders. Their mean IQ is generally low. Intellectually subnormal persons are more often involved in criminal activities – or they run a higher risk of being apprehended. Is the supposedly higher crime rate of XYY men only a result of their reduced average intelligence?

This option appeared to be less likely according to one study, which found an increased rate of criminality in XYY men even after adjusting for social class and intelligence [66]. However, this study was also not conducted on unbiased samples: only men taller than 183 cm were screened. This resulted in a considerable discrepancy between expected and diagnosed numbers of XYY men: assuming an incidence of 1 in 1,000 of the male population the study should have found at least 30 individuals in this cohort of 31,000, whereas in the height-restricted group only 12 XYY men were found. There is the possibility that this resulted in an identification of a subgroup more likely to receive a criminal conviction in court, perhaps because of perceived threat on account of their greater height. In fact, it has been hypothesized that the characteristic tall stature of XYY men may increase the probability of being apprehended [26].

Thus, these important questions can only be addressed by unbiased samples which also look at other behavioral aspects of XYY men.

23.6.5.4 Behavioral Aspects of XYY Men

Broader, less biased studies have been performed on males with XYY syndrome, showing that behavior disorders are not a primary feature in childhood [7]. It is evident that environmental factors play a great role in the development of personality and behavior in males with karyotype 47, XYY as well as in males with a normal chromosome constitution [9].

The aforementioned study by Götz et al. [20] identified no XYY man who would have fitted diagnostic criteria for major psychiatric disorder. However, compared with controls significant differences in antisocial behavior in XYY men were found for unstable occupational history (defined as frequency of job changes, absences from work, and periods of unemployment) and antisocial behavior during adolescence and adulthood (defined as smoothness of school careers; school performance, lying at school and at home, disruptive behavior). Overall, this study suggested a slightly increased liability to antisocial behavior in XYY men [20].

Another study observed longitudinally 38 XYY males, 12 of whom were diagnosed prenatally. XYY males were at a considerably increased risk for delayed language and/or motor development. From birth onward, weight, height, and head circumference were above average values. The majority attended kindergarten in the normal education circuit, although in 50% of these cases psychosocial problems were documented. From primary school age on, there is an increased risk for child psychiatric disorders such as autism. Moreover, although normally intelligent, many of these boys are referred to special education programs [18].

In contrast, 47,XYY boys from families with better socioeconomic status had slightly higher IQs and fewer language problems than those from families of lower socioeconomic status [31].

As a consequence of these studies there can be little doubt that men with the chromosome constitution XYY run a higher relative risk of showing antisocial behavior and coming into conflict with the law than normal XY men.

23.6.5.5 Association of Criminal Behavior and Lowered Intelligence in XYY Men

Whether part of the increased risk of coming into conflict with the law can be traced to the impaired intellectual function of XYY men can only be found if we cultivate a more complex, holistic appreciation of the intervening variables, as opposed to making simple assumptions about genotype-phenotype relationships as has so often been done in the past. While the study conducted by Götz et al. [20] in unselected men confirmed an increase in antisocial and criminal behavior in XYY men, multiple regression analysis showed that this is mediated mainly through their lowered intelligence. Additional background variables, such as the socioeconomic status of the parents, may also account for some of the differences in criminality between the XYY and XY groups [31].

23.6.5.6 Social and Therapeutic Consequences

The evidence shows that the legal consequences for preventing crimes by XYY men as proposed in the heyday of the aggression hypothesis have no basis at all. Still, problems remain. If the XYY status is discovered in a study on newborns, should the parents be informed? Could such information have the effect of a self-fulfilling prophecy in that parents would treat their boy differently, and could this enhance his tendency to deviating behavior? In our opinion, all information should be provided; however, great care is needed in conveying the facts to the parents in a form that causes as little embarrassment as possible and, above all, no damage. The parents should understand that their child might possibly need somewhat more special attention during his education than an XY boy, but that given a stable environment and the same amount of parental protection as other boys enjoy, normal social adjustment is the most likely outcome.

The behavioral problems with XYY individuals (as well as with other persons having deviant sex-chromosomal karyotypes) could probably be alleviated if these conditions had been diagnosed at birth, and if they (and their parents) had received special care during their childhood. Children show marked improvement with appropriate care, e.g., training of their motoric abilities, not only in psychomotoric but also in intellectual development. In an increasing number of countries support groups have been founded to help with these problems.

Inevitably, with the widespread use of antenatal diagnostics XYY, XXY, and XXX karyotypes are discovered by amniocentesis. Parents usually should be fully informed of the findings and the implications of the sex chromosome constitution. The option of abortion as a possibility needs careful discussion; genetic counseling should be nondirective, and the decision should be left to the parents. However, investigations of the rate of pregnancy termination for various fetal aneuploidies suggest that about 57% of pregnancies with a 47,XYY chromosomal constitution are terminated [55].

23.6.5.7 XXYY Syndrome

Phenotypic features of this syndrome are described in Sect. 3.6.4.3. Importantly, the traditional view of the XXYY syndrome as a variant of the Klinefelter syndrome is obsolete, as medical problems are more severe in the former syndrome. Specifically, neurodevelopmental and psychological difficulties are a significant component of the XXYY behavioral phenotype, with developmental delays and learning disabilities universal but variable in severity. A review of 95 males with XXYY syndrome reported that 26% had full-scale IQs in the range of intellectual disability, and adaptive functioning was significantly impaired, with 68% having adaptive composite scores < 70. Overall, rates of neurodevelopmental disorders were elevated to 55.9% and included ADHD (attention-deficit/hyperactivity disorder) (72.2%), autism spectrum disorders (28.3%), mood disorders (46.8%), and tic disorders (18.9%) [59].

23.6.6 Other Chromosomal Variants

Specific behavioral phenotypes exist not only for whole chromosome aneuploidies, but also for numerous other smaller chromosomal aberrations resulting in segmental aneuploidies. The identification of specific cognitive and behavioral associations within a genetic syndrome is important for the characterization of potential etiological pathways of behavior at both the cognitive and the neurobiological level. Therefore, the study of the behavioral phenotype in a known chromosomal disorder provides an important and promising strategy for understanding the genetics and pathogenesis of these disorders in the wider population.

Here only a few syndromes associated with structural chromosomal rearrangements with relatively well-characterized behavioral phenotypes have been selected for discussion. First, the behavioral phenotype of several well-characterized microdeletion syndromes is presented. Microdeletions are so small in size that they usually escape detection in standard banding analysis. However, in many syndromes associated with larger segmental aneuploidies, which are visible by standard chromosome analysis, attempts have been made to identify genotype-phenotype correlations, but cognitive-behavioral aspects of individuals associated with the genotype have not yet been studied systematically. Therefore, data is relatively limited and discussion of behavioral consequences of segmental aneuploidies is confined to two syndromes, i.e., cri-du-chat and Wolf–Hirschhorn syndromes.

23.6.6.1 22q11.2 Deletion Syndrome

The 22q11.2 deletion syndrome (22q11.2DS) refers to a group of related syndromes including velo-cardio-facial syndrome (VCFS), Di George syndrome, and conotruncal anomaly face syndrome. The cause of the deletion is usually nonallelic homologous recombination (NAHR) (Sect. 3.5.5). Many features of this syndrome are discussed in Sect. 3.6.2.2.3. Here the focus will be on the behavioral aspects of this syndrome.

Various behavioral disorders and psychiatric illnesses have been reported within the context of the 22q11.2DS. There is an especially well-documented association between 22q11.2DS and schizophrenia [29], but multiple other behavioral features are linked to this syndrome in addition. Several studies described temperamental and behavioral difficulties, such as poor social skills, problems with social interaction, social withdrawal, and others in early childhood [57]. Other studies found that children with 22q11.2DS exhibit significant attachment to the mother or other caregivers and display clinging behavior and separation anxiety [58]. However, at present longitudinal studies with age-, gender-, and IQ-matched controls are lacking to test whether these early behavioral problems are indicators of future psychiatric disorders.

Attention deficit hyperactivity disorder (ADHD) is the most prevalent psychiatric disorder in children with 22q11.2DS and may occur in up to 40% of affected children [3, 21]. By comparison, the prevalence rates of ADHD in nondeleted school-age children are in the range of 3–5%. When a 22q11.2DS group was compared with another group characterized by similar IQ scores, degree of facial dysmorphism, and cardiac and cleft anomalies, the prevalence of

ADHD was significantly higher in the 22q11.2DS group. This supports the ideas that ADHD in this syndrome may have a genetic basis and that developmental and physical factors may play a smaller role [21]. Furthermore, several studies reported high rates of affective disorders, anxiety disorders, and obsessive-compulsive disorders in children and adults with 22q11.2DS. In addition, a high prevalence (in the range of 14%) of autism spectrum disorders in children with 22q11.2DS was described [40].

Shprintzen et al. [50] were the first to report psychotic symptoms, which they described as resembling "chronic paranoid schizophrenia" in 12 of 90 children and adults with 22q11.2DS [50]. This observation was indeed confirmed by multiple subsequent studies. For example, a longitudinal study comparing adolescents with 22q11.2DS and a control group with idiopathic developmental disability who were matched for age and IQ found that individuals with 22q11.2DS developed psychotic disorders significantly more frequently than control individuals. The available evidence suggests that about 30% of adults with 22q11.2DS have schizophrenia and that the underlying deletion contributes to these high rates [22, 40].

Therefore, the study of 22q11.2DS provides an exciting opportunity to understand the neurobiological basis of psychiatric disorders in both 22q11.2DS and in the wider nondeleted population. Current research is directed towards elucidating the contribution of several susceptibility genes within the 22q11.2 region, such as catechol-*o*-methyltransferase (*COMT*), proline dehydrogenase (*PRODH*), *GNB1L*, and *TBX1*. In 22q11.2DS mouse models, haploinsufficiency of *Tbx1* and *Gnb1L* is associated with a schizophrenia endophenotype [37]. Thus, *TBX1*, a transcription factor, the mutation of which is likely to be sufficient to cause most of the physical features of 22q11.2DS, may also be associated with the behavioral/psychiatric phenotype. However, further studies in persons with 22q11.2DS will be needed to examine the contribution of *TBX1* and *GNB1L* and other genes and their interaction with other candidate genes to the 22q11.2DS behavioral phenotype.

23.6.6.2 Smith–Magenis Syndrome

Smith–Magenis syndrome (SMS) is generally a sporadic disorder caused by either a 17p11.2 deletion encompassing the retinoic acid-induced 1 (*RAI1*) gene or a mutation of *RAI1* [53, 54]. Approximately 90% of all reported cases with SMS have a 17p11.2 deletion, while the remaining 10% have a mutation in the *RAI1* gene. The 17p11.2 SMS deletions are frequently caused by the NAHR mechanism (Sect. 3.5.5). In fact, chromosome 17p11.2p12 is one of the most recombination-prone regions of the genome and is also associated with hereditary neuropathy with liability to pressure palsies (HNPP) and Charcot–Marie–Tooth disease type 1A. The incidence is estimated at in the range of 1:15,000–25,000; however, this syndrome may be often underdiagnosed.

The physical phenotype is frequently described as consisting of craniofacial anomalies including brachycephaly, frontal bossing, hypertelorism, synophrys, upslanting palpebral fissures, midface hypoplasia, or a broad square face with depressed nasal bridge [15]. However, although the phenotype has some distinctive features, diagnosis is often made because of the behavioral rather than the physical phenotype.

Most SMS individuals have mild-to-moderate mental retardation with IQ ranging between 20 and 78. School-age children with IQs in the low normal range have been identified; however, IQ decreases as the child ages [15]. Sleep disturbance is one of the cardinal features and has been reported in 75–100% of SMS cases. In fact, sleep disturbances are one of the earliest diagnostic indicators of SMS and include reduced 24-h and night sleep, fragmented and shortened sleep cycles with frequent nocturnal and early-morning awakenings, and excessive daytime sleepiness. These abnormal sleep patterns are due to an inverted circadian rhythm of melatonin. An aberrant melatonin synthesis/degradation pathway has been proposed as the underlying cause for the inverted circadian rhythm. Management of sleep disturbances has been one of the challenging tasks. No well-controlled treatment plan has been reported [15, 39].

A number of additional behavioral issues belong to the characteristic features of SMS. Some of these features are unique to SMS, such as onychotillomania (pulling out of fingernails and toenails) and polyembolokoilamania (insertion of objects into bodily orifices). Stereotypical behaviors also unique to SMS include the spasmodic upper body squeeze or "self-hugging," and page-flipping or "lick and flip" behavior often seen in association with excitement. In addition, children with SMS frequently display maladaptive behaviors, including frequent outbursts/temper tantrums, attention seeking, aggression, disobedience, distraction, and self-injurious behaviors. The behavioral phenotype of SMS escalates with age, typically with the onset of puberty [15].

All SMS patients with a 17p11.2 deletion are deleted for *RAI1,* and mutations in *RAI1* are likely to result in a truncated and/or nonfunctional protein, thus leading to haploinsufficiency [55]. While *RAI1* has been shown to be responsible for most SMS features, other genes in the 17p11.2 region may contribute to the variability and severity of the phenotype in 17p11.2 deletion cases.

23.6.6.3 Prader-Willi and Angelman Syndrome

A loss of chromosomal region 15q11-q13 results in one of the two most common microdeletion syndromes, i.e., Prader–Willi (PWS) or Angelman (AS) syndrome. In both syndromes, microdeletion of the respective region on chromosome 15 is observed in about 70% of cases, again caused by NAHR (section 3.5.5). Prader–Willi syndrome results from the absence of certain paternally inherited genes on the long arm of chromosome 15, whereas Angelman syndrome is associated with loss of maternal genes (for a detailed discussion of these syndromes, see Chap. 9: Epigenetics).

PWS is most commonly known for its food-related characteristics of hyperphagia, food-seeking behavior, and consequent obesity. Overall, the behavioral phenotype of Prader–Willi syndrome affects four domains: food-seeking-related behaviors; traits indicating lack of flexibility, oppositional behaviors, and interpersonal problems. Treatment should be offered by a multidisciplinary approach with anticipatory medical and psychiatric care. Importantly, the management requires lifelong dietary restrictive supervision to prevent morbid obesity. Psychopharmacologic management may be exacerbated by metabolic abnormalities [6].

Almost all manifestations of Angelman syndrome seem to be related to lack of *UBE3A* gene expression in the brain. The *UBE3A* gene is an imprinted gene located within the aforementioned 15q11-q13 deletion region. The behavioral phenotype is characterized by a happy demeanor with prominent smiling, poorly specific laughing, and general exuberance, associated with hypermotor behavior and stereotypies. In addition, a number of characteristic features of Angelman syndrome may be seen in the context of the autistic spectrum, including virtual absence of speech, impaired use of nonverbal communicative behaviors (facial expression, body postures/gestures to regulate social interaction and decoding of emotional facial expressions), attention deficits, hyperactivity, feeding and sleeping problems, and delays in motor development [39].

23.6.6.4 Williams–Beuren Syndrome

Williams–Beuren syndrome (WBS) is caused by recurrent de novo microdeletions at 7q11.23, which are also mediated by NAHR (Sect. 3.5.5) between low copy repeats flanking this critical region. WBS is a multisystem disorder with a characteristic dysmorphic face, short stature, particularly typical cardiovascular lesions (e.g., supravalvar aortic stenosis), hypercalcemia, and neurological problems.

Individuals with WBS have mild to moderate intellectual disability or learning difficulties; however, this masks an uneven cognitive profile. The WBS neuropsychological profile is striking, characterized by strengths in certain complex faculties (language, music, face processing, and sociability) alongside marked and severe deficits in visuospatial abilities. Children and adults with WBS also have characteristic personality traits, preferring the company of adults to peers and lacking shyness with strangers, over-friendliness and charismatic speech rich in vocabulary. Approximately 70% also suffer from attention deficit disorder, and many experience anxiety and simple phobias. An interesting feature is the musical creativity observed in WBS individuals [62]. It is likely that dosage-sensitive genes within the region are important for the proper development of human speech and language [37].

23.6.6.5 Cri-du-chat Syndrome

Deletions on chromosome 5p lead to a variety of developmental defects, with most cases classified as cri-du-chat syndrome. These deletions may be terminal or interstitial and occasionally occur in the context of a cytogenetically complex karyotype. Cri-du-chat syndrome has several phenotypic components, including the characteristic cry that gives the syndrome its name, facial dysmorphology, speech delay, and mental retardation (MR). While the physical symptoms have frequently been documented, the developmental and behavioral aspects of the syndrome have not been adequately explored [63].

Array-CGH revealed that in patients with only 5p deletions different deleted regions exist, each having a different effect on retardation. Depending on size and location of the deletion, the level of mental retardation may range from moderate to profound [69].

In a study of 10 children a high rate of distractibility and a low level of object-directed behavior were observed in the play sessions. This demeanor may be an early precursor of hyperactivity, distractibility, and stereotypy, which have been reported to be the characteristic features of the behavioral phenotype of older individuals with 5p-Syndrome [47].

23.6.6.6 Wolf–Hirschhorn Syndrome

Wolf–Hirschhorn syndrome (WHS) is associated with microdeletions in the 4p16.3 region, which are variable in size but may produce similar clinical features in the phenotype that characterizes WHS. A recent study examined the cognitive skills and behavioral repertoire of 12 children, ages 4–17 years, who were diagnosed with WHS and who had some speech and expressive language. It was found that their cognitive deficits ranged from mild to severe MR with a mean IQ score of 44.1 (range: 33–64). Children with WHS exhibited relative strengths in Verbal and Quantitative Reasoning, and relative weaknesses in Abstract/Visual Reasoning and Short-term Memory. The adaptive behavior skills of all the children with WHS we assessed were lower than adequate. However, children with WHS exhibit significant relative strength in socialization compared with their communication and daily living skills. In addition, hyperactivity levels and inattentiveness consistent with a diagnosis of ADD or ADHD were noted. However, ADHD and ADD are frequently observed as comorbid features of individuals with MR [16].

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Genetics of Schizophrenia and Bipolar Affective Disorder 23.7

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Abstract Schizophrenia and bipolar affective disorder (bipolar disorder, manic depression) are the paradigmatic illnesses of psychiatry. They profoundly affect thought, perception, emotion, and behavior, and their symptoms cause significant social and/or occupational dysfunction. Schizophrenia and bipolar disorder have been recognized for several millennia, and the WHO (2001) ranks both among the top ten leading causes of the global burden of disease for the age group 15–44 years.

Schizophrenia and bipolar disorder are illnesses with a largely unknown pathophysiology and etiology. Evidence of a clear genetic contribution to the development of these disorders has led to important endeavors to discover the responsible genes. This chapter provides a concise and comprehensive review of the current state of genetic research into schizophrenia and bipolar disorder, and also of its limitations and possible future directions.

Contents

23.7.1 Schizophrenia

Schizophrenia (for diagnostic criteria see Table 23.7.1) is characterized by fundamental and characteristic distortions of thought and perception, inappropriate feelings and/or blunted emotions, and a restricted capacity to act and interact appropriately. The hallmark symptoms of schizophrenia are psychotic phenomena, which include delusions, delusional perceptions, and hallucinations.

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Table 23.7.1 Symptoms required for a DMS-IV diagnosis* of major depressive episode, manic episode, and schizophrenia

Major depressive episode	Manic episode	Schizophrenia
Five symptoms during a *2-week* period	*Three (four)* symptoms *during 1 week* of abnormally elevated expansive (or irritable) mood or any duration if hospitalized	*Two* symptoms during a *1-month* period or less if successfully treated
• Depressed mood	• Inflated self-esteem or grandiosity	• Delusions
• Loss of interest or pleasure	• Decreased need for sleep	• Hallucinations
• Change in appetite and or weight	• More talkative than usual or pressure to keep talking	• Disorganized speech
• Insomnia or hypersomnia	• Flight of ideas or racing thoughts	• Grossly disorganized or catatonic behavior
• Psychomotor agitation or retardation	• Distractibility	• Negative symptoms, i.e., affective flattening, alogia, or avolition
• Fatigue or loss of energy	• Increase in goal-directed activity or psychomotor agitation	Only one symptom is required if delusions are bizarre or hallucinations consist of a voice keeping up a running commentary on the person's behavior or thoughts, or two or more voices conversing with each other
• Feelings of worthlessness or excessive or inappropriate guilt	• Excessive involvement in pleasurable activities	Continuous signs of the disturbance present for at least *6 months*, which may include periods of prodromal or residual symptoms
• Diminished concentration or indecisiveness		
• Recurrent thoughts of death/suicidal ideation or suicide attempt		

From [9]

The symptoms are not due to the direct physiological effects of a substance (e.g., a drug of abuse, a medication or other treatment) or a general medical condition

The symptoms cause clinically significant distress or impairment in social, occupational, or other important areas of functioning

Negative symptoms, thought disorders, and neuropsychological deficits, while less striking in nature, are usually more persistent and more indicative of the course of the disorder [50]. Although cognitive deficits and a decline in intellectual capacities are observed in most patients, consciousness and a substantial level of intellectual capacity are maintained. The course of the disorder is often characterized by recurrent episodes and an increased mortality rate. Approximately two-thirds of all affected individuals have persistent or fluctuating symptoms even if they receive optimal treatment [9].

The onset of schizophrenia typically occurs in early adulthood, although premorbid symptoms have often been present for many years [121]. On average, the age at onset is 3–5 years earlier in men than in women, but this gender difference is not observed in patients with a family history of schizophrenia [4, 44, 121].

23.7.1.1 Prevalence

The life-time prevalence of schizophrenia in developed countries is around 0.5–1%. Early studies indicated that schizophrenia occurs at the same rate world-wide, but more recent studies have suggested that the prevalence may vary between countries, a higher prevalence being observed in developed nations [43, 119]. The prevalence in females and males is similar [6, 119].

23.7.1.2 Environmental Risk Factors

A substantial body of epidemiological research has established that there is a set of nongenetic risk factors for schizophrenia. These include being a first- or second-generation migrant, being born or living in an urban area, having had a winter or spring birth, advanced paternal age, prenatal and/or obstetric complications, cannabis use during adolescence, parental unemployment, and socio-economic status [21, 23, 35, 37, 89, 91, 93, 103, 105, 120, 145]. The effect size of individual risk factors is modest; a typical odds ratio is ≤2, except for migration, which appears to confer a higher risk of around 4. The observation that the incidence of schizophrenia may fluctuate over time is consistent with the influence of environmental factors on disease risk [92].

23.7.1.3 Formal Genetic Studies

23.7.1.3.1 Family Studies

A large number of family studies have shown that schizophrenia runs in families. The average risk for the sibling of an affected person is around 9%, that for an offspring is around 13%, and the risk for a parent is around 6% [130]. The lower risk of developing the disorder observed in parents has been explained by the reproductive disadvantage conferred by schizophrenia. Only one study has directly assessed second- and third-degree relatives, and this has reported risks of 3% and 1.5%, respectively [88]. It has been suggested that the relatives of both female patients and patients with an early age at onset may have an increased risk of developing the illness, although findings have been inconclusive [130]. Relatives of schizophrenia patients show an increased risk for the following disorders: schizophrenia-related personality disorders, i.e., schizotypal and paranoid personality disorders; nonschizophrenic psychotic disorders, i.e., schizophreniform disorder, schizoaffective disorder, delusional disorder, and psychotic disorder not otherwise specified; unipolar depression; and bipolar disorder [69, 88]. Symptoms and symptom dimensions showing familiality are age at onset, course of disorder, impairment during disorder, mode of onset, premorbid functioning, psychomotor poverty, disorganization, and manic features [150].

23.7.1.3.2 Twin Studies

Nearly all of the twin studies conducted to date have shown that concordance rates are higher for monozygotic twins than for dizygotic twins. In studies conducted prior to 1980, pooled concordance rates for monozygotic and dizygotic twins were estimated as 53% and 15%, respectively [67]. Reviews of more recent studies, which were conducted using modern methodological standards (blinded and structured recruitment), have found pooled concordance rates of 40–65% for monozygotic and 0–28% for dizygotic twins [24, 25, 79]. Heritability of the liability to schizophrenia is reported as 81% (95% confidence interval: 73–90%) according to meta-analytical estimates from the pooled data of 12 twin studies [137].

23.7.1.3.3 Adoption Studies

Adoption studies have shown that the biological relatives of patients with schizophrenia (adopted-away children of schizophrenia patients, biological relatives of adopted-away children who later develop schizophrenia) have an increased risk of developing the disorder [57, 70, 72–74, 85, 140, 141]. These studies also demonstrate that nongenetic factors influence vulnerability. The adopted-away children of mothers with schizophrenia only developed schizophrenia when placed in adoptive families with psychological abnormalities [140, 141, 153]. Children of healthy parents adopted by a parent who later developed a schizophrenia spectrum disorder did not show increased risk, demonstrating that nongenetic factors alone are not sufficient to cause the disorder [149].

23.7.1.4 Gene–Environment Interaction

Given the importance of environmental risk factors in the development of schizophrenia (see above), it is important to address the issue of whether these factors act with genetic susceptibility in an independent (additive) fashion, or in a synergistic (interactive) fashion in which the effect of one factor is conditional upon the other [139, 145]. Indirect evidence for a gene–environment interaction has been obtained from twin and adoption studies, which have shown, for example, that the disease risk for adopted-away children of mothers with schizophrenia is dependent on the psychological functioning of the family in which such children are placed [140, 141, 153]. Research has also shown that individuals with a familial genetic loading for schizophrenia may be more likely to develop psychosis as a consequence of cannabis use [95].

23.7.1.5 The Evolutionary Paradox of Schizophrenia

Researchers have long been confused by the fact that so debilitating a disorder as schizophrenia has remained so common in the human population despite its negative effect on reproductive fitness. In their in-depth review of the theoretical and empirical evidence for different evolutionary models, Keller and Miller [68] conclude that only a polygenic mutation-selection balance model would appear consistent with the available data on schizophrenia prevalence rates, fitness costs, the probable rarity of susceptibility alleles, and the increased risk of mental disorder associated with brain trauma, inbreeding, and paternal age. While conceding that the alternative models of ancestral neutrality and balancing selection almost certainly play a part in maintaining some susceptibility alleles, they argue that these models lack sufficient convincing support from empirical studies to serve as a general explanation. The authors suggest that these arguments may also be applicable to other neuropsychiatric disorders, such as bipolar affective disorder.

23.7.1.6 Molecular Genetic Studies

The high heritability of schizophrenia has stimulated intense research into the identification of susceptibility genes. Two main approaches are employed in the search for these genes: linkage and association studies using genetic polymorphisms.

In linkage studies, the objective is to identify regions of the genome that are cotransmitted with the disease in families with two or more affected individuals. Using a few hundred evenly spaced genetic markers across the whole-genome, linkage has the potential to locate disease genes by virtue of chromosomal position alone, without any prior knowledge of disease etiology. Linkage studies are ideally suited to detecting genes of large effect, as in monogenic diseases. Their power to detect genes of moderate to small effect is limited, however, and large numbers of families are needed to locate genes for such complex disorders as schizophrenia.

Association studies aim to detect alleles that are more (or less) common in patients than they are within the general population. These studies are more powerful than linkage studies in detecting genes of small effect, assuming that a high proportion of the disease alleles are relatively common in the population and that they are attributable to a common founder [116]. Until recently, one disadvantage of association studies was that they were restricted to candidate genes only, leaving a large proportion of the genome uninvestigated. These candidate genes are selected on the basis that they are either functional candidates, i.e., they encode a protein implicated by an etiological hypothesis, or positional candidates, i.e., they map chromosomal regions implicated by previous linkage studies, or a combination of the two.

The recent introduction of array technology, which permit genome-wide association studies that investigate several hundreds of thousands of markers at a time, has meant that association studies may now be performed in a hypothesis-free fashion, with systematic testing of all genes and most intergenic sequences in the genome for association.

Molecular genetic approaches became widely available in the 1980s, and many groups have since performed linkage and association studies to identify genetic variants conferring susceptibility to schizophrenia. Difficulties in replicating early findings had led to increasing skepticism that such approaches would ever be successful. However, increasing knowledge of the human genome and its variation, as well as the development of new technologies and their successful application to the study of the genetics of schizophrenia, has led to renewed optimism.

23.7.1.6.1 Linkage Studies

The results of early linkage studies into schizophrenia were greeted with some disappointment. Hopes of finding Mendelian forms of the illness, i.e., forms in families showing almost monogenic effects, have not materialized, and most studies have failed to achieve stringent "genome-wide" levels of significance or to replicate pre-existing findings. This is probably attributable to small genetic effects, inadequate sample sizes (<100 families), and marker maps whose size was insufficient for the extraction of genetic information. Replication studies in complex diseases are also hampered by the fact that experimental conditions cannot be reproduced identically, since samples almost certainly differ in genetic architecture as a consequence of ethnicity and recruitment. It is not therefore to be expected that all studies will yield identical findings.

Despite these difficulties, the 20 or more genome-wide linkage studies (genome-scans) published to date have provided some consistent patterns of positive linkage. Three of the best-supported regions are 1q21–q22, 6p24–p22, and 13q32–q34, which have shown genome-wide significance in independent studies, i.e., a linkage value that is expected less than once by chance in 20 complete genome-scans. Further promising regions with evidence for linkage are 1q42, 5q21–q33, 6q21–q25, 8p21–p22, 10p15–p11, and 22q11–q12. Two meta-analyzes of schizophrenia linkage studies have been conducted to address issues of power [10, 84]. Such meta-analyzes enable the identification of genes that make a relatively small, but widespread, contribution to the development of an illness. These studies support the existence of susceptibility genes on 1q, 2q, 3p, 5q, 6p, 8p, 11q, 13q, 14p, 20q, and 22q. Proof that these linkage regions are correct will be obtained when the disease genes are identified.

Studies in isolated populations have been performed as an alternative approach to linkage studies in the hope that founder effects will render the contribution of individual genes larger and thus easier to detect. Some of these studies have suggested new chromosomal regions that may harbor disease genes [146].

23.7.1.6.2 Candidate Gene Studies

The list of candidate gene analyzes of schizophrenia is very comprehensive. Many of these findings are likely to represent false-positive findings since they have not been replicated in independent studies. The convergence of positive linkage findings, however, has led to several detailed mapping studies of linked regions. Some of these positional candidate genes are considered to be the most promising susceptibility genes for schizophrenia, having received the strongest support from independent studies. These include the genes *dystrobrevin-binding protein 1* (*DTNBP1*) at 6p, *neuregulin 1* (*NRG1*) at 8p, *disrupted in schizophrenia 1* (*DISC1*) at 1q, *D-amino acid oxidase activator* (*DAOA, G72/G30*) at 13q, and *catechol-O-methyl-transferase* (*COMT*) at 22q [29, 101, 129, 134, 136]. No consistent evidence of association has been observed across studies for any of these genes, however, and causative mutations still await identification [2, 11, 28, 51, 52, 90, 109, 115, 117, 151] .

It is difficult in some cases to judge the replication status of a gene, since different studies report associations with opposite risk alleles for the same marker [2, 106]. Such “flip-flop” phenomena are difficult to interpret, particularly when they are observed in comparably recruited samples from a common population. Differences in linkage disequilibrium (LD) architecture across populations with different ancestral origins may be one plausible theoretical explanation. In populations with a similar ancestral background, however, such differences seem less likely. However, LD patterns may vary even within (sub)populations, either as a consequence of differences in local recombination rates, genetic drift, and population history, or as a result of sampling variation. Lin et al. [86] have applied theoretical modeling to demonstrate that flip-flop associations are possible within samples recruited from the same population when the investigated variants are correlated through interactive effects or LD with a second causal variant. They showed that such flip-flop associations are particularly often observed when the risk allele at the genotyped locus is a relatively common allele in weak LD with an as yet unknown second causal variant. Under such circumstances, the observed direction of allelic association may be especially susceptible to sampling variation, and remarkable variation in LD patterns between the investigated samples is not required.

While further replication of the implicated genes remains the priority for research, the respective contributions of each gene, their relationship to aspects of the phenotype, the possibility of epistatic interactions between genes, and functional interactions between the gene products all await investigation.

23.7.1.6.3 Genome-Wide Association Studies

Genome-wide association studies have recently become possible as a result of enormous technological advances. Such studies involve the use of array technology that can simultaneously genotype up to 1 million single nucleotide polymorphisms (SNPs) per individual. As with complex genetic illnesses in general, this approach holds promise for the identification of common genetic risk variants for schizophrenia. It enables a search for the sites of illness genes on every gene and in most intergenetic regions of the genome in samples of unrelated patients and controls, independent of speculation regarding pathophysiology. In this respect they resemble genome-wide linkage studies (genome-scans), but they are not dependent on the recruitment of families and they have markedly better resolution since, in contrast to linkage, they detect the linkage disequilibrium to the illness-relevant genetic variants.

The first genome-wide association studies for schizophrenia have recently been reported [76, 80, 107, 108]. They demonstrate that schizophrenia is, in principle, amenable to systematic genetic association approaches, but that genetic effect sizes are small (OR in the 1.1–1.3 range), as is the case with other common genetic disorders. The most convincing association reported to date has been for variation in *ZNF804A*, which encodes a zinc finger transcription factor [108]. A recent functional magnetic resonance brain imaging study in healthy individuals demonstrated abnormal functional coupling between hippocampus formation and the dorsolateral prefrontal cortex in carriers of the *ZNF804A* risk variant [38]. Disturbed interaction between these brain areas has been observed in schizophrenia, and this strongly suggests that the identified genetic risk factor of small effect is highly penetrant in the brain and produces pathophysiological patterns observed in overt disease. It is anticipated that future studies involving the use of even larger samples and the pooling of datasets will identify additional specific risk factors for schizophrenia with higher statistical power.

23.7.1.6.4 Submicroscopic Chromosomal Aberrations

The fact that small chromosomal aberrations (copy number variations, genomic imbalances) may confer a risk for schizophrenia is exemplified by the 22q11.2 deletion syndrome (22q11.2DS), which is a common microdeletion syndrome with congenital and late-onset features, including a high risk for neuropsychiatric diseases (up to 25% risk for schizophrenia) [12, 63]. Interestingly, it has not been possible to correlate the extent of the deletion with the occurrence of schizophrenia in 22q11.2 DS patients and there is experimental evidence that altered expression of several genes within the 22q11.2 region may be necessary to increase susceptibility [98, 131]. This may explain why attempts to implicate individual genes from the deletion region as general susceptibility genes for the development of schizophrenia have not led to replicable results [41].

With the use of new technologies, such as comparative genomic hybridization (CGH) or SNP arrays applied in genome-wide association studies, it became possible to identify small chromosomal aberrations on a genome-wide scale. Initial studies found increased overall rates of aberrations in schizophrenia [147, 154], while later studies have been able to implicate specific chromosomal regions [58, 75, 107, 118, 135]. The implicated aberrations include microdeletions in chromosomal regions 1q21.1, 2p16.3, 15q11.2, and 15q13.3, as well as a microduplication in chromosomal region 15q13.1. Although each of these variants is more frequently observed among patients than among controls, the frequency of each individual variant in schizophrenia patients is still low (<1%). Further studies will be required to determine the penetrance, the mutation rate, and the full phenotypic spectrum associated with the aberrations. It has already been shown that some variants occur more frequently in patients with other CNS phenotypes, such as autism, mental disability, and epilepsy [15, 56, 99, 102], suggesting that common etiological factors exist among these disorders.

23.7.1.7 Endophenotypes

Endophenotypes, which are quantitative risk factors that are correlated with disease, have gained importance in molecular genetic analyzes over recent years [8, 14, 45]. It is hypothesized that endophenotypes bear a closer relationship to genetic variation than clinical symptoms and that their use in research may render gene identification more straightforward and successful. In order to be of value in genetic studies it has been suggested that endophenotypes should be (1) associated with illness in the population, (2) heritable, (3) detectable in an individual irrespective of whether or not the illness is active, (4) found to co-segregate with illness in families, and (5) found in unaffected relatives of probands at a higher rate than in the general population [53]. Endophenotypes from a variety of domains have been proposed for schizophrenia, including neurocognition, neurodevelopment, metabolism, and neurophysiology [19, 22]. The fact that only relatively small samples of patients with a specific endophenotype are available may limit their use in gene identification efforts owing to limited power. They will certainly have great potential once disease susceptibility genes are identified and attempts are made to understand the impact of risk variants on specific functions of the human brain.

It may be concluded that molecular genetic studies aiming to identify genes that contribute to the risk for schizophrenia have recently made substantial progress. However, the great majority of genetic risk factors still await identification. A general lesson learnt from genome-wide studies is that the genetic heterogeneity is greater than previously thought, a situation which is, however, similar to that for other common multifactorial disorders.

23.7.2 Bipolar Disorder

Humor, temper, tune, spirit, vibes, sentiment, disposition: the many descriptions of mood attest to its natural unsteadiness. Fluctuations in mood and mood swings are indeed inherent in human nature and are considered normal when they are restricted in intensity and/or duration, but excessive deviations from normal are defined as mood disorders. Mood disorders have been recognized and described for more than 2,000 years [30] and rank among the most common diseases of mankind. The WHO [152] has described mood disorders as a serious global health burden.

Modern classification systems define specific types of mood disorders, depending on the presence of specific patterns of symptoms and signs over specified periods of time (for DSM-IV criteria see Table 23.7.1). This explains the occurrence of many mood syndromes which do not meet diagnostic thresholds. The most common and important mood disorders are major depression (unipolar depression) and bipolar disorder. Mood in a depressed phase is sad and despondent. Other characteristic features are loss of drive, slowness of thought, feelings of guilt, and loss of interest in life. These features are typically accompanied by appetite and sleep disturbances. Depressive phases may occur as single episodes, but tend to recur. Bipolar disorder is characterized by changes in mood between the two poles of depression and mania. An episode of mania is characterized by expansive mood with an exaggerated estimation of ability, increased drive, and rapid thoughts and speech. Marked irritability is another frequent feature of the manic phase. The alternation between depressive and manic episodes may be rapid, taking place within hours or days, or may occur at longer intervals, separated by months or years.

The DSM-IV classification system [9] distinguishes between bipolar I and bipolar II disorder, this distinction being based on the severity of the manic symptoms. Bipolar I disorder has clear manic symptoms and may include delusions. Bipolar II disorder has less distinct symptomatology, with so-called hypomanic symptoms.

In terms of genetic studies, bipolar disorder is the most extensively studied mood disorder to date. The onset of bipolar disorder typically occurs in the midteens or twenties, and an episode of major depression or hypomania is usually its first manifestation.

23.7.2.1 Prevalence

Unipolar depression affects women twice as often as men, and has a life-time prevalence of around 10–15%. Bipolar disorder has a life-time prevalence of 0.5–1.5% and affects both sexes equally.

23.7.2.2 Environmental Risk Factors

To date, fewer studies investigating external risk factors have been conducted for bipolar disorder than for schizophrenia, and their findings have been inconclusive. There is some evidence that season of birth may have an influence on the development of bipolar disorder, as has been reported for schizophrenia [143]. Residence in large cities seems to increase the risk for psychotic bipolar disorder, although it does not influence the risk for bipolar disorder per se [65]. No conclusive evidence has been found to suggest that migration [138] or a history of obstetric complications [127] leads to a significant increase in the risk of developing the disorder. A large Danish study found no influence of external factors on the occurrence of bipolar disorder with the exception of parental loss; maternal loss before the age of 5 years was found to increase the risk 4-fold [104].

23.7.2.3 Formal Genetic Studies

Results from a large number of formal genetic studies suggest that genetic factors contribute substantially to the development of bipolar disorder [39, 62, 133].

23.7.2.3.1 Family Studies

All completed family studies have shown that the first-degree relatives of patients with bipolar I disorder have an increased risk of developing the disorder. These studies have used standard diagnostic methods. The relative risk for first-degree relatives is approximately 7. These studies have also shown that first-degree relatives have an increased risk of developing unipolar depression, the risk being approximately double that for the general population. It is not possible in individual cases to determine whether the prevailing depressive illness is related to the familial genetic loading with bipolar disorder, since unipolar depression is very common in the general population. It has been estimated that 70% of the unipolar cases among relatives of bipolar patients share a common genetic background [18]. Other disorders observed in relatives of patients with bipolar I disorder are bipolar II disorder and schizoaffective disorder, raising further suspicions of overlapping etiology.

Many attempts are being made to identify clinical characteristics that influence the risk of illness in families. The identification of such characteristics would be of value in molecular genetic research, since this would make it possible to identify subgroups of patients in whom a higher genetic loading could be assumed. One such characteristic may be early age at onset, for which a large number of studies have reported an increased risk of illness in relatives [83]. Formal segregation analyzes have provided further support for considering age at onset as an important variable in genetic studies of bipolar disorder, with evidence having been obtained for a major gene effect in early-onset families [50, 114].

Other clinical features reported to increase the risk for illness in families include response to lithium [5], a history of psychotic bipolar disorder [112, 113], puerperal manic or hypomanic episodes [63], and co-morbidity with panic disorder [86, 87]. Some of these features have been reported to be familial traits [123], suggesting that they may breed true to some extent.

23.7.2.3.2 Twin Studies

Findings from twin studies also demonstrate the strong contribution of genetic factors to the etiology of bipolar disorder. The average concordance rate for bipolar disorder obtained for monozygotic twins from studies employing a modern concept of bipolar disorder is 50%, as against a rate of 10% for dizygotic twins [7, 17, 25, 71, 78, 142]. For unipolar disorder, the concordance rate is approximately 80% in monozygotic twins and 20% in dizygotic twins [17]. These findings are in accordance with twin studies conducted prior to 1960, which did not distinguish between unipolar and bipolar disorder but which supported the involvement of genes in broadly defined mood disorders (for review see [144]). Heritability estimates range between 60% and 80% for bipolar disorder and 33% and 42% for unipolar disorder [31].

23.7.2.3.3 Adoption Studies

To date there has been only one adoption study from which meaningful conclusions can be drawn concerning bipolar disorder [100]. This study included 29 bipolar and 22 healthy adoptees and their biological and nonbiological parents. In order to test the influence a chronic disease exerts on parental affective status, 31 parents of children with bipolar disorder and 20 parents of children with poliomyelitis were investigated: 31% of parents whose children were affected with bipolar disorder displayed an affective disorder, as opposed to only 12% of those whose children did not suffer from bipolar disorder.

23.7.2.4 Molecular Genetic Studies

The biological mechanisms responsible for the development of bipolar disorder remain largely unknown.

Biological psychiatric research has proposed a multitude of possible mechanisms, including a disturbance of neurotransmitters, intracellular , and neuroendocrine regulation. Whether these mechanisms are genuinely implicated, and to what extent they are involved, is still unclear. The advantage of the molecular genetic approach is that the identification of vulnerability genes reveals causal factors. This in turn allows an understanding of the function of a given gene product and gradually reveals the functional context which ultimately produces the clinical phenotypes.

23.7.2.4.1 Linkage Studies

Linkage studies have proposed a large number of chromosomal regions that are likely to contain genes contributing to bipolar disorder: 4p16, 4q35, 8q24, 10q25–q26, 12q23–24, 13q32–q33, 18p11.2-cen, 18q21–q23, 21q22, and 22q12–q13. Although all of these chromosomal loci have been found by two or more independent research groups, no locus has been consistently replicated by all groups. This reflects both the high degree of locus-heterogeneity and the variability of studies with regard to their definition of phenotype, sample sizes, and the type and density of genetic markers used. The largest linkage study in bipolar disorder combined the original genotype data from 11 genome-wide linkage scans comprising 5,179 individuals from 1,067 families, and established that loci on chromosomes 6q and 8q show genome-wide significance and loci on 9p and 20p show suggestive evidence of linkage [99]. Recently, a first genome-wide interaction linkage scan in bipolar disorder provided evidence of interaction between disease genes on chromosomes 2q22–q24 and 6q23–q24 [3].

Two large meta-analyzes of all published genome-wide linkage studies have been conducted [10, 128]. Since the original studies were not uniform with respect to a number of methodological issues (e.g., diagnostic criteria, genetic markers), the meta-analyzes involved some loss of information. A specific advantage of such meta-analyzes, however, lies in their sensitivity for genes with a relatively small, but widespread, contribution to the development of the illness. These genes may remain undetected in an individual study, but become detectable when results from a large number of studies are combined. It is therefore not surprising that the meta-analyzes, as well as confirming previously implicated loci, have also suggested new loci (10q11–q22, and 14q24–q32). Linkage with schizophrenia has also been reported for some of the chromosomal regions highlighted by the meta-analyzes. Since there is a possible etiological overlap between bipolar disorder and schizophrenia, the genes identified for schizophrenia in these regions are excellent candidate genes for bipolar disorder. Using this strategy, the G72/G30 locus (chr. 13q33) has recently been found to be associated with bipolar disorder [54, 125], although the responsible gene has not yet been unequivocally identified [2].

As with schizophrenia, studies in isolated populations have been performed for bipolar affective disorder, and they have suggested some new chromosomal regions [146].

23.7.2.4.2 Candidate Gene Studies

There have been a large number of studies on candidate genes for bipolar disorder, and a simple interpretation of their findings is difficult. Many candidate gene studies are disadvantaged by small sample sizes and the lack of a systematic approach to the investigation of specific candidate genes (e.g., low number of investigated polymorphisms and insufficient knowledge of haplotype structure). There are also (theoretically) a multitude of biological mechanisms that could be responsible for the illness, and there are therefore a large number of potential candidate genes to be examined. A candidate gene has a higher plausibility when it is located in a chromosomal region that has been shown to have linkage with the disorder.

Among the most discussed candidate genes are the *serotonin transporter gene* (*5-HTT*), the *catechol-*O-*methyl-transferase gene* (*COMT*), and the *brain-derived neurotrophic factor gene* (*BDNF*) [60]. Reported findings have been inconsistent, and no judgement on their relevance can be made at this time.

23.7.2.4.3 Genome-Wide Association Studies

Although the initial genome-wide association (GWA) studies in bipolar disorder highlighted interesting candidates, none of the findings reached the threshold of genome-wide significance [13, 132, 148]. An important future step towards maximizing the total statistical explanatory power of these samples is likely to be the performance of joint meta-analyzes, as suggested by the results of a first joint analysis of the data from the Wellcome Trust Case-Control Consortium [148] and the Sklar study [132], which were obtained from a total sample of 4,300 bipolar patients and 6,200 controls [40]. The analysis produced a genome-wide significant finding for rs10994336, located in the ankyrinG gene (*ANK3*) (coding for ankyrinG), which was observed to have a *p*-value of 9.1×10^{-9}. The same study also reported a strong association with variability in the gene *CACNA1C* (coding for the alpha 1C subunit of the L-type voltage-gated calcium channel; rs1006737 $p = 7.0 \times 10^{-8}$). An independent study by Schulze et al. [124] has recently replicated the association finding in *ANK3* in independent samples of US American and German origin and found evidence for the presence of independent risk variants for bipolar disorder at this locus. No functional evidence is available for any of the variants, however, to suggest that they are true causative variants.

23.7.2.5 Endophenotypes

It has been suggested that some of the most promising endophenotypes for the study of bipolar disorder are neuropsychological deficits, circadian rhythm instability, dysmodulation of motivation and reward, neuropathological abnormalities, and symptom provocation responses [53].

23.7.3 Schizophrenia and Bipolar Disorder: Approaches Beyond a Diagnostic Dichotomy

In 1896 Kraepelin proposed the categorization of major psychoses into dementia praecox and manic-depressive insanity [77]. Although it has been argued that the two disorders are not distinct entities but should be viewed rather as disorders along a psychosis continuum [33, 34], the distinction made by Kraepelin [77] has influenced all current operational classification systems. There is high diagnostic reliability between psychiatrists, and family studies have shown that relatives of index patients have a higher risk of being assigned the same diagnosis. Psychiatrists are well aware of the limitations of these categorical classification systems, however, since there is substantial overlap in clinical symptoms between the disorders. Around 50% of patients with bipolar disorder display psychotic symptoms, and more than 15% of patients assigned a diagnosis of schizophrenia will develop affective symptoms during the course of the disorder, while around 10% of patients diagnosed as being manic later develop persistent symptoms of schizophrenia [27]. Family and twin studies have also shown that relatives of index patients with bipolar disorder have an increased risk of schizophrenia and vice versa [26, 33]. An increasing amount of molecular genetic evidence over recent years has challenged the validity of the dichotomous classification. Linkage studies have mapped a large number of identical chromosomal loci to schizophrenia and bipolar disorder, and association studies have suggested variants that may increase the risk for both schizophrenia and bipolar disorder [16, 32]. For some of these loci/genes, detailed analysis has revealed that the linkage/ association finding was actually due to subgroups of patients who suffered from symptoms which were common to both diagnostic groups. A linkage finding for schizophrenia and bipolar disorder on chromosome 13p, for example, increased when only those bipolar families who suffered from mood-incongruent psychotic symptoms were included [42]. Similarly, an association found for schizophrenia and bipolar disorder with *G72* markers was found to be due to those bipolar patients with persecutory delusions [122], and an association finding between affective disorder and *BDNF* alleles was also detected in schizophrenia patients with affective symptoms [126]. Analyzes in future genetic studies into bipolar disorder and schizophrenia must therefore include symptoms and symptom dimensions both within and across the categorical diagnoses [36].

23.7.4 Outlook

Genetic epidemiology has demonstrated that modern diagnostic criteria (see Table 23.7.1) define disorders that are highly heritable. It is generally accepted that the inheritance of psychiatric disorders is complex, with multiple genetic as well as environmental factors contributing to the development of a disorder

[1, 20, 46, 55, 64, 81, 94] and with possible interactions occurring among them [139, 145]. When the magnitude of the overall genetic contribution is considered this must be borne in mind.

Any consideration of the magnitude of the overall genetic contribution must take due account of the fact that an unselected sample of schizophrenia or bipolar disorder patients will always include a diverse mixture of individuals whose genetic loading ranges from no or very little genetic contribution to a strong genetic contribution with very little influence from nongenetic factors. It is possible that rare mutations with high penetrance exist in some families in which pronounced clustering and a Mendelian pattern of inheritance is observed, although no such high penetrance mutation has yet been identified.

It has been suggested that complex genetic mechanisms such as imprinting [49, 96], anticipation [97], mitochondrial inheritance [98], and epigenetics [110, 111] account, at least in part, for the irregularities in disease transmission observed within families, but no convincing molecular proof has yet been provided for these hypotheses.

Until recently, systematic genome-wide searches for the genes involved in psychiatric disorders were only possible through the use of a linkage approach. A series of chromosomal regions in which susceptibility genes are likely to be located have been identified through linkage studies. Highly promising association findings have been obtained for some genes in regions with positive evidence for linkage. To date, however, no genetic variant that directly confers a functional effect and is consistently associated with disease across populations has been identified for any of these genes. A similarly cautious conclusion must be drawn for the investigation of numerous candidate genes, although promising findings have been obtained for some of them.

Given the nonreplication of findings that seemed very convincing in the original studies, some authors have challenged the requirement that the true causative variant must show consistent association across populations by the proposal of population specific gene-gene or gene-environment factors. This is pure speculation at present, however, since these factors (if they exist at all) are completely unknown and it is more likely that the true causative variants still await identification [59].

The pace of progress in molecular genetic research is enormous. The first GWA studies in schizophrenia and bipolar disorder have recently been completed, with many more still in progress, and large international meta-analyzes to increase statistical power are being planned. Research findings obtained to date suggest that the variants identified through GWA studies will confer only small individual risks. The major limitation of GWA studies is that they only investigate variants that are common within the population. If a large fraction of the genetic contribution is conferred by rare variants, other approaches will be necessary to identify these factors. A successful first step in this direction has been the identification of rare submicroscopic chromosomal aberrations as causes of schizophrenia. However, owing to methodological restraints, this approach is still limited to aberrations comprising at least several thousand base pairs. Rapid technological developments will provide future studies with increasing resolution. Ultimately, the availability of low-cost whole-genome sequencing technology will make it possible to obtain the complete genomic sequences of large patient samples and compare them with controls. In principle, this will allow the systematic identification of rare variants associated with disease risk, although the existence of a myriad of rare variants in the human genome will render this a complex task. It is hoped that some rare variants confer a larger disease risk, which will facilitate the detection of association in large case-control samples. Rare variants with small disease risk may be extremely difficult to detect because prohibitively large samples sizes may be required to demonstrate significant association.

Once disease susceptibility genes have been identified, future studies will be required to understand the phenotypic dimensions most strongly associated with a specific gene. This will include the analysis of clinical symptoms as well as endophenotypes. The latter may be particularly suited to guiding researchers in the selection of the most promising phenotypes for animal studies [49]. For example, promising endophenotypes for schizophrenia which have already been successfully studied in animals include sensorimotor gating deficits, as indexed by measures of prepulse inhibition of the startle reflex, P50 auditory evoked potential suppression, and antisaccade eye movements.

It is expected that the identification of disease-associated genes will increase our knowledge of the pathophysiology underlying psychiatric disorders in an as yet unforeseen way. The identification of biological pathways has the potential to revolutionize diagnostics and treatment. These developments may well also challenge the existence of the field of psychiatry. A diagnosis of schizophrenia or bipolar disorder cannot be assigned if the disorder is due to the direct physiological effects of a substance or a general medical condition such as organic brain disease, and management of patients has traditionally been taken away from psychiatrists and handed over to specialists from other medical fields when an underlying biological cause for psychiatric symptoms has been identified for which appropriate therapy exists, as in the case of mania due to syphilis, or psychotic symptoms due to hyperthyroidism or porphyria. It will be interesting to see how the field of psychiatry will evolve once genetic research has unraveled the biological pathways responsible for schizophrenia and bipolar disorder. Awareness of the biological causes of psychiatric diseases has increased enormously among psychiatrists over recent decades, and this will continue with the identification of the genetic causes. This may ultimately lead to the development of a very different self-image for the field of psychiatry.

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Model Organisms for Human Disorders

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Introductory Note by Michael R. Speicher

Given the physical and ethical problems involved in performing experiments on humans, model organisms are vital for our understanding of human biology and disease. Animal models in genetically tractable organisms are indispensible tools for the analysis of the pathogenesis and the development of therapeutic avenues in many human diseases. With the increasingly available number of genomic sequences for multiple organisms spanning the evolutionary tree it is now possible to use comparative genomics to build or select better animal models and to facilitate gene discovery. In addition, for many well-established model systems abundant genetic tools are available.

The most common model organisms are small mammals, usually rats and mice, which offer a variety of tailored model systems with controlled genetic backgrounds. As well as other mammals, e.g., dog, other organisms, such as the nematode *Caenorhabditis elegans*, the fruitfly, *Drosophila melanogaster*, yeast, and the zebrafish, *Danio rerio*, are also extremely important popular model genetic organisms. Their low cost, their smallness, and their external development make these latter organisms excellent tools for development biology and genetic screens for human diseases.

Techniques for large-scale genome mutagenesis and gene mapping, transgenesis, protein overexpression or knockdown, cell transplantation and chimeric embryo analysis, and chemical screens have increased the power of many model organisms immeasurably. It is now possible to rapidly determine the developmental function of a gene of interest in vivo, and then identify genetic and chemical modifiers of the processes involved under normal and pathologic conditions.

In the following chapters a number of model organisms are reviewed and their potential roles for human genetics are discussed. Among various eukaryotic microorganisms, the yeast, *Saccharomyces cerevisiae*, has become legendary for its ease of simple genetic analysis [1]. This organism thus became an important simple model for human genetics. A separate chapter on yeast was therefore planned for the fourth edition of this book, but logistical problems unfortunately prevented its inclusion.

Reference

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Mouse as a Model for Human Disease

24.1

Antonio Baldini

Abstract Precisely targeted genetic manipulation of the laboratory mouse has been one of the most significant achievements in the field of modeling human genetic diseases. The availability of an ever-increasing number of mutants, and the ability of generating ever-more complex manipulations beyond the "simple" gene knockout, offer unprecedented possibilities for genetic experiments addressing complex questions. Thus, the mouse is getting closer and closer to the most powerful genetic models such as *Drosophila*.

This chapter provides a view of the commonly used or most promising approaches to modeling genetic disease in the mouse. It also provides a discussion on which approach is more suited to modeling different types of mutations.

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24.1.1 Introduction

Since the development of mouse embryonic stem cell technology [5, 17] combined with homologous recombination [8, 18, 19] was applied to modify genes in those cells, modeling of human genetic diseases in the mouse has been by far the most powerful method used to study the effect of genetic mutations in mammals. With all the limitations related to species-specific characteristics, mouse models remain a virtually "obligatory" step in genetic disease research. Genetically and phenotypically accurate models are essential to understand pathophysiology and experiment therapies.

Gene targeting in the mouse has become routine in many research Institutions, and protocols have become so robust that gene targeting is not only the method of choice for the generation of models of human genetic disease, but also an essential method for studying the function of genes in mammals. The trend of using gene knockout as a tool for mammalian genetics has culminated in multinational initiatives to fund the generation of knockouts for all the mouse genes (the NIH Knockout Project, or KOMP; http://www.nih.gov/science/models/mouse/knockout/index.html and the European EUCOMM initiative; http://www.eucomm.org/info/).

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In parallel with all this, the level of sophistication of gene manipulation technologies has increased to a point where a "simple" knockout, i.e., gene inactivation, may be considered a relatively modest research investment for individual research projects if we consider the range of options at our disposal for gene manipulation. More complex strategies, though laborious and time consuming, may, in the long run, provide a significantly greater scientific return.

In this chapter, I will review the tool kit at our disposal to generate mouse models as close as possible to the genetic diseases of interest.

24.1.2 Gene and Genome Manipulation Strategies

24.1.2.1 *Knockout*

This is a targeted mutation that leads to complete inactivation of the gene. Thus, this method is suitable for modeling diseases caused by loss of function of a gene. Typically, this is obtained by replacing the gene of interest with extraneous DNA, generally a segment encoding a drug resistance protein used as a positive selection cassette (Fig. 24.1.1a). Gene targeting is carried out using a "replacement" type of vector made of two DNA segments identical to genomic DNA flanking the gene of interest (homology arms), separated by a positive selection cassette and followed by a negative selection cassette positioned at one extremity of the vector (Fig. 24.1.1a). After introduction of the vector DNA into ES cells, homologous recombination occurs within the homology arms and the genomic region flanked by the homology arms sequence is replaced by the DNA flanked by the homology arms in the vector. Because the methods used to introduce DNA into ES cells are relatively inefficient, cells that have incorporated vector DNA are selected using a drug to which resistance is conferred by the positive selection cassette carried by the targeting vector. However, only a small percentage of cells that have incorporated vector DNA have actually undergone homologous recombination. Indeed, most of the cells will carry the vector DNA randomly inserted in their genome. The use of a negative selection system is designed to enrich the ES cell population with cells that have undergone homologous recombination. Because the negative selection cassette is positioned externally to the homology arms ("–sel" in Fig. 24.1.1a), it will not be incorporated into the genomic DNA after homologous recombination, but it will if the vector DNA is simply inserted at random in the genomic DNA. Thus, if one uses a drug that kills cells that have incorporated the negative selection cassette, the cell population will become richer in cells that have undergone homologous recombination.

Once ES cells with the desired mutation have been obtained, these are injected into mouse blastocysts and reimplanted into foster mothers. Because the injected ES cells' genome encodes for a different coat color than the blastocyst's genome, the resulting chimeric animals' coats have a characteristic patchy color (Fig. 24.1.2). Germ cells of these animals will also be partly derived from the modified ES cell's genome and from the blastocyst's, wild type genome. These animals are then crossed with wild type animals, and some of the progeny will inherit ES cells' chromosomes, including the one with the targeted mutation. Once established in a mouse, the mutation will be transmitted as a Mendelian trait.

24.1.2.2 *Knockin and the Use of Site-Specific Recombinases*

Many gene mutations may not be readily classifiable as gain or loss of function. For example, functional interpretation of missense mutations may be difficult. This is just one example of a case in which the use of a knockin gene targeting strategy would be beneficial, perhaps one of the most powerful and flexible approaches to gene manipulation. The method allows the introduction of subtle modifications to the endogenous gene, including point mutations. The basic mechanism is very similar to the one described above, but there are some important differences. In particular, the structure of the targeted gene is not altered, so that it can still be transcribed normally, and no selection cassette is left in the targeted allele (Fig. 24.1.1b). This approach has been made possible thanks to the use of site-specific recombinases. Currently, two recombinases are used in gene manipulation, Cre recombinase, which is by far the more commonly used, and Flp recombinase [6, 13]. For both recombinases, the structure of the target site is made up of two 13-bp palindromic sequences that flank an 8-bp core sequence.

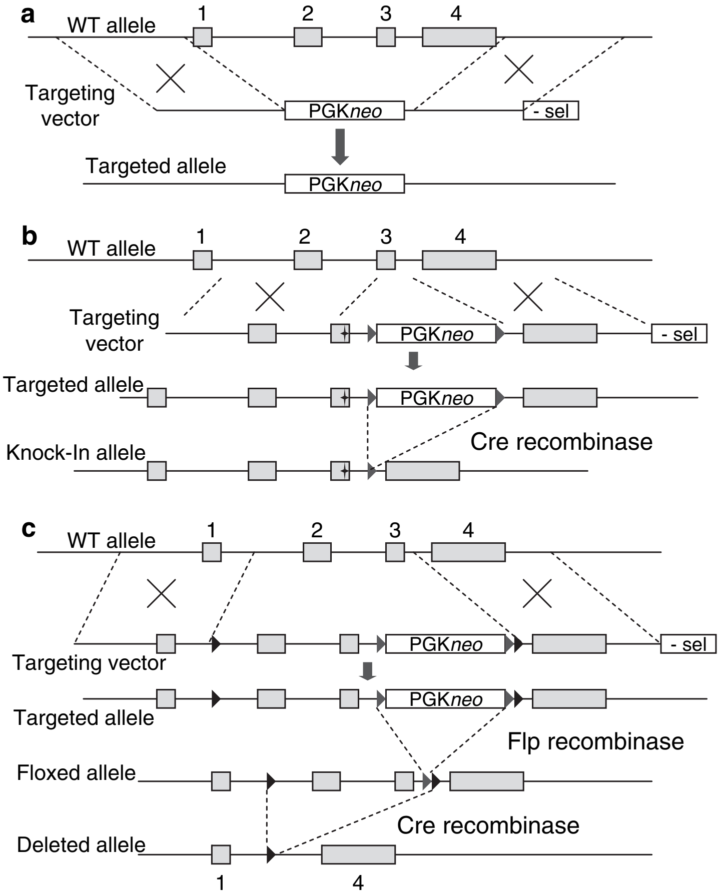

Fig. 24.1.1 (**a–c**) Schematic representation of gene-targeting approaches to obtain different types of alleles. *Numbers* indicate exons of an hypothetical gene of interest. *PGKneo* is a positive selection cassette, *-sel* is a negative selection cassette. *Small triangles* indicate recombination sites for site-specific recombinases Cre or Flp. (**a**) Generation of a knockout allele; **b** generation of a knockin allele (+ in exon 3 point mutation introduced artificially into the targeting vector); **c** generation of a conditional allele

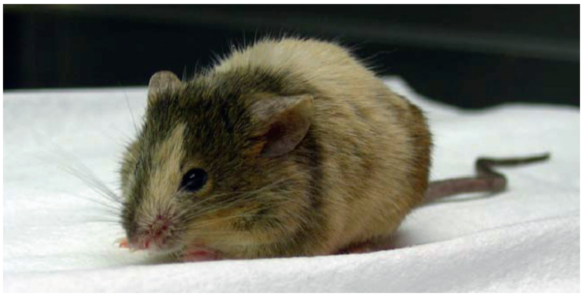

Fig. 24.1.2 A chimeric mouse

However, the sequence of the Cre target site (named *lox*P) is different from that of Flp (named FRT). The target sites are not present in the wild type mouse genome. Therefore, these sites can be introduced artificially at the desired location (using homologous recombination) so that Cre- or Flp-mediated recombination can occur exactly and exclusively at the desired points. In addition, the expression of these recombinases in various tissues in vivo appears to have no phenotypic consequences. The introduction of these site-specific recombinases has been one of the most significant advances in the mouse genetics field, the variety of applications being limited only by the creativity of the investigator.

The knockin strategy uses homologous recombination to target modifications to the endogenous gene, and then uses site-specific recombinases to remove from the targeted allele extraneous sequences that may interfere with the normal function of the allele (e.g., the selection cassette) (Fig. 24.1.1b). This final step, however, leaves behind a *lox*P or FRT site (34 bp) that usually does not harm gene functionality but might do so in some cases, as will be discussed in the next section.

24.1.2.3 Conditional Mutations

The engineered mutations described above, once established in mice, are inherited as Mendelian characters unless they reduce mouse viability. Most autosomal gene mutations are viable in the heterozygous state, but homozygous mutants may be so severe as to make the model practically unusable for studying the pathogenesis of a disease, e.g., if lethality occurs in early embryogenesis (the issue of gene dosage sensitivity in humans and mice will be addressed later in this chapter). In addition, certain genes may have broad expression and their loss may cause a complex phenotype that is difficult to dissect and interpret. Conditional mutations can address these issues, at least partially. Mutations are not present in the germline, but are generated in somatic tissues and at a particular developmental stage.

This strategy, in the great majority of cases, is based on the site-specific recombinase Cre and carefully positioned *lox*P sites, the target sites of Cre. It requires two genetic components, one of which is a modified allele of the gene of interest, generally referred to as "floxed" (or *lox*P-flanked) allele. This can be generated as shown in Fig. 24.1.1c. The *lox*P sites are positioned flanking an essential segment of the gene. The floxed allele must be functional in order to generate truly conditional mutations. While this is often the case, the insertion of *lox*P sites could have deleterious consequences for gene function even when positioned in apparently "neutral" segments of the gene, for example in nonevolutionary conserved regions of introns. Interference of *lox*P sites with transcription may be difficult or impossible to predict and may only be discovered after the allele is established in vivo.

The second component is an artificial gene expressed in the desired tissue and time, encoding Cre recombinase. The expression of Cre will cause recombination between the two *lox*P sites of the floxed allele and delete the intervening DNA, thus effectively causing the loss of function of the gene of interest, but only in the tissues where Cre is expressed. Today there are many mouse lines available that have been engineered to express Cre in different tissues (Cre-drivers) (see, e.g., http://nagy.mshri.on.ca/cre/index.php). These animals can be crossed with mice carrying the floxed allele of the gene of interest, and it is then possible to study the phenotypic consequences of the loss of the gene in specific tissues.

An additional category of Cre drivers uses special types of Cre that normally are not "active" (i.e., are localized in the cytoplasmic compartment of the cell), but they become active on subministration of a drug that causes the Cre protein to translocate to the nucleus, where it can carry out its recombinase activity. These special recombinases are fused to mutant estrogen receptor (mER) sequences that can bind tamoxifen (an estrogen receptor modulator), but they cannot bind the endogenous estrogens. Tamoxifen can be injected into mice to translocate Cre-mER into the nucleus. Because tamoxifen crosses the placenta, it can also be used to activate Cre during embryonic development. The tamoxifen-inducible Cre system is very powerful for testing gene function at specific time points and developmental stages [20].

24.1.2.4 Multigene Deletions and Duplications

Site-specific recombinases can be used to generate large and precisely targeted genomic rearrangements involving many genes [16, 21]. This approach can be used to model segmental aneuploidy syndromes. The strategy includes the positioning *lox*P sites at the extremities of the chromosomal segments that are to be deleted or duplicated. Cre recombination will cause the deletion of the segment if the two *lox*P sites are located in the same chromosome, or both deletion and duplication of the segment if they are located one in each homologous chromosome.

24.1.2.5 Transgenics

Transgenics are obtained by injecting nude DNA (transgene) into one-cell mouse embryos. The DNA is generally incorporated (at random) into the genomic DNA of the embryo, in one or multiple copies. Embryos are then reimplanted in a foster mother, and a percentage of the newborn will carry the transgene and transmit it as a Mendelian trait. In contrast to the gene-targeting strategies described above, transgenics are not designed to modify or disable endogenous genes but to force the expression of an additional gene coded by the transgene. The transgene is generally made up of a promoter/enhancer (to drive expression in the desired tissues) and a cDNA encoding the gene of interest or a mutated form. The use of transgenics in modeling human disease will be discussed later. Transgenics, although very useful in several circumstances, have limitations. For example, the expression of the gene carried by the transgene is difficult to control, because of common multiple copy insertions and because the locus of insertion may affect expression; another potential problem is that insertion of the transgene may disrupt an endogenous gene.

24.1.3 Generating Genetically Accurate Models

24.1.3.1 Genetic Diseases Caused by Loss of Function Mutations

This is the simplest scenario, in which ablation (knockout) of the gene of interest is required. Many such attempts may fail (there is no reliable estimate of how many, because negative results are not easily published), either because the loss of function does not have any phenotypic consequences in the mouse or because the consequences are too severe to be informative or to be representative of the disease of interest (e.g., very early embryo lethality). There are a number of strategies that could help in addressing some of these potential problems. The lack of phenotypic consequences of ablation of a human disease gene in a mouse knockout may be due to functional redundancy that is more "effective" in mice than in humans. A possible way to bypass this problem is to ablate the functionally redundant gene (double knockout).

Excessively severe phenotypes in mouse models may occur for different reasons. For example, the mouse gene may have a broader expression of the human gene and broader functions, or there may be partial functional redundancy in humans but not in mice. To get around excessively severe phenotypes, there are at least two approaches. One is to generate hypomorphic alleles (i.e., alleles that have lower functionality than the wild type (wt) allele but not null functionality), while the other is to use a conditional mutation strategy. The latter has been described above (Sect. 24.1.2.3). The generation of hypomorphic alleles is generally done by inserting a positive selection cassette (e.g., PGKneo) into an intron of the gene of interest, using homologous recombination. The insertion of the cassette may affect transcription and or splicing of the targeted gene. The result is a net reduction of mature mRNA available for translation. Unfortunately, the functionality of the modified allele is not easily predictable, so that the outcome of PGKneo insertion may be variable, from total disruption of gene function to very mild reduction of transcription. Nevertheless, hypomorphic alleles are a very important addition to the toolbox for mouse modeling of human diseases.

Another important issue to consider is that the genetic background of the mouse strain used for modeling may have a strong influence on phenotypic presentation. This eventuality may be used to the researcher's advantage, as genetic background-dependent phenotypes may be used to map genetic modifiers.

24.1.3.2 Gene Dosage Mutations

Dominant diseases caused by heterozygous mutations inactivating a gene are relatively common and may pose special challenges to the generation of mouse models. Loss of one of the two copies of an autosomal gene causes phenotypic consequences if the gene is haploinsufficient. Genes that are haploinsufficient in humans are not necessarily so in mice; hence the possibility that a mouse model of this type of mutation may not recapitulate the human phenotype, and this is often the case. Heterozygous mutation in mice may cause a phenotype that only partially resembles the human disease [2, 4, 12, 15], while homozygous mutation may provide a more complete phenotype but it will often be too severe to be an accurate model of the disease. The molecular basis of gene haploinsufficiency is not clear. In general, a gene product needs to be at a sufficient concentration for it to carry out its biological function. Some proteins, e.g., many enzymes, may be able to function even at a much reduced concentration, and other proteins may not function well after a modest reduction of concentration (some transcription factors belong to this category). To complicate the issue further, different biological processes or developmental programs may differ in their sensitivity to the dosage of a given gene, within the same organism. This is why heterozygous mouse mutants of haploinsufficient genes may present with only some of the phenotypic abnormalities. The gene product concentration threshold beyond which certain biological processes start to fail may be different in mouse and humans, which gives rise to the difficulty in accurately modeling disorders caused by gene dosage abnormalities. The use of hypomorphic alleles is the most powerful strategy for modeling haploinsufficiency disorders when heterozygous mutation in the mouse does not recapitulate the disease phenotype. Crossing these alleles with a null allele allows the reduction of gene product dosage below the level afforded by heterozygous mutation.

24.1.3.3 Gain of Function Mutations

Genetic disorders caused by gain of function mutations can be modeled by expressing the mutant form of the gene in the mouse. The accurate strategy that can be used to achieve this objective is to modify the endogenous gene by targeting the mutation into it (see knockin description in Sect. 24.1.2.2). With this strategy, the mutant gene is expressed in the appropriate tissues and at the appropriate transcription level. However, the less laborious transgenic approach is commonly used to model these disorders. Examples of successful use of transgenics are seen in models of disorders caused by expansion of CAG repeats, such as Huntington disease and spinocerebellar ataxia 1 [3, 7].

24.1.3.4 Segmental Aneuploidies

Syndromes caused by deletion or duplication of a chromosomal segment (or an entire chromosome, as in Down syndrome) are relatively frequent [1, 9]. Until recently, genetic disorders of this type could not be modeled accurately in mice. Chromosome engineering has made this possible (see Sect. 24.1.2.4), and the first engineered model of a microdeletion syndrome (the del22q11.2 deletion / velocardiofacial / DiGeorge syndrome) was reported in 1999 [11]. Attempts at modeling Down syndrome, associated with aneuploidy of a much larger region of DNA, have also met with partial success [14], but a new model carrying three copies of most of the region involved in this syndrome has been reported only recently [10].

24.1.4 Future Perspectives

The completion of the sequence of such complex genomes as the human and mouse genomes and many others has initiated the so-called postgenomic phase of biomedical research, when DNA sequence of entire genomes can be analyzed with increasingly sophisticated computational tools. Of course, that does not tell us how those genes work and what they do. By analogy, the generation of inactivating mutations of every mouse gene will inaugurate the post-knockout era. We will be able to look at the phenotypic consequences of the loss of any gene of interest from our desktop computers and develop new hypotheses concerning gene function, possible roles in diseases, interactions, etc. In a way, the post-knockout era has already started for many genes, and, in many cases (especially for developmentally important genes and for genes required in very early development or extra embryonic tissue) the subsequent step has been conditional or other, “specialized” mutations.

Mouse mutants (like any models based on complex organisms) have limitations that are mainly due to species-specific characteristics (e.g., the aforementioned differences in gene dosage sensitivity) or to difficulties in interpreting the consequences that the loss of a critical gene may have in a complex organism. For example, the loss of a gene may perturb multiple genetic networks and have far-reaching consequences. One solution might be to reduce the complexity, either by using a different model (e.g., a tissue culture model) or by restricting the mutation to a tissue or time window.

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Caenorhabditis elegans, A Simple Worm: Bridging the Gap Between Traditional and Systems-Level Biology

24.2

Morgan Tucker and Min Han

Abstract *Caenorhabditis elegans* is a simple invertebrate roundworm that has served as a model for exploring basic biological questions concerning multicellular organisms and has made numerous contributions to our understanding of human biology. In this chapter we explore some of the approaches C. elegans researchers have employed in an effort to elucidate conserved molecular mechanisms and understand the basic biology underlying many human disorders.

Contents

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24.2.1 A Primer

The principal goal of this chapter is to introduce the reader to *Caenorhabditis elegans*, a simple roundworm that has been the focus of intense research over the past 40+ years. As a model organism, this small invertebrate has proven to be a valuable tool for exploring basic biological questions concerning multicellular organisms and has also made numerous contributions to our understanding of human biology. We will explore some of the approaches *C. elegans* researchers employ in an effort to elucidate conserved molecular mechanisms and understand the basic biology underlying many human disorders.

24.2.1.1 A Short History

In 2002, the Nobel Prize for Medicine was awarded to Sydney Brenner, Robert Horvitz, and John Sulston for their genetic research on development and programmed cell death in *C. elegans*. The path that led to this award began in the 1960s when Brenner chose this small roundworm as a model for neuronal development and the genetics of behavior. At this time, many of the fundamental processes of molecular biology were just

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beginning to come to light. The structure of DNA was deciphered a decade earlier, thereby prompting intense investigation of the processes of DNA replication, mRNA transcription, and protein translation. A few pioneering scientists instead focused their attention on new frontiers in biology. These investigators possessed a basic understanding of how the eukaryotic cell works, but their understanding of how these cells propagate and come together to form a complex organism at the molecular level was shadowed in mystery.

Brenner set out to find the perfect model organism in which to study development. His specific interest was centered on how the nervous system was assembled and how this structure subsequently processed intercellular signals to control behavior. *C. elegans* proved to fit the bill. Brenner found that its economy, ease of maintenance in the laboratory, and capacity for long-term storage made it a tractable organism. More importantly, its transparent cuticle allowed each cell to be observed through the light microscope, and the organism was small enough for slices to fit easily under an electron microscope.

In an amazing tour de force, Brenner's colleague John Sulston was able to observe all the cell divisions and map the developmental fate of every single cell in *C. elegans* [22, 23]. The complete cell fate of each of the 959 somatic cells in the adult hermaphrodite was demonstrated to be largely invariant between individuals. Use of the electron microscope was pivotal in generating a complete map of the nervous system, consisting of only 302 neurons, and allowed the connectivity of each of these neurons to be determined [25]. Although this early work was founded in the tradition of descriptive biology, it laid the foundation for more advanced experiments and in many ways made *C. elegans* the powerful tool that it is today. Having a detailed description of every single cell fate and position enables *C. elegans* researchers to link the requirement of a single gene product to the formation of a specific anatomical structure, thus permitting the elucidation of the development processes regulated by a given gene.

C. elegans has provided considerable insights into the mechanisms of basic biology: programmed cell death, organ formation, cell signaling, cell polarity, gene regulation, metabolism, and sex determination have all been studied intensively. However, analyses of the complete *C. elegans* genome sequence suggest that this organism has a broader application as a human disease model. *C. elegans* was the first multicellular organism to have its genome completely sequenced. The sequence of all five autosomes and the single sex chromosome was first published in 1998 [2]. The genome sequence is frequently re-annotated and updated online, thus providing an indispensable bioinformatics resource [3]. The complete sequence consists of 100 million base pairs, encoding approximately 20,000 genes and greater than 1,300 noncoding RNAs. This is comparable to the approximately 23,000 genes predicted in human genome, of which nearly 40% have direct homologs in *C. elegans*. Considering the high degree of structural and functional conservation with mammalian homologs, continued work in *C. elegans* will likely provide considerable insight into the mechanisms of human development and disease at the molecular level.

24.2.1.2 The Worm, Its Life Cycle, and Its Cultivation

C. elegans is a free-living, soil-dwelling nematode that is approximately 1 mm in length – just barely visible to the naked eye. These roundworms have a simple body plan that primarily consists of a gut and a gonad. The intestine is connected at its anterior to the pharynx, a muscled organ that drives the feeding process, and at its posterior to the anal opening (Fig. 24.2.1). The midbody is marked by the vulva, which connects the symmetrical gonad to the external environment. The worm's sinusoidal movement is driven by contraction of the body wall musculature under control of a simple nervous system. The exterior of the worm is covered with a collagenous cuticle, which provides protection from the environment and functions as an external skeleton.

The *C. elegans* life cycle is fairly straightforward. After fertilization of a single oocyte, embryogenesis occurs over the next 13 h. This process consists of standard cell divisions followed by morphogenetic rearrangements and elongation of the embryo just prior to hatching. Under most conditions, larval development consists of four stages (L1–L4), punctuated by cuticle molts and a dramatic increase in overall size, followed by adulthood (Fig. 24.2.2). Alternatively, during conditions of stress, including starvation and/or overcrowding, an alternative third larval stage called

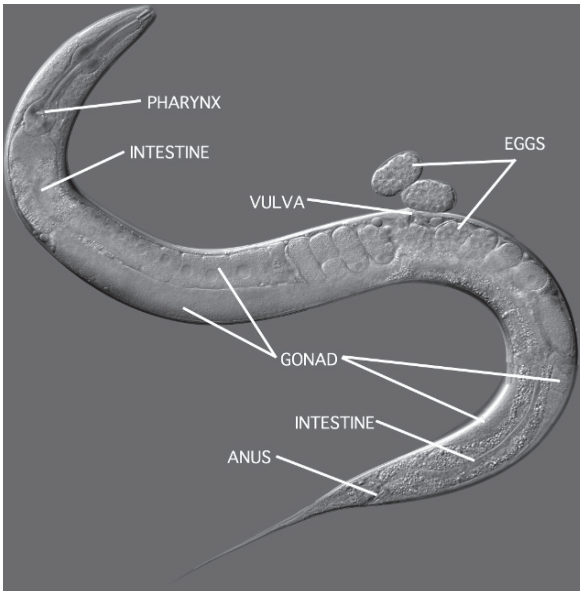

Fig. 24.2.1 Differential interference contrast image of an adult *C. elegans* hermaphrodite. Major anatomical landmarks are indicated. The muscled pharynx pumps food into the intestinal lumen, which is connected at its posterior to the anal opening. The hermaphrodite gonad is comprised of functionally independent anterior and posterior arms. Each U-shaped arm, connected at its proximal end to the vulval opening, produces both sperm and oocytes. *C. elegans* propagate by self-fertilization, and a single hermaphrodite is capable of producing 300+ progeny in only 2–3 days

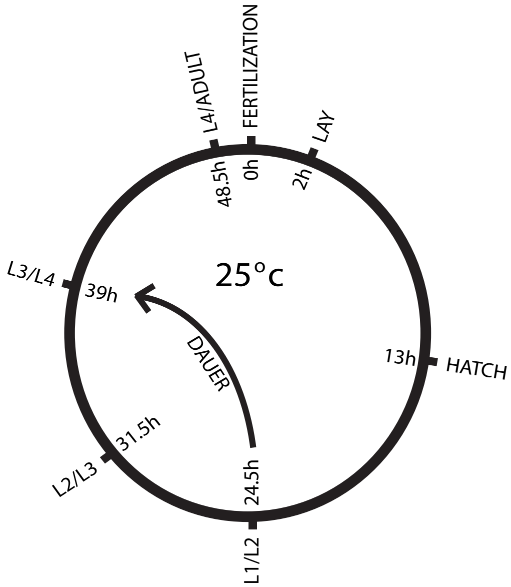

Fig. 24.2.2 *C. elegans* life cycle. In approximately 50 h at 25 C a single fertilized oocyte will complete embryogenesis, grow through four larval stages, and begin laying eggs of its own. At each of the four larval molts, a new cuticle is secreted and the old one is shed. Under conditions of stress or starvation larva can enter diapause, an alternative L3 larval state called dauer. Dauer larva are capable of surviving in the absence of food for as long as 6 months. In the presence of a food supply, dauer larvae will re-enter the development cycle to become fertile adults

the dauer state can be achieved. Dauer larvae do not feed and are highly resistant to stress. These larvae are capable of living for 3–6 months (4–8 times the normal life span) in a dormant state. When growth conditions improve, the larvae can resume the developmental cycle to become fertile adults.

In the wild, *C. elegans* feed on available microorganisms; in the laboratory, they are maintained on agar plates, subsisting on a diet of the common bacterium *Escherichia coli*. Hermaphrodite (XX) worms primarily propagate by self-fertilization. This allows researchers to maintain genetically identical populations over multiple generations with little or no effort. Spontaneous males (XO) do infrequently arise in a population, thus allowing for cross-fertilization between strains and facilitating complementation tests and the construction of double mutants. Transgenic worms containing non-native DNA can be created by microinjecting DNA constructs that are subsequently maintained as extra-chromosomal arrays or that are integrated directly into the *C. elegans* genome [17].

During its life span of approximately 2–3 weeks, an individual hermaphrodite is capable of producing more than 300 progeny. With a generation time of approximately 2–3 days, it is possible for a single worm to produce a large population of genetically identical worms very quickly. This ability means that *C. elegans* is not only suitable for genetic research, but it is also amenable to large-scale genomic experiments and biochemistry.

24.2.2 The How and Why of Screening

24.2.2.1 Genetic Screens: A Traditional Single-Gene Approach

In the postgenomic era, many researchers are quick to dismiss traditional genetic screens as outdated. Nothing could be further from the truth. Forward genetic screens still constitute the most powerful, nonbiased tools biologists have in their quest to assign function to a given gene. In brief, genetic screens are used to identify new genes involved in a given process, either by looking for new mutations that correspond to a previously characterized phenotype or by identifying mutations that can enhance or suppress a phenotype associated with a known gene. This approach allows for identification of gene networks that have a specific relationship, defined based on the screening approach.

C. elegans possesses many advantages that make it a powerful genetic model. Its hermaphroditic lifestyle, rapid generation time, simplicity, ease of manipulation, and fully sequenced genome all facilitate rapid screening and mapping of new mutations. The basic approach to screening is straightforward: mutations are induced in the genome with an appropriate mutagen, and individuals in the population that display the phenotype of interest are subsequently identified. Mapping the mutation of interest is carried out by linkage mapping relative to mutations with known phenotypes, by measuring the recombination frequency between the mutation of interest and single nucleotide polymorphisms (SNPs) found throughout the genome, or by combining these two approaches [7].

This simple approach has been successfully applied in the study of many human disorders. For example, mutations in the human dystrophin–glycoprotein complex (DGC) have been shown to result in muscular dystrophies [1] – one of the most common forms of human genetic disorder. The etiology is characterized by progressive muscle degeneration, although the exact mechanism responsible for such pathogenesis is not well defined. Mutations in the *C. elegans* homologs of the DGC components result in a characteristic hyperactive phenotype and muscle degeneration [10]. An extensive genetic screen for worms that displayed phenotypes identical to those of DGC mutants resulted in the identification of a previously uncharacterized protein, SNF-6 [12]. The *snf-6* gene encodes a novel acetylcholine/choline transporter that is required for the uptake of acetylcholine at neuromuscular junctions. A direct interaction between the DGC and SNF-6 is necessary for proper localization of SNF-6 at neuromuscular junctions [13]. These findings have considerable implications for the treatment of muscular dystrophies. The authors of this study suggest that inhibiting the process of acetylcholine clearing from the neuromuscular junction likely results in prolonged muscle excitation and that this may be the underlying cause of the muscle degeneration that is observed in *C. elegans* mutant for other components of the DGC [11]. The results also suggest that the development of therapeutic agents that affect acetylcholine levels at the neuromuscular junction may be of significant use when treating muscular dystrophy disorders.

24.2.2.2 RNAi Screens: A High-Throughput Approach

In 2006, the Nobel Prize for Medicine was awarded to Andrew Fire and Craig Mello for their research on RNA interference (RNAi) in *C. elegans*. This discovery [10], during the late 1990s, challenged the way gene regulation was conceptualized and also proved to be a powerful tool in many organisms. RNAi is the process by which fragments of double-stranded RNA (dsRNA) interfere with the expression of any genes that share homologous sequences. The molecular mechanism of RNAi has been described in some detail elsewhere [19]. dsRNAs entering a cell, through a mechanism that is not well understood, are bound by the protein Dicer, an RNAse III nuclease, which cleaves the dsRNAs to produce smaller fragments 21–23 bp in length, called small interfering RNAs (siRNAs). These siRNAs are loaded into the RNA-induced silencing complex (RISC), where they guide mRNA degradation or translation silencing, depending on the complementarity of the target. The RNAi process is systemically propagated through transport and replication of the siRNAs. The process is heritable in both plants and *C. elegans* but not, however, in *Drosophila* or mammals, though the exact reason for this difference is unclear.

Researchers have designed a collection of bacteria that individually express dsRNA encoding each gene in the entire *C. elegans* genome [12]. This bacterial library enables worms to be screened for specific phenotypes by essentially knocking out gene function via RNAi. When worms are fed the dsRNA-expressing bacteria, RNA is taken up by the intestinal cells, spreads from cell to cell, and subsequently generates effects that are heritable for multiple generations. The resulting progeny display an approximation of the knockout phenotype for a particular suppressed gene, thereby allowing researchers to quickly assay gene function.

Although RNAi is a powerful screening tool, it is limited in a number of ways. Not all genes are equally responsive to RNAi, and some tissues, notably neuronal cells, are not amenable to RNAi. Also, RNAi only assays for the loss of gene function. Therefore, unlike traditional techniques used in genetics, this method does not make it possible to assay gain of function or dominant phenotypes. With these considerations, RNAi should be regarded as a complement to traditional methods, and not as a complete replacement for genetic screens.

That said, the ease of applying RNAi and the immediate knowledge of the gene target facilitates phenotypic analysis that would simply be unimaginable using traditional methods. Phenotypes that have a very low penetrance or require time-consuming techniques are strong candidates for RNAi-based screens. For example, a technically complicated RNAi-based screen was recently performed in an effort to identify all of the gene products required for the first two rounds of cell division following fertilization of a *C. elegans* oocyte [21]. This screen targeted 98% of all known open reading frames of the *C. elegans* genome via RNAi. Defects in cell division were recorded with the use of differential interference contrast time-lapse microscopy. Approximately 40,000 time-lapse recordings from more than 19,000 individual RNAi experiments were acquired and assayed for phenotypic defects. These experiments identified at least 661 genes that were required for the earliest cell divisions during embryogenesis, about 14% of which had no known prior function. Furthermore, half of the previously uncharacterized gene products had readily indentifiable homologs in other organisms, thus demonstrating how experiments that are only possible in *C. elegans* can have broader implications that extend to other organisms.

24.2.2.3 Compound Screens: Identifying Therapeutics

Many researchers are pursuing the identification of new bioactive compounds because of their value as possible pharmaceuticals for treating human disease and their subsequent market potential. *C. elegans* has many features that make it appealing to those interested in large-scale drug screening. Not only is it a multicellular animal that is easy to grow and reproduces rapidly; its small size allows it to be readily cultured in a 96-well plate measuring only 5 by 3.5 in, thus facilitating process automation. *C. elegans* also has a large number of conserved genes and disease pathways that have potential therapeutic value when considering the treatment of human disorders. Furthermore, the added ability to carry out traditional genetic screens allows for the rapid identification of molecular targets for potential therapeutics.

The combined ability to screen compound libraries for bioactive small molecules and the ability to efficiently characterize new drug targets makes *C. elegans* a powerful tool for defining the mechanisms of drug action. A recent screen of 14,100 small molecules in *C. elegans* identified 308 compounds that produced discernable phenotypes [14]. Growing worms in the presence of one of these compounds, referred to as nemadipine-A, resulted in reproducible defects in body morphology and inhibited the ability of the worms to lay eggs. Nemadipine-A shares a high degree of structural similarity to the common anti-hypertension drugs 1,4-dihydropyridines (DHPs). DHPs have been shown to function by binding to L-type calcium channels to inhibit their function. A genetic screen for mutations that could suppress the effects of nemadipine-A identified multiple dominant mutations in a single gene, egl-19. egl-19 encodes the alpha subunit of an L-type calcium channel. Sequence analysis of the dominant egl-19 mutations demonstrated amino acid changes in regions of the channel that have been previously shown to be required for interaction with DHPs in mammalian systems. These and other results indicate that the primary target of nemadipine-A is egl-19. This example validates the prospect of using *C. elegans* to rapidly identify active small molecules and their molecular targets, thus facilitating drug discovery.

24.2.3 Beyond the Simple Screen

24.2.3.1 A Systems Approach: Protein Interaction Maps

Proteomics refers to the large-scale analysis of all the proteins within a given cell or organism. When and how these proteins are expressed and post-translationally modified and how they interact with each other and with other components within the cellular milieu is information that falls under the proteomics umbrella. In *C. elegans*, many of these areas of systematic research are still in the early stages. However, significant progress has been made in defining the protein–protein interaction map using large-scale two-hybrid analysis. In these experiments, a yeast-based reporter system is used to determine if two proteins are capable of directly interacting with one another [9]. The overall goal is to generate a genome-wide protein interaction map that links uncharacterized proteins to those with known functions in defined biological processes. Although incomplete, mapping projects centered on a subset of metazoan-specific proteins have provided validation of this experimental approach in *C. elegans* [15].

This approach has also been adapted to identify direct interactions between transcription factors and their associated promoter regions, with the overall goal of creating a genome-wide transcription factor-promoter map for *C. elegans*. In theses experiments, a yeast-based reporter system, similar to the two-hybrid assay, is used to determine which proteins within the genome bind to a specific gene promoter and subsequently activate transcription of a reporter gene [9]. In the initial experiments testing this approach, 72 promoters were analyzed [5]. Each was chosen because of its ability to drive gene expression specifically in the gut of the worm. The analysis identified 283 individual protein-DNA interactions involving 117 proteins. Interestingly, a hierarchical nature of the system was observed: overall, there were three or more layers of transcriptional control regulating the genes. A handful of the identified transcription factors were highly connected within the network, binding to many different promoters. These transcription factors were designated ‘global regulators.’ Another set of transcription factors, termed ‘master regulators’ bound to fewer, but still multiple, genes. Finally, there were a number of ‘specifiers’ that only bound to a small number of genes. This hierarchical system, as the authors point out, is similar to the multi-layered transcriptional regulatory networks observed in bacteria, suggesting that the overall structure of the system may be evolutionarily conserved. Further such studies, conducted on a larger scale and coupled with extensive expression data, can provide the first look at the overall developmental transcriptional regulatory cascades of multicellular organisms. These kinds of network maps will likely lay the foundation for our understanding of more complex systems, including neural development and numerous neurological disorders in humans.

24.2.3.2 Exploring Complex Traits: Aging

Aging is a fundamental and universal process that affects all living organisms. At its core, aging in humans is an incredibly complex trait and is unlikely to fit a simplistic framework of inheritance. Aside from overwhelming environmental influences, the genetic factors affecting longevity are likely multifaceted at best. Remarkably, however, it has been shown that individual mutations can dramatically extend the lifespan of *C. elegans*. These observations, implicate the insulin/insulin growth factor-1 (IGF-1)-like signaling pathway as a critical determinant of lifespan [24]. The insulin/IGF-1-like signaling pathway in *C. elegans* consists of a number of genes, including daf-2 (an insulin/IGF receptor homolog), age-1 (a phosphatidylinositol-3 kinase), daf-18 (a phosphatase and tensin homolog deleted on chromosome X [PTEN] phosphatase), and daf-16 (a Forkhead box class O [FOXO] family transcription factor). Impeding this pathway, by either mutation or RNAi, can significantly extend the *C. elegans* adult lifespan without significantly affecting reproductive fitness. These observations demonstrate that lifespan is clearly subject to regulation and not driven solely by entropy.

Similar results in both flies and mice indicate a level of conservation in the molecular mechanisms affecting longevity, suggesting the possibility of one day developing pharmacological agents to extend the human lifespan. However, the prospect of identifying drugs that can positively influence aging in humans is complicated by the extensive drug-approval guidelines set forth by the Food and Drug Administration (FDA) and the fact that an individual researcher would likely only be able to observe a single cohort of patients, given the time required to complete a clinical trial on aging. With this in mind, a recent study assayed a number of known drug compounds that had already received FDA approval for the treatment of various unrelated human disorders to determine their ability to extend lifespan in *C. elegans* [6]. A class of anticonvulsants traditionally used to modulate neural activity in humans was found to significantly extend both the mean and the maximum lifespan of worms. These results not only serve to support previous findings showing that neural activity in worms can affect longevity; they also suggest a feasible approach for identifying age-extending pharmaceuticals for use in humans by exploiting short-lived model organisms. Testing compounds that have already passed through clinical-trial development mitigates some of the need and cost of this process, although clearly determining the efficacy of these drugs on human longevity will still require multiple generations and some clinical analysis.

24.2.3.3 Modeling Human Disorders: Alzheimer's Disease

Protein misfolding with aberrant protein aggregation is a hallmark of many age-related neurodegenerative disorders, including Alzheimer's, Parkinson's, triplet repeat disorders, and prion-related diseases. Individuals with Alzheimer's disease present with significant and progressive memory loss owing to aberrant cell death in the central nervous system. These clinical traits are usually only observed in individuals in their late fifties or older. The molecular mechanism underlying this age-related neurodegeneration is not completely understood. The histopathology indicates the presence of aberrant protein aggregates, including the presence of amyloid plaques formed by the beta-amyloid peptide (Aβ), a proteolytic fragment of the amyloid precursor protein [20]. The leading hypothesis suggests that Aβ aggregates are neurotoxic and likely play a key part in disease progression. To date, there are no effective therapies to combat the progression of Alzheimer's.

C. elegans has proved to be an influential means for modeling proteotoxic disorders such as Alzheimer's. Ectopic expression of Aβ in the body wall muscles of *C. elegans* results in progressive paralysis [16]. This paralysis is paralleled by Aβ aggregation and deposition along the muscle fibers, as assayed using both amyloid-specific antibodies and dyes [8, 16]. Although *C. elegans* does not encode an amyloid gene homologue, the rationale for constructing such a model is that it allows researchers to utilize the experimental tools available in *C. elegans* to understand how fundamental cellular processes may be affecting the progression of this neurodegenerative disease. As such, this model provides a system in which to address a number of basic questions about aggregate formation and toxicity. For example, it has allowed researchers to address the question of whether aggregate formation is simply a stochastic development that occurs over time during the life of the animal or whether it is a progressive mechanism that is influenced by the aging process. To address this particular question, paralysis of Aβ-expressing worms was measured following down-regulation of insulin signaling via RNAi directed against daf-2 (insulin/IGF receptor homolog) in order to extend the lifespan of these worms [4]. Interestingly, the onset and extent of subsequent paralysis paralleled the altered aging profile of the worms. These results are similar to those showing that slowing the aging process in *C. elegans* can decrease Huntington-associated proteotoxicity [18]. Hence, it appears that there is a direct link between the mechanism of aging and the progression of proteotoxicity. One possible model is that aging reduces an organism's ability to detoxify aggregates, thus suggesting a direct link between longevity and the ability to maintain protein homeostasis. Extrapolating observations such as these to humans may prove useful for identifying potential targets for drug research.

24.2.4 Conclusions and Perspectives

The life sciences have undergone extraordinary advances in the last decade. The dawn of the genomic era in biological research has taken traditional and experimental biology to new heights. No longer are researchers asking what a single component of a cell does at a specific time and place; many now possess the tools to address the interconnections between various components of a cell, how assemblages interact, and how the attributes of living things are derived from the sum of their whole. Recent technological advances are driving this new systems-level approach to biology. In many ways, automated methods based on industrial models have allowed for the development of high-throughput experiments designed to leverage the power of the fully sequenced genome. Despite these advances, it is important to realize that a simple list of genes involved in some aspect of biology is often of little use.

Although such a list can be extremely useful for quickly generating new ideas and research directions, hypothesis validation still requires a return to the basics of experimental biology in the long run. In the end, to fully understand the intricate complexity of a living system, we will need to understand what every single component of the system does at any given time and place. It is with this in mind that we have considered the nematode, *Caenorhabditis elegans*. *C. elegans* research has not only provided a solid foundation in traditional, basic biological inquiry, but is also uniquely suited to modern systems-level approaches. Indeed, many advances in our understanding of human disease mechanisms are likely to revolve around this simple worm over the next decade.

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Drosophila as a Model for Human Disease 24.3

Ruth Johnson and Ross Cagan

Abstract *Drosophila melanogaster* has proved a remarkable genetically tractable model organism that continues to provide significant contributions to our understanding of numerous biological processes. In this chapter we discuss insights into a variety of human diseases that have been gained directly from studies conducted in fly labs. These include discoveries relating to the basic biology of diseases (the signaling pathways, for example, that may contribute to disease states), new loci implicated in disease progression or susceptibility (uncovered in large-scale screens and verified in situ using often ingenious assays) and the identification of pharmacological reagents to treat diseases (also identified and tested in well-designed screens and assays). Because genomes, biological processes, and responses have been well conserved, and particularly with the current trend in translational research, studies in flies continue to build a strong foundation for disease studies. Those discussed in this chapter include cancer, neurodegenerative diseases, heart disease, diabetes and metabolic diseases, addiction, and sleep disorders.

Contents

24.3.1 Why Flies?

The diminutive fruit fly, *Drosophila melanogaster*, may not be an intuitive model organism for studying human disease. Yet its contributions to this broad topic have been extensive, particularly in the era of genome sequencing, which has simplified the identification of orthologs.

Typically, multiple loci contribute to the susceptibility to or etiology of a single disease. *Drosophila* has a fully sequenced and annotated genome of approximately 14,000 genes and provides a model system that

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can identify and manipulate loci with relative ease. This is made possible by a remarkable array of available tools that enable large-scale screening of the *Drosophila* genome for mutations in primary and susceptibility loci. Through the efforts of individual laboratories, the Berkeley Drosophila Genome Project (BDGP), and commercial enterprises such as Exelixis, the community has ready access to fly lines that contain individual targeted mutations that together disrupt nearly 90% of the predicted loci. Deficiencies that cover approximately 70% of the fly genome are available for screening a few dozen genes per fly line. The FLP/FRT and "MARCM" systems are used to create "mitotic clones" of discrete mutants, allowing for the precise study of a mutation within even individual isolated cells [50, 88]. The UAS/GAL4 system and other related systems can be harnessed to express a gene of interest in a tissue- and time-specific manner [54]; with the ease of use of RNA interference in *Drosophila*, these powerful mis-expression systems can also be used to knock down the activity of targeted genes in specific tissues or individual cells [68]. Most of these fly lines are readily available: a key strength of the *Drosophila* community has been a community ethos that emphasizes developing and quickly sharing useful tools and engineered fly lines.

Until recently, most *Drosophila* researchers have focused on broad questions of cell-cell signaling and cell biology as they relate to development. The tiny fruit fly has provided many of the fundamental insights into these issues, and Christiane Nusslein-Volhard, Eric Wieschaus, and Edward Lewis were honored with a Nobel Prize in 1995 for their genetic approaches to development. Recently, an increasing number of *Drosophila* laboratories have turned their attention to specific issues of disease, bringing their powerful tools and a unique vision that emphasizes in situ studies of specific tissues and individual cells.

How similar is *D. melanogaster* to mammals, and how useful is the fruit fly for studying disease? For specific diseases we often do not really know until studies have progressed in both, but the overall signs are encouraging. The fly genome shows a startling degree of homology to that of humans, with better than 60% of disease genes showing a clear ortholog by simple sequence gazing [2] and more careful studies indicating a still higher percentage with functional homology. These similarities extend to diseases that are often thought of in a social context, such as alcoholism, drug abuse, sleep apnea, and also behavioral issues, such as eating habits or aggression. Observing flies reacting to alcohol in ways that mimic human behavior teaches us something about the biological basis of our own responses to intoxication. With a life-span of 2–3 months, a generation time of 10 days, a propensity for each female to produce hundreds of progeny, and easy and inexpensive maintenance, flies provide a cheap, rapid, and powerful approach to the study of specific diseases. And, critically, *Drosophila* are sufficiently complex to permit meaningful generalizations to mammals and humans.

24.3.2 Cancer

Perhaps the first in vivo tumor suppressor identified was the *Drosophila* gene *lethal giant larvae* (*lgl*), which was identified more than 70 years ago by Bridges: recessive loss-of-function mutations led to dramatic overgrowth [13, 14]. *Drosophila* workers bring a somewhat unusual, and potentially very useful, perspective: *Drosophila* is traditionally strong in developmental biology, and the defects observed during oncogenic progression have many aspects that are familiar to *Drosophila* developmental biologists. For these reasons and more, cancer is the disease with the largest efforts in the *Drosophila* field, and the length of this section reflects those efforts.

Of course, *Drosophila* is not a perfect model for human cancer, and a consideration of its similarities and differences is useful. Decades of research have indicated that humans and flies have remarkable similarities in their basic epithelial architecture: junctional proteins are well conserved with some important differences [47, 87]; most major signaling, growth, and death pathways are also well conserved. For example, nearly all of the genes most commonly found altered in tumorigenesis are conserved both structurally and (mostly) functionally in flies, including P53, Ras, Raf, Pten, Src, etc. Perhaps the most important difference lies in the inflammatory response, which has important differences including a lack of the immunoglobulin system. And of course flies do not have bones, which are a major target of breast and prostate metastases, for example. Finally, not all cancer-related genes have clear *Drosophila* orthologs: for example, the BRCA1 and BRCA2 (genes cursive) linked to breast cancer and the mechanisms of telomere maintenance appear to differ.

While potential models of *Drosophila* hematopoietic tumors have been observed (e.g., the emergence of "melanotic tumors" derived from the hematopoietic system), most fly cancer research to date has focused on models of solid tumors. These studies have been extensively reviewed (e.g., [4, 12, 44, 71, 79, 85, 89, 97]), and the purpose below is to introduce the non-fly worker to some of the more notable advances.

24.3.2.1 Cell Cycle

24.3.2.1.1 The Cell Cycle Machinery

The basic cell cycle machinery has been conserved in a broad array of animals from single-cell yeast to mammals, and *Drosophila* shares most of the standard regulators. *Drosophila* has made many of its most important contributions to our understanding of the connections between cell cycle and signaling. A large body of work on *Drosophila* has focused on the spatial regulation of the cell cycle during development (reviewed in [20, 22]), and recent screens have included saturation screens for cell cycle regulators [9]. Recently, *Drosophila* biologists have directed more of their work at aspects that impinge more directly on tissue overgrowth.

Regulation of the Cell Cycle

The cell cycle is regulated at multiple steps by the E2F/Rb pathway, which directly modulates expression of factors such as the cyclins (reviewed in [31]). *Drosophila* has two E2F isoforms: dE2F1 promotes cell proliferation, while dE2F2 opposes it and can also act in other pathways [29, 82]. Both in mammals and in flies, E2Fs act with their heterodimeric partner Dp1; this complex is in turn regulated by physical interactions with Rb. During development, the E2F complex appears to control cell cycle progression through its regulation by a host of other cell cycle-relevant factors (e.g., [19]). As in mammals, deleting the function of the E2Fs indicates they are not essential for cell cycle progression [29]. *Drosophila* Rb acts both to regulate cell cycle and to promote cell death; this balance appears to be at least in part due to regulation by epidermal growth factor receptor (EGFR; [27, 56]), an ortholog of the ErbB family of oncogenes. One goal of E2F/Rb research is to understand the factors that influence Rb to promote cell death vs proliferation and to identify factors that can emphasize the former in advancing tumors that contain activated E2F.

24.3.2.2 Cell Death

Control of cell death is a central aspect of cancer, and *Drosophila* models have provided important contributions to our understanding of cell death pathways, again with the added advantage of being able to examine cell death events in situ.

24.3.2.2.1 Apoptosis Pathway

The primary effectors of apoptotic cell death are the "caspases", cysteine aspases that cleave dozens of downstream targets to initiate the apoptotic process (reviewed in [92]). *Drosophila* has seven caspases, including upstream "initiator" caspases (Dronc, Strica/Dream, and Dredd) and downstream "effector" or "executioner" caspases (Drice, Dcp-1, Damm, Decay) [35]. Regulation of caspase activity represents the central control point of apoptosis. In mammals, caspases are primarily regulated by members of the Bcl-2 superfamily. A second regulatory system is anchored by the IAP (inhibition of apoptosis protein) superfamily, cytoplasmic proteins that inhibit caspase activity through their binding of intermediaries including Smac, Diablo, etc. [35]. Though considered less important as caspase regulators in mammals, IAPs – particularly Survivin and XIAP – represent some of the most strongly overexpressed genes in malignant tumors [70, 100].

In flies, both the Bcl-2 and the IAP systems are active during regulation of apoptosis. Unlike in mammals, IAPs play the dominant role during development [35]. For example, loss of the IAP family member Diap-1 during embryogenesis leads to widespread apoptosis and rapid organism lethality, whereas reduction of either of the characterized Bcl-2 family members (Buffy, Dborg-2) has mild effects. Diap-1 is in turn bound and inhibited by the "H99" group of proteins – Reaper, Hid, and Grim – and other proteins such as Jafrac2, Sickle, and Morgue, in some cases by targeting an

N-terminal IAP-binding motif (IBM). Similar mechanisms appear to be at work in the mammalian IBM motif-containing proteins Smac/DIABLO and HtrA2/Omi, and *Drosophila* has proved a successful model for understanding how this class of proteins acts.

24.3.2.2.2 Neighboring Signals and Cell Death

The Myc complex, composed of Myc, Max, and Mnt family members, regulates cell growth and proliferation in numerous organisms. These are potent oncogenes. Mutations that reduce Myc function lead to small mice and flies, and Myc appears to regulate overall tissue growth. In a similar way to mammals, *Drosophila* Myc (dMyc) represents an important point of cell cycle regulation. For example, the Wg signaling pathway controls Myc expression in the developing wing, and Wingless (Wg) activity at the dorsal-ventral boundary leads to down-regulation of dMyc to create a discrete "zone of nonproliferating cells" (ZNC) at the boundary (e.g., [21]).

Drosophila cells that overexpress dMyc are at a competitive advantage; that is, they grow more quickly than their neighbors. Interestingly, cells neighboring these dMyc overexpressers grow more slowly than expected, indicating that dMyc mediates a signal between cells that keeps overall tissue size within its normal range. In a series of elegant experiments, the Johnston and Basler laboratories used clonal analysis to demonstrate how cells with reduced dMyc activity are cued to die by their wild type neighboring cells [18, 43, 57]. This death signal is mediated at least in part by the Wg and Decapentaplegic (Dpp) pathways, orthologs of mammalian Wnt and BMP signal transduction pathways, respectively. Cell competition and the signals traded between cells are a field of growing interest, holding out promise of improved understanding of some of the local signaling aspects between normal and transformed cells during oncogenesis. These local effects within the epithelium are likely to be better appreciated in mammals as tools improve to permit finer resolution of mammalian tumors.

24.3.2.3 Hyperplasia and Neoplasia

Similar to their mammalian counterparts, *Drosophila* tumor suppressor genes are typically partitioned into "hyperplastic" and "neoplastic." Hyperplastic genes are also known as "growth control" genes. They are thought to be key mediators of setting organ size, and loss of their activity can lead to large imaginal disc epithelia that otherwise develop reasonably normally. Hyperplastic loci include *hippo*, *salvador*, *warts*, *tsc1*, *tsc2*, *pten*, and *src*. Changing a tissue's size is not as simple a task as it might seem: for example, increasing cell proliferation typically leads to compensatory apoptosis, which brings tissue size back to normal. Mutations in hyperplastic loci have the distinct property that they can direct overproliferation and simultaneously block apoptosis. Neoplastic loci (*scribble*, *lgl*, *dlg*) yield a more dramatic overgrowth phenotype: cells lose their apical-basal polarity and masses of expanding mutant tissue are sometimes seen invading other regions. Unlike hyperplastic tissue, neoplastic tissue is poorly constructed.

24.3.2.3.1 Hyperplasia and Growth Control

The primary pathway that appears to mediate size regulation in developing epithelia is the "Hippo pathway" (Fig. 24.3.1) (reviewed in [34]). This pathway couples events at the cell surface – e.g., through the cell surface FERM family proteins Merlin and Expanded and the protocadherin Fat – to regulation of cell growth and cell death. Hippo and Lats/Warts encode Ste-20 and NDR-type serine-threonine kinases, whereas Salvador encodes

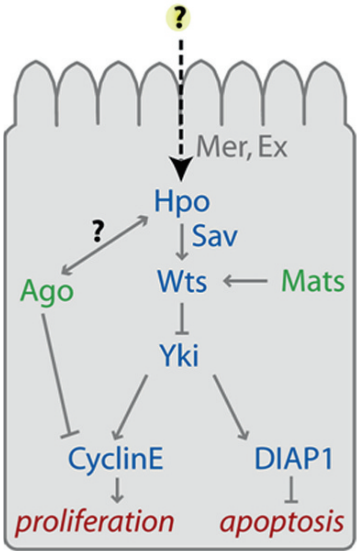

Fig. 24.3.1 The hippo signaling pathway

a scaffolding protein with a WW domain. The kinase activity of Lats/Warts is, in turn, regulated by Mats, an ortholog of the Mob superfamily of tumor suppressors. Reducing activity of any of these proteins leads to activation of the transcription factor Yorkie, which then directs expanded tissues by simultaneously increasing proliferation (via cyclin E) and blocking programmed cell death (via dIAP1). This remarkable ability to simultaneously regulate both aspects of tissue sizing suggests that the Hippo pathway represents a fundamental "ruler" that measures and adjusts tissue size. Critically, human cancer-derived cell lines have germline mutations in the locus encoding the Hippo ortholog *Mst2*, and mutations in murine and human *Lats1* lead to sarcomas and ovarian tumors (e.g., [38, 83]). Similar size-regulation properties have been described in *Drosophila* for the Ste-20 ortholog Slik [37], the F-box protein Archipeligo [55], and the micro-RNA Bantam [10], although the mechanisms by which they act are less clear.

24.3.2.3.2 Neoplasia and Cell Polarity

Neoplastic growth is characterized by uncontrolled proliferation and a failure of cells to enter or maintain terminal differentiation. As carcinomas (malignant epithelial tumors) mature, they are increasingly characterized by a loss of cell morphology (cell structure becomes more relaxed) and tissue polarity (cells pile up on one another). In recent years, *Drosophila* cancer biologists have established models that seek to understand the mechanisms that direct this transition.

Three proteins have taken center stage in *Drosophila* models of neoplasms: Lgl, Dlg, and Scribble. Reducing the activity of any of these proteins leads to massive overgrowth of imaginal disc epithelia and nervous system components (Fig. 24.3.2). These three proteins are associated with apical junctions, and Dlg and Scribble contain multiple protein-protein binding domains that indicate they act in part as molecular scaffolds. Dlg and Scribble act together – likely as a multi-protein complex – to help build and maintain proper apically based polarity [7]. They do so through complex interactions with other apical junction-associated proteins including dPatJ, Crumbs, Stardust, Par-6, Bazooka/Par-3, and Atypical PKC in addition to Lgl [6]. Loss of *lgl*, *dlg*, or *scribble* leads to (a) a loss of morphological and molecular markers of apical cell polarity; (b) a failure of cells to differentiate; and (c) intensive overgrowth. What is especially fascinating about these loci is that they clearly point to an intimate association between epithelial architecture and growth control. Importantly, all three have mammalian orthologs, although the precise role played by these loci in human tumors is not yet understood.

Curiously, a group of fly proteins that regulate endocytosis also direct neoplastic growth when mutated. Four of these factors have been carefully studied in *Drosophila*: Vps25, Rab5, Tsg101 (also known as Vps23 or Erupted), and Avalanche (reviewed in [33]). Reducing the activity of any of these four factors results in overgrowth that is strikingly similar to mutations in *lgl*, *dlg*, or *scribble*, but their mechanism of action appears to be significantly different. Each of these four factors is a component of the endocytic machinery that regulates protein presence at the cell surface. Reducing activity of any of the four loci leads to a block in proper endocytic trafficking, leaving proteins such as signaling receptors (e.g., Notch, EGF Receptor) and polarity components (e.g., Crumbs, a protein that helps establish a cell's apical domain) stranded inappropriately at the cell surface. Depending on the cellular context, this can lead to overgrowth. Furthermore, reducing activity of Vps25 and Tsg101, components of the ESCRT complex that direct protein sorting from the early endosomes, can lead to overgrowth of surrounding nonmutant cells. These mutants point to the importance of regulating growth factor stability in controlling normal tissue growth and maturation.

24.3.2.4 Models of Metastasis

24.3.2.4.1 Ras

Ras signaling represents a canonical signal transduction pathway that is directly activated in perhaps 20–40% of all solid tumor types. The Ras pathway typically coordinates information from upstream signaling receptors, leading to changes that depend on cell context but include proliferation, migration, and differentiation. Recent work on *Drosophila* has extended previous data demonstrating that Ras can act cooperatively and potently with other factors to direct neoplastic overgrowth and even metastasis.

Strong overexpression of activated isoforms of the *Drosophila* Ras ortholog dRas1 leads to overgrowth, but

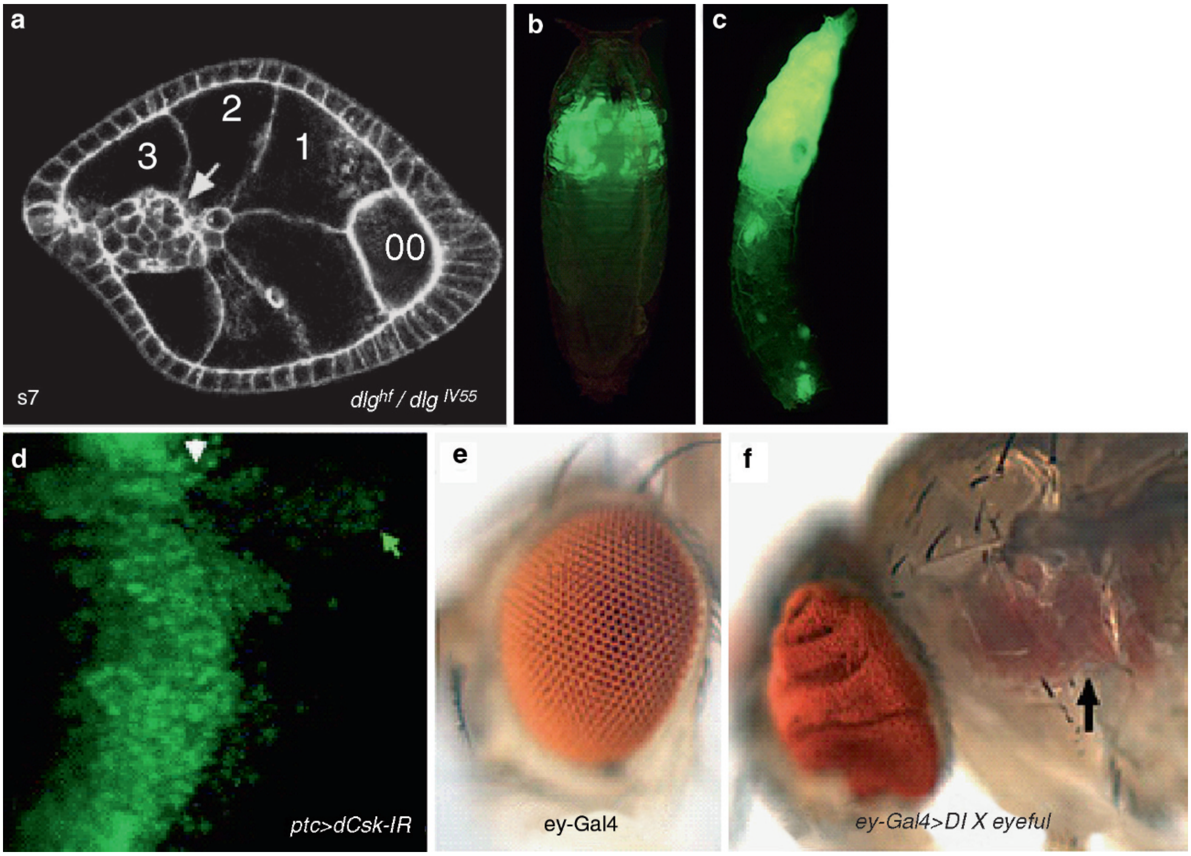

Fig. 24.3.2 (**a–f**) *Drosophila* cancer models. (**a**) dlg Mutant 'follicle cells' expand as a tumor into the egg chamber, a structure that houses and patterns the emerging oocyte. Cells at the anterior and posterior poles of the egg chamber are especially susceptible to transformation by *dlg*. From [30a] (**b**) Expressing the oncogenic Ras isoform dRas1^{G12V} led to low-level, localized overgrowth. (**c**) Reducing *scribble* function in the presence of dRas1^{G12V}, however, was sufficient to produce strongly overgrown tissue that expands away from the head region. Transformed tissue marked with EGFP. From Pagliarini et al. [60] (**d**) At the boundary of a region with reduced dCsk activity, Src activity increases and cells migrate away (*green arrow*) from the original region of the dCsk "tumor." The white arrow indicates the tumor edge; cells are marked with EGFP. (**e**, **f**) Coupling overexpression of the Notch ligand Delta with the epigenetic silencers Lola and Pipsqueak (which together constitute "eyeful") led to massive overgrowth of the eye (**f**) compared with controls (**e**) in addition to secondary growths of eye-like material (red material indicated by *arrows* in F)

also to compensatory apoptosis (see discussion of this phenomenon above). This triggering of cell death is both autonomous and nonautonomous (that is, neighboring wild type cells are also affected) and often leads to a tissue that is eventually reduced in size. Activated dRas1 (dRas1^{G12V}) strongly cooperated with members of the hyperplastic and neoplastic groups of tumor suppressors. The outcome for each is different in important ways, however. Pairing dRas1^{G12V} with a hyperplastic mutation in *lats* (see above) in the eye led to dramatically increased overgrowth, but the cells remained within the tissue [60]. By contrast, dRas1^{G12V} plus reducing function in *scribble* led to overgrowth and, in addition, degradation of the basement membrane and cell migration in a JNK-dependent manner (Fig. 24.3.2) [11, 40].

24.3.2.4.2 Src

Increase in Src activity has been linked to increased risk of metastasis in dozens of solid tumor types including breast, colorectal, melanomas, etc. While the precise role played by Src in metastasis is not well understood, recent work has linked changes in Src activity to changes in junctions, cell adhesion, cytoskeleton, and cell motility (reviewed in [41]).

Drosophila has two Src orthologs, dSrc42A and dSrc64B, and the Src-like Btk ortholog dSrc29 that is a likely downstream Src effector. Reducing activity of the Src repressor *Drosophila* C-terminal Src Kinase (*dCsk*) led to Src activation, tissue overgrowth, an arrest in larval development, and eventual death of the fly. Cells with reduced *dCsk* function showed increased overall proliferation, a block in cell death, and an increase in organ size, placing *dCsk* – and by extension Src – in the category of hyperplastic mutations described above. Indeed, in vitro and genetic studies suggest a link between *dCsk* and Lats.

While reducing dCsk activity throughout a tissue led to an increase in organ size, reducing *dCsk* activity in discrete patches of cells led to basal release of cells and migration away from the *dCsk* "tumor"; these cells eventually died by apoptosis (Fig. 24.3.2) [91]. Interestingly, only cells at the border of the *dcsk* patch showed this behavior, suggesting that interactions with neighboring wild type cells provoke migration. Further genetic studies suggested a pathway – triggered specifically in *dcsk* cells at the "tumor" border – that extends from Src through E-cadherin, P120-catenin, RhoA, JNK, metal metalloproteases, and caspases. While some evidence exists for a change in E-cadherin and P120-catenin at the boundaries of human squamous cell carcinomas [80], further work will be required to determine whether Csk/Src-mediated metastasis commonly utilizes this pathway in human tumors.

24.3.2.4.3 Notch

The Notch signal transduction pathway is broadly utilized during development. Notch is a transmembrane signaling receptor that is activated by Delta and signals through cleavage-dependent translocation of its intracellular domain to the nucleus, where it acts as a transcriptional activator [58]. In humans, activating mutations in Notch can lead to disease, including hematopoetic cancers such as acute lymphoblastic leukemia (e.g., [23]). Indeed, Notch itself is required for proper regulation of cell proliferation in *Drosophila*, and unregulated activation of Notch activity – for example by ectopic expression of its ligand Delta – can lead to uncontrolled proliferation of the larval wing.

Dominguez et al. [25] found a link between Notch and HDAC regulators Lola and Pipsqueak; the latter two act as epigenetic chromosome silencers by regulating HDAC activity. Expressing Delta, Lola, and Pipsqueak together led to a dramatic overgrowth of the eye and, remarkably, "eye tumors" in distant regions such as the abdomen (Fig. 24.3.2). This work provides a wonderful demonstration of how signal transduction pathways can combine with epigenetic elements to drive metastasis-like cell behavior. One surprise is that many of these distant "tumors" retained the ability to develop as eye tissue, suggesting they are not fully transformed or can "revert" once inserted into an epithelium (Fig. 24.3.2).

24.3.2.5 Models of Specific Cancers

While most cancers are polygenic, a few cancers progress through a single monogenic event. Not surprisingly, monogenic cancers have been the first to be examined in *Drosophila*, as the triggering events are easily duplicated.

24.3.2.5.1 Tuberous Sclerosis

In humans, mutations that reduce the activity of Tsc1 or Tsc2 lead to a benign tumor syndrome in which hamartomas emerge in the brain and other organs such as the heart and kidney (reviewed in [61]). Loss-of-function mutations in *Drosophila tsc1* or *tsc2* (originally known as *gigas*) led to an increase in cell size and cell growth due to increased expression of cyclins A, B, D, and E [42, 86], indicating that Tsc activity normally opposes cell proliferation. Work in both mammals and *Drosophila* has linked cell size effects to Tsc-2 GAP activity for the small GTPase Rheb, which in turn acts through the Tor pathway. Tor (target of rapamycin) activity regulates cell metabolism through multiple pathways, including generalized protein biosynthesis [61], and rapamycin is currently in clinical trials in tubular sclerosis patients [28]. Recently, the Tsc/Rheb/Tor pathway has been linked to PI3K signaling, a central mediator of growth response in epithelia. Tuberous sclerosis is an important example of the link between metabolism and oncogenesis, and research on other diseases will no doubt need to account for metabolic processes during the search for effective therapeutics.

24.3.2.5.2 Neurofibromatosis 1

Neurofibromatosis 1 (NF1) is one of the most common inherited nervous system cancer syndromes. Neurofibromas are benign tumors found in peripheral nerves, especially Schwann cells. In addition, *NF1* patients show a palate of problems that include skin pigmentation defects, hamartomas of the iris, optic pathway gliomas, and mental retardation [49]. In a minority of patients tumors advance to become metastatic and can prove fatal.

The *NF1* gene encodes a large protein that includes a functional RasGAP (Ras GTPase activating protein) domain. RasGAP domains stimulate Ras-GTP hydrolysis to inactivate Ras function, and at least some of NF1's activities can be assigned to its regulation of Ras function in mammalian cell culture tumorigenesis models [48]. Work in *Drosophila* provided some of the first evidence that NF1 can act on pathways independently of Ras: mutations in *Drosophila* NF1 lead to small body size that is dependent on cyclic AMP-dependent protein kinase A (PKA). These effects on both Ras and on PKA have also been identified in mammalian systems, leading to the question of whether this large and structurally complex protein acts in other pathways as well.

24.3.2.5.3 Multiple Endocrine Neoplasia Types 1 and 2

Multiple endocrine neoplasia type 1 (MEN1) and type 2 (MEN2) are both cancer syndromes that result in dispersed tumors with strongest representation in hormone-producing endocrine tissues. *MEN1* is an autosomal dominant cancer syndrome characterized by a palate of tumors that include parathyroid, anterior pituitary, and pancreatic islet tumors in addition to nonendocrine tumors such as angiofibroma. The *men1* locus encodes Menin, a large nuclear protein whose the function remains mysterious (reviewed in [98]). Some of the strongest in situ data for Menin's links to both cell cycle and genomic integrity have come from flies. Loss of *Drosophila* Menin (Mnn1) activity led to a viable adult that nonetheless shows poor ability to repair its DNA after ionizing irradiation-induced breaks or handle various physiological stresses and a failure of cells to arrest at the G1-S checkpoint after ionizing irradiation, an observation that was confirmed in mammalian cell lines [15, 62]. Further genetic screens have identified or confirmed functional interactions between *Drosophila* Mnn1 and components of the JNK/AP-1 pathway as well as Ches1, a forkhead/winged helix transcription factor linked to DNA checkpoint regulation. Based on the fly and mammalian data, a picture is beginning to emerge in which Menin is a tumor suppressor that functions as a co-factor for multiple transcription factors and regulators of chromatin integrity during DNA repair.

24.3.2.5.4 Multiple Endocrine Neoplasia 2

MEN2 patients typically have one of several activating mutations in the Ret receptor tyrosine kinase. Patients consistently display medullary thyroid carcinoma (MTC) and can also display pheochromocytomas, parathyroid adenomas, mucosal neuromas, etc. Targeting either *MEN2A*- or *MEN2B*-equivalent Ret mutations to the developing *Drosophila* eye led to a series of tissue defects that mimicked particular aspects of human *MEN2* tumors, including increased cell proliferation, compensatory apoptosis, and disruption of cell fates [63]. A genetic modifier screen identified 140 functionally linked loci; human orthologs of two modifier loci – TNIK and CHD3 – are located within deletions associated with patients displaying secondary pheochromocytomas, suggesting these loci are at least candidate biomarkers of susceptibility to adrenal tumors [63]. This demonstrates how fly genetics can help identify human susceptibility loci.

The anilinoquinazoline ZD6474 inhibited Ret activity in mammalian tumor cell lines. *Drosophila* provided further in situ evidence of its utility: feeding the compound to *MEN2* flies strongly suppressed the eye *MEN2* phenotypes with minimal toxicity to the animal (Fig. 24.3.3) [90]. This compound is being tested in phase III clinical trials to treat patients with medullary thyroid carcinoma (S. Wells, personal communication), pointing to the utility of using *Drosophila* both as a genetic tool and also as a whole-animal screen for therapeutic compounds.

24.3.3 Neurodegenerative Diseases

Neurodegenerative diseases are increasingly prevalent within the aging human population. They have proved especially difficult to treat owing to the complexity of

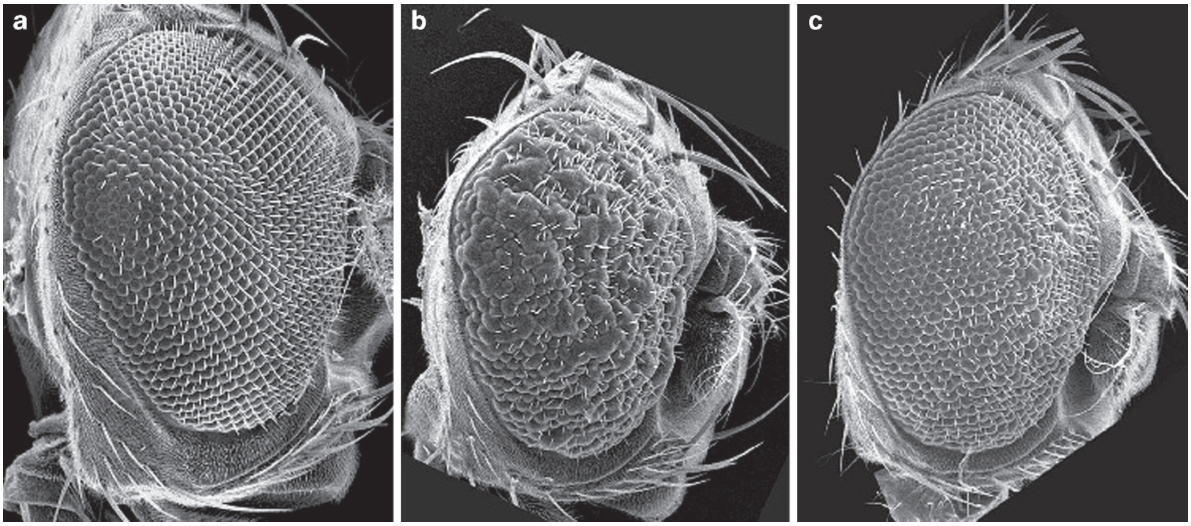

Fig. 24.3.3 Using Drosophila to screen for therapeutic compounds for multiple endocrine neoplasia (MEN). (**a**) Control flies display a smooth eye surface. (**b**) Expression of oncogenic Ret(MEN2) isoforms phenocopies several aspects of tumors including overproliferation, compensatory apoptosis, etc. (**c**) Feeding flies: the compound ZD6474 strongly suppressed Ret(MEN2)-mediated defects

repairing nervous system defects and also to our poor understanding of the biology behind the most common of these diseases. *Drosophila* can help in each of these regards; indeed, some of the first fly models developed for the study of specific human diseases were generated to model neurodegenerative diseases. These include models for Parkinson's disease (PD), Alzheimer's disease (AD) and several triplet-repeat diseases. In humans these late-onset neurodegenerative diseases are often accompanied by the accumulation of aberrant protein deposits: Lewy bodies (PD), insoluble β-amyloid plaques and neurofibrillary tangles (AD), or nuclear inclusions/aggregates of polyQ and other repeat proteins. The relationship between these protein aggregations and neural degeneration is unclear.

24.3.3.1 Parkinson's Disease

Parkinson's disease is characterized by tremors and progressive loss of muscle function owing primarily to loss of dopaminergic neurons within the substantia nigra. At least six genetic loci linked to familial PD in humans have been cloned [24, 95]: *parkin*, *dj-1*, *pink1*, *α-synuclein, leucine rich repeat kinase 2 (LRRK2)*, and *UCH-L1*. In *Drosophila*, mutations in orthologs of many of these loci also result in nervous system defects. Mutations in *pink1* lead to aberrant mitochondrial morphology and function and reduced ATP levels, male sterility, apoptotic muscle degeneration, and disorganized muscle fibers. These mitochondrial and sterility defects are rescued by ectopic expression of the E3 ubiquitin ligase Parkin. Genetic studies have linked Pink/Parkin to JNK signaling and to *glutathione S-transferase S1*, which may function in the cellular response to oxidation. Also linked to oxidative stress were *Drosophila dj-1*. While *Drosophila* does not have a clear α-Synuclein ortholog, expression of human α-Synuclein in flies led to neuronal degeneration and α-Synuclein positive aggregates a phenotype rescued by overexpression of Parkin or feeding the HSP90-associated chemical geldanamycin.

24.3.3.2 Alzheimer's Disease

Insoluble β-amyloid – in Aβ plaques and neurofibrillary tangles – is commonly found in patients with AD, a late-onset disease also characterized by neural degeneration. The Aβ precursor protein (APP) has been conserved in flies and is encoded by the *Appl* locus, but no endogenous Aβ has been detected in flies. However, expression of a human *Aβ* "minigene" in flies led to neural degeneration, and modifier screens using this disease model yielded a gain of function allele of *neprilysin*, which is able to degrade Aβ and suppress its ability to provoke neuronal cell death [26].

Mammalian presenilins function as essential components of the γ-secretase complex that generates Aβ; mutations in the human *PS1* and *PS2* genes are associated with familial AD [51, 77]. Experiments in *Drosophila* indicated that endogenous presinilin has several processing targets including the transmembrane receptor Notch that is cleaved during signaling activation [84, 99], observations confirmed in mammalian systems. This important observation raises the concern that therapeutic strategies designed to target γ-secretase function would adversely affect Notch signaling, a pathway fundamental to the development and maintenance of many tissues. Expression of another component of the AD-associated tangles, the microtubule-associated protein Tau, also led to neurodegeneration (reviewed in [8, 69]. Fly experiments confirmed that Tau is phosphorylated in vivo by the serine-threonine kinases GSK-3β, PAR-1 and cdk5; cdk5 and GSK-3β associate with neurofibrillary tangles in the vertebrate brain.

24.3.3.3 Triplet-Repeat Diseases

Triplet nucleotide repeat diseases are associated with the further expansion of a domain composed of a tandem string of three amino acids. For example, CAG (glutamine) repeats in the *Huntingtin* locus can expand to 39 or more glutamines in HD [39]. These expanded polyQ-containing proteins form aggregates and nuclear inclusions. In *Drosophila* ectopic expression of either polyQ-containing proteins or even a simple expanded polyQ peptide alone leads to extensive and progressive neural degeneration mirroring the fatal loss of neuronal tissue seen in human patients with different PolyQ diseases (Fig. 24.3.4) [69]. Such experiments suggest that neural toxicity may for the most part be attributed to the polyQ repeat itself and that the mechanism of neural atrophy is likely to be common to the family of triple repeat diseases.

The function of wild type Huntingtin protein (Htt) is largely unknown [39]. In vertebrate systems, expanded Htt isoforms have been detected in nuclear aggregates that localize with the transcriptional coactivator CBP, a histone acetyltransferase. This role for mutant polyQ proteins in transcriptional dysregulation has been confirmed in several independent studies in *Drosophila*. Other molecules shown to modify poly-Q repeat peptides in flies include molecules associated with ubiquitination, chaperone proteins, components of phosphatidylinositol 3-kinase/AKT signaling, ataxin1, and the ATPase VCP. This diverse list illustrates the involvement of many cellular components in mediating or regulating the toxicity of polyQ proteins. A clearer perspective of the biology of triplet-repeat disease biology is just beginning to emerge in flies, representing an exciting new tool in the study of these devastating disease syndromes and demonstrating the power of fly genetics to address complex diseases.

24.3.4 Heart Disease

Heart failure is the most common source of death in Western societies. Although diet and stress can lead to cardiac damage, it is also clear that heart function progressively degrades through simple aging. The prevalence of heart disease has sparked increasing interest in the *Drosophila* community. Although these are early days in studies of heart dysfunction in *Drosophila*, some interesting parallels have already been noted. *Drosophila* has a "single-chambered" heart, really a specialized thickening of the endothelium that consists of just two cell types: the contractile myocardial cells are surrounded by pericardial cells (Fig. 24.3.5). The fly has an open circulatory system (no veins or arteries), and its heart serves to promote blood ("hemolymph") circulation.

Despite these basic differences, the early molecules that define the heart region and direct early heart development are markedly conserved. Another similarity with humans is that the adult fly heart shows an increase in spontaneous arrhythmias and progressively poor response to cardiac stress as it ages [93, 94]. Classic genetic approaches demonstrated that these aspects of cardiac aging could be slowed or suppressed by reducing insulin pathway function, including the insulin receptor and the downstream effector Foxo as well as the downstream metabolic pathway regulator target of rapamycin (TOR [53]). The tools for examining issues of cardiac aging and diseases are advancing rapidly, and though the field is young the study of cardiac dysfunction in *Drosophila* should provide important insights in the coming years.

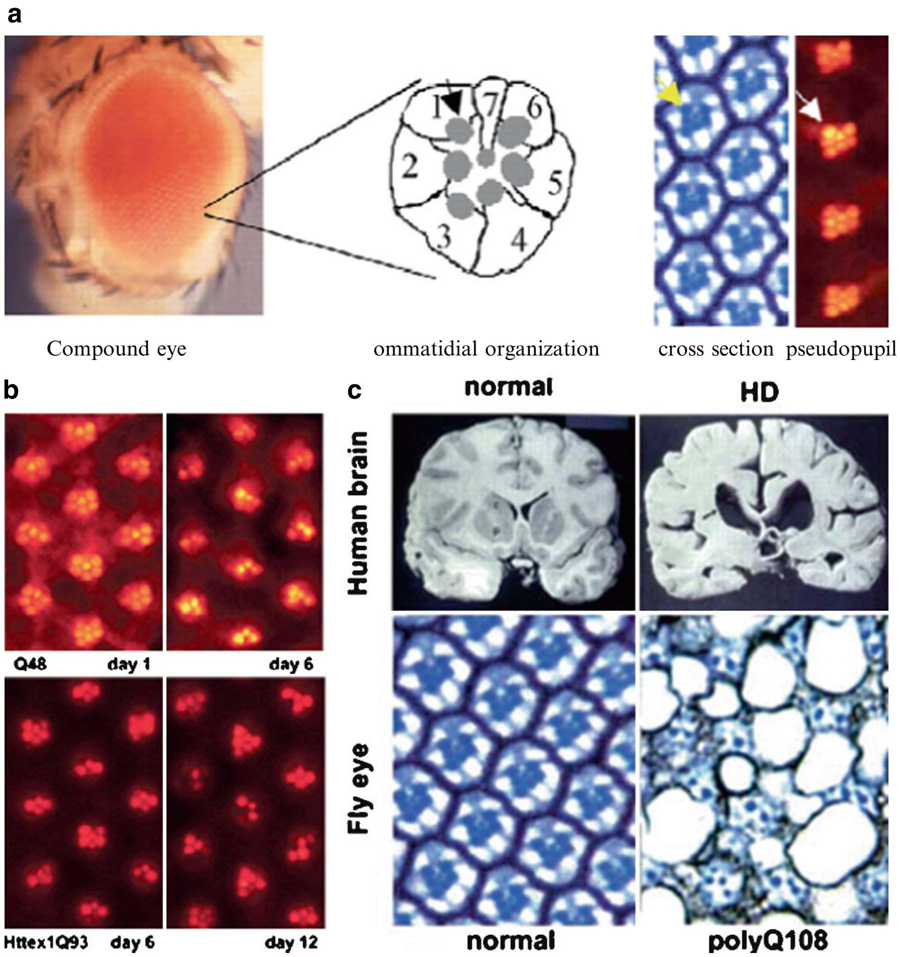

Fig. 24.3.4 (**a**) The Drosophila eye as a model for neurodegeneration. Structure of the adult Drosophila eye, showing the external eye, a diagram of the structure of the photoreceptor cells in an ommatidiuma, a section of the adult eye showing ommatidia in cross-section or using the pseudopupil technique. Adapted from [52a] (**b**) The rhabdomeres of flies at different ages expressing a pure polyQ peptide (Q48) and expressing a mutant exon1 fragment of a human Htt gene with 93Qs (Httex1Q93). Note the rhabdomere constellations get progressively worse. (**c**) Cross-sections through a normal and postmortem HD patient brain demonstrate the dramatic degeneration and loss of neuronal tissue. Cross-sections through the eye of a fly expressing polyQ108 in the photoreceptor neurons show similar significant loss of neuronal tissue

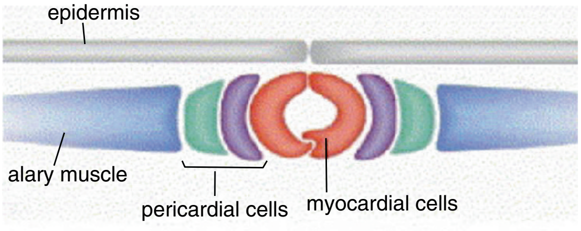

Fig. 24.3.5 (**a**) Drosophila has a single-chamber heart composed of pericardial and myocardial cells. From [5] (**b**) M-Mode traces prepared from high-speed movies of dissected flies with exposed hearts at 1 week (*top*) and 5 weeks of age (*bottom*). Hearts were recorded for 10 s. As in humans, the incidence and severity of arrhythmicity increases with age. From [59]

24.3.5 Diabetes and Metabolic Diseases

Drosophila has made important contributions to the study of cellular and organismal metabolic regulation, and interest in disease-related issues such as diabetes is rising. The majority of advances in *Drosophila* to date have emerged from studies of organ and cell size. As in mammals, the insulin signaling pathway plays the primary role in size regulation. Work primarily in yeast, *C. elegans*, flies, and mammals has outlined a basic pathway (Fig. 24.3.6). With the exception of seven insulin-like peptides (Dilp1-7), *Drosophila* contains a single ortholog for each step in this growth-control cascade. This parsimony has simplified the identification and ordering of pathway components.

24.3.5.1 Body Size

The developing *Drosophila* larva needs to gain sufficient weight to advance to the pupal stage while precisely matching the size of each of its organs to make a viable adult. Figure 24.3.4 demonstrates how starvation leads to smaller body size, but overexpression of *Drosophila* insulin-like peptides (see below) can direct larger body size. Insulin itself appears to achieve this growth regulation through its inhibition of the TSC/TOR pathway, a growth pathway known to respond in flies and mammals to amino acids (Fig. 24.3.6) [17, 30]. This inhibition is mediated through the amino acid transporter Slimfast (Fig. 24.3.6) [30], an effector of insulin signaling provided by the "fat body" that serves as the fly's liver and storage of adipose tissue.

24.3.5.2 Models of Diabetes

In addition to studies of nutrition and aging, *Drosophila* is an emerging model system for the study of diabetes and metabolic syndromes. In *Drosophila*, the functional equivalent to beta cells are 14 "insulin-producing cells" (IPCs), which are neurosecretory cells located in the brain that secrete at least three Dilps into the circulatory system ("aorta") and the corpora cardiaca, the source of the glucagon-related hormone adipokinetic hormone. These Dilps carry out the functions of both insulin and IGF1/2, and ablation of the IPCs leads to an interesting model of type I diabetes characterized by hyperglycemia and small body size [67].

Drosophila metabolic controls may differ in some respects from their mammalian counterparts. In particular, ablation of IPCs leads to severe hypoglycemia and lethality, leading to the suggestion [46] that glucagon plays the more central role in regulating carbohydrate metabolism, with insulin activity involved in glucose tolerance. Similar conclusions have been reached based on pancreatectomy of birds, such as geese [45, 78], but the relative importance of glucagons in mammals is less clear. Further, the sulfonylurea receptor subunits of

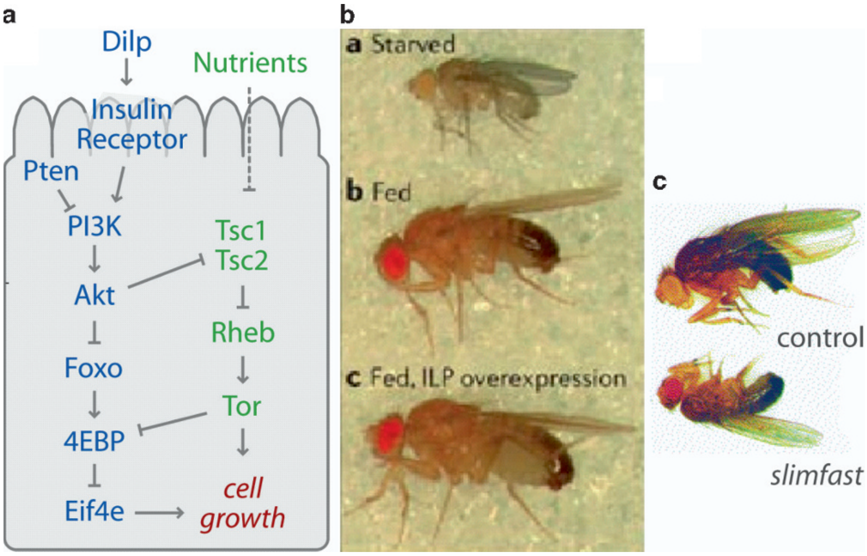

Fig. 24.3.6 (**a–c**) Insulin signaling. (**a**) The insulin and TOR pathways. (**b**) Starvation leads to flies with a smaller body size (*top panel*) compared with controls (*central panel*); overexpression of insulin-like peptides increases body size (*bottom*). (**c**) This reduction in body size is also seen in flies with reduced activity of the amino acid transporter Slimfast

the ATP-sensitive potassium channels – points of insulin regulation in mammalian beta cells – are expressed in the corpora cardiaca in flies [46]. This suggests that glucose acts to regulate itself through glucagon release, although a role has not been ruled out for the IPCs as well. Furthermore, cross-talk between the glucagon and insulin pathways can complicate interpretations of experiments in flies, birds, and possibly mammals.

24.3.6 Addiction

Addiction is a disease of enormous societal and economic cost. Many still equate addiction with a lack of willpower or a weakness of character; addiction research in model organisms such as flies plays a central role not only in elucidating the physiological mechanisms that underlie addiction, but also in overturning such attitudes. *Drosophila* has been used to investigate addiction to alcohol, cocaine, and nicotine [65, 96]. The effects that these drugs have in flies, rodent models and humans are remarkably similar.

In humans, exposure to low doses of alcohol leads to euphoria and loss of inhibition. Increasing doses result in decreased coordination, confusion, and sedation. Similarly, when exposed to ethanol vapor *Drosophila* become hyperactive, then lose coordination and finally become sedated. This behavior translates into a decreased ability of flies to balance on a slanted surface and is used in the design of the somewhat amusingly named "inebriometer" test chamber (Fig. 24.3.7a). The "crackometer" Fig. 24.3.7b) is used to expose flies to free-base cocaine; low doses lead to hyperactivity, moderate doses cause hypokinesis and stereotypic locomotion, and higher doses lead to spasmodic activity, tremors, and finally complete loss of movement (Fig. 24.3.7c). Exposure to volatilized nicotine produces similar hyperactivity, hypokinesis, or akinesia.

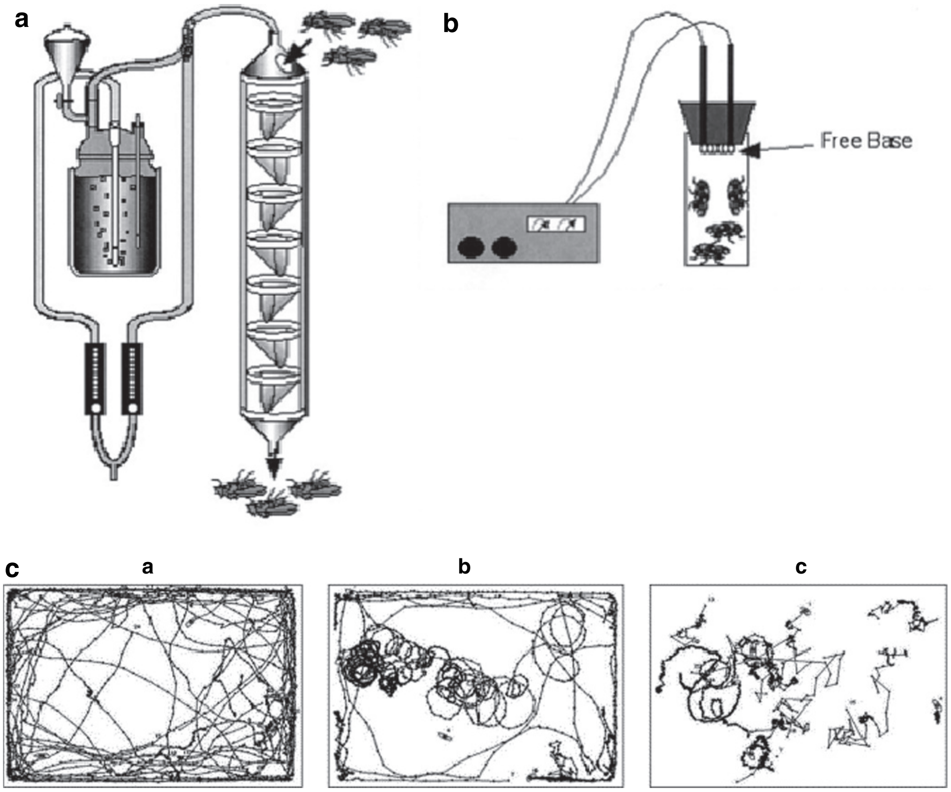

Fig. 24.3.7 (**a–c**) Assaying intoxication in flies. (**a**) The inebriometer. Ethanol vapor is blown on flies at the entrance to the funnel; the time required for flies to fall through the baffles is then assessed. (**b**) The crackometer. Cocaine is volatized and administered to flies. (**c**) Computer-generated traces of the locomotor behavior of a group of five flies exposed to volatilized free-base cocaine. Each panel corresponds to a 1-min period starting 2 min after the end of the cocaine exposure. Ca Mock exposure; Cb exposure to 100 µg of cocaine; Cc exposure to 200 μg of cocaine. Flies have become hyperactive but unresponsive. Adapted from [65, 96]

24.3.6.1 The Genetics of Addiction: Sensitivity and Tolerance

Increasingly sophisticated behavioral tests are being developed to assess the complex movements and behaviors of flies to addictive substances. With these assays, Drosophila provided the first in vivo evidence that cAMP signaling mediates the body's response to ethanol. Mediators involved include the neuropeptide amnesiac (christened "Cheapdate") that acts through adenylate cyclase to increase cAMP levels, adenylate cyclase (Rutabaga), and protein kinase A (PKA). Flies were similarly used to confirm the role of dopamine and serotonin in intoxication responses and, recently, to demonstrate a role for Rho-type GTPases in regulating sensitivity to alcohol intoxication as well as to nicotine and cocaine [66]. These data support a model in which drugs such as alcohol directly affect organization of the cytoskeleton, a structure known to be regulated by small GTPases [32].

Tolerance to narcotics and alcohol is an additional factor contributing to the potency and development of addiction. In flies, adaptation is evident after just one acute exposure to ethanol, termed rapid tolerance [3, 72, 89]. By contrast, prolonged or repeated exposure to small doses of ethanol leads to chronic tolerance: higher levels of alcohol are required to cause intoxication on each consecutive exposure, a hallmark of neuronal habituation. Targeting mutants to particular brain areas permitted mapping of the development of tolerance to a set of neurons within the fly brain's "central complex." Octopamine, the *Drosophila* noradrenaline analog, mediated this tolerance, demonstrating a role similar to noradrenaline in mice. Tolerance also required the nuclear zinc-finger protein *hangover* [73] that mediates a cellular stress response induced on exposure to ethanol. In each case, the majority of mutant fly lines abnormal for ethanol sensitivity or tolerance do not display changes in the rate at which alcohol is absorbed or metabolized [64], indicating that, for the most part, ethanol sensitivity and tolerance are separable from ethanol metabolism.

24.3.7 Sleep Disorders

Though a third of our lifetime is spent in sleep, we understand astonishingly little about why we sleep or what the function of sleep is at the cellular level. Sleep appears to be a common feature of all brain-endowed animals, and flies are proving a useful model system (for reviews see, e.g., [16, 36, 75]). Several drugs that reduce the requirement for sleep in humans, such as caffeine, modafinil, metamphetamine, and antihistamines, also affect the sleep patterns of *Drosophila.* Increasing the release of dopamine and inhibiting its re-uptake stimulates arousal in both humans and flies: for example, feeding flies metamphetamine reduced the frequency and duration of sleep; inhibiting dopamine synthesis led to narcoleptic-like behavior [1]. This is mediated through the voltage-dependent potassium channel Shaker and the dopamine transporter Fumin. Similar to us, sleep-deprived flies tend to sleep more the next day and show reduced performance in several behavior tests. Furthermore, complete sleep deprivation is fatal in flies (which die within 60 h), rats (which survive for 2–3 weeks without sleep), and humans (patients with fatal familial insomnia die 7–36 months after they first present with symptoms) [52, 76]. As we age our need for sleep is reduced and sleep becomes increasingly fragmented, and aging flies display these same sleep pattern changes [48].

Recent work in flies has provided intriguing insights into sleep disruption. The Drosophila Activity Monitoring System (DAMS) instrument enables researchers to simultaneously monitor the activity and sleep patterns of a large number of individual flies; activity is monitored by an infrared light beam bisecting the tube. Devices such as DAMS have allowed researchers to demonstrate roles for homeostatic and circadian mechanisms in regulating sleep in flies. For example, when fed a low dose of the free-radical generator paraquat, aging flies displayed an enhancement of sleep disruption, suggesting a role for oxidative stress in sleep fragmentation; similar effects have been observed in rats [48]. Other factors include serotonin, PKA and cAMP and, interestingly, diet [81].

Finally, recent *Drosophila* work suggests a method to detect biomarkers of sleepiness in humans [74]: researchers found that the levels of amylase mRNA isolated from whole fly heads increased with progressive sleep deprivation, and went on to show an analogous increase in amylase mRNA levels in saliva from sleep-deprived human subjects.

24.3.8 Conclusions

Drosophila offers a host of advantages for studying human disease. A century of study has yielded a dizzying array of tools, giving the fly field perhaps the most powerful, most sophisticated toolset currently available. Although initially focused on development, the shift to human disease reflects both new demands by the public and the natural maturation of the fly field. Once considered pioneers and even outsiders, fly researchers who focus on disease are now considered an important part of the future of fly research. As with every book chapter, some of the data presented here are no doubt out of date, as fly researchers tend to move quickly. Despite their myriad advantages, perhaps the true future of *Drosophila* lies in close cooperation with researchers in mammalian fields. By informing the *Drosophila* community of the details of human diseases, fly researchers can in turn focus on creating more sophisticated models. This is particularly true of polygenic diseases, in which *Drosophila* offers one of the best opportunities to translate the accumulation of complex human mutation data into useful models by altering multiple genes and signaling networks in a manner that few other complex model organisms can match.

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Human Genetics and the Canine System 24.4

Heidi G. Parker and Elaine A. Ostrander

Abstract With constant advances in canine genomics, the dog has found a permanent position as a source of genetic information for the inheritance of morphologic traits and disease susceptibility. The modern domestic dog is not a typical model organism. They share our environment, our life-styles and often our food. In addition, they experience many of the same diseases that people do and are diagnosed and treated using the same medical procedures and pharmaceuticals. However, unlike humans, the purebred dog maintains a highly structured population organization that, if used correctly, can simplify the genetics of complex traits and disorders. In this chapter, we will discuss the history of canine genomics along with recent advances in resource development. Specific examples will be provided to demonstrate strategies for using population stratification to the best advantage in mapping traits both simple and complex. Together, these data highlight the utility of the canine system for mapping traits and finding mutations important in both human and companion animal science.

Contents

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Abbreviations

AKC	American kennel club
BHD	Birt–Hogg–Dube syndrome
DLA	Dog leukocyte antigen
Gb	Gigabases
HLA	Human leukocyte antigen
IBD	Identical by descent
IDID	Inherited diseases in dogs
LD	Linkage disequilibrium
Mb	Mega bases
OMIM	Online mendelian inheritance in man
QTL	Quantitative trait loci (locus)
PC	Principle component
PCA	Principle component analysis
PME	Progressive recessive myoclonic epilepsy
PRA	Progressive retinal atrophy
PWD	Portuguese Water dog
rcd(1,2)	Rod-cone dystrophy
RCND	Canine hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis
SINE	Short interspersed nuclear element
SINEC_Cf	Canine-specific short interspersed nuclear element
SNP	Single nucleotide polymorphism

24.4.1 Introduction to the Canine System

Human genetics has found a new partner in an old friend, the domestic dog. While rodent systems are, for the time being, the mainstay of comparative genomics, the sequencing of several mammalian genomes, including the canine genome, offers human geneticists a new set of resources for mapping traits, identifying functional elements, and understanding chromosome structure and evolution. Indeed, as geneticists increasingly exploit the principles of comparative genomics, we can expect the dog to play a significant role in mapping loci for disease susceptibility, morphology, and behavior.

In this chapter, the utility of the canine system for mapping traits and finding mutations important in both human and companion animal science will be discussed. We will describe the population structure of the dog and how it relates to canine genetics along with recent advances in resource development. Moreover, specific examples will be provided to demonstrate the benefits of mapping within and across dog breeds. Finally, the genetics of complex traits such as morphology and behavior will be discussed. In aggregate, the ideas and data presented here support the use of the canine system and the properties of comparative genomics for understanding the genetic basis of traits of interest to mammalian biologists.

24.4.1.1 *Origins of the Domestic Dog*

The domestic dog is the most recently evolved species in the family Canidae, whose collective history spans nearly 50 million years. Dogs are believed to have evolved from wolves about 40,000 years ago, with the initial site of domestication still under debate [25, 64, 74].

24.4.1.2 *Dog Breeds*

There are over 400 breeds of dog in the world, about 155 of which are recognized by the American Kennel Club (AKC) [3]. Distinct breeds differ by as much as 100-fold in mass and display amazing levels of morphologic variation, as evidenced by differences in overall body size, skull shapes, leg lengths, and more (Fig. 24.4.1). In fact, the diversity in skeletal size and proportion between dog breeds is greater than that observed within any other terrestrial mammalian species [75, 76]. Equally amazing are the behaviors that characterize specific breeds, such as herding, tracking, retrieving, and guarding.

24.4.1.3 *Variation Between Breeds*

Because of their unique population structure, domestic dogs are ideal for genetic mapping studies. In a study of 85 dog breeds, Parker et al. showed that while humans and dogs have similar levels of overall nucleotide diversity, i.e., 8×10^{-4} nucleotide substitutions per basepair per generation, the amount of total variation

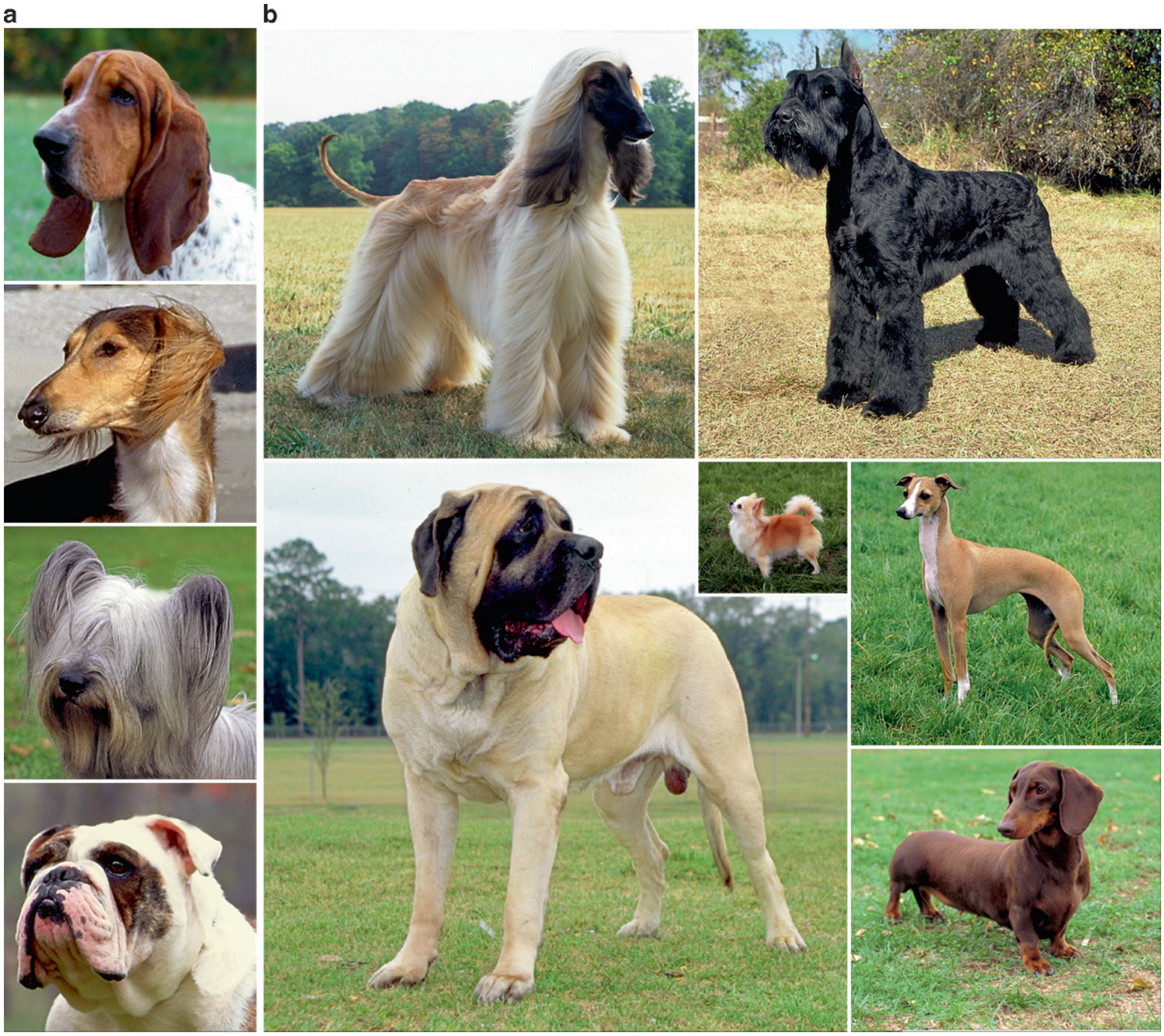

Fig. 24.4.1 Morphologic variation found in AKC recognized dog breeds. (**a**) Examples of gross morphology in skull shape, ear size and carriage, and coat type and patterning. Breeds from *top to bottom*: Basset Hound, Saluki, Skye Terrier, Bulldog. (**b**) Examples of variation in overall body size, coat-type, leg length and proportion. Breeds *clockwise from top-left*: Afghan Hound, Giant Schnauzer, Italian Greyhound, Standard Dachshund, Mastiff, and Chihuahua (*center*) Photographs by Mary Bloom, copyright AKC

that is accounted for by differences between the breeds is much greater than that associated with differences between human populations (27.5% vs. 5.4%, respectively) [50]. Conversely, the degree of genetic variation found within individual dog breeds is much lower than that observed within distinct human populations. For a dog to be a member of a dog breed, therefore, is a more meaningful genetic distinction than for a human to be associated with a particular nationality.

24.4.1.4 Lack of Variation Within Breeds

Dogs are valued and judged in the show ring by the closeness to which each approximates their own breed standard [3]. To be a registered member of a breed, both of a dog's parents must have been documented members of the same breed. Each dog breed effectively represents a closed breeding population defined by small numbers of founders, population bottlenecks, and restricted breeding programs. Owing to the competitive nature of dog shows, sires that perform well in exhibition are bred repeatedly and may contribute excessively to later generations, creating bottlenecks that further reduce genetic variability [50, 51].

The level of intrabreed homozygosity and interbreed heterozygosity is sufficient to allow genetic distinction between breeds based on analysis of small numbers of genetic markers [31, 45]. For example, in the Parker et al. study, data from 96 microsatellite markers spanning all dog autosomes at approximately a 30 MB resolution were tested on 414 dogs to determine the degree to which dogs could be assigned to their breed based solely on genetic data [50]. Using an unsupervised clustering analysis, 95% of the dogs could be correctly assigned to either a single breed or a pair of closely related breeds, such as the Belgian Sheepdog/Belgian Tervuren pairing. Additionally, using the same data set, 99% of the dogs were assigned to the correct breed group by calculating the highest probability of an individual's genotype fitting any of the populations, in a leave-one-out analysis. As a result, the connotation of a "breed" is meaningful at not only the phenotypic level but the genetic level as well.

24.4.1.5 Benefits of Mapping in a Breed-Based System

These results make two functional predictions. First, dog breeds should display not only specific morphologic and behavior traits, but specific disease susceptibilities as well. Within any single breed the number of genes responsible for a trait is apt to be small, reflecting the lack of genetic variation within a breed, which stems from a small number of founders and the ensuing bottlenecks. Thus, mapping disease genes of interest within dog breeds may be a way to simplify the locus heterogeneity that plagues the mapping of many complex human traits. Second, since all dogs share recent common ancestors, the genetic signature of founders used to create related breeds is likely still to exist in the form of common ancestral disease mutations. Thus, one way to move from linked marker to gene may be to take advantage of such common variants. The study of disease susceptibility both within and between breeds supports these hypotheses.

24.4.2 Navigating the Canine Genome

24.4.2.1 Maps

In a few short years the canine mapping community has advanced from the first meiotic linkage map of the dog, composed of 150 markers divided into 30 linkage groups [46] to the development of whole-genome radiation hybrid maps [27], preliminary comparative maps [63], integrated linkage and RH maps [7], and finally, a detailed comparative RH map composed of 10,000 canine gene sequences [30]. These maps provided the foundation for all canine genetics studies, including the much anticipated whole-genome sequencing of the domestic dog.

24.4.2.2 Sequence

The first high-quality draft sequence of the dog, derived from a female boxer, was published in 2005 and comprised over 2.4 Gb estimated to cover ~99% of the euchromatic genome [41]. The sequence was assembled from 31.5 million sequence reads, which was sufficient to cover the genome 7.5 times. The gene count was listed as ~19,000, nearly all of which are orthologs of known human genes. This number is slightly lower than that reported for humans (~22,000 genes), a difference that is likely explained by the frequent occurrence of splice variants and pseudogenes and in some cases, species specific gene gains and losses [21]. The completed canine genome sequence has a critical role in advancing our knowledge of canine disease susceptibility, as discussed in Sect. 24.4.4.

24.4.3 Canine Disease Gene Studies

The domestic dog is second only to human in the number of recorded naturally occurring genetic disorders [4, 53]. Indeed, scientists have appreciated the importance of genetic predisposition in canine diseases for several years [53-55], but only recently have the resources become available to map diseases of interest and identify the underlying DNA variants [30, 36, 41]. Even more recent have been the advances made in studies aimed at identifying genes which regulate truly complex genetic traits, such as morphologic variation [10, 11, 69] and behavior [28, 29].

Careful phenotyping from the veterinary medical community combined with available genetic resources has resulted in the successful mapping of many canine disease genes and, in some cases, identification of the underlying variant (reviewed in [49, 51, 68, 70]). Specific examples include metabolic and endocrine disorders [14, 72], diseases related to the digestive system [16, 60], blindness [1, 26, 65], cancer [43] neurologic disorders [40, 42, 44], disease of the skin and muscle [15], and skeletal and developmental disorders [12, 13, 71]. The largest and most complete listing of documented canine inherited diseases is the Inherited Disease in Dogs database (IDID) [62], which is modeled on OMIM (Online Mendelian Inheritance in Man).

24.4.3.1 Canine Disease Mirrors Human Disease

The above studies demonstrate several principles of interest to human and companion animal scientists. Most studies have focused on diseases that are important in both canine and human medicine and in doing so have expanded our knowledge of the underlying biology of the disease. The most frequently cited example remains the work of Lin et al., who examined a family of Doberman Pinschers in order to show that the sleep disorder, narcolepsy, is caused by the insertion of a short interspersed nuclear element (SINE) in the *hypocretin 2 receptor* gene, resulting in a splicing defect [40]. While inherited narcolepsy is rare in both humans and dogs, the identification of a new class of molecules controlling sleep patterns suggested novel directions for the study of common sleep disorders, such as several forms of insomnia.

Another excellent set of studies are those of progressive retinal atrophy (PRA) in the dog. PRA is a collective term referring to a group of ocular conditions similar to retinitis pigmentosa in humans (reviewed in [57]). Different forms of PRA predominate in different dog breeds. For a number of canine retinal disorders, causative mutations have been found in genes that were previously implicated in clinically similar human diseases (Table 24.4.1). Examination of the disorders as they arise in individual breeds is akin to reading a flowchart of gene pathways and interactions important in eye development. For example, rod-cone dysplasia (rcd) is an early onset form of PRA observed in Irish setters and Cardigan Welsh corgis [58, 66]. In the Irish setter the disease (rcd1) is caused by a mutation in the *PDE6B* gene which encodes the beta subunit of cyclic GMP phosphodiesterase [66]. A similar disease in the corgi, rcd3, is caused by a null mutation in *PDE6A*, the gene encoding the alpha subunit of cyclic GMP phosphodiesterase [58]. Both of these mutations inhibit the enzymatic function of the phosphodiesterase, leading to death of the rod and

Table 24.4.1 Canine retinal disorders

Breed	Canine disease	Human disease	Gene	Reference
Siberian Husky	X-Linked progressive retinal atrophy	X-Linked retinitis pigmentosa	RPGR	[79]
Mastiff	Dominant progressive retinal atrophy	Retinitis pigmentosa 4, autosomal dominant	RHO	[35]
Alaskan Malamute	Cone degeneration	Achromatopsia	CNGB3	[65]
German Shorthaired Pointer	Cone degeneration	Achromatopsia	CNGB3	[65]
Multiple breeds [a]	Progressive rod-cone degeneration	Autosomal recessive retinitis pigmentosa	PRCD[b]	[78]
Irish setter	Rod-cone dysplasia 1	Autosomal recessive retinitis pigmentosa	PDE6B	[66]
Sloughi	Rod-cone dysplasia 1	Autosomal recessive retinitis pigmentosa	PDE6B	[20]
Cardigan Welsh corgi	Rod-cone dysplasia 3	Autosomal recessive retinitis pigmentosa	PDE6A	[58]
Briard	Retinal dystrophy	Leber's congenital amaurosis type-2	RPE65	[2]

[a] Poodle, Cocker spaniel, Labrador retriever, Portuguese water dog, Chesapeake Bay retriever, Nova Scotia duck tolling retriever, and Australian cattle dog all carry the same mutation

[b] Novel gene

cone cells in the retina [57]. In human RP families, mutations in *PDE6A* and *PDE6B* are predicted to account for ~8% of disease [23]. While the phosphodiesterase family of genes had been associated with retinal disorders in humans before they were identified in the dog, another form of rcd (rcd2), has recently been mapped in the collie to a gene of unknown function that is orthologous to human and mouse rd3 [38, 39]. Analysis of splice variations and expression of this gene in the dog may reveal a unique step in the pathway leading to photoreceptor death.

24.4.3.2 Canine Disease and Mechanisms of Mutation

24.4.3.2.1 SINE Insertions

In addition to facilitating our understanding of human disease, canine studies can reveal unique molecular mechanisms involved in genetic disease. Canine narcolepsy was caused by insertion of a canine-specific short interspersed nuclear element (SINEC_Cf) [6, 47, 73]. These elements are retrotransposons derived from a tRNA-Lys that occur frequently throughout the canine genome [6, 18, 36]. As with *Alu* repeats in the human genome, SINE elements are often located in positions affecting gene expression. Other examples include the aberrant insertion of the SINEC_Cf element into the canine *PTPLA* gene, which has been found to cause centronuclear myopathy in the Labrador retriever by causing splicing errors during maturation of the mRNA [56]. In addition, merle coat patterning in several breeds is the result of a SINE element insertion into the *SILV* gene, which plays a part in the biogenesis of premelanosomes, the precursors of pigment organelles [17].

24.4.3.2.2 Simple Repeats

Other diseases have been found to be associated with unique mutagenic events in the dog. For example, Lohi et al. reported that the cause of progressive recessive myoclonic epilepsy (PME) in miniature wirehaired dachshunds was the expansion of a canid-specific dodecamer repeat in the *Epm2b* (*Nhlrc1*) gene [44]. While trinucleotide repeat expansion has been reported in association with several human disorders (reviewed in: [8, 24]), this is the first report of a dodecamer repeat expansion causing a disease in any species. PME is common in multiple dog breeds. An affected basset hound was analyzed, and a shorter repeat expansion mutation was found at the same locus, showing that this mutation is not a single-breed anomaly. This poses an interesting question: will this type of mutation cause disease in humans?

24.4.3.2.3 Single Base Mutations

Finally, the ability to sample multiple generations within canine families has allowed more progress in understanding the role of missense changes in disease susceptibility than has been possible in humans. For instance, canine hereditary multifocal renal cystadenocarcinoma and nodular dermatofibrosis (RCND) is a naturally occurring autosomal dominant form of cancer characterized by bilateral tumors in the kidney and numerous collagen nodules in the skin [32]. This is found exclusively in German shepherd dogs and was mapped to CFA5 in 2000 (Fig. 24.4.2) [32]. Shortly after publication of the canine linkage mapping result, a similar disease in humans, Birt-Hogg-Dube syndrome (BHD), was mapped to chromosome 17q22.1 [34]. Because RCND localized to a portion of CFA5 that corresponded to 17q22.1 it was likely that both the canine and human diseases were due to mutations in the same gene. Indeed, this proved to be the case [43]. The gene implicated in both diseases, *BHD,* encodes a protein called folliculin. Protein-truncating mutations in this gene account for disease in approximately one third of BHD families [48]. However, in the dog, the disease is caused by a single base change in exon 7, creating a conservative missense change, H255R [43]. While it is often difficult to unambiguously determine whether a given missense change is truly disease associated, in this case three lines of evidence support this conclusion [43]. First, only RCND-affected dogs, regardless of country of origin, carried the H255R mutation, while all unaffected German shepherds and dogs of other breeds lacked the variant. Second, a multiple alignment of the folliculin protein sequence in 13 divergent species showed that the histidine !!! residue was completely conserved from dog and human through yeast. Finally, test matings demonstrated that the mutation is embryonic lethal in homozygotes,

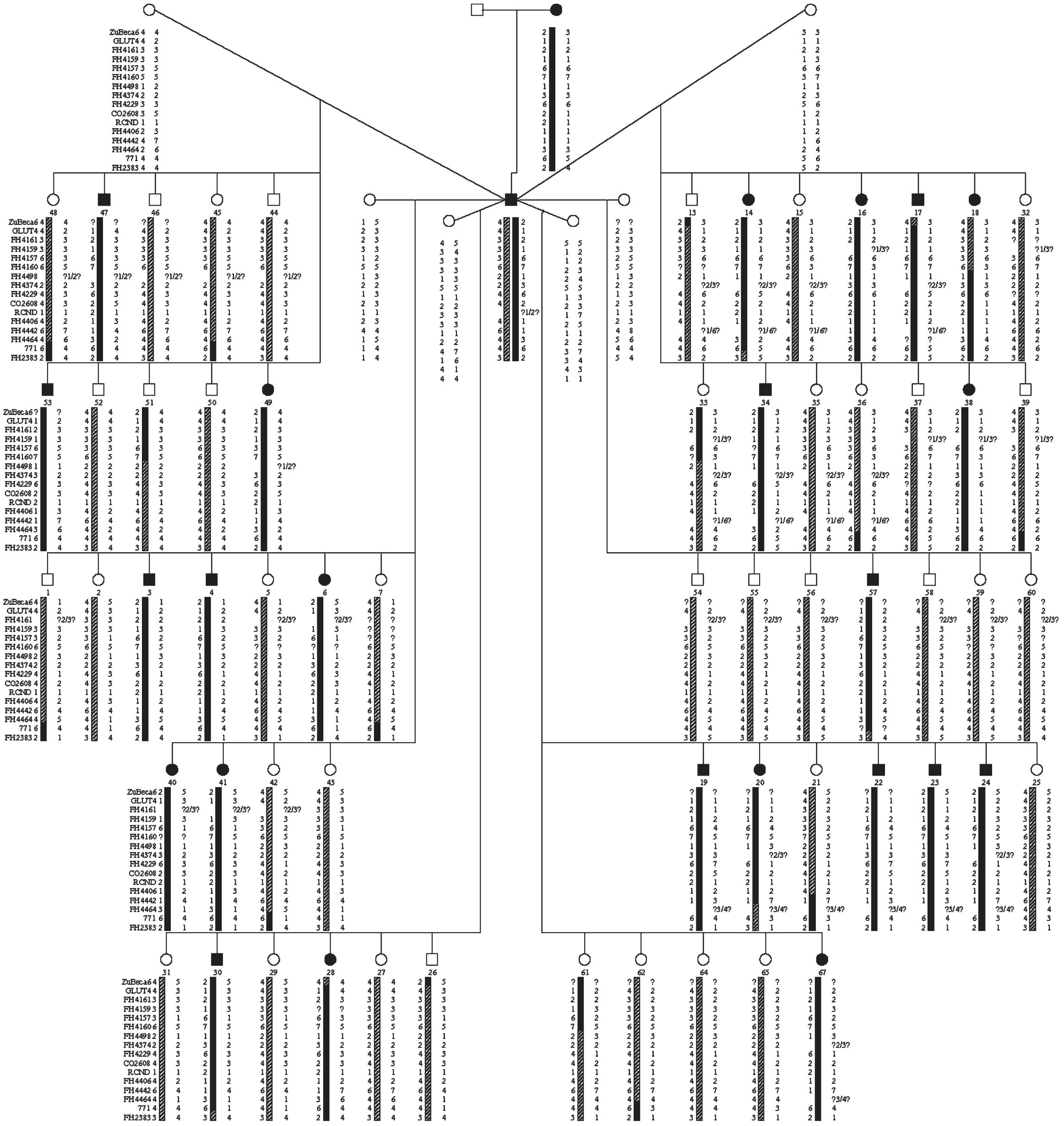

Fig. 24.4.2 The canine pedigree segregating RCND. Affected dogs are represented with black shading and unaffected dogs are unshaded. Marker names are indicated to the left of each row of genotypes. The genotypes of all markers are shown, but the vertical bar representing the haplotypes in the offspring is only shown for the affected proband's side. For the RCND locus, a '1' indicates the wild-type allele (unaffected) and a '2' indicates the mutant allele (affected). (Reprinted from [43], by permission of Oxford University Press)

implying a critical loss of function in the mutant protein. Thus, while it has been nearly impossible to draw conclusions on missense changes in folliculin from studies of humans, it is clear that a single ancestral variant causing the canine disease informs us about a key regulatory portion of the protein.

24.4.4 Genome Structure in the Domestic Dog

While the aforementioned examples (Sect. 24.4.3) illustrate many advantages of the canine system, the future promise of the dog as a genetic system lies in its application to complex traits. Successful mapping of polygenic traits in the dog requires an understanding of linkage disequilibrium (LD) [31, 41, 45, 67]. LD refers to the nonrandom association of two or more usually adjacent loci segregating together through several generations. Two major studies have undertaken the task of describing the extent of LD in the dog [41, 67].

24.4.4.1 Linkage Disequilibrium

In 2004 Sutter et al. examined the extent of LD in five breeds: the akita, Labrador retriever, Bernese Mountain dog, golden retriever, and Pekingese. Five genomic regions were examined and the results averaged. Both this study and the subsequent study of Lindblad-Toh et al., in which ten breeds were examined at ten distinct loci, found that the average length of LD in the dog exceeded 2 Mb [41, 67]. This is 40–100 times greater than the extent of the range of LD found in the human genome [19]. Significant variation was also noted between breeds. Those with long population bottlenecks or small numbers of founders displayed a longer range of LD (e.g., the Irish wolfhound) than did breeds associated with more founders and greater popularity (e.g., the labrador retriever). As we learn more about the canine genome this conclusion will likely bear some modification. We already know that LD varies enormously across the genome, and some of the large differences seen between breeds may reflect the small number of genomic regions examined [41].

24.4.4.2 Haplotype Structure

In addition to LD measurements, both studies looked at haplotypes within and across breeds and found that haplotype diversity was low, while haplotype sharing was high. The data presented by Sutter et al. show that at any given locus, approximately 60% of the chromosomes in each breed carried common haplotypes and 30% carried haplotypes found in every breed studied [67]. Examination of the assembled DNA sequence data from 7.5× whole-genome assembly provides additional insights. Comparison of the two boxer haplotypes, as well as the resequencing and genotyping of nine additional breeds, demonstrated mega-base-sized portions of the genome that are alternatively homozygous and heterozygous. Detailed experiments using 24 dogs from each of ten breeds support these data, suggesting that megabase-sized haplotypes will be common in many dog breeds [35].

The implications of these findings are two-fold. While a typical whole-genome association study in humans may require up to 500,000 SNPs [37], extensive LD suggests that only 10,000-30,000 SNPs will be required for the same study in dogs. In addition, the haplotype results suggest that a single set of SNPs will likely be informative for mapping studies in all breeds and that individual SNP marker sets or SNP chips do not need to be developed for individual breeds or groups of breeds. These facts, coupled with all of the benefits of mapping in the dog discussed in Sect. 24.4.1.4, suggest that for diseases prevalent in both humans and dogs, such as cancer, deafness, blindness, heart disease, epilepsy, cataracts, and motor neuron disorders, it is far easier to conduct initial mapping studies in dogs than in humans.

24.4.4.3 Single Nucleotide Polymorphisms

As a result of the canine sequencing effort approximately 2.1 million single nucleotide polymorphisms (SNPs) have been identified [41] and SNP chips for whole genome analysis have been released (Affymetrix, Santa Clara, CA; Illumina, San Diego, CA). The Affymetrix product supplies approximately 45,000 verified SNPs that are reproducible in multiple dog breeds while the Illumina product comprises approximately 20,000. This density, approximately one SNP every 100 kb, is more than sufficient to cover the range of LD expected within any domestic dog breed, sounds not logical to me in a variety of recent genome wide association studies aimed at finding the genes responsible for both morphologic traits and diseases [5, 22, 33, 61, 77].

24.4.5 Population Structure in the Domestic Dog

24.4.5.1 Canine Breed Clusters Facilitate Mapping Efforts

With the advances described above, the challenge now is to identify methods for mapping disease genes that make full use of the canine population structure as a mechanism for simplifying the mapping process. As an extension of the study described previously (Sect. 24.4.1.2), Parker et al. used microsatellite allele patterns to determine the ancestral relationships between various dog breeds [50]. Using the same data from 96 microsatellite markers analyzed on five unrelated dogs out of 85, breeds they performed an unsupervised clustering analysis using the computer program *structure* [59]. The 85 breeds were ordered into four clusters based on similar patterns of alleles, presumably from a shared ancestral pool. Asian and African breeds grouped together with the gray wolves in what is believed to be the ancient breed cluster. The mastiff-type working dogs formed a distinct cluster. A subset of sighthounds, primarily of European development, grouped with herding dogs of the same regions, and hunting dogs such as hounds, gun dogs, and terriers formed the last cluster (Fig. 24.4.3).

24.4.5.2 Combining Breeds to Improve Mapping

The Parker clusters offered a first look at relationships between breeds that did not rely on historical lore and pedigree records [50]. It is presumed that dog breeds from the same cluster share common ancestors and are therefore more likely to share traits that are identical by descent (IBD). These data, coupled with the findings of the haplotype studies (Sect. 24.4.4.2) suggest a study design for mapping traits that involves multiple breeds [41, 67]. Extensive LD will allow for quick identification of disease-associated regions of the genome within a single breed. However, the extensive LD means that the initial mapping segments will be large, on the order of megabases. Causative mutations will therefore be most easily identified by comparison of haplotypes in affected individuals from multiple breeds [26]. Furthermore, choosing affected dogs from breeds within a single Parker cluster will increase the chance that the breeds in question share an ancestral mutation and improve the likelihood for successful fine mapping [52].

24.4.5.3 Homozygosity and Population Bottlenecks

Analysis of haplotype sharing in the canine genome sequence (Sect. 24.4.4.2) revealed two major bottlenecks in the history of all breeds, a detail that is critical

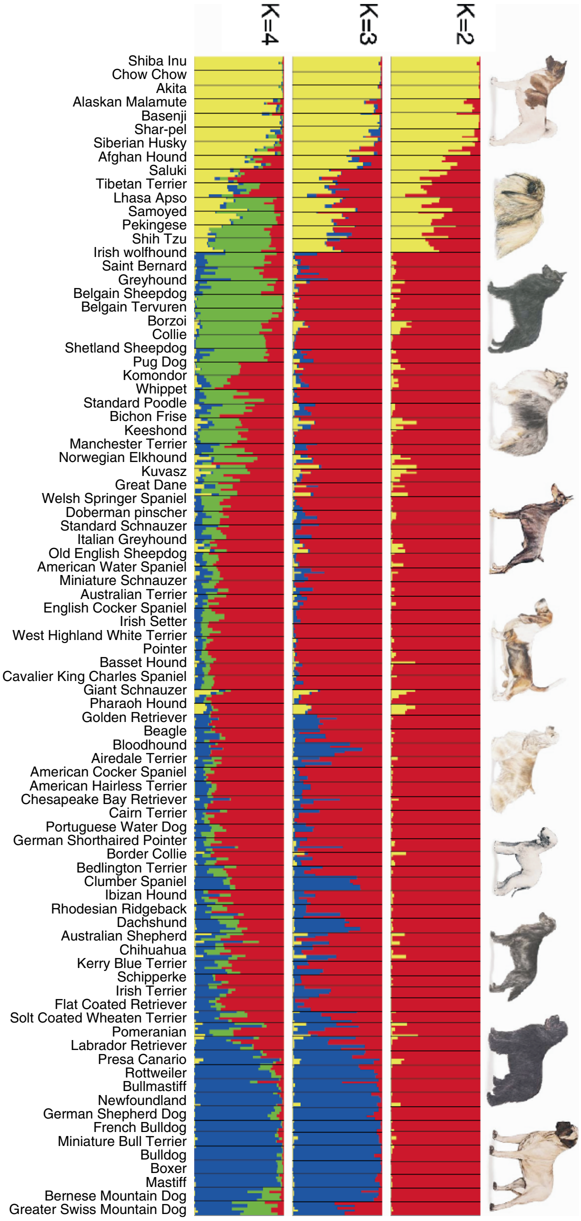

Fig. 24.4.3 Population structure of 85 domestic dog breeds. Each individual dog is represented by a single vertical line divided into K colors where K is the number of clusters assumed. Each color represents one cluster, and the length of the colored segment shows the individual's estimated proportion of membership in that cluster. Thin black lines separate the breeds that are labeled below the figure. Pictures of dogs from representative breeds for each grouping are shown at the top. Results shown are averages over 15 *structure* runs at each value of K. (Reprinted, with permission, from [50], [©AAAS])

for experimental design [41]. The first bottleneck is ancient and is presumed to have occurred at the time dogs were domesticated from wolves. The associated short, ancient haplotypes are common to all breeds. The second bottleneck is specific to the individual breed, and likely occurred during selection for formation of the breed. The haplotypes associated with this event are much longer and often comprise multiple ancient haplotypes. These findings serve as a reminder that all dogs, regardless of modern appearances and breed membership, are related, and the distance of the relationship will be inversely proportional to the length of the haplotypes shared between them.

24.4.6 Mapping Multigenic Traits in the Dog

24.4.6.1 Quantitative Trait Loci

In considering problems to which to apply the above advances in canine genomics, none is more challenging then those aimed at understanding the genetics of canine morphology. Domestic dog breeds differ more than five-fold in height alone and vary far more in over all body mass and appearance (see Sect. 24.4.1.2 and Fig. 24.4.1) [3]. Understanding how members of the same species can tolerate that level of morphologic variation is one of the most interesting questions in the field. Towards this end, studies of quantitative traits in the dog have been initiated that are based on methods developed for studies of inbred plant strains [10].

Quantitative traits are phenotypes controlled by many genes acting in concert not "working in concert". Because each gene contributes only fractionally to the trait, genomic variability in the mapping populations increases the challenge of finding causative genes. The ideal population for quantitative trait loci (QTL) mapping is one in which the phenotypes under question show high levels of variation. Additionally, the population as a whole should ideally derive from a small number of founders, which maximizes mapping power by reducing the number of haplotypes associated with any trait. The Portuguese water dog (PWD) was chosen as the primary focus of the morphology study because it meets the above criteria [11].

24.4.6.2 Establishing a Cohort

In order to identify QTLs for morphologic traits in the dog, investigators at the University of Utah established a cohort of more than several hundred AKC-registered PWDs [11]. DNA samples, X-rays, pedigrees, and detailed health data were collected on each dog. Ninety measurements derived from the X-rays were subjected to principal component analysis (PCA), which groups individual phenotypes into correlated traits. Nine heritable principle components (PCs) relating to overall body size and proportion of the skeleton were identified. The four major PCs are described in Table 24.4.2. Follow-up analysis of the postcranial skeleton has revealed additional PCs related to the trade-off between speed and strength in the canine physique [9]. A genome scan of ~500 microsatellite markers was completed on all dogs and several QTLs were found to be associated with the majority of PCs. Based on these studies, a single gene locus around *insulin-like growth factor 1 (Igf1)* has been identified that is a major contributor to small size in at least 14 diverse small dog breeds [69]. Additional studies are currently under way to find the genes

Table 24.4.2 Top four principle components (PCs) identified from skeletal measurements accounting for 61% of variation [11].

PC	Description	% of total variation	Heritability, %	Heritable variation, %
1	Overall size of skeleton	43.6	23 ± 6	10.0
2	Inverse relationship between the size and strength of the musculoskeletal systems in the pelvis versus the head and neck	8.1	55 ± 8	4.5
3	Inverse relationship between the length of the skull and limbs and the volume of the skull	4.6	24 ± 6	1.1
4	Inverse relationship between length of skull and limbs and the strength of limbs and axial skeleton	4.5	70 ± 6	3.2

responsible for the remaining PCs. Once identified, the data will be invaluable for understanding the genetics of morphologic development. For instance, dogs tend to have either long thin limbs or short squat limbs. Finding the genes controlling growth regulation for these correlated phenotypes will provide a wealth of data about growth regulation during early development.

24.4.6.3 Complex Disease

In addition to finding QTLs for morphology, the PWD study is well poised to identify disease susceptibility loci as well. Because the dogs were selected randomly from the population of living AKC registered PWD, and about 10% of the overall population participated, a representative set of complex genetic disorders is represented in the dataset. Using the already completed genome-wide scan, and health records provided by owners and veterinarians, loci for Addison's disease, osteoarthritis, and hip laxity have been identified [12-14]. Each of these traits has important implications for understanding human disease. For instance, Addison's disease in dogs appears to be immune mediated and, as with humans, occurs late in life with a female to male ratio of 2:1. Two canine loci have been identified to date, one of which is in the canine locus termed DLA (for dog leukocyte antigen) on CFA12 that is comparable to the human HLA locus [14]. The other is in a region of CFA37 that encodes genes for immune suppression. Understanding the exact variation responsible for the disease in dogs will provide insights into the poorly understood human condition as well.

The hip dysplasia/hip laxity study is equally provocative. Nearly 1/1000 human babies are born with hip dysplasia. In the PWD study, two predisposition loci, both on CFA1 have been identified and investigators are currently using haplotype analysis to find the underlying genetic variants. Additionally, investigators working with a mixed population of Greyhounds and Labrador retriever have identified 12 QTLs associated with hip dysplasia, none of which is on CFA1 [71]. The net result of these studies is expected to be a major advancement in both companion animal and human health and to provide insight into mammalian development.

24.4.7 Conclusion

We have argued in recent months that advances in genomic sciences have brought canine genetics into its prime; the dog genome has in effect "come of age". The dog community has now earned the right to take its place alongside other model systems, such as the rat and mouse. In fact, this is a fallacy. The critical experiments in canine genetics were not accomplished through the use of new technologies nor were they designed by scientists in a laboratory. The critical experiments in canine genetics were done by dog breeders and fanciers in the last 200–300 years, in their quest to create dogs of ideal appearance or behavior. Dog breeders are some of the most sophisticated geneticists practicing the craft today. They understand at a very real level the consequences of QTLs, incomplete penetrance, and complex traits. In creating a species that carries within its genome a truly extraordinary level of variation, dog breeders have provided for scientists a mechanism to truly understand the fundamental properties of mammalian biology. It somehow degrades centuries of careful manipulation if the dog is referred to simply as a "model system" for anything. It is an elegant experiment that, in reaching fruition, is of mutual benefit to dogs and humans alike.

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During preparation of this article, the 12-year-old Border Collie of one of the authors died after a short and unexpected illness. Only the loss of a parent or child can bring more tears than the loss of a pet. We dedicate this article to the many pet owners who, in similar situations, have shown us how to deal with our loss with grace and dignity.

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Fish as a Model for Human Disease

24.5

Siew Hong Lam and Zhiyuan Gong

Abstract As a vertebrate, fish shares many conserved physiological and molecular features with mammals, and its position at the other extreme end of the vertebrate taxon from mammals makes fish an excellent complementary model to existing mammalian disease models for comparative analyses to identify molecular conservation of disorders. Fish, especially small freshwater species that have short generation time, high fecundity, externally and rapidly developing transparent embryos, and low husbandry cost, such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), platyfishes, and swordtails (*Xiphophorus* spp.), are particularly valuable for modeling human disorders at the molecular genetics level. These fish, especially zebrafish and medaka, are amenable to various molecular techniques and are supported by vast genomic resources allowing large-scale mutagenesis screens for the first time in vertebrates, making them highly versatile models of genetic diseases. *Xiphophorus* spp. are particularly valuable for cancer research owing to their ability to generate several spontaneous or induced tumor varieties through interspecies crossing between platyfish and swordtail. In order to fully exploit the fish system for modeling human disorders, various strategies, including both forward and reverse genetics and the use of physical manipulation to induce disease-like states, have been employed. Through a combination of these strategies, these fish are increasingly used to model human genetic diseases caused by both single gene mutation and multiple gene defects, and involving almost all the tissue-organ systems that are found or are homologous in both fish and human. Some of the fish models of human blood or heart disorders, and of cancer are highlighted. The use of these fish models of human diseases for screening of genetic and chemical modifiers of a disease phenotype which can lead to the discovery of drugs and therapeutic targets is also discussed.

Contents

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24.5.1 Introduction

24.5.1.1 Brief Historical Background and Current Status

Fish considered as "lower" vertebrates have long been used by biologists to investigate and clarify some of the "complex" physiological processes and pathological problems observed in "higher" vertebrates such as in humans [10]. Researches with fishes provide a conceptual framework and evolutionary reference point for comparative studies. Using fish as a model to unravel some of the fundamental biological mechanisms common in both fish and human could provide insights into normal human biological processes and the pathogenesis of human disorders. Small freshwater species that are amenable to various molecular techniques and have short generation time, high fecundity and low husbandry cost such as zebrafish (*Danio rerio*), medaka (*Oryzias latipes*), platyfishes and swordtails (*Xiphophorus* spp.), are particularly valuable for modeling human disorders at the molecular genetics level.

Medaka (Fig. 24.5.1) came into genetic research as early as 1913 when it was used to demonstrate Mendelian inheritance in vertebrates and in 1921, it became the first vertebrate in which the occurrence of crossing over between X and Y chromosomes and Y-linked inheritance was shown (for reviews see [55, 65]). Medaka was subsequently used to study pigmentation, sex determination, development, and toxicology. It was the first fish species in which stable transgenesis and stable embryonic stem-like cells were established. In 1999, an international consortium, Medaka Genome Initiative (http://www.dsp.jst.go.jp/MGI/), was formed to sequence the medaka genome and to establish genetic and physical mapping resources. The use of *Xiphophorus* spp. in cancer research can be dated back to 1920s when it was discovered that interspecies hybrids between platyfish *Xiphophorus maculatus* and swordtail *Xiphophorus helleri* develop melanomas spontaneously (for reviews see [37, 62]). The *Xiphophorus* Genetic Stock Center (XGSC; http://www.xiphophorus.org/xgsc.htm) was established in 1939 with the primary interest of developing genetically inbred stock for the identification of genes responsible for cancer. Presently, XGSC maintains 65 pedigreed *Xiphophorus* lines representing 23 species and selected backcross mating between these lines can produced several spontaneous or induced tumor varieties such as melanoma, renal adenocarcinoma, retinoblastoma, fibrosarcoma and schwannoma. Construction of a complete genetic linkage map has been initiated in *Xiphophorus* and development of expressed sequence tags and microsatellite methodologies are ongoing to facilitate genetic studies.

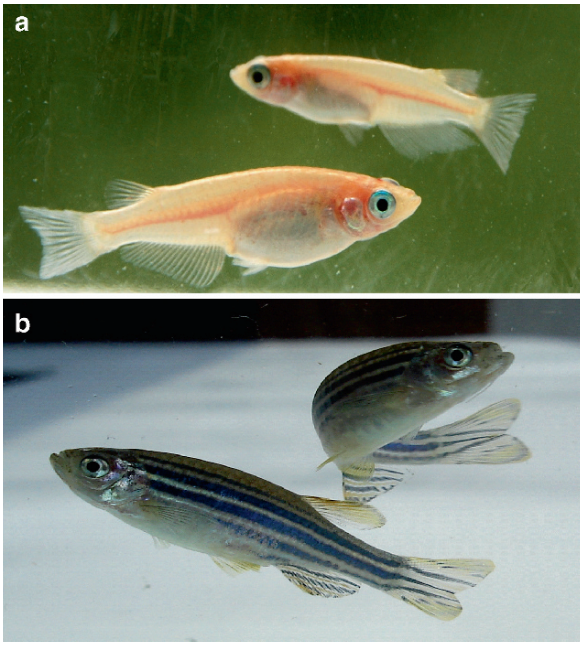

Fig. 24.5.1 (**a**, **b**) Two fish models that are widely used for disease modeling. (**a**) Medaka, *Oryzias latipes*; (**b**) zebrafish, *Danio rerio*

The zebrafish (Fig. 24.5.1) has been used as an experimental model since 1930s to study various environmental stress-related disorders ranging from developmental, anatomical to behavioral, by manipulating the external environment of the fish (for review, see [16]). In 1965, zebrafish became the first fish species used as a chemical carcinogenesis model demonstrating the development of hepatic neoplasia after exposure to carcinogen diethylnitrosamine [57]. However, it was not until the 1970s and 1980s that the zebrafish was developed as a genetic and developmental model. This set the stage for the historic large scale mutagenesis screens in the 1990s which, according to Amsterdam and Hopkins [2], generated more than 6,600 mutations initially but presently only 1,740 genetic mutants with specific embryogenesis defects were maintained and many had been described in the landmark publication of the zebrafish issue in the journal *Development* (volume 123) [9, 19]. Many of these mutant phenotypes resembled human disease conditions and several were found to be affected by orthologues of the human disease genes and serve as zebrafish models of these disorders (see Sect. 24.5.3.1). Within a decade, from a premier vertebrate developmental model, the zebrafish has now positioned itself as a biomedical model supported by vast genomic resources (including the zebrafish genome sequencing project; http://www.sanger.ac.uk/Projects/D_rerio/), a zebrafish stock center (Zebrafish International Resource Center; http://zfin.org/zirc/home/guide.php) and a centralized web-based database Zebrafish Information Network (http://zfin.org/) to facilitate the rapid exchange of genomic data and genetic resources. As a reflection of the growing confidence in the scientific community, Trans-NIH Zebrafish Initiative and European ZF Models consortium were set up to invest and consolidate efforts in developing zebrafish as human disease models for discovering insights into human disorders and novel therapeutics.

Over the span of 100 years, fish has came of age as a species used for model systems; from an experimental animal used for addressing various biological questions it progressed to become a premier model for developmental studies, and now a genetic biomedical model for addressing human disorders, and it even promises to find cures for human diseases. It is now clear that the biology that is observed in fish is likely not restricted to fish alone but may also be found to be applicable to humans. Thus, it is no longer just fish that we are beholding but human lives.

24.5.1.2 Why Use Fish to Model Human Disorders?

As vertebrates, fish and human share most developmental processes, physiological mechanisms, and organ systems. In fact the zebrafish has been described as a "canonical vertebrate" owing to the many similarities shared with mammalian biology [12]. Many of the genes or molecules with essential functions that are found in human are also found in fish. A comparison between the Fugu fish and human genomes revealed that 75% of predicted human proteins have a strong match to Fugu [4]. A number of zebrafish homologues of human disease genes such as those involved in cancer, Alzheimer's disease, muscular dystrophy, diabetes, thrombosis, blood, and heart disorders have been cloned or identified [29, 44, 57]. Thus, many diseases found in human are also observable in fish, allowing it to be used as an experimental animal to probe pathological problems in human.

As the oldest vertebrate, with over 500 million years of evolutionary history, fish is phylogenetically positioned at the opposite extreme end of the vertebrate taxon from human. Given the extreme phylogenetic separation between fish and human, it is reasonable to assume that only important genes, molecules, pathways, and processes that are involved in a particular disease phenotype observed in both fish and human would be conserved. This strategic phylogenetic position in the vertebrate taxon, then, offers unique comparative opportunities for identifying genes and molecules, pathways, and processes that are strongly associated with a disease phenotype. In cases where clinical samples or data are limited, the power of comparative disease modeling between two phylogenetically distant vertebrates such as the fish and rodent is evident; any identified molecular conservation of a disease phenotype between fish and a mammalian model would immediately underscore its fundamental importance in association with the disease and highlight its clinical potential. This comparative advantage was demonstrated when a number of genes that were identified as strongly associated with human and zebrafish liver tumors had also been suggested to have diagnostic, prognostic, or therapeutic values [27].

As the largest and most diverse group of vertebrates, with more than 28,000 species, fish offer a large number of models of adaptation and response to various natural and anthropogenic environments. Some of the specialized physiologies, traits, and adaptations can be exploited to address specific disorders. For example, the short 3-month lifespan of the fish *Nothobranchius furzeri* has been exploited for investigations into aging-related dysfunction of organ systems and drug validation [14].

Fish are very sensitive to change in the environment because of their close physiological contact with the surrounding water. Fish are therefore quick to respond to specific physiological challenges in order to maintain homeostasis, which otherwise will affect susceptible cells, tissues and organs. The intimate physiological-environment relationship and the rapid homeostatic response are more easily defined and their impacts more readily studied in fish than in terrestrial species. Fish are therefore excellent models for studying environment-induced health disorders and for inference of health risk, particularly as a result of environmental pollution, stress, or substance abuse (see Sect. 24.5.2.3).

In addition to the unique advantages mentioned above, the distinct features of some of the small aquarium fishes are what have propelled fish into the forefront of biomedical research. In particular, the zebrafish and medaka are excellent experimental animals owing to their short generation time, high fecundity, externally and rapidly developing transparent embryos, and ease of breeding and maintenance. For example, zebrafish are easily available in large numbers because of their short generation time (3 months), and they spawn throughout the year in a laboratory environment, producing large numbers (100–200) of external transparent embryos that rapidly develop from single-cell stage (with organogenesis occurring within 24 h) to free-feeding larvae with fully functioning organ systems within 5 days. They are inexpensive and easy to maintain in large numbers in a relatively small space. These attributes made large-scale mutagenesis screens feasible for the first time in vertebrates and paved the way for disease modeling in zebrafish and medaka. As a result, the zebrafish and medaka systems are now supported by vast genomic resources and amenable to many molecular tools making them highly versatile for genetic disease modeling (see Sect. 24.5.2).

Moreover, the combination of the above factors placed zebrafish and medaka in a strategic position to bridge the gap between in vitro cell-based models and the in vivo rodent model in biomedical research. The in vitro cell-based model, which is suitable for high-throughput applications, lacks the relevant physiological whole-organism setting, while the rodent model, which provides more relevant in vivo data, is less suited to high-throughput applications. Other established in vivo high-throughput screening systems such as *Drosophila* and *Caenorhabditis elegans* lack important vertebrate organ systems. Zebrafish and medaka are therefore presently the only vertebrate model systems that can be used for medium- to high-throughput bioassays while at the same time providing physiologically relevant data derived from a whole-organism setting. As disease models, these fishes are therefore highly suited for whole-organism-based therapeutic target and small molecule screening for drug discovery [67] (see Sect. 24.5.4).

24.5.2 Strategies for Modeling Human Disorders in Fish

In order for the zebrafish and medaka systems to be fully exploited for modeling human disorders, various genetic manipulations, including both forward and reverse genetics, and many molecular tools have been developed. The amenability of the zebrafish system to these powerful molecular strategies for studying human disorders will be highlighted in this section. For detailed description of each method, the reader is advised to refer to some of the cited literature.

24.5.2.1 Forward Genetics

Forward genetics is a powerful approach to the discovery of dysfunctional genetic control of biological processes that result in diseases without prior knowledge of the genes involved. By generating mutants that phenocopy human diseases, they can serve as experimental models to aid pathological investigations or used for screening of therapeutics (see Sect. 24.5.4). Although there are several ways of performing forward genetic screens, chemical and insertional mutagenesis have been most successful in zebrafish [2, 47].

24.5.2.1.1 Chemical Mutagenesis

Chemical mutagens could induce point mutations in single genes in rapidly dividing premeiotic germ cells at very high efficiency, and *N*-ethyl-*N*-nitrosourea (ENU) has proved to be most effective in zebrafish. Conventionally, chemical mutagenesis is carried out by a three-generation screening approach. Adult males are treated with ENU several weeks before crossing with wild-type females to generate F1 fish, which potentially carry a specific heterozygous mutation. These F1 fish are then crossed either with siblings or with wild-type females to generate F2 families. F2 siblings are then crossed with each other to reveal recessive mutations in the F3 homozygotes. Phenotype screening is then performed in F3 generation. "Targeted" screening may also be performed by focusing on specific organ systems or biological processes. Once a phenotype of interest is detected, positional cloning and candidate gene testing are performed to identify the mutated gene.

ENU screens have successfully generated a number of mutants that are now used for modeling blood and cardiovascular, neurosensory, musculoskeletal, and skin disorders (see Table 24.5.1) [2]. Subsequent cloning of the mutated genes in some of these zebrafish mutants revealed that many of the genes were also known to be mutated in human disease conditions (see Sect. 24.5.3). Likewise, this strategy has also been used to generate mutants for addressing disorders that are more biochemical or pharmacological in nature, such as cocaine addiction [7], d-amphetamine addiction [41], and disorders associated with the endocrine system [36], lipid metabolism [20], and hemostasis [22]. A large-scale ENU mutagenesis screen was also performed in medaka, resulting in 2031 embryonic lethal mutations being identified [13]. These included 312 embryonic lethal mutations causing defects in organogenesis, which were analyzed, and 126 mutations which were characterized genetically and assigned to 105 genes. Seven blood, liver, and thymic mutants have been generated and are potential models for the corresponding disorders in humans.

24.5.2.1.2 Insertional Mutagenesis

Retrovirus-mediated insertional mutagenesis is less efficient in generating mutants than the ENU approach, but it has the advantage of rapid identification of the mutated gene owing to the presence of a molecular tag inserted at the mutated site. Injection of murine retrovirus into a large number of blastula-staged (1,000- to 2,000-cell stage) embryos causes insertion of the viral sequence into the genome of primordial germ cells, causing specific genes to be mutated and transmitted to the next generation. Founder fish are used to produce F1 families, which will be screened for viral inserts. As the inserted viral sequence is known, the mutated genes can be rapidly identified by inverse PCR methods once a phenotype of interest is identified in subsequent generations [2].

By means of this strategy, gene loci or genes involved in cystic kidney disease [59], liver disease [52], and visual system disorders [15] have been identified. In addition, genes associated with craniofacial birth defects and zebrafish models for campomelic dysplasia and Ehlers-Danlos syndrome were also identified using an insertional mutagenesis screen [42]. It has also been found that many insertional mutants with ribosomal protein genes mutated are susceptible to cancer, suggesting similar tumor-suppressing roles of ribosomal proteins in humans that may have escaped detection or been overlooked owing to the many ribosomal proteins present in human [3]. In the same report, a neurofibromatosis type 2 mutant, a known tumor suppressor gene in human, has also been identified with elevated tumor incidence and can therefore be used for modeling tumorigenesis.

24.5.2.2 Reverse Genetics

Reverse genetics is a useful approach to generating disease models when the underlying genetic basis or gene involved in the human disorder is known. However, unlike the mouse model, gene knockout by targeted gene disruption through homologous recombination is still not possible in fish, because there are still no suitable embryonic cell lines. Nevertheless, advances in the derivation of pluripotent germline-competent embryonic cells from medaka [21] and zebrafish [11], and the feasibility of cloning by nuclear transfer in both species [33, 61] are making the prospect of targeted knockout in fish closer to reality. In the meantime, reverse-genetic strategies such as "gene knockdown" of a targeted gene using morpholinos and reverse-genetic screening by TILLING (*t*argeted *i*nduced *l*ocal *l*esion *in* *g*enome) and also tissue-specific overexpression of a foreign gene using transgenic technology have been employed for modeling gene dysfunctions and generating disease models in the zebrafish system.

24.5.2.2.1 Morpholinos

Morpholinos are modified antisense oligonucleotides that are resistant to cellular RNase and are injected into single- or two-cell stage embryos to knock down expression of a target gene product by either blocking translation initiation or interfering with the splicing of a particular exon [39]. Injected morpholinos are unlikely to result in complete loss of function, but usually generate phenotypes with several degrees of severity. This may be seen as an advantage where complete loss of function results in early lethality, and for modeling disorders that display different degrees of severity as a result of sensitivity to the dose-effect of a gene product. Owing to the stability of morpholinos and the rapid development of the zebrafish, phenotypes resulting from the knockdown of the targeted gene can be rapidly observed for several days, hence providing a quick and economical approach for generating disease models.

It has been demonstrated in several cases that injection of morpholinos results in developing embryos/larvae that phenocopy some human disease states. For example, morpholinos against *urod* caused cases of porphyria similar to those seen in humans, including autofluorescence of erythrocytes in ultraviolet light [39], dystrophin morpholinos resulted in muscular degeneration similar to that seen in human muscular dystrophy [18], and *invs* morpholino produced a renal cystic phenotype mimicking cystic kidney disease as observed in children [46]. Similarly, injection of morpholinos targeted against *Pit1* resulted in loss of the lactotroph, somatotroph, and thyrotrophic cells in the developing pituitary and a lack of growth in the juvenile (dwarfism) phenocopying human *Pit1* mutants of combined pituitary hormone deficiencies (CPHD) [40]. Thus, morpholino has become a cost-effective tool for verifying gene function in association with a disorder.

24.5.2.2.2 Reverse Genetic Screening by TILLING

Although the morpholino gene knockdown approach is quick and simple, it is not a germline mutation, so that the effect is only stable for a short period. In addition, some genes may be more difficult to knock down owing to the abundant maternal protein and/or to the late expression during development. On the other hand, mutational screens are efficient in generating mutants, but they are resource intensive in terms of the space, labor, and time required. In order to overcome these limitations, reverse-genetic screening by TILLING has been applied to the zebrafish system. This involves the combination of classic high-saturation ENU screen with high-throughput screening for point mutations in targeted genes of the mutagenized genome [56].

As in ENU screening, adult males are exposed to ENU to induce point mutation in their germ cells and mated with wild-type females to produce F1 generation. DNA can be extracted from tail biopsies of F1 fish maintained in small groups (live library) or from cryopreserved sperm of sacrificed F1 males (frozen sperm library) for scanning of mutation in a targeted gene. TILLING provides a cost-effective high-throughput approach for scanning of ENU-induced point mutation in PCR products of a targeted gene by using the celery mismatched-repair enzyme CEL-1, an endonuclease that cleaves DNA after single-base-pair mismatches, and subsequently allowing for electrophoresis-based detection of the mutation. Once a potential mutant is detected and the nature of the mutation is determined by sequencing, the live fish can immediately be outcrossed to propagate the line, or otherwise cryopreserved sperm is thawed for in vitro fertilization of wild-type eggs to regenerate the line.

Using this approach, Wienholds et al. [63] have identified several *rag1* mutants with amino acid substitutions or with premature stop codon. The mutant with a premature stop codon was intercrossed to produce homozygous fish which are defective in V(D)J recombination, confirming the loss of Rag1 function. In another screen, Wienholds et al. [64] identified 255 mutations, 14 of which had a premature stop codon, 7 had a splice donor/acceptor site mutation, and 119 had an amino acid substitution, and generated 13 potential knockout fish in a few months. This approach has also been used to recover two tp53 mutant lines with missense mutations similar to those found in human cancers, one mutant line being prone to the development of malignant peripheral nerve sheath tumor, which is useful for identifying genes that are associated with the tumor phenotype [5].

24.5.2.2.3 Transgenesis

Overexpressing a gene to mimic or induce a disease state, either by transient overexpression or by transgenesis, can be a useful way to study the gene-disease relationship and to develop a disease model. Transient overexpression of genes in zebrafish embryos is performed by injection of a DNA construct or in vitro-transcribed mRNA into embryos of 1-to 2-cell stage. Transient overexpression is usually performed in parallel with knockdown studies or gain-of-function rescue experiments to decipher gene function. However, it can have distinct advantages over the knockdown approach, as it can be designed to express protein subunits or mutated forms of proteins to decipher structure–function relationships and determine important protein domains associated with a dysfunction. Transient expression of the zebrafish equivalent to a human muscular dystrophy mutant, *CAV3P104L*, which has been identified in human patients with limb girdle muscular dystrophy (LGMD-1C) and rippling muscle disease, causes severe disruption of muscle differentiation in zebrafish and produces a similar dominant phenotype to that in humans [43].

In order to generate stable germline-transmissible foreign gene in zebrafish, DNA-injected embryos are raised to adulthood to produce the next generation. Only a small percentage (1-10%) of the injected embryos will stably integrate the foreign DNA construct into the genome of their germ cells and become transgenic founder fish (F0). Co-injection with an easily screenable reporter, such as a green or red fluorescent protein (GFP or RFP), could facilitate the identification of potential founders. F1 generation is screened for transgene expression by direct fluorescent visualization or other molecular expression. Once a transgenic F1 individual is identified, it can be used to develop a stable transgenic line. Using transgenic technology, several excellent zebrafish leukemia models had been developed [31, 32] (see Sect. 24.5.3.3).

24.5.2.3 Physical Manipulation: Chemical Treatment, Environmental Stressor, and Infection

Another strategy for modeling human disorders in zebrafish is through physical manipulation by using chemical agents, an environmental stressor, or microbial infection to induce disease-like states. Zebrafish are being used to model Parkinson disease by treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and for screening of environmental pollutants, which induce selective loss of dopaminergic neurons in the brain, eliciting symptoms characteristic of Parkinson's disease [6]. MPTP-induced neurodegeneration can be prevented by co-incubation with l-deprenyl (monoamine oxidase-B inhibitor) or nomifensine (dopamine transporter inhibitor), indicating that the mechanism for MPTP-induced dopaminergic neuron toxicity in mammals and zebrafish is conserved [28]. Similarly, zebrafish are being used to model several substance-abuse-related disorders by exposing the fish to cocaine [7], d-amphetamine [41] and ethanol [35]. Likewise, in environmental toxicology, zebrafish liver exposed to arsenic shares a comparable molecular signature with those reported in mammalian systems, suggesting that the zebrafish liver coupled with the available microarray technology presents an excellent in vivo toxicogenomic model for investigating arsenic toxicity [30]. The acute arsenic-induced liver transcriptome changes in zebrafish can be used to infer possible liver damage that could occur in individuals exposed to arsenic in endemic areas where there is a high incidence of liver diseases in these populations. Zebrafish has also been used as a model to investigate molecular mechanisms and signaling molecules that are involved in the physiology and disorders resulting from hypoxic conditioning. In these hypoxic studies, zebrafish were maintained in an artificially induced hypoxic environment by decreasing air saturation in the tank water or by bubbling nitrogen gas in water to reduce oxygen levels. These studies have relevance to human disorders such as stroke, chronic ischemia, tumorigenesis, and intrauterine growth restriction, which increases fetal and neonatal morbidity and mortality [23]. Zebrafish has also been used to model infections, such as mycobacterial, streptococcal, aeromonad, and staphylococcal infections, which are caused by similar bacteria that are common in both human and fish. It has been shown that the zebrafish acute phase responses to *Aeromonas salmonicida* and *Staphylococcus aureus* infections are strikingly similar to the responses in mammals in terms of the type of proteins involved and how they are induced, although additional novel proteins have also been identified in fish only [34].

24.5.3 Fish Models of Human Disorders

Using a combination of strategies, as in sect. 24.5.2, zebrafish are increasingly employed to model human genetic diseases caused by both single gene mutation and multiple gene defects and involving almost all the tissue-organ systems that are found or are homologous in both zebrafish and human. These disease models range from hematopoietic, cardiovascular [44], musculoskeletal [43], and germ cell chromosome disorders [50] to complex neurosensory behavioral [17], cancer [57], environmental health [6] and aging-/degeneration-related disorders [26]. Table 24.5.1 lists some of the selected zebrafish disease models of human disorders, which include mutants with similar genes mutated in corresponding mammalian/human disease states. Some of the fish models of human blood or heart disorders and of cancer will be highlighted in this section (see Fig. 24.5.2).

24.5.3.1 Blood Disorders

The hematopoietic process involving the generation of multi-blood lineages (erythroid, myeloid, lymphoid, platelets),and the expression of many blood-specific genes (*cmyb*, *gata1*, *gata2*, *globin*, *hhex*, *ikaros*, *lmo2*, *pu1*, *rag1*, *runx1*, *scl*, and *vegf*) are conserved between zebrafish and human, although some hematopoietic sites may differ between the two species [8]. As the developing zebrafish embryo can survive without blood for several days, it allows for the detection of mutant larvae with blood-related defects. More than 50 mutants that affect hematopoiesis have been recovered in large-scale mutagenesis screens (for reviews see [2, 44]). The validity of using zebrafish as a model for human blood disorders is demonstrated by the many blood mutants that phenocopy human blood disease conditions.

The zebrafish *sauternes* (*sau*) mutant is the first animal model for human congenital sideroblastic anemia caused by mutations in δ-aminolevulinate synthase (ALAS2), an erythroid-specific enzyme required to initiate heme biosynthesis. The *sau* mutant is characterized by delayed erythroid maturation and abnormal globin expression, resulting in a microcytic, hypochromic anemia, and positional cloning discloses that the mutant gene encodes ALAS2, as in human conditions. The hypochromic mutant *weissherbst* (*weh*) has significantly reduced cellular hemoglobin and iron levels although the red blood cell count is near normal. Positional cloning of the gene responsible for the *weh* mutant led to the discovery of *ferroportin1*, which encodes a novel iron transporter that is conserved in vertebrates. Soon after the discovery of *ferroportin1* in zebrafish, it was found that similar mutations also occurred in patients suffering from a severe form of hereditary iron overload (hemochromatosis type IV). This is the first demonstration of the potential of using zebrafish to discover a previously unknown gene that is similarly mutated in the corresponding human disease.

Photosensitive blood mutants, such as *yquem* (*yqe*) and *dracula* (*drc*), have erythrocytes that autofluoresce and lyse when exposed to ambient light, phenocopying human congenital erythropoietic porphyrias. By using porphyrin and enzymatic assays, uroporphyrinogen decarboxylase (UROD) is identified as the enzymatic deficiency in the *yqe* zebrafish mutants that prove to be the first animal model for hepatoerythropoietic porphyria in which patients are deficient in UROD. The *drc* mutant has a mutation in *ferrochelatase* which encodes a heme enzyme and is characterized by photosensitive, autofluorescent erythrocytes, accumulation of protoporphyin IX, and liver disease, similar to patients with erythropoietic protoporphyria, a disorder of ferrochelatase. Both the mutants are excellent models for studying the pathogenesis and progression of protoporphyrin-induced liver disease using controlled light conditions.

Several of the blood mutants are characterized by fragile, hemolytic red blood cells attributable to mutations in cytoskeletal proteins. By using positional cloning and candidate gene cloning methods, the *merlot* (*mot*) and *chablis* (*cha*) mutations have been found to be located in the same gene-encoding erythrocyte protein 4.1 (also called band 4.1 or 4.1R), which is a structural membrane protein that anchors the spectrin-actin cytoskeleton to the erythrocyte cell membrane, thus conferring morphological stability to the erythrocyte. Homozygous *mot* or *cha* adults have decreased erythrocyte count, with immature erythrocytes arrested in the basophilic erythroblast stage and erythrocytes displaying abnormal morphology and osmotic fragility suggestive of hemolytic anemia. These mutants provide a model for hereditary elliptocytosis in humans, a rare cause of hemolytic anemia characterized by elliptical red blood cells caused by deficiency of protein 4.1.

Table 24.5.1 Selected zebrafish models of human disorders

Human disorder/disease	Gene product	Fish model: mutant, morphant or transgenic (phenotype)[a]
Blood		
Hypochromic microcytic anemia	SLC11A2	*chardonnay* (Hypochromic blood)
Hereditary elliptocytosis	EBP41	*chablis/merlot* (Hemolytic anemia)
Erythropoietic protoporphyria	Ferrochelatase	*dracula* (Porphyria; blood cell photosensitivity)
Congenital dyserythropoietic anemia type 2	SLC4A1	*retsina* (Hemolytic anemia)
Hereditary spherocytosis; elliptocytosis	beta-spectrin	*riesling* (Hemolytic anemia)
Sideroblastic anemia	Alas2	*sauternes* (Hypochromic anemia, decreasing blood count)
Hemochromatosis type 4	Ferroportin 1	*weissherbst* (Hypochromic anemia)
Hepatoerythropoietic porphyria	Urod	*yquem* (Porphyria)
Dyserythropoietic anemia; thrombocytopenia	Gata1	*vlad tepes* (Fewer blood cells)
Heart		
Holt-Oram syndrome	Tbx5	*heartstring* (Cardiac dysfunction; lacks pectoral fins)
Cardiomyopathy	Titin	*pickwick* (Heart contraction defect)
Cardiomyopathy	Tnnt2	*silent heart* (Absence of heart beat)
Cardiomyopathy	ACTC	*cardiofunk* (Defective endocardial cushion formation)
Cardiomyopathy	Myh6	*weak atrium* (Large and poorly beating atrium)
Timothy syndrome	CACNA1C	*island beat* (Defective heart function)
Long QT syndrome 2	KCNH2	*breakdance; kcnh2* morphant (Defective heartbeat)
Musculo-skeletal/skin (pigmentation)		
Duchenne muscular dystrophy	Dystrophin	*sapje* (Muscle degeneration, decreased motility)
Brody myopathy	ATP2A1	*accordion* (Delayed, prolonged trunk muscle relaxation)
Congenital slow-channel myasthenic syndrome	CHRNA1	*nicotinic receptor* (Nonmotile)
Campomelic dysplasia	Sox9a	*jellyfish* (Cartilage defects)
Progeroid type Ehlers-Danlos syndrome	B4galt7	*b4galt7* (Cartilage defects)
Exostoses, multiple type II	Exostosin 2	*dackel* (Abnormal fin, retinotectal projection, jaw, head, ear)
Oculocutaneous albinism type 1	Tyrosinase	*sandy* (No melanin pigment in body and eyes)
Piebaldism	Kit receptor	*sparse* (Fewer and smaller melanophores than wild type)
Waardenburg-Shah syndrome	sox10	*sox10* (Lacks pigment cells; abnormal ears)
Neuro-/organ-sensory		
Deafness	Myo6b	*satellite* (Uncoordinated swimming owing to vestibular defect)
Usher syndrome type 1b (hearing impairment)	Myo7a	*mariner* (Circular swimming owing to vestibular defect)
Usher syndrome type 1d	Cadherin 23	*sputnik* (Hearing defects, circular swimming, vestibular defect)
Achromatopsia	GNAT2	*no optokinetic response* (Lack optokinetic response)
Congenital cataracts with facial dysmorphy	Ctdp1	*ctdp1* (Small head and eyes)
Pituitary anomalies; holoprosencephaly-like	Gli2a	*you too* (Neural tube, hindbrain, midline axon guidance defects)
Kidney / pancreas / endocrine		
Polycystic kidney disease 2	PKD2	*pkd2* morphant (Cystic kidney)
Glomerulocystic kidney disease	Tcf2	*tcf2* (Cystic kidney)
Maturity-onset diabetes of the young type 5	Tcf2	*tcf2* (Smaller pancreas)
Pancreatic agenesis; neonatal diabetes mellitus	Ptf1a	*ptf1a* morphant (Exocrine pancreas agenesis)
Pituitary-hormone deficiency	Pit1 / POU1F1	*pit1 / pouf1* (Lacks several pituitary cell types)
Adrenal agenesis	Sf-1 / Ff1b	*ff1b* morphant (Impaired interenal development)
Cancer and other conditions		
Neurofibromatosis type 2	NF2	*nf2* (Predisposed to malignant peripheral nerve sheath tumors)
Most commonly mutated in human cancers	p53	*p53* (Predisposed to tumor formation)
Melanoma	hBRAF(V600E)	*mitfa-BRAF (V600E)* in *p53* mutant (Invasive melanoma)
T-cell acute lymphoblastic leukemia	Myc	*rag2-EGFP-Myc* transgenic (T-cell leukemia)
B-cell acute lymphoblastic leukemia	TEL-AML1	*XEF-EGFP-TEL-AML1* transgenic (B-cell leukemia)
DiGeorge syndrome	Tbx1	*van gogh* (Ear, thymus, pharyngeal arches defects)
Telangiectasia, hereditary hemorrhagic type 2	ACVRL1	*violet beauregarde* (Defective circulatory system)

[a] Unless indicated as morphant (morpholino knockdown) or transgenic, all models are mutants generated by forward genetics. The affected genes in these mutants have been cloned and are known to be mutated in human disorders [2, 44]. Only disease-relevant phenotype is indicated

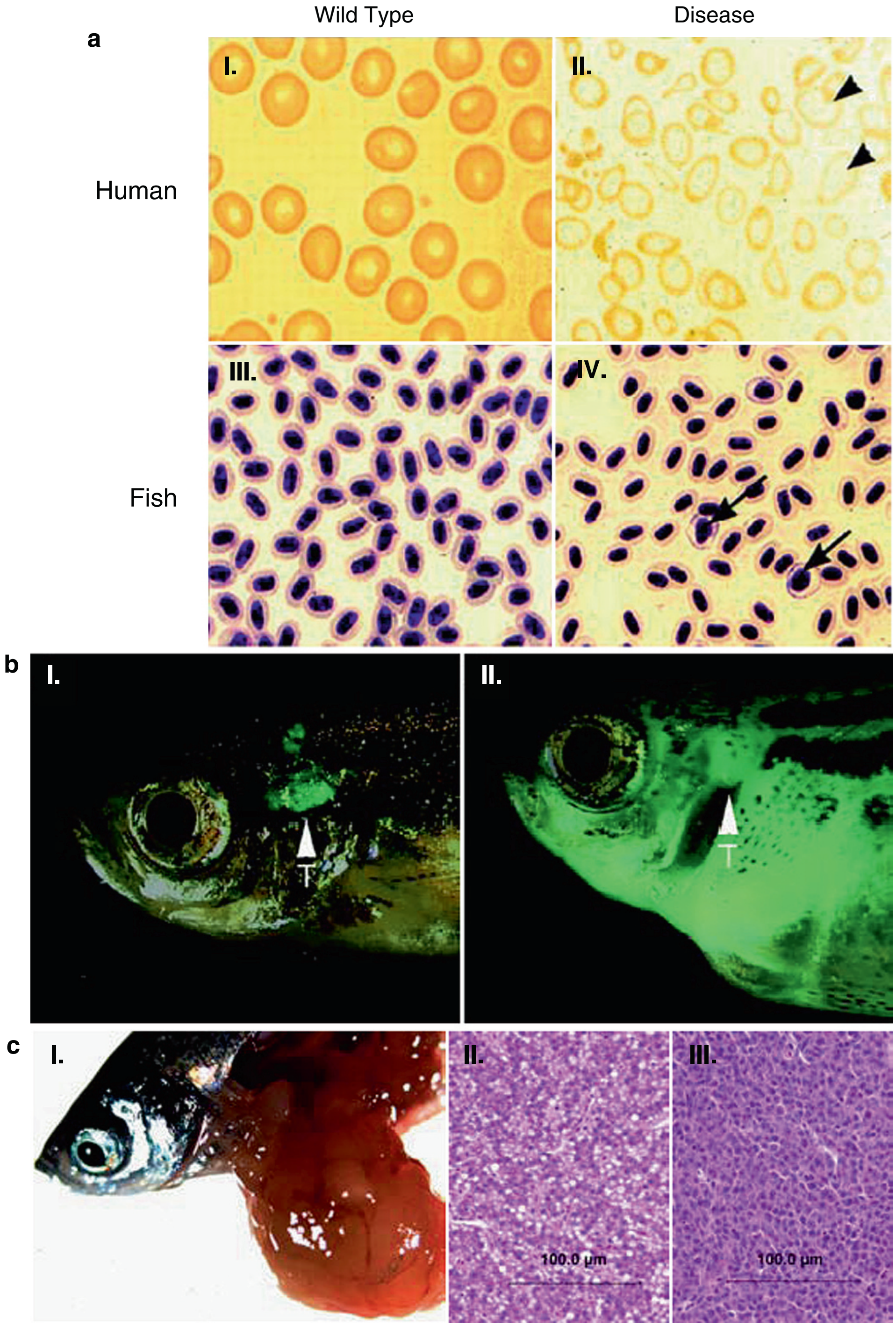

Fig. 24.5.2 (**a**–**c**) Fish models of human disorders. (**a**) Comparison of the human and zebrafish blood disorders associated with mutations in *ALAS-2*. The red blood cells in the peripheral blood are normally round and uniform (*I*). In human congenital sideroblastic anemia (*II*), cells have abnormal shapes and are hypochromic (owing to a low level of hemoglobin). Red cell precursors, such as reticulocytes, are larger (*arrowheads*). The increase in reticulocytes indicates a response of the marrow to the anemia. A similar hypochromia and increase in precursors is evident in the peripheral blood smear of *sau* homozygous mutants (*arrows* in *IV*) compared to wild-type fish (*III*). (Reproduced with permission from [66]). (**b**) Stable transgenic *rag2-EGFP-mMyc* zebrafish develop GFP-labeled thymic lymphoma, which progresses to T-ALL. Fluorescence microscopic analysis at 50 days of life, showing the thymus of control *rag2-GFP* transgenic fish (*I*) and massive GFP-labeled cellular dissemination of leukemic lymphoblasts in *rag2-EGFP-mMyc* transgenic fish (*II*). *Arrowheads* mark location of the thymus (*T*). (Reproduced with permission from [32]). (**c**) Carcinogen-induced liver tumor in zebrafish. Gross morphology of liver tumor (*I*) and histologic sections showing normal liver (*II*) and the dedifferentiated state of the liver with hepatoblastoma (*III*)

Zebrafish mutant *riesling* (*ris*) is severely anemic owing to membrane instability and rapid hemolysis caused by a mutation in the erythroid *β-spectrin* gene, which encodes the largest cytoskeletal component of the erythrocyte. The *ris* mutant has erythrocytes that are abnormally shaped with large nuclei resembling those seen in the human erythroid disorder hereditary spherocytosis (HS) caused by mutation in human *β-spectrin*. The mutant *retsina* (*ret*) is characterized by the presence immature erythrocytes with bilobed nuclei and is anemic owing to the arrest of erythrocyte maturation at the late erythroblast stage as a result of defective cytokinesis. This is caused by a *band 3* (also called *SLC4A1*) mutation, which produces a dysfunctional cytoskeletal protein responsible for the failure of mitotic chromosomal segregation in developing erythroblast. These features are also observed in patients with congenital dyserythropoietic anemia type 2 and thus *ret* mutant provides a model for investigating the underlying mechanism of the disorder.

It is worth noting that similar blood mutants, such as those with hypochromic anemia, progressive anemia, and erythropoietic porphyrias, had been generated in medaka through ENU mutagenesis screening [53, 60]. For example, the medaka *whiteout* (*who*) mutant identified in an ENU screen displays a normal erythrocyte count initially, but this gradually decreases during the embryonic and larval stages. The erythrocytes in the *who* mutants have elongated morphology and little hemoglobin activity. A missense mutation in a gene for delta aminolevulinic acid dehydratase (ALAD), the second enzyme in the heme synthetic pathway, has been identified in the *who* mutant, which now represents a model for the human disease ALAD deficiency porphyria [53]. There are still many medaka and zebrafish blood mutants that phenocopy human blood disorders and have yet to be cloned, and further characterization of these mutants may yield novel insights into these diseases.

24.5.3.2 Heart Disorders

The anatomy of the two-chambered (atrio-ventricular) zebrafish heart lined by an inner endothelial layer and an outer myocardial layer resembles that of the human heart at 3 weeks of gestation. However ,unlike the situation in mammals, the zebrafish embryonic heart function can be assessed visually and the fish embryo is not dependent on blood circulation for survival during embryogenesis, thus allowing many zebrafish mutants with cardiac defects to be identified through mutagenesis screens (for reviews see [2, 44]).

The mutant *heartstrings* (*hst*), which encodes the human ortholog *TBX5*, a member of the T-box family of transcription factors, does not develop pectoral fins (analogous to mammalian limbs) and has severe heart defects. In humans, mutations in *TBX5* result in Holt-Oram syndrome, a genetic disorder characterized by heart and upper limb defects. Low-level morpholino knockdown of *tbx5* also results in a variety of bilateral and asymmetric fin (limb) deformities similar to haploinsufficiency of *TBX5* in humans. The study demonstrated the versatility of zebrafish to model different degrees of severity of a human disorder that is sensitive to dose-effect of a gene product.

The *silent heart* (*sih*) mutants have a heart that fails to contract although embryonic cardiac development proceeds normally. The mutations causing *sih* are found in the sarcomere component *cardiac troponin T* (*tnnt2*) and were confirmed by morpholino and DNA rescue experiments. The dysfunctional heart muscle of *sih* is caused by failure in cardiac sarcomere assembly attributable to the defects in thin filament stability in the absence of Tnnt2. The *sih* mutants are the first animal model of Tnnt2 deficiency, as they phenocopy human familial hypertrophic cardiomyopathy characterized by sarcomere loss and myocyte disarray as a result of mutations in *TNNT2*. Similarly, the *pickwick* (*pik*) mutant has embryonic, thin, cardiac myofibrils that can contract, but fail to assemble into normal sarcomeres and thus suffer contractility defects resulting in very low systolic pressure and insufficient blood being pumped out from the heart. Use of a positional cloning approach showed that a mutation in cardiac-specific exons of *titin* (*ttn*) caused *pik*. The *pik* mutant phenocopies the defects seen in human heritable dilated cardiomyopathy caused by mutations in *TTN*.

The zebrafish mutant *island beat* (*isl*) has a defective heartbeat, the atrium exhibiting rapid discoordinated contractions that are not propagated to the ventricle. Besides not beating, the ventricle has a lesser number of cardiomyocytes and is small. The mutation of *isl* embryos is in the *alpha-1C L-type calcium channel subunit* (*C-LTCC*) encoding the primary ion-conducting pore-forming subunit of the L-type calcium channel in cardiac tissue. In humans, mutations of the gene (also known as $Ca_V 1.2$) cause cardiac arrhythmias that can lead to syncope and sudden death and are part of a multisystem disorder known as Timothy syndrome. The cardiac arrhythmia is characterized by extreme prolongation of the QT interval on electrocardiogram, as a result of prolongation of cardiomyocyte action potentials and delayed secondary depolarizations owing to defective voltage-dependent channel inactivation and abnormal Ca^{2+} signaling. Similarly, cardiomyocytes in zebrafish mutant *isl* are absent from L-type calcium currents owing to a defect in calcium channel function, thus modeling human arrhythmia disorder caused by cardiac L-type calcium channel mutations.

In addition, there are many zebrafish heart mutants that phenocopy human heart disorders, such as cardia bifida (*miles apart*), cardiac valve defects (*jekyll*), cardiac hyperthrophy (*liebeskummer*), bradycardia (*slow mo*), coarctation of the aorta (*gridlock*), and atrium and/or ventricle defects (*heart and soul*, *pandora*, *foggy*, *acerebellar*). in which their respect mutation has been identified in zebrafish but a similar gene mutation has yet to be found in the corresponding human disease counterpart. Even so, with the combination of the phenotype and knowledge of the mutated gene, these mutants are useful for understanding the molecular basis of these pathologies and for screening of potential drug candidates that can suppress the disease phenotypes (see Sect. 24.5.4).

24.5.3.3 Cancers

The discovery that melanomas can be generated in the *Xiphophorus* (swordtail and platyfish) hybrid model opened a new frontier for melanoma and tumorigenesis research. A gene encoding a novel receptor tyrosine kinase (Xmrk2), which has homology to the human oncoprotein epidermal growth-factor receptor, has been identified as associated with melanoma formation. Molecular signaling pathways induced by Xmrk consist of essential steps in tumor development, such as preventing cell differentiation, activating unrestricted proliferation, exerting an antiapoptosis effect, and inducing migration [37]. Furthermore, a second genetic locus that is involved in *Xiphophorus* melanoma development has been narrowed down to a tumor suppressor gene called *CDKN2AB* (because of its similarity to both human *CDKN2A* and *CDKN2B* loci), which is mutated in many human cancers, including melanomas [25]. The *Xiphophorus* melanoma, which can be initiated by simple crossings and the well-defined signaling pathways governing its tumor growth and progression, provides a model for a comprehensive study of the molecular changes and regulatory networks underlying tumor formation involving different levels of organization from molecules to where the tumor interacts with healthy organ systems of the whole organism [37]. This should provide further insights into some basic principles of cancer biology and identify new areas for melanoma research.

Zebrafish is susceptible to various chemical carcinogens and produces many neoplasm types in various tissues, showing remarkable histopathologic resemblance to human and mammalian cancers [1]. Comparative expression profile analysis of human and zebrafish tumors revealed that both tumors shared a similar molecular hallmark, having a large number of differentially expressed genes coding for proteins involved in cell cycle/proliferation, apoptosis, DNA replication and repair, cytoskeletal organization, cell adhesion and motility, RNA processing and protein synthesis, and metastasis [29, 30]. Moreover, when microarray data from histopathologically graded liver tumor samples of humans and zebrafish were compared, a large number of genes were observed to share strikingly similar expression profiles correlating with tumor progression (Fig. 24.5.3). This study provides the first validation of a large set of genes that are associated with liver tumor and its progression in fish and human, and highlights the use of comparative expression genomics between two phylogenetically distant species for identifying biomarkers with clinical potential based on the strong association with a disease phenotype such as liver cancer [27].

The zebrafish has several excellent transgenic leukemia models. The transgenic rag2–EGFP-*mMyc* zebrafish line develops GFP-labeled T-cell acute lymphoblastic leukemia (T-ALL) that expresses the zebrafish orthologs of the human T-ALL oncogenes *tal1*/*sel* and *lmo2*, similar to the most prevalent molecular subgroup of human T-ALL [31]. These fluorescent leukemic cells allow monitoring of the pathogenesis of the disease, as it can be transplanted into irradiated wild-type adult fish and will home to the thymus to produce a pervasive leukemia. A conditional transgenic zebrafish model of myc-inducible T-cell acute lymphoblastic leukemia has also been developed using the Cre/loxP system for the breeding and maintenance of stable transgenic lethal disease models [32]. This

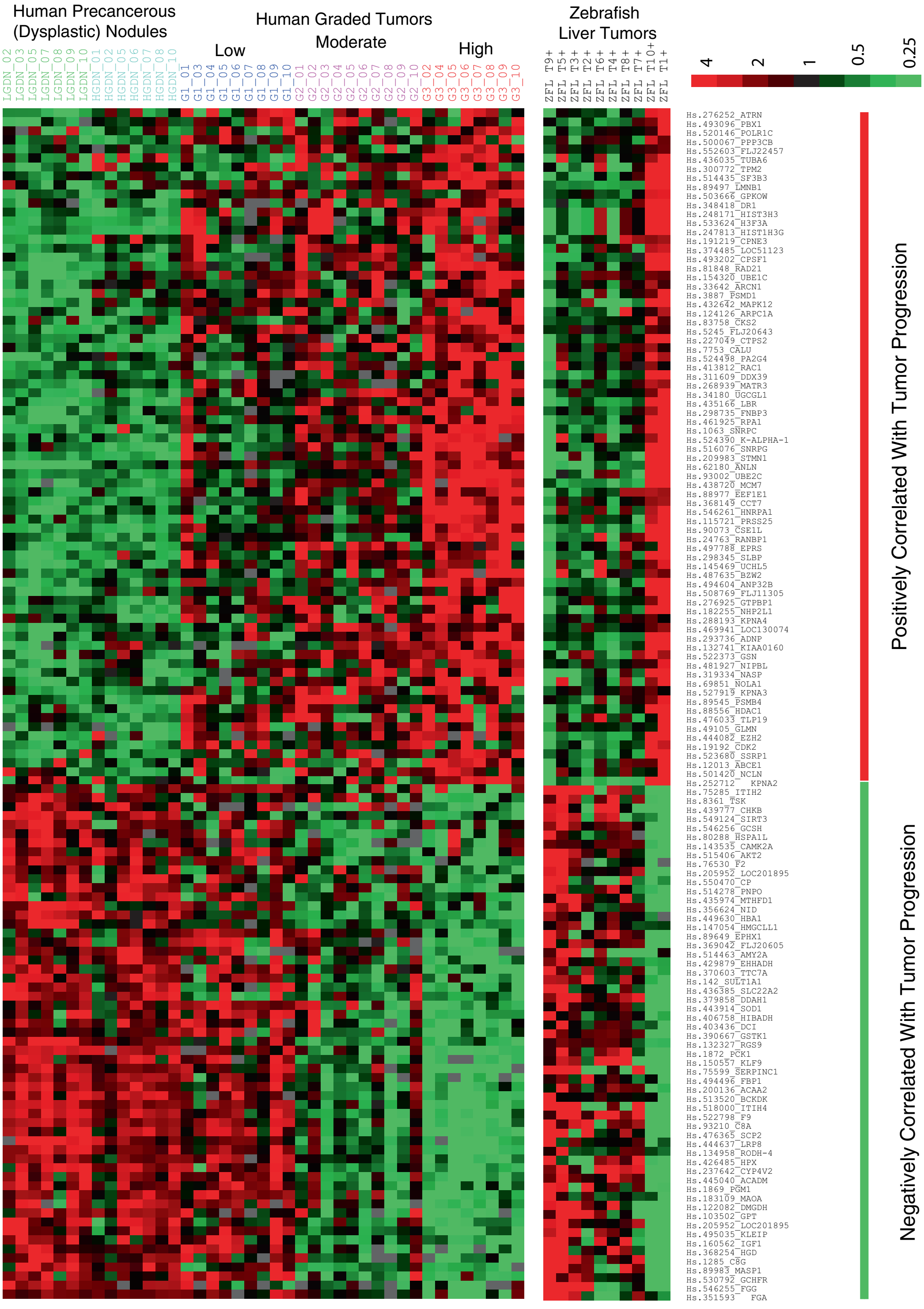

Fig. 24.5.3 Expression profiles of genes showing similar correlations, with tumor progression in both zebrafish and human liver tumors. The *color* in each cell reflects the expression level of the corresponding gene in the corresponding tissue sample relative to its mean expression level across the entire respective set of human and zebrafish tissue samples. *Gray cell* indicates missing or excluded data (*LGDN* low-grade dysplastic nodule, *HGDN* high-grade dysplastic nodule, *G1-G3* grades 1–3)

stable *rag2-loxP-dsRED2-loxP-EGFP-mMyc* transgenic zebrafish line has red fluorescent thymocytes and will not develop leukemia until *Cre* RNA is injected into the single-cell stage embryos or crossed with an activator *Cre* transgenic line. Besides T-ALL, a zebrafish model of human precursor B (pre-B) ALL, commonly caused by a t(p13;q22) chromosomal translocation resulting in a TEL AML1 (ETV6 RUNX1) fusion in pre-B cells, has also been generated by transgenesis [51]. This leukemia was transplantable to irradiated wild-type fish with pathology observable between 6 and 9 weeks after transplantation. The study showed that TEL AML1 induces the arrest of B-cell differentiation and found that the loss of TEL expression and an elevated Bcl1/Bax ratio contributed to leukemia development. This transgenic model provides the opportunity to study the various genetic events associated with *TEL-AML1*-induced leukemia, a prevalent childhood cancer. Apart from stable lines, transient expression of a zebrafish *tel-jak2a* fusion oncogene similar to that seen in human chronic myeloid leukemia, under the control of the *spi1* promoter, which is strongly active in myeloid precursors [45], and transient expression of a human *RUNX1-CBF2T1* [24], were able to generate models useful for the experimental study of blood malignancy. Likewise, a melanoma model is generated when *mitfa-BRAF*^{V600E}, the most common human melanoma BRAF mutant form (V600E) under the control of the melanocyte *mitfa* promoter, is injected into p53-deficient embryos causing invasive and transplantable human-like melanomas to develop in these animals at 4 months of age [48]. Besides transgene expression, there are several zebrafish mutants that exhibit increased tumor incidence that are useful models for cancer research, namely tumor suppressor mutants tp53 [5] and neurofibromatosis type 2 [1], in addition to *crb* mutants that display cell cycle defects and genome instability attributable to a mutation in a transcriptional regulator *bmyb* [54].

24.5.4 Modeling Human Disorders in Fish for Drug Discovery

The zebrafish disease models that are generated using forward and reverse genetics or by a chemical/environmental stressor are not only useful for studying the underlying genetic basis of a disease but also provide excellent systems for screening of genetic and chemical modifiers of a disease phenotype which can lead to the discovery of drugs and therapeutic targets along the drug development pipeline [57, 67]. Identification of the genes that are responsible for the phenotypes of disease models might lead directly to the identification of therapeutic targets. Positional cloning of ENU-induced mutant or inverse PCR cloning of insertional mutant followed by validation using morpholino knockdown or rescue experiment by transient overexpression can help to pin down potential targets. Another approach is to apply large-scale morpholino screening by systematically knocking down genes using a morpholino library in order to identify gene knockdowns that prevent or slow the development of a disease phenotype. Therapeutic targets identified by forward genetic and morpholino screens could become the focus of conventional, target-based drug discovery efforts, including in vitro high-throughput screening.

Alternatively, small-molecule screens to identify suppressors of zebrafish disease phenotypes can be initiated before the identification of a validated target. The feasibility of performing phenotype-based small-molecule screens in zebrafish has been demonstrated by the identification of chemical suppressors of the *gridlock* mutant phenotype, which is a vascular defect caused by a *hey2* mutation [49], and *crb* mutant phenotype, which is a cell cycle defect caused by a *bmyb* mutation [58]. The zebrafish mutation *gridlock* causes impaired aortic blood flow similar to aortic coarctation in humans. After screening 5,000 small molecules, two structurally related novel classes of compounds previously not known to affect blood vessel formation, which completely suppress the *gridlock* coarctation phenotype, hence enabling survival to adulthood, were identified [49]. The *crb* mutation decreased the expression of cyclin B1 that is responsible for driving cell progression from G2 phase through mitosis, thus resulting in mitotic arrest and genome instability. Approximately 16,000 compounds were screened in 16 weeks before one novel compound, persynthamide (psy 1), which suppresses bmyb-dependent mitotic defects, was discovered. Psy-treated embryos showed an S-phase delay and up-regulation of *cyclin B1* mRNA, which promotes the progression of cells through mitosis [57]. Since most cell-cycle genes are conserved between zebrafish and humans, this approach could be applied to a number of zebrafish cell-cycle mutants or cancer models to screen for cancer suppressors.These studies show that the phenotype-based whole-organism screen allows us to approach biological questions that simply cannot be addressed in vitro, such as suppression of the *gridlock* coarctation phenotype and reversal of the cardiac dilation or contractility defects in a zebrafish model of heart failure [67]. Even when an in vitro screen is available, the results obtained by whole-organism screens might be more relevant than those obtained using the in vitro screen, because physiologically relevant organismal setting may be required for the mechanisms of action of the compounds. Likewise, in vitro screens, while suited to high-throughput applications, lacks the relevant physiological whole-organism setting for toxicology, and toxicity or unwanted side effects are major reasons for the high attrition of compounds in the drug development pipeline. By using zebrafish model, in vivo toxicology can be performed earlier, if not simultaneously, in the drug discovery process at a lower cost than in a rodent model. Zebrafish has been noted to show a similar toxic response to that of mammals with regard to chemicals that cause endocrine disruption, reproductive toxicity, behavioral defects, teratogenesis, carcinogenesis, cardiotoxicity, and liver toxicity [67]. Zebrafish also responds to several drugs in a similar manner to that in humans. For example, 100 compounds was screened in a high-throughput assay developed for bradycardia in zebrafish embryos, and 22 of 23 drugs known to cause QT prolongation in humans were also found to cause bradycardia in zebrafish. In addition, known drug-drug interactions between erythromycin and cisapride and also between cimetidine and terfenadine, which lead to QT prolongation, were also detected by the assay, highlighting the fact that complex pharmacokinetic and pharmacodynamic processes occurring in humans can also be reproduced in zebrafish [38]. Moreover, with the availability of more fluorescent transgenic zebrafish, disease models that allow for easy visualization and quantification of the degree of severity of a disease phenotype, the zebrafish will become even more attractive for drug screening.

In summary, fish, especially small species such as zebrafish, medaka, and *Xiphophorus* have proved valuable in disease modeling at the molecular genetics level. Currently, the zebrafish is the most intensively studied fish model for human disorders, and it allows for disease pathogenesis, forward genetic, reverse genetic, small molecule screening, and toxicology to be performed in the same organism at higher throughput and lower cost than in rodent models. As a vertebrate, the zebrafish shares many conserved physiological and molecular features with mammals, and its position at the other extreme end of the vertebrate taxon makes zebrafish an excellent complementary model to existing mammalian disease models for comparative pathology to identify molecular conservation of disorders. Thus, it is not surprising that the zebrafish model is beginning to be used at various preclinical stages of disease modeling and drug discovery processes. Given the strong interest within the scientific community, as reflected by the setting up of the Trans-NIH Zebrafish Initiative and European ZF Models Consortium, and the many genetic screens that are ongoing in several laboratories, it is expected that the zebrafish will gain more prominence for modeling human disorders.

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Genetic Counseling and Prenatal Diagnosis 25

Tiemo Grimm and Klaus Zerres

Abstract The expanding knowledge in human genetics has led to practical applications at an increasing rate – especially in genetic counseling and genetic screening. Conventional and invasive diagnostic procedures have been complemented or entirely replaced by genetic-testing. DNA tests allow us to predict diseases and to modify risk figures. With increasing numbers of both diagnostic and predictive genetic tests available, genetic counseling is becoming more important in virtually all fields of clinical practice. The traditional areas of genetic counseling included pediatrics (assessment of children with developmental delay and dysmorphic features) and obstetrics (prenatal diagnosis). However, as the genetic basis of more and more diseases is unraveled, genetic counseling is now increasingly requested from other disciplines, including neurology, oncology, ophthalmology.

Contents

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25.1 Genetic Counseling

Genetic counseling is an important area of applied human genetics. Patients request advice or are referred by their physicians for counseling to help them to understand biological facts and the medical implications and recurrence risks of genetic diseases. As the public media and the medical literature disseminate more news about genetics, public and medical interest in genetic disease is expanding [25, 30, 31, 53, 55].

25.1.1 Origins and Goals of Genetic Counseling

Genetic counseling is a communication process that deals with human problems associated with the occurrence or the risk of occurrence of genetic disorders in individuals or families. This process involves an attempt by one or more appropriately trained specialists to help an individual or family to:

1. Comprehend the medical facts, including origin, diagnosis, and probable course of a given disorder and the available management

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2. Appreciate the way heredity contributes to the disorder and the risk for recurrence in specific relatives
3. Understand the alternatives for dealing with the risk of recurrence

25.1.1.1 Definition of Genetic Counseling

Genetic counseling refers to the totality of activities that:

1. Establish the diagnosis
2. Assess the recurrence risk
3. Communicate the likelihood of recurrence for the patient and family
4. Provide information about the many problems raised by the disease and its natural history, including the potential medical, economic, psychological, and social burdens
5. Provide information regarding potential reproductive options, including prenatal diagnosis
6. Provide for referral of patients to appropriate specialists

The range of problems and questions arising during genetic counseling is wide. Generally, only 30–50% of patients and families turn out to have classic genetic diseases such as monogenic diseases or chromosomal aberrations. Many consultations deal with various birth defects, mental retardation, delayed development, dysmorphic-looking children, short stature, and similar problems, which may or may not have a genetic cause.

Genetic counseling is usually carried out by physicians who have specialist training in medical genetics. They usually work in medical genetics clinics and carry out much of the information gathering, counseling, and follow-up. In several European countries trained social workers, or genetic counselors in the USA, are also involved in genetic counseling. In fact, in the USA nonphysician genetic counselors have an increasingly important role. Genetic counseling services are often provided by post-baccalaureates (largely women) with 2 years' specialized training in medical genetics. These persons usually work as team members with clinical geneticists or with obstetricians, and increasingly with commercial genetic-testing laboratories.

Genetic counseling is increasingly applied in an interdisciplinary manner. Important examples are seen in the case of neuromuscular disorders. A rational diagnostic approach in this field should be discussed with specialist neurologists, pathologists, and geneticists. In clinical oncology, e.g., in families with familial breast cancer or hereditary nonpolyposis colorectal cancer (HNPCC), close contact of involved experts trained in different fields and regular interdisciplinary exchange are essential. The role of genetic counseling in familial or potentially familial cancer in particular has gained significantly in importance owing to the identification of more and more cancer-predisposing genes. With increasing knowledge about the complex genetic basis of diseases, clinical geneticists attach great importance to the provision of information and to the medical care of affected families.

25.1.1.2 Origins of Genetic Counseling

Genetic counseling is medically oriented. It is considered inappropriate to include eugenic considerations. Couples asking for advice are encouraged to make their own reproductive decisions. Even though under 2% of human afflictions of medical relevance follow a monogenic pattern of inheritance, an increasingly large number of common diseases have a genetic component that can be assessed with genetic tests that in most cases do not yet have clinical utility. There are good medical and genetic reasons for genetic counseling, which needs to be provided in humane and ethically responsible ways.

25.1.1.3 Indications for Genetic Counseling

Important questions in genetic counseling are as follows:

25.1.1.3.1 Birth of a Child with a Congenital or Developmental Disorder

If a child with birth defects or developmental delay is born to healthy parents, the most common question concerns the risk to other children. For example, the recurrence risk for carrier status of balanced chromosomal translocations is an important issue (see Sect. 25.2.1.3). In order to answer this question, a correct diagnosis of the child's condition must be established.

25.1.1.3.2 A Parent Is Affected

The diseases of a parent are a frequent concern leading to the question of recurrence risk in the parents' children. If the consultand is affected or even pregnant, the question of the impact of pregnancy on the course of the disease is another important issue.

25.1.1.3.3 Diseases or Developmental Disorders in Relatives of a Consultand

As a rule, this situation requires communication with multiple members of a family. Formal genetic aspects are often crucial. Careful pedigree analysis and risk calculations frequently yield relatively low risks. Occasionally an increased risk can be ruled out entirely. The issue of predictive testing for late-onset diseases in unaffected but at-risk family members must be considered carefully.

25.1.1.3.4 Age Risks

Owing to an increasing number of pregnancies in older women in western societies, the demand for counseling on this topic is increasing. Most consultands are aware that advanced maternal age increases their risk of having a child with a chromosomal disorder. Even though the age-related risk for trisomy 21 only amounts to less than 1% at age 35, and to less than 5% at age 45, these age-related risks are usually overestimated and considerably lower than perceived by the counselees [3]. Maternal serum screening for aneuploidy and ultrasound (see Sects. 25.2.1.1 and 25.2.2.2) have become part of routine prenatal care in many countries. Information on the limitations and risks of antenatal diagnostic measures should be provided by trained professionals. Elevated paternal age increases the risk for point mutations, but again, this risk is relatively low (1% or less) [71].

25.1.1.3.5 Recurrent Pregnancy Loss or Stillbirths

Approximately every eighth pregnancy ends in miscarriage, the cause of which remains unknown in many cases. Chromosomal aberrations are the most frequent finding among early pregnancy losses, with triploidy, trisomy 16, and monosomy X accounting for around 10% of spontaneous abortions prior to week 12. However, the vast majority are lethal conditions which occur spontaneously, with usually low recurrence risks that mean routine chromosome analysis of tissues from spontaneous pregnancy loss is of limited clinical value. Parental chromosome analysis should, however, be carried out after three unexplained abortions in order to exclude carrier status for balanced chromosomal translocations. Such translocations are found in about 5% of couples with two or more previous abortions. In addition to frequent and recurrent pregnancy losses, the family history in translocation carrier families includes births of both clinically normal individuals and children with birth defects. Male carriers may have oligo-astheno-teratozoospermia syndrome with fertility problems.

25.1.1.3.6 Teratogenic or Mutagenic Effects

The question of a child's risk attributable to exposure to exogenous factors such as drugs, radiation, alcohol, or prenatal infections during pregnancy (teratogenic risks) is a frequent indication for genetic counseling [24]. Again, potential risks for adverse effects are often overestimated. The number of drugs with proven teratogenic or mutagenic effects is rather small [39]. Risks inherent in radiation exposure are also often overestimated.

In order to mitigate excessive concerns of pregnant women, accurate information should be provided. A history of drug and alcohol consumption should be carefully evaluated and discussed. It has become clear that even low doses of alcoholic beverages can have adverse effects on intrauterine development. Because there is no known safe amount of alcohol consumption during pregnancy, the American Academy of Pediatrics recommends abstinence from alcohol for woman who are pregnant or who are planning a pregnancy [1]. Prenatal infections represent complex situations which require interdisciplinary management.

25.1.1.3.7 Consanguinity

First cousins and more remote relatives who are contemplating marriage occasionally ask for advice about the risks of having children with inherited diseases. Marriages between first cousins are illegal in 30 states of the United States of America. Consanguinity definitely increases the risks of disease caused by homozygosity for recessive genes (Chap. 13), but the absolute risks are relatively low in families without evidence for genetic disorders. It has been estimated that the rate of various diseases, birth defects, and mental retardation among offspring of first-cousin matings is at most twice the background rate faced by any given couple; thus, the chance that a child from such a mating will be normal is around 93–95%. These risks are still lower for more remote consanguinity, and thus are difficult to separate from the population background rate for such disorders [15]. There are no additional risks for offspring of a normal person married to an unrelated person when one partner has consanguineous parents. On the other hand, the risks are considerable for children of incestuous matings involving first-degree relatives, such as sib-sib and father-daughter matings; there may be up-to a 50% risk that such a child will be affected by severe abnormality, childhood death, or mental retardation [2, 33, 58]. It is remarkable that defects in offspring of consanguineous matings mostly manifest themselves as nonspecific congenital malformations, childhood death, and mental retardation rather than as well-defined autosomal-recessive disease entities. However, detailed searches for the many different recessive inborn errors of metabolism have rarely been carried out, and it is likely that a significant proportion of childhood deaths involve such unrecognized recessive disorders.

25.1.2 Genetic Diagnosis

Accurate diagnosis of a genetic disease using all the modalities of modern medicine is the cornerstone of genetic counseling. Diagnostic accuracy is emphasized, since similar phenotypes may sometimes have different modes of inheritance or may not be inherited at all. The family history is important, because a clear-cut pattern of inheritance such as that in the case of autosomal-dominant traits often provides the basis for counseling when a definitive diagnosis may not be clear. Previous medical and hospital records are helpful in arriving at a correct diagnosis. Since many genetic diseases are associated with somewhat characteristic facial features, inspection of photographs of family members may be helpful. Chromosomal examinations are frequently required in the diagnosis of complex birth defects. Since many genetic diseases are rare, even trained medical geneticists and specialists in a given field of medicine may have difficulty in arriving at an accurate diagnosis. They cannot be equally knowledgeable about all genetic diseases in every area of medicine, but do need to be aware of recent monographs and computerized expert systems to establish the appropriate diagnosis. The Catalog of Mendelian Traits in Man published by McKusick [43] and its computerized version OMIM are helpful [51]. It is essential in clinical genetics to have a good and up-to-date library and to know how to consult the current literature. Because of the rapid expansion of knowledge, utilization of the journal literature, as opposed to textbooks and monographs, is more important in clinical genetics than in most fields of medicine. This is facilitated today by computerized searches with such syndrome identification programs as POSSUM [52] or the London Dysmorphology Database [42]. However, their proper use requires knowledge, experience, and a critical mind.

Parents whose infants are stillborn or die in the neonatal period often request genetic counseling about recurrence risks. Usually little or no information is available on the specific abnormality of the stillbirth, since no pathological or other diagnostic study has been conducted. It has been recommended that as a minimum a gross autopsy, photographic and radiographic records, and bacterial cultures be performed in all cases of stillbirth or early neonatal death. Such information is essential for subsequent genetic counseling [27]. A definitive diagnosis often cannot be made even by experienced specialists owing to the enormous complexity of development and its possible perturbation by frequently unknown genetic, epigenetic, and environmental factors. Fewer diagnostic uncertainties occur with monogenic diseases than with various birth defects. However, even in this area the growth of the McKusick catalog over the years, i.e., from 866 defined gene loci in 1971 to 11,867 in April 2009 [51] attests to the rapid expansion of knowledge in this field.

25.1.2.1 Molecular Diagnosis

As more genes are cloned and the molecular nature of mutations causing disease becomes known, direct DNA diagnosis of genetic disease is increasingly possible. Unlike indirect diagnosis using linked DNA markers, a family study is not required for direct genetic-testing. However, the exact nature of the mutation to be detected must usually be known. It is good practice to isolate and store DNA from patients with genetic diseases in DNA banks for appropriate future analysis. The resultant information may be of great help in counseling family members in the future.

If the gene in question has been isolated, but the mutation remains unknown, a closely linked or intragenic DNA polymorphism (RFLP, VNTR, CA repeat, SNIP) can be used to track the mutant gene by cosegregation of the marker when the exact nature of the mutation is unknown. The possibility of nonallelic genetic heterogeneity must be kept in mind, however, as this may be a source of error.

Where neither the gene nor its mutations have been defined, linked DNA polymorphism can still be helpful. However, as with intragenic DNA polymorphisms, the exact "phase" of the DNA marker in relation to the disease gene must be known. In such situations, a sufficient number of family members is often not available, precluding the reliable application of indirect genetic-testing. Problems of genetic heterogeneity and possible recombination between the linked DNA marker and the disease gene render the indirect approach less than 100% accurate.

The examples of Duchenne and Becker muscular dystrophy illustrate these principles. About two-thirds of all Duchenne and Becker mutations are caused by deletions and about 7% by duplications of the X-linked dystrophin gene [16, 70]. Direct DNA diagnosis for these deletions and duplications is relatively simple [e.g., by in situ hybridization (see Sect. 3.4.4) and multiplex ligation-dependent probe amplification (MLPA; see Sect. 3.4.3.4)] [56, 57]. These techniques can be used for prenatal diagnosis and for carrier detection. If a deletion cannot be detected, screening for one of the many different missense point mutations that can cause the disease is not always practical. However, indirect DNA diagnosis using DNA markers of the dystrophin gene combined with a family study can be attempted. Since the gene is very large, intragenic crossovers are relatively frequent (about 5–10% [29]). This problem can be overcome by using flanking markers on either side of the disease gene. In view of these complexities, measurement of creatine phosphokinase levels that are elevated in Duchenne muscular dystrophy carriers (but less so in Becker muscular dystrophy carriers) may aid further in carrier detection [35].

Genetic advice concerning multifactorial conditions, such as many birth defects, common diseases of late-onset, and major psychoses, lacks the precision that can be achieved in counseling patients with Mendelian disorders. Empirical risk figures need to be used based on the frequency of recurrence of the disease in many affected families. These recurrence risks are usually lower than those in the Mendelian diseases, ranging between 3 and 5% for many common birth defects, such as neural tube defects, cleft lip, and cleft palate. Risks to first-degree relatives (sibs, parents, and children) for common diseases such as hypertension, schizophrenia, and affective disorders are in the order of 10–15%.

In contrast to monogenic disorders, the risk in multifactorial disorders increases with an increasing number of affected relatives and with increasing severity of the disease; risk is usually negligible for distant relatives.

A careful search for the rare monogenic variety of a disease that appears multifactorial must always be kept in mind. For example, rare patients with gout may have an X-linked monogenic disease caused by hypoxanthine-guanine phosphoribosyl-transferase deficiency. Alternatively, their gout may be caused by autosomal-dominant phosphoribosylpyrophosphate synthetase deficiency. Among male patients under the age of 60 years with coronary heart disease about 5% have familial hypercholesterolemia – an autosomal-dominant trait.

Transmitted chromosome abnormalities, such as translocations, do not segregate by Mendelian ratios, and counseling must be based on empirical risk figures.

Citing percentage figures of absolute recurrence risk is more meaningful to a family than relative risks based on the relative likelihood of the disease compared with that in the general population. A 100-fold increase in risk for a condition that occurs in the population with a frequency of 1:10,000 carries an actual risk of only 1:100 – a small recurrence risk. For Mendelian conditions the recurrence risks are fixed regardless of whether several affected children or none have previously been born. Chance has no memory! In multifactorial diseases such as congenital heart disease or cleft lip and palate, the following considerations apply: if two or more first-degree relatives are affected in a given family, more disease-producing genes are likely to be involved in that family, and the risk for future offspring becomes higher than the usual 3–5% [30]. However, differentiation from an autosomal-recessive variety of the condition with a recurrence risk of 25% may sometimes be difficult. Detailed discussions of the approaches to genetic counseling and risk data for many different types of diseases can be found elsewhere [30].

25.1.2.2 Heterozygote Detection

It is particularly important to detect heterozygotes in sisters of boys affected with X-linked recessive diseases, such as hemophilia and Duchenne- or Becker-type muscular dystrophy. Regardless of their partner's genetic constitution, there is a 50% risk that the sons of female heterozygotes will be affected. In contrast, autosomal-recessive diseases become evident only when both parents are heterozygotes; a heterozygote sib of an affected patient must mate with another heterozygote for the disease to occur. The probability that an unrelated mate of a person who is a carrier of an autosomal-recessive disease carries the same mutation is usually quite low.

Biochemical and functional tests must be carefully standardized on normal subjects and obligate heterozygotes before they are applied for individual carrier identification. The detection of heterozygotes is accurate and simple in the hemoglobinopathies. An increasing number of heterozygote conditions for various autosomal enzyme deficiencies (e.g., hexosaminidase deficiency causing Tay-Sachs disease) can be recognized by enzyme assays as well as by DNA tests [34]. However, measuring mutant enzyme activity in a group of heterozygotes will often demonstrate a wide range of enzyme activity in such subjects. Thus, both low and high enzyme activity may be found in different heterozygotes for the same mutation. Such subjects can reliably be characterized as heterozygotes only by DNA testing. In contrast, enzyme levels, which may be useful genetically and clinically, will not be demonstrated by DNA tests.

If there is overlap in laboratory results of tests for enzyme activity between normal subjects with a low value and carriers with a high value, the significance of an identical laboratory result in various individuals may differ depending upon the a-priori probability of the tested person being a carrier. Tests that are excellent for carrier detection in sisters of males affected with X-linked diseases may give too many false positives in screening studies of extended kindreds or particularly in the general population, where the probability that the tested subject is a carrier is low.

For example, 5% of the average female population would be identified as having a high risk of being carriers of hemophilia using the same standards that identify sisters of hemophilic boys as heterozygotes.

In some of these situations additional statistical techniques may be helpful for refinement of a genetic prognosis. Assume a woman's brother is affected with an X-linked recessive condition; a maternal uncle is also affected. She therefore has a 50% risk of being heterozygous. Assume that she already has two nonaffected sons, and that a test for heterozygote detection is not available. The information that her two sons are normal reduces her chance of being a carrier. Alternately, such a woman may have a negative result for a test that detects 90% of heterozygotes. In this case her risk of being a carrier is very low. Special statistical methods are needed for calculation of the exact recurrence risks in such complex situations [47, 72] (Appendix).

The increasing availability of DNA markers facilitates carrier diagnosis in X-linked diseases. In any given diagnostic problem the simplest and most direct approach should be selected, which increasingly is direct DNA diagnosis. However, use of biochemical tests is often necessary and complementary. Information from DNA markers can be combined with biochemical tests and pedigree information. The fragile X mental retardation syndrome is a good example of how DNA diagnosis has become very helpful. Since the number of CGG triplet repeats responsible for the syndrome can be easily assessed, DNA diagnosis can discriminate between affected males who have a large triplet expansion (i.e., more than 200 CGG repeats) and normal transmitting males who carry a premutation (i.e., between 55 and 200 CGG repeats). The nonaffected carrier daughters of such transmitting males will have only moderate expansion of CGG triplets, if any at all. They are usually not affected, but do carry an increased risk for their sons. For women who have inherited the expansion from their mothers and carry a full mutation there is a 50% risk of at least mild learning difficulties.

Carrier detection for mutations for autosomal-recessive disorders is often required for close relatives of affected children (i.e., healthy siblings, aunts, uncles, nephews, and nieces). Depending on the disease and the detection rate, sensitivity and specificity of possible heterozygote tests, genetic counseling is essential for discussion of the limitations and possible consequences of the available tests and the assessment of possible risks. The availability of a mutational analysis of the affected relative is essential for risk assessment.

Owing to the possibility of compound heterozygosity for mutations a prediction of possible consequences can be difficult or even impossible and might be an important point in genetic counseling. A good example is cystic fibrosis (CF). CF is caused by mutations in the gene on chromosome 7 encoding the CF transmembrane conductance regulator (CFTR) gene, which regulates sodium transport and potassium channels and conducts chloride across the cell membrane. The primary pathophysiology that leads to chronic illness in CF patients is mucosal obstruction of exocrine glands. The most common mutation in the CFTR gene is ΔF508; however, over 1,400 other mutations have also been reported (see www.genet.sickkids.on.ca/cftr/app). The ΔF508 mutation has been reported in a compound heterozygous state with several other mutations, so that reliable genotype-phenotype correlations can be made for some compound heterozygotes. For example, in one study patients with the genotype R117H/ΔF508 significantly differed from age-matched and sex-matched ΔF508 homozygotes because they were older at the time of diagnosis, were more likely to have pancreatic sufficiency, and had lower sweat chloride concentrations [13]. For other compound heterozygotes there are fewer reports in the literature, making prediction of phenotypic consequences difficult.

Population screening, for example for hemoglobinopathies in populations of African origin or Tay-Sachs disease and some other recessive conditions in Jewish communities, addresses difficult ethical issues regarding abortion. Religious tradition can impact on decision making around genetics testing and requires a detailed understanding of culturally sensitive ethical care.

25.1.3 Recurrence Risk

Genetic risks in Mendelian diseases are clearly defined and depend on the specific mode of inheritance. The actual clinical risks to the patient, particularly in autosomal-dominant inheritance, depend upon variable penetrance and expression, especially in late-onset disorders. Patients are more interested in the actual recurrence risk of the clinical feature than in the formal genetic risks alone. In diseases with decreased penetrance the actual recurrence risk is lower than the formal risk of genetic transmission. For example, an offspring's risk of an autosomal-dominant disease with 70% penetrance is 35% rather than 50% ($0.5 \times 0.7 = 0.35$). The risk declines with late-onset diseases, as a person remains unaffected beyond the age at which the disease first becomes manifest.

The McKusick catalog is available as a computerized data base on-line (OMIM) and is updated continuously [51]. In addition, keywords can be introduced, so that more comprehensive cross-searches that also allow access to abstracts of recent articles may be possible and helpful.

25.1.3.1 Recurrence Risk for Multifactorial Conditions

For most complex "common" diseases with a polygenic basis, empirical risks for close relatives exist. These risks usually increase with an increasing number of affected relatives and with worsening severity of the disease. With increasingly remote relationship to the affected person the risk usually decreases. In several disorders with different incidences depending on the sex of affected persons, recurrence risks for close relatives can be different, with the higher risk for persons whose gender has the lower incidence. This phenomenon is known as the Carter effect: the incidence in relatives is higher when the index case is of the less commonly affected sex. For example, in pyloric stenosis the incidence is highest in the sons of affected women and lowest in daughters of affected men.

25.1.3.2 Reproductive Options and Alternatives

If a couple decides that the risks of having children of their own are too high, several options besides contraception should be discussed. Adoption is becoming less practicable because fewer babies are available. Artificial insemination by a donor (heterologous insemination) may be acceptable for some couples to avoid autosomal-recessive disease or autosomal-dominant disease contributed by the male partner, but often raises difficult ethical questions.

25.1.3.3 Predictive Testing

Predictive testing in families in which genetic diseases may not be obvious at birth but may manifest sometime later is an important reason for genetic counseling. For some conditions the identification of gene carriers may be lifesaving if followed by suitable therapy. A sib of a patient with Wilson disease has a 25% chance of being affected, but may be too young to exhibit overt symptoms. Children of patients with the autosomal-dominant condition familial adenomatous polyposis have a 50% chance of being gene carriers, and if they inherit the mutant *APC* gene they have a nearly 100% risk of malignant transformation in one of the many polyps seen in this condition. In general, attempts should be made to examine relatives when a genetic condition causes serious preventable or treatable diseases. Information should always be given to a relative who is at-risk by the family members themselves.

Predictive testing in genetic diseases of late-onset has been performed for many years in Huntington's disease, since the identification of the underlying mutation in 1993. Much literature is available with a focus on the psychological impact for tested persons at-risk and their families [31]. Huntington's disease is an example of a disease with no relevant therapeutic options for persons who test positive, while therapeutic options themselves are in the focus of research in hereditary cancer syndromes such as polyposis coli, Lynch syndrome, and familial breast cancer. Growing data collections including data on affected families and systematic studies about the experience and follow-up with different therapeutic options have made genetic counseling one of the important keystones in family care of these families [66].

An interdisciplinary concept in genetic counseling is useful for many diseases, which has been shown for many years in Huntington disease with detailed guidelines for the application of predictive testing [65].

25.1.4 Communication and Support

The meaning of genetic risks must be conveyed in terms that can be understood by patients. The probability that 3–4% of all children of healthy parents are born with birth defects or possible genetic diseases should be communicated as a baseline risk figure that applies to the general population. There may be problems in communicating the extent of uncertainty. For example, with a sporadic case of an undiagnosable birth defect the risk might be zero if the disease is nongenetic, 2–3% if there is a multifactorial etiology, and 25% if it is caused by an autosomal-recessive trait. Therefore, an empirical risk is based on the probability of the various possibilities. Such a risk figure might be 5% on the assumption that monogenic recessive varieties of this birth defect tend to be rare. Both the physical and the emotional burden of the disease must be discussed. It is well-known that very severe conditions that are invariably fatal in early life often place a less severe burden to the family than do those associated with chronic or slowly progressive diseases. Various reproductive options and alternatives need to be discussed.

Since problems may be complex and may prove to be emotionally difficult for the counselee, it may be necessary to have several counseling sessions. In any case, the counselor should provide a written summary of the counseling session using lay language.

25.1.5 Directive Vs. Nondirective Genetic Counseling

In the tradition of paternalistic medicine, which is now obsolete, occasional physicians are still accustomed to giving directive advice for or against future pregnancies. The practice of medical genetics has clearly evolved in the direction of nondirective counseling. Some of this nondirectiveness may have sociological reasons. When genetic counseling began in the United States some 40 years ago, it was usually carried out by nonphysician geneticists, who lacked the medical profession's tradition of giving directive (paternalistic) advice. Nondirective genetic counseling mirrors recent trends to increasing patient autonomy. Since each family is unique and reactions to risks vary, nondirectiveness fosters mature decision making. However, absolutely neutral advice is rarely possible or even desirable. The person or family requesting advice usually wants and needs more than a computer-like professional who only dispenses facts. The counselor may unconsciously emphasize the more positive or the more frightening aspects of a given disease. These feelings tend to affect the counseling process directly or indirectly, often through nonverbal clues. Not all couples have the necessary educational background and social or emotional maturity to make fully informed decisions. In addition, many couples expect the medical geneticist, whom they consider an experienced expert on their disease, to assist them in arriving at a decision they can live with. "What would you do if you were in our position?" is a frequent question from counselees, regardless of background. However, since a couple's economic situation, religious affiliation, and cultural background (for example) may differ substantially from that of the counselor, the counselor's choices for his/her own circumstances would usually not be appropriate. Reproductive decisions differ widely among individual couples even if the genetic facts and the disease burden are identical.

As predictive testing for late-onset diseases becomes increasingly possible, nondirective advice no longer applies when a disease can be prevented or treated by appropriate measures. The relevant medical recommendations on preventing and treating the disease must be given (including all options).

25.1.6 Assessment of Genetic Counseling and Psychosocial Aspects

Most professionals engaged in genetic counseling agree that counselees should achieve sufficient understanding of the medical significance and social impact of the disease to allow them to make appropriate decisions. Some observers have measured the success of genetic counseling with regard to increased recurrence risks in a further pregnancy by subsequent reproductive behavior. If more couples with a high risk (>10%) than couples with a low risk were deterred from reproduction, genetic counseling would be considered to have been successful. This result has in fact been noted in several studies. Such a narrow end-point is considered an inadequate evaluation of genetic counseling. It would be better to know whether full sharing of information and comprehension of the disease and its recurrence risks have been achieved, and whether all needs for information and psychological and social support have been met.

Various studies of genetic counseling agree that many patients are confused about recurrence risks and do not fully understand the nature of the disease. In the late 1970s a large study was carried out by a group of sociologists in 47 genetic counseling clinics in the United States; 205 counselors and over 1,000 female counselees were involved [61]. Many different conditions were included, and both counselors and counselees were questioned about their experiences and assessment of the counseling process. The results showed that counselors tended to emphasize recurrence rates during the counseling sessions, while counselees were often interested in causation, prognosis, and treatment of the disease – an area which, according to the counselees, was seldom discussed as fully as desired. While both counselors and counselees were generally more interested in the medical and genetic aspects of the consultation, counselees occasionally had psychosocial concerns which were not addressed by the professionals.

This study found that 54% of counselees who were given a risk level and 40% of those given a diagnosis were unable to report these data shortly after counseling. This failure to correctly memorize risk or diagnosis occurred regardless of whether MDs, PhDs, or genetic counselors had carried out the counseling. It was unrelated to the experience of the counselor. Counselors with many years of experience had no better results than more recent graduates. Several other studies have yielded results that are substantially better, but by no means perfect [21]. Usually, but not always, education is found to be correlated with a better level of understanding.

Genetic counseling services have been used more extensively by families with good educational backgrounds than by less advantaged population groups. Couples who are motivated to learn about the disease and the risk of its recurrence are more likely to be affected in their reproductive decisions by the information provided than those who have been referred and are not always certain about the purpose of the genetic consultation. Thus, self-referred patients also tend to have better comprehension of genetic counseling information.

Another study investigated perception of counseling information [40, 41]. Perception of recurrence rates was often not used by the counselees in the probabilistic sense represented by the figures given. Percentage risks were more frequently perceived as binary, i.e., even with lower risks it was believed that the disease either would or would not occur, with all the attendant fears of recurrence. Parents were then overwhelmed by multiple uncertainties, such as how to make reproductive choices, how others would react to their decision, what it would mean to have an affected child, and whether they would be able to fulfill their role as parents. Such perceptions appeared more important for decision making than the actual facts of diagnosis, prognosis, and risk. These data show that there is often a discrepancy between the mind-set of the scientifically oriented counselor and the reflection processes of the counselees, who find it difficult to deal with probabilistic information. Bridging this gap is a real challenge.

Genetic counseling – as currently practiced in most countries – places less specific emphasis on emotional aspects than "counseling" activities in other areas, such as psychological and marriage counseling. Some observers have recommended that more attention be given to the psychodynamic aspects of genetic disease [8, 20, 36]. If there are deep-seated psychological problems, referral to a psychiatrist or psychotherapist is the most appropriate course of action. An empathic and understanding approach to families with awareness of the many social and psychological aspects of the disease and support in these matters, however, needs to be encouraged. Genetic counseling is more than mere diagnosis, risk assessment, and factual dispensation of information.

There are imperfections in the genetic counseling process as currently practiced. Nevertheless, most educated counselees who receive definite information about the matters troubling them usually appear to be satisfied. The majority of counselees with low risks are relieved to find that their actual risks are much lower than they had feared.

The interaction of patients and professionals in any encounter has many variables, and scientific study of this process is difficult. Nevertheless, genetic counseling, as a new field, demands further investigation of the process, its psychosocial effects, and outcomes, so that optimum results can be worked out. Controlled studies comparing patients who received counseling with those with a similar disease who did not would be interesting.

25.2 Prenatal Diagnosis

In 1956 Edwards discussed for the first time the possibility of "antenatal detection of hereditary disorders" [17]. The first use of amniotic-fluid examination in the diagnosis of genetic diseases was reported by Fuchs and Riis in 1956 [26]. They were able to determine the fetal sex from cells found in amniotic-fluid based on the presence or absence of the Barr body, the inactivated X-chromosome.

The improvement in cytogenetics and in somatic cell genetics led to the introduction of prenatal diagnosis in the late 1960s [19]. In 1966 Steele and Breg showed that cultured amniotic-fluid cells were suitable for fetal karyotyping [63]. In 1968 Nadler reported one of the first cases of trisomy 21 diagnosed after amniocentesis [48].

In cases where prenatal diagnosis is being considered in genetic counseling, several risk factors for the unborn child must be examined:

1. Family history of the pregnant woman and her pregnancy history
2. The possibility of consanguinity, depending on the ethnical origin
3. Exposure to mutagenic and teratogenic agents
4. Use or abuse of medicines and drugs

25.2.1 Indications for Prenatal Diagnosis

In most cases prenatal diagnosis is performed with the aim of diagnosing or excluding a serious disease or condition, and may be followed by termination of pregnancy. In this situation prenatal diagnosis is usually requested if there is a known risk for a clearly defined genetic disorder, which can be examined in the fetal cells, in the amniotic-fluid, in the fetal blood, or in the morphology or the skin of the fetus. Also some basic factors should be taken into consideration [30]:

1. Severity of disorder
2. Possibility of treatment
3. Acceptance of termination of an affected pregnancy
4. Availability of an accurate prenatal diagnostic test
5. Assessment of the genetic risk

In a second group, prenatal diagnosis is performed with the aim of diagnosing a fetal condition prenatally in order to start a therapy immediately after birth or even prenatally. An example is adrenal hyperplasia. Prenatal diagnosis may also be helpful to define the risks of prenatal infections.

The different problems of prenatal diagnosis require technical experience and proven authority, and also early and efficient cooperation between medical specialists in many different fields of activity.

25.2.1.1 Risk Assessment for Chromosomal Disorders

25.2.1.1.1 Maternal Age

The most common indication for prenatal diagnosis is above-average maternal age. The chief condition for which children born to women who become pregnant

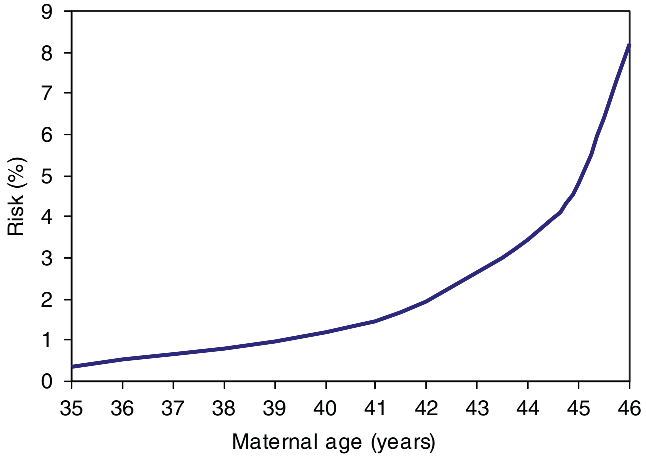

Fig. 25.1 Approximate incidence of trisomy 21 at time of amniocentesis (16 weeks). (Data after [23])

when already at a relatively advanced age are at-risk is Down syndrome (trisomy 21; Fig. 25.1).

There is no standard criterion for determining at what age a pregnant woman should be offered the option of invasive prenatal testing for fetal chromosome analysis. In most countries amniocentesis is offered somewhat arbitrarily to women aged 35 years or over. In the USA courts have considered a physician to be negligent if he fails to offer prenatal diagnostics to older mothers. With the advent of high-resolution ultrasound and first-trimester maternal serum screening, age has become a less important indication for invasive prenatal diagnosis.

About 8.1% of all trisomy 21 cases are caused by paternal nondisjunction in meiosis I or II [32]. However, in these cases no correlation with advanced paternal age has been reported.

25.2.1.1.2 First-Trimester Screening

First-trimester screening facilitates noninvasive risk assessment for certain chromosomal disorders. Assuming a 5% false-positive rate, the detection rate is 30% for age-based screening (age cut-off at 37 years). If nuchal translucency measurement and maternal serum concentrations of free beta-human chorionic gonadotroplin (hCG) and pregnancy-associated plasma protein A (PAPP-A) are included, the detection rate increases to about 90% of all pregnancies with trisomy 21 (Down's syndrome; see also "Maternal Blood Sampling" in Sect. 25.2.2.2). The increased predictive value of these integrated noninvasive tests seems to be resulting in a decline of invasive prenatal diagnosis procedures. At present, most institutions do not perform abortions for Down syndrome based on the results of noninvasive tests only, but require in addition confirmation by amniocentesis.

25.2.1.2 Previous Aneuploidy

A previous child with Down syndrome or other autosomal trisomy slightly increases the risk of recurrence. The recurrence risk for Down syndrome is 0.5–1% higher than the mother's age-related risk. Maternal germline mosaicism may be a possible explanation for this increased risk.

25.2.1.3 Parental Chromosomal Rearrangements

Balanced parental chromosomal rearrangements (translocations or pericentric inversions), which are relatively frequent in the normal population (frequency: 1:500), can lead to variable risks of offspring with chromosomal syndromes. These risks do not correspond to those expected from chromosomal segregation patterns, but are based on empirical data, presumably because of selection against unbalanced gametes [64]. The risk for translocation trisomy 21 is about 15% when the mother is the carrier and only 1–2% if the father is the carrier of a Robertsonian translocation involving chromosome 21 (e.g., t14q21 and t21q22q) [22]. The risk is 100% if one of the parents carries a 21q21q Robertsonian translocation. In reciprocal translocations the risk of future affected offspring is significantly higher (~20%) if ascertainment occurs via an affected live offspring as opposed to ascertainment by way of recurrent abortions (5%) [64]. More extensive unbalanced duplications/deletions (3–6 chromosome bands out of a total of 200) are associated with a lower recurrence risk (9–16%) than those with duplications/deletions affecting only 1 or 2 bands (34%). Presumably larger defects are often not viable and the affected fetuses are aborted spontaneously prior to amniocentesis. Possible recurrence risks have to be assessed individually.

25.2.1.4 Family History of a Monogenic Disorder

In many families monogenic disorders have risks of 25% (autosomal-recessive and X-linked with a 50% risk for boys only) or of 50 % (autosomal-dominant) in sibs of affected children. The accurate diagnosis of a mutation is possible through DNA analysis of fetal cells, in the same way as in postnatal genetic-testing. Therefore molecular prenatal diagnosis is now available for a large and ever-increasing number of monogenic disorders. If direct mutation analysis is not possible, prenatal diagnosis may be offered using linkage analysis with polymorphic DNA markers. However, such analyzes are often complicated by family availability, crossovers, allelic heterogeneity, etc.

It is the exception for prenatal diagnosis to be requested in autosomal-dominant diseases.

25.2.1.5 Neural Tube Defects

Amniocentesis (not chorionic biopsy) for amniotic-fluid AFP is carried out in women at high risk, such as those with previously affected children, or following a confirmed maternal high blood AFP level. The empirical risk for neural tube defects after the birth of an affected child is about 5%. It rises to more than 10% if the parents have two affected children. Ultrasound is very efficient in these circumstances. Other markers in amniotic-fluid (e.g., increases in acetyl cholinesterase) may provide further clues to the presence of neural tube defects and other anomalies.

25.2.1.6 Psychological Indications

Each prenatal investigation can refer only to a defined genetic or teratogenic risk. If no such defined risk is present, from the viewpoint of medical genetics parents' fear of having a handicapped child is not in itself strictly regarded as an indication for an invasive prenatal diagnosis procedure that itself carries a risk of at least 0.5% for a subsequent pregnancy loss. Moreover, a "normal" conventional chromosomal result does not guarantee a healthy child. The genetic counselor has to convey these limitations to the parents. Nevertheless, there may be individual cases in which the genetic advisor comes to the conclusion that a prenatal test may be justified as "psychologically indicated" in order to alleviate exaggerated anxiety and fears.

25.2.2 *Techniques of Prenatal Diagnosis*

The principle of the different procedures for obtaining fetal tissue is shown in Fig. 25.2 and Table 25.1. Weeks of gestational age are usually counted starting from the first day of the last menstruation.

25.2.2.1 Investigations Prior to Implantation

25.2.2.1.1 Preimplantation Genetic Diagnosis

For some couples at high risk of transmitting a serious genetic disorder conventional prenatal diagnosis may be unacceptable, as it potentially implicates termination of pregnancy at an advanced stage. In this situation preimplantation genetic diagnosis (PGD) may be an acceptable alternative. There is increasing demand for PGD for late-onset autosomal-dominant disorders for which conventional prenatal diagnosis is not requested. Following in vitro fertilization and blastomere biopsy at the 4- to 8-cell stage, molecular or cytogenetic analysis can be performed. After the in vitro analysis only those embryos shown to have an unaffected genotype are transferred into the mother's uterus. The success rate of this procedure is often less than 25% per cycle of treatment [18]. The other group of PGD users is made up of those who have been found to be subfertile or infertile and who wish to combine assisted reproduction with mainly cytogenetic testing of the early embryo.

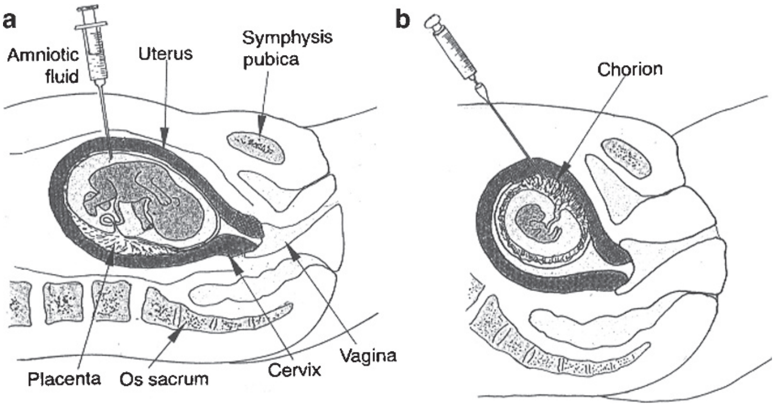

Fig. 25.2 (**a**) Amniocentesis: puncture of the amniotic cavity through the abdominal wall; (**b**) chorionic villus sampling: puncture of the chorion by the abdominal approach

Table 25.1 Techniques used in prenatal diagnosis

Prior to implantation		
Technique	Optimal time (in weeks)	Main indications
Preimplantation genetic diagnosis (PGD)		a) Very high risk for a well-known disease without an effective therapy b) For parents who carry a high risk for a chromosomal aberration
Polar body diagnosis		Investigation from second polar body before fertilization of the oocyte for genetic diagnostics of certain maternal monogenic disorders and structural chromosomal aberrations
After implantation		
Noninvasive prenatal diagnosis		
First-trimester screening	11–13	Down syndrome and other chromosomal disorders
Ultrasound		Structural abnormalities, e.g., CNS, heart, kidneys and limbs
Invasive prenatal diagnosis		
Chorionic villus sampling (CVS)	10–12	Chromosome abnormalities, metabolic disorders, molecular defects
Amniocentesis	15–16	Neural tube defects, chromosome abnormalities, metabolic disorders, molecular defects
Cordocentesis		Chromosome abnormalities, hematological disorders, prenatal infections
Fetobiopsy(e.g., skin)		e.g., Hereditary skin disorders

Frequent indications for PGD are:

1. Monogenic disorders
2. Sex selection in X-linked disorders
3. Chromosomal anomalies (e.g., Robertsonian or reciprocal translocation)

Children conceived with the aid of IVF procedures, however, have a slightly higher rate of major birth defects (1–2%) [50]; whether this risk is due to culture conditions or to the mechanical treatment of the embryo cannot be determined at present.

A further restriction is diagnostic accuracy. The probability that a result is obtained on the basis of investigation of the genome of a single isolated cell is about 90–95%. The probability that this result is correct is again 90–95%. The biggest problem is a false-negative test result resulting from contamination with foreign DNA or from allelic dropout, i.e., the analysis limited to a single allele.

PGD is prohibited by law in some countries (e.g., Austria, Germany, Italy, and Switzerland).

25.2.2.1.2 Polar Body Analysis

The symmetrical cell division during female meiosis results in the production of polar bodies. The two polar bodies are relatively small cell structures inside an ovum and can be obtained by zona drilling or laser drilling. Genetic analysis of the polar body can be an alternative to PGD for certain monogenic disorders and maternal structural chromosomal aberrations. This analysis represents an indirect method of genotyping; the unfertilized oocyte and the first polar body arise from each other during meiosis I. They thus contain different members of each pair of homologous chromosomes. For example, if in a carrier of a heterozygous autosomal-recessive mutation the mutation is detected in the polar body, the oocyte will contain the wild-type copy of the gene. Polar body diagnosis has been successfully used in couples at-risk for having a baby with cystic fibrosis and hemophilia, but like PGD, polar body diagnosis is technically difficult and unlikely to become a routine procedure.

25.2.2.2 Noninvasive Diagnosis During Pregnancy

25.2.2.2.1 Ultrasound

Almost all pregnant women are routinely offered an ultrasound scan at around 12 gestational weeks, and repeated ultrasound examination has become part of routine pregnancy surveillance in some countries. Current studies indicate that ultrasound is harmless to the developing fetus. During later stages of pregnancy, ultrasound examination allows the detection of a variety of fetal structural anomalies. Often a detailed ultrasound screen is performed around the 20th gestational week. Below is a list of reasons for prenatal ultrasound and of fetal conditions that can be detected prenatally by this means in most cases [30]:

- Obstetric indications:
  - Accurate gestational dating
  - Multiple pregnancies
  - Placental localization before amniocentesis or chorionic villus sampling
- CNS disorders:
  - Anencephaly
  - Hydrocephaly
  - Encephalocele
  - Meningomyelocele
  - Spina bifida
  - Holoprosencephaly
  - Microcephaly
- Skeletal defects:
  - Severe neonatal bone dysplasias
  - Congenital types of osteogenesis imperfecta
  - Severe limb defects
- Internal abnormalities:
  - Severe congenital heart defects
  - Gastroschisis
  - Omphalocele
  - Duodenal atresia
  - Esophageal atresia
  - Renal agenesis
  - Congenital polycystic kidneys
  - Severe obstructive uropathy
  - Various fetal tumors

Obscure or pathologic ultrasound findings are often an indication for fetal chromosomal analysis. Many ultrasonographic markers may be of transient and of physiological nature rather than indicative of fetal pathology. Others may indicate abnormality in only a proportion of cases, e.g., nuchal edema and assessment of nasal bone in trisomy 21 (Table 25.2). This increased nuchal fold results from water stored under the skin of the embryo, which is most prominent between the 10th and 14th weeks of gestation.

Table 25.2 Multiplication factor for the modified age risk for trisomy 21 with ultrasonographic markers[a] [4, 60, 62]

Ultrasonographic markers	Time of the occurrence	Multiplication factor for the risk for trisomy 21
Nuchal translucency thickness and fetal nasal bone assessment	10–14 weeks of gestation	Dependent on width of edema, between 3 and 22
Nuchal fold thickening>mm	Second trimester	10
Echogenic bowel	Second trimester	5.5
Shortened femur length	Second trimester	2.5
Dilation of the renal pelvis	Second trimester	1.5
Choroid plexus cysts	Second trimester	1.5

[a]Example: A 29-year-old woman in week 13+2 of gestation (body weight: 56.1 kg) with fetal crown-heel length of 71.1 mm; nuchal transparency of 1.3 mm, PAPP-A (MoM) of 0.45, and free Beta-HCG (MoM) of 1.27 carries an adjusted risk for trisomy 21 of 1:3,500, which is much lower than the age-related risk of 1:730. (PAP-A-MoM and free beta-HCG-MoM are adjusted for body weight.)

25.2.2.2.2 Maternal Blood Sampling

The screening of maternal blood for elevated α-fetoprotein (AFP) levels to detect neural tube defects and some other fetal anomalies is standard practice in many centers [11, 28]. AFP screening as one parameter may also be useful to detect Down syndrome, since fetuses with trisomy 21 have lower AFP levels than normal fetuses [12]. Abnormalities that increase or decrease amniotic-fluid levels of AFP include:

Increased AFP:

Neural tube defects (anencephaly; open spina bifida)
Spontaneous intrauterine death
Abdominal wall defect (omphalocele; gastroschisis)
Congenital nephrotic syndrome
Incorrect gestational age
Lowered AFP:
Down syndrome

Other useful biochemical markers for Down syndrome screening include unconjugated estriol (μE3; decreased), human chorionic gonadotropin (β-hCG; increased), inhibin-A (increased) and pregnancy-associated plasma protein A (PAPP-A; decreased). Multiple marker screening for fetal Down syndrome (2nd trimester) using AFP, μE3, β-hCG in addition to maternal age achieves a detection rate of about 60–70%, with a false-positive rate of approx. 5%. Using ultrasound to detect increased fetal nuchal translucency (NT), the two biochemical markers AFP, β-hCG and the age of the pregnant woman, the detection rate would increase to 86% if 5% of all pregnancies were tested [9, 67, 68].

25.2.2.2.3 Detection of Fetal Cells and Fetal DNA in the Maternal Circulation

Using fetal cells in the peripheral blood of pregnant women to diagnose or screen for fetal chromosome abnormalities is still in the test stage [54, 69]. Analysis of cell-free fetal DNA in the circulation of the pregnant woman is also being tested as a noninvasive prenatal diagnostic method that might be developed [73]. Preliminary examination of principal results with next-generation sequencing suggests that detection of fetal aneuploidies may become possible through the analysis of cell-free fetal DNA in the circulation of pregnant women [10]. The use of fetal cells from transcervical samples collected in early pregnancy may also be a means of noninvasive prenatal diagnosis in the future [7].

25.2.2.3 Invasive Diagnostics During Pregnancy

All invasive diagnostic procedures during pregnancy involve a defined risk and should only be applied after detailed genetic consultation has been offered.

25.2.2.3.1 Amniocentesis

Amniocentesis [49] is the most common form of invasive prenatal diagnostic technique offered to pregnant women. It is carried out at the beginning of the second trimester of pregnancy (13th–15th week of pregnancy; Fig. 25.2a). It involves aspiration of 10–20 ml of amniotic-fluid by transabdominal puncture under ultrasound guidance. The amniotic-fluid is spun down to yield a pellet of cells and supernatant fluid. The fetal cells are derived from amnion, fetal skin, fetal lung, gastrointestinal tract, and urinary tract epithelium. The cell pellet is cultured with fetal calf serum, which stimulates cell growth, and some cells will start to divide and form cell colonies. After 8–12 days of incubation there are usually enough dividing cells for chromosome or DNA analysis. In many centers, a FISH test taking less than 24 h is performed on uncultivated amniotic-fluid cells. This test involves identification of chromosomes 13, 18, 21, X, and Y via fluorescence in situ hybridization (FISH). Alternatively, quantitative polymerase chain reaction (PCR) or multiplex ligation-dependent probe amplification (MLPA) can be used to identify numerical aberrations involving these chromosomes. However, these rapid tests yield only limited information, so that they cannot replace a complete chromosome study as the gold standard using amniocyte cultures.

25.2.2.3.2 Chorionic Villus Sampling

Chorionic villus sampling (CVS) [5, 44, 59] was first developed in China. This procedure may be performed by collecting chorionic villi by the cervical or by the abdominal approach (Fig. 25.2a). The abdominal approach is now generally preferred because of the lower risk of infection. Chorionic villus sampling must be performed under ultrasound guidance. Chorionic tissue of fetal trophoblastic origin can be used for cytogenetic analysis, DNA testing, or biochemical analysis. CVS can be carried out around the 12th week of gestation. The procedure therefore has psychological and practical advantages over amniocentesis, which is not carried out until the 13–16th week of gestation. In addition, cytogenetic results are available much sooner. However, chromosome analysis is less reliable and the risk of miscarriage after chorionic villus sampling is higher than after amniocentesis (1–2% vs 0.5–1%) [37, 46]. There is also evidence that CVS can cause limb abnormalities if carried out before the 12th week of gestation. Placental biopsy is the term used if the puncture is performed at later stages of pregnancy.

25.2.2.3.3 Cordocentesis

Cordocentesis [14] examines blood from the fetus to detect fetal abnormalities. The sample of fetal blood is taken from the umbilical cord. Cordocentesis is usually done when diagnostic information cannot be obtained through amniocentesis, CVS, or ultrasound or these tests have yielded inconclusive results. Cordocentesis is usually performed after the 20th week of gestation. It may be performed to help find whether any of the following concerns is justified:

1. Fetal infection (e.g., toxoplasmosis or rubella)
2. Fetal anemia
3. Chromosome abnormalities

The risk of miscarriage after cordocentesis is between 1 and 2%.

25.2.2.3.4 Fetoscopy

Fetoscopy [38] is usually done after the 18th week of gestation in women who have a high risk of having a child with a major birth defect that can be found only using fetoscopy. Because fetoscopy, which was initially performed to detect congenital birth defects, has been replaced by high-resolution ultrasound scanning, the main use of fetoscopy today is to collect samples of tissue (usually skin) from the fetus. The tissue can then be tested for hereditary skin diseases in rare conditions where a DNA analysis is not yet available. Fetoscopy is associated with a 3–5% risk of miscarriage, and there are very few indications for this procedure.

25.2.2.4 Special Problems with Prenatal Diagnosis

25.2.2.4.1 Failure to Obtain Fetal Material

With each prenatal diagnosis it must be pointed out that there is a <1% probability that it will not be possible to obtain any fetal cells or that any cells obtained will subsequently fail to grow.

25.2.2.4.2 Mosaic Constellation for Chromosome Aberrations

Chorionic villus sampling. In approximately 1% of cases, CVS shows evidence of apparent chromosome mosaicism. This can have various reasons:

1. Contamination of the fetal sample with maternal cells. This arises more frequently from cultured cells than with direct preparations.
2. Mosaicism as a culture artifact. Because of this possibility, at least two separate cultures are usually established for diagnostic use. If the aberrant cell lineage occurs only in one of the culture bottles, it may not be representative of the fetus.
3. Limitation of mosaic to a portion of the placenta. This arises through a replication error during the formation and development of the trophoblast. These mosaics are only of importance for the development of the fetus if they lead to placental insufficiency.
4. True fetal mosaicism. This situation may have effects on the development of the fetus.

Amniocentesis specimens are normally set up as two or three separate cultures. If a single abnormal cell is identified in only one of the cultures, it is highly probable that it represents a culture artifact. This is referred to as level 1 mosaicism or pseudomosaicism. If an aberrant karyotype is present in two or more cells of a single culture bottle, level 2 mosaicism is present. In this situation, 80% of such cases represent culture artifacts and only 20% reflect true fetal mosaicism. If the aberrant cell lineage is present in two or more cells in two separate culture bottles, level 3 mosaicism is present.

This level most likely represents true fetal mosaicism, requiring further diagnostic steps (e.g., high-resolution ultrasound).

25.2.2.4.3 Chromosome Abnormality

The most frequently diagnosed numerical chromosome abnormalities of concern are trisomies 21, 13, and 18, and other autosomal trisomies are found only occasionally. Gonosomal aneuploidies are usually discovered by chance and, with appropriate genetic counseling, are of less concern than autosomal aberrations.

Except for trisomy 21, almost all autosomal trisomies are associated with a poor prognosis as far as the probability of survival of the fetus is concerned. The situation is altogether different in sex chromosomal aneuploidies (47,XXX, 47,XXY, or 47,XYY). The life expectancy of the affected children is normal, malformations are discrete, and the intelligence, while somewhat below sibling scores, is usually within the normal range. The search for sex chromosomal abnormalities is therefore not a standard indication for invasive prenatal diagnostics. Following the coincidental discovery of a gonosomal aberration only a minority of parents opts for termination of pregnancy [6].

25.2.2.4.4 Structural Chromosomal Rearrangements

The incidence of structural chromosomal rearrangements amounts to approx. 1:500 in the newborn population. Half of these are Robertsonian translocations (translocations involving acrocentric chromosomes). Structural chromosomal rearrangements can be balanced or unbalanced. In case of a balanced structural chromosomal rearrangement it is helpful to analyze the parental chromosomes. If one of the parents is a carrier of the rearrangement, this will not usually cause problems in the fetus. If the balanced structural chromosomal rearrangement occurs as a de novo event in the fetus, there is a 5% empirical risk of a developmental handicap, which means that 19 of 20 children will be born without any impairment. The 5% risk is attributed to submicroscopic loss of genetically relevant material at the breakpoints.

25.2.2.4.5 Marker Chromosome

A marker chromosome is a small additional chromosome fragment which carries portions of one or more chromosomes. If the marker is present in one of the parents, it is unlikely that it will interfere with pre- or postnatal development.

If the parents do not have the marker chromosome, a rough estimate of an a-priori risk of 15% for a developmental handicap can be given for a marker with euchromatic material. However, with currently available methods in molecular cytogenetics, including FISH, further analyzes can clarify whether the marker chromosome contains mostly heterochromatic or euchromatic material. The origin of the marker can frequently be determined such that more precise risk information can be given to the parents.

25.3.2.5 Consultation and Aftercare

Prenatal diagnosis should be preceded by genetic counseling, during which the pregnant woman should be given information about the following aspects:

1. Indication for and goal and risks of the investigation
2. Possibilities and limitations of the prenatal diagnostic procedures offered
3. Basis risk, individual risk, and risks inherent in the particular diagnostic procedure under discussion
4. Interpretation and validity of prenatal test results, including possible problems with mosaicism
5. Types and severity levels of diagnosable pathologic conditions
6. Possible therapeutic options if pathologic findings are present
7. Psychological and ethical conflicts

Informed consent of the pregnant women is a prerequisite for any type of prenatal diagnosis. The risks, time of analysis, possible limitations, and possible consequences of this investigation have to be pointed out, and furthermore information about risks, e.g., bleeding, infection, or abortion, of invasive prenatal procedures must be made explicit. Moreover, the geneticist must inform the woman that a normal cytogenetic, molecular-genetic, or biochemical result does not exclude all possible diseases and handicaps. The counselee has to understand that a normal outcome of any of the diagnostic procedures does not guarantee the birth of a normal child. The abrupt confrontation with pathologic serum findings causes undue anxiety in the majority of women. In order to avoid such overreactions, maternal serum screening and ultrasound investigations require the careful provision of information to the patient prior to performance of these procedures. One of the main objectives of prenatal diagnosis is to alleviate fears and anxieties in the pregnant woman rather than provoking such feelings.

Following a pathologic finding, an unhurried counseling session with the pregnant woman, preferably together with her partner, should take place and include the following information:

1. Origin type and natural history (prognosis) of the disorder or developmental disturbance of the unborn child, and possible complications
2. Intrauterine therapy / operational measures (for the pregnant woman, or the fetus)
3. Postnatal therapy and supportive measures
4. Preparation for the special circumstances following the birth of a child with handicap/genetic disorder
5. Aspects of living with a handicapped child (establishment of contact with other parents, and support groups if requested)
6. Modalities and legal/emotional implications of termination of pregnancy
7. Possibilities of psychotherapeutic and psychosocial assistance

Informing pregnant women about pathologic findings requires expertise and experience. The decision of the counselee and the extent of the information provided need to be documented in a written report.

25.3.2.6 Aftercare

In the case of termination of pregnancy or the birth of an affected child, the following points should be considered:

1. Careful documentation of clinical findings (photographic, X-ray pictures and/or fetal pathologies)
2. Confirmation or correction of the result of prenatal diagnosis
3. Genetic counseling of parents (e.g., about possible prenatal diagnostic procedures in subsequent pregnancies; discussion of implications of the findings for other family members)
4. Encouragement of acceptance of findings and provision of psychotherapeutic support

25.3.2.7 Psychological Aspects and Outlook

Antenatal diagnosis is widely used in today's industrialized countries. The possibility of early detection of an affected embryo or fetus often encourages parents to start a pregnancy incircumstances in which the fear of having an affected infant would previously have deterred them from childbearing. While medical termination of pregnancies has become acceptable in many countries and cultures, a significant proportion of the population in the United States and elsewhere strongly opposes prenatal diagnosis for religious or other reasons. Some of the opponents are particularly concerned about the value judgments of human life implicit in these procedures. They feel that such practices are the beginning of a "slippery slope" that would ultimately lead to rejection of relatively minor defects and encourage the search for the "perfect" baby, possible resulting in the resurgence of eugenic and racist ideas. Fears have also been expressed that society would be less inclined to pay large sums of money to take care of children with genetic diseases when abortion could have prevented the birth of the disabled child. Largely because of the realization that most human disabilities arise during postnatal life, utilitarian or eugenic views have not become a driving force in prenatal diagnosis. The fact that in recent years many countries have made provisions to meet their obligations towards handicapped citizens argues against the validity of these fears [45].

New challenges for genetic counseling arise from the recent general availability of DNA testing that provide risk data for a variety of traits and diseases based on DNA genotyping. Several companies offer an "exploration of your genome" at low costs (usually in the range of $400). These companies provide test kits to collect cheek cells in saliva or cells from a buccal swab. The DNA is then genotyped within 4 or 6 weeks, and the user can check results on a designated internet page. Data used for genotyping comes often from published whole-genome association studies and provides information about certain conditions (e.g., risk for obesity) or common diseases (e.g., diabetes, breast cancer). However, the data employed is often incomplete, as it has often not been validated by repeated analysis or not confirmed for populations with different ethnic backgrounds, and it may therefore provide misleading information. The relative risks for phenotypic features or diseases are usually very small (odds ratios are often below 2). Although these companies often have well-prepared and informative websites and now often employ trained genetic counselors, people now have the chance to obtain some information about their genome without any assurance about whether they understand possible implications and whether they can interpret the data and draw correct conclusions from the results.

Further challenges come from new technologies allowing the sequencing of entire genomes at low costs (see Sect. 4.4), which make it likely that whole-genome data will be introduced into routine clinical genetics in the near future. Numerous new bioinformatics tools will be needed for data interpretation, but once such tools exist these technologies will provide a wealth of information, which will include susceptibilities for both common conditions and diseases. Together with the aforementioned web-based genetic-testing offerings, these new technologies will change genetic counseling tremendously. The main focus of genetic counseling is at present concerned about a certain disease in an individual or in the individual's family. However, presymptomatic, predictive testing will become more important as more information about our genome becomes available. Such predictive testing will not only concern diseases that might be prevented by appropriate means, but also such common conditions as the likelihood of obesity, baldness, and so on. Professional genetic counselors have to meet these challenges and have to provide appropriate services. Such services may be in competition with companies that may offer similar information at a different standard.

25.3 Conclusions

Knowledge of human genetics can be applied for genetic counseling of individuals and families at-risk for hereditary anomalies and diseases. Genetic counseling refers to the sum of activities that (a) establish the diagnosis of such diseases, (b) assess the recurrence risk, (c) communicate to the client and family the chance of recurrence, and (d) provide information regarding the many problems raised by the disease, including natural history and variability of the disease. Formal methods applied in risk assessment include the use of genetic algorithms, statistical considerations, and empirically derived risk estimates if the mode of inheritance is unknown. Laboratory techniques involve prenatal diagnosis by ultrasonography, amniocentesis, and chorionic villus sampling and the diagnostic application of techniques such as cytogenetics and molecular genetics. Genetic counseling is not merely concerned with scientific issues that are at the root of genetic disease. It must provide understanding and empathy for the clients´ concerns, and it must deal appropriately with the many psychological aspects of the process. As such, genetic counseling is an important part of comprehensive medical care.

25.4 Appendix Example of a Bayesian Table

Assuming a woman's brother and her maternal uncle are affected by an X-linked recessive condition, her prior probability of being a carrier will be ½, or 50%. If this consultand has two healthy sons, her risk of being a carrier will be reduced to 1/5 or 20% (posterior probability) according to Bayes' theorem.

	Consultand is a carrier	Consultand is not a carrier	Sum: 1/8 + ½ = 5/8
Prior probability of the consultand	½	½	
Two healthy sons	½ × ½ = ¼	1	
Joint probability	= ½ × ¼ = 1/8	= ½ × 1 = ½	
Prior probability of the consultand	½	½	
Two healthy sons	½ × ½ = ¼	1	
Joint probability	= ½ × ¼ = 1/8	= ½ × 1 = ½	
Posterior probability	=1/8/5/8 = 1/5 = 0.2	=1/2/5/8 = 4/5 = 0.8	

	Consultand is a carrier	Consultand is not a carrier	Sum: 1/80 + ½ = 41/80
Prior probability of the consultand	½	½	
2 healthy sons	½ × ½ = ¼	1	
Negative test result	1/10	1	
Joint probability	= ½ × ¼ × 1/10 = 1/80	= ½ × 1 × 1 = ½	
Prior probability of the consultand	½	½	
2 healthy sons	½ × ½ = ¼	1	
Negative test result	1/10	1	
Joint probability	= ½ × ¼ × 1/10 = 1/80	= ½ × 1 × 1 = ½	
Posterior probability	=1/80/41/80 = 1/41 » 0.024	=1/2/41/80 = 40/41 » 0.976	

	Consultand is a carrier	Consultand is not a carrier	Sum: 1/20 + ½ = 11/20
Prior probability of the consultand	½	½	
Negative test result	1/10	1	
Joint probability	=½ × 1/10 = 1/20	= ½ × 1 = ½	
Prior probability of the consultand	½	½	
Negative test result	1/10	1	
Joint probability	=½ × 1/10 = 1/20	= ½ × 1 = ½	
Posterior probability	=1/20/11/20 = 1/11 » 0.09	=1/2/11/20 = 10/11 » 091	

Assuming a woman's brother and her maternal uncle are affected by an X-linked recessive condition, her prior probability of being a carrier will be ½, or 50%. If this consultand has a negative result for a test that detects 90% of heterozygotes (sensitivity = 0.9), her risk of being a carrier will be reduced to 1/11 or 9% (posterior probability) according to Bayes' theorem.

Assuming a woman's brother and her maternal uncle are affected by an X-linked recessive condition, her prior probability of being a carrier will be ½, or 50%. If this consultand has two healthy sons and a negative result for a test that detects 90% of the heterozygotes (sensitivity = 0.9), her of risk being a carrier will be reduced to 1/41 or 2.4 % (posterior probability) according to Bayes' theorem.

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Gene Therapy

26

Vivian W. Choi and R. Jude Samulski

Abstract The integrity of the human genome is essential for maintaining the well being of an individual. Mutations, either caused by extrinsic or intrinsic means, that alters the genetic code can lead to genetic diseases, including, but not limited to, immature aging, developmental disorder, neurological disorder, cancer, etc. Traditional therapeutics may be able to treat or attenuate the condition of the genetic disease, but the mutated gene is still maintained in the patient's genome and the root of the problem is not addressed. Gene therapy, or genetic replacement therapy, aims to treats the disease at the genetic level, either by introducing a wild-type sequence gene over the defective one or a gene that encodes a therapeutic protein. There have been great challenges in developing gene therapy treatment in a safe, effective and specialized way for treating different genetic diseases. We will discuss (1) what diseases are considered to be suitable for gene therapy, (2) currently available methods used in gene delivery, (3) the choice of different viral and non-viral gene delivery tools and how they can be introduced into the target tissues or organs, (4) the safety aspects of gene therapy and (5) challenges yet to be solved in increasing the success rate of gene therapy. We hope to introduce to the readers the latest advancement of the gene therapy field and further discuss challenges and difficulties in having a successful outcome of gene therapy.

Contents

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26.1 Advent of Gene Therapy

Gene therapy, or genetic replacement therapy, is one of the most recent medical treatments for previously incurable genetic diseases [33]. The idea of gene therapy is to treat diseases by supplying genetic materials to modulate the pathophysiology caused by a malfunctioning gene in the patient's genome, with the ultimate goal of achieving long-term cure in a single treatment. The completion of the human genome project complemented the field of gene therapy by providing an incredibly vast amount of genetic information for medical research [9, 10], and the better tools for diagnosis and the advancement in molecular biology assisted in accelerating the development of gene therapy in the clinics.

26.2 Diseases Considered Suitable for Gene Therapy

Several types of genetic diseases are considered suitable for gene therapy. Monogenic hereditary disorders, such as cystic fibrosis, adenosine deaminase (ADA) deficiency, hemophilia A and B, familial hypercholestrolemia, Canavan disease, muscular dystrophy, and X-linked SCID [39, 57], are good candidates for gene therapy treatment, because the disease treatment can be carried out by introducing the correct sequence of the mutated gene. With a better understanding of more complicated diseases, the feasibility of using gene therapy in treating inflammatory joint diseases, such as arthritis, neurological diseases, such as Batten's, Parkinson's and Alzheimer's diseases, and various types of cancer, such as glioma, Lewis lung carcinoma, chronic lymphocytic leukemia, and cervical carcinomas [5, 30], has been demonstrated.

26.3 Gene Therapy Vectors

Even though the technology needed to synthesize genetic materials is readily available, the problem of developing gene delivery vehicles sufficiently efficient to transfer the DNA to all the target tissues remains challenging. An efficient gene delivery vector should fulfill the following criteria:

1. High safety level with minimal side effects
2. High efficiency and specificity
3. Large packaging capacity
4. Scalable to produce large quantities
5. Regulable to control gene expression

The two main categories of gene delivery vectors are viral and nonviral, and the most commonly used vectors, are discussed below.

26.3.1 Viral Vectors

Viral vectors are genetically modified viruses that have reduced pathogenicity while retaining the ability to infect cells. They usually consist of a modified viral genome, with a minimum amount of genetic material from the wild-type (wt) virus, packaged inside a capsid. Different viral gene delivery vectors (or viral vectors) offer various advantages that can be exploited to treat different diseases. In general, optimization of tissue tropism can be achieved by capsid mutagenesis, while long-term transgene expression is determined by the nature and the composition of the viral genome. Therefore, choosing the most suitable viral vector for each treatment is crucial to safe and effective gene therapy.

26.3.1.1 Adenovirus

Adenovirus (Ad) is a nonenveloped, double-stranded DNA virus with an icosahedral capsid diameter of 70–100 nm. Its vector genome is nonintegrating but remains episomal (to be discussed in Sect. 26.4.3) [44]. Among the few versions of Ad vectors developed in the past decade, the latest generation, the gutless vector, carries only the 5′ and 3′ inverted terminal repeats (ITRs) and the packaging signal (Ψ) as a minimal required *cis* element to generate the vector [40]. One of the few advantages of Ad vector is its large packaging capacity of up to 36 kb to accommodate either large or multiple transgene expression cassettes, ideal for the treatment of polygenic diseases. However, the major disadvantage of the Ad vector is that its systemic delivery can induce adaptive humoral and innate immune response, thus causing tissue damage and removal of infected cells by macrophages [18, 61]. This prohibits the use of Ad vector in establishing long-term transgene expression for life-long therapy. Another challenge of using Ad vector is the potential contamination of wt or replication competent Ad, causing serious side effects.

26.3.1.2 Retrovirus

Retrovirus is an enveloped virus carrying a single-stranded RNA genome. Moloney murine leukemia virus (MMLV), and human immunodeficiency virus (HIV) are examples of retroviral gene delivery vectors. These vector genomes, with packaging sizes of 8–10 kb, carry the minimal *cis* elements, including the long terminal repeats (LTRs) and the packaging signal from the wt virus and a transgene expression cassette. The other components needed for reverse transcription and packaging are provided in *trans*, including gag/pol, rev, and envelope [49]. Unlike Ad vector, retroviral vector genomes can lead to long-term gene expression by vector genome integration into the host cell chromosomes. This is also a disadvantage for retroviral vectors because the mechanism of integration can cause insertional mutagenesis. For example, it has been well documented that retroviral vectors can cause oncogenic transformation [31]. The newest generation of HIV vector, the self-inactivating vector (SIN), does not carry the viral LTR enhancer/promoter activity and is anticipated to provide a higher safety profile than the prior generations of retroviral vectors [32].

26.3.1.3 Adeno-associated Virus

Adeno-associated virus (AAV) belongs to the family *Parvoviridae*, whose members are among the smallest of the DNA animal viruses. It infects vertebrates and cannot replicate on its own; therefore it is characterized as a *Dependovirus*. An AAV virion is 26 nm in diameter and is composed of 60 subunits of capsid proteins, forming an icosahedral structure encapsidating a single-stranded DNA genome. AAV is regarded as one of the most promising gene therapy vectors for clinical use owing to its lack of pathogenicity in humans, effectiveness in transduction, and inability to self-replicate [46]. Additionally, both naturally occurring serotypes and artificially mutated AAV capsids can infect a wide host-cell range of tissues with high transduction efficiency [7, 15]. AAV can also lead to long-term gene expression owing to episomal persistence of AAV genomes, even though site-specific integration at human chromosome 19 AAVS1 site is possible for wt AAV2 at a very low efficiency [6, 8, 43, 47–59]. In addition, a newer generation of AAV vector, self-complementary AAV (scAAV) is developed as an improved vector, as it carries a genome that resembles a double-stranded DNA template ready for transcription upon infection [34]. Even though the genome packaging size of AAV vector (4.7 kb) is smaller than other viral vectors, improved transgene expression cassette designs and the use of split vector system have overcome this hurdle [8, 28].

26.3.1.4 Herpes Simplex Virus

Herpes Simplex Virus (HSV) is an enveloped and double-stranded DNA virus, which consists of an external envelope surrounding an icosadeltahedral capsid containing a wt genome of 152 kb [2-4]. HSV vectors are used for targeting neuronal tissue (e.g., Parkinson's, malignant gliomas, and cerebral ischemia). In a similar way to Ad and AAV vectors, the HSV genome is also maintained episomally. Despite the high efficiency of targeting central nervous tissues, one of the drawbacks of HSV vector is that wt HSV contamination is highly pathogenic and cerebral injection of such a vector can cause fatal encephalitis [22, 38]. Even though there are concerns in using HSV vector for gene therapy, a replication-defective HSV vector is considered to be one of the most desirable vectors for cancer gene therapy [4].

26.3.2 Nonviral Vectors

Nonviral vectors are chemically assembled vectors made up of lipids, nucleic acids, peptides, and/or inorganic materials. To carry out gene delivery successfully, these vectors need to:

1. Be of biocompatible composition
2. Bind to cell surface and get internalized
3. Perform endosomal escape
4. Traffic through the cytoplasm

5. Deliver the genome to the nucleus

A great advantage of nonviral vectors is that it can possibly carry a transgene expression cassette of no size limitations. In addition, it has been suggested that nonviral vectors induce fewer inflammatory responses or have a higher safety level compared to viral vectors, but this still requires further validation [11]. However, nonviral vectors are far less efficient than viral vectors, because viruses have evolved to infect cells efficiently in order to complete their life cycles. Therefore, further improvements to the nonviral vectors are necessary to generate a gene-targeting vector whose efficiency approaches that of viral vectors.

26.3.2.1 Naked DNA

Naked DNA is the cheapest and easiest material to be produced for gene therapy. By using the hydrodynamic delivery method (large-volume injection into the bloodstream over a short duration), DNA is forced into the bloodstream and then into the internal organs. However, rapid degradation by nucleases and clearance by the mononuclear phagocyte system reduce the inefficiency of in vivo transfection of naked DNA [11].

26.3.2.2 Liposomes

The most popular liposomes are the cationic amphiphile and neutral phospholipids [14]. The plasmid DNA is condensed and packed inside the liposomes through interactions with the polar headgroups. The hydrophobic tail assists the formation of micelles, which fuse with the cellular membrane at the time of transfection, leading to endocytosis [16]. One variant form of improved liposomes is seen in the DNA-ligand conjugates, which guide the complex to bind to specific cellular surface receptors for more efficient gene transfer [25]. Commonly used lipid vector backbones include 2,3-dioleyloxy-*N*-[2(spermine-carboxamido) ethyl]-*N*,*N*-dimethyl-1-propanaminiumtrifluoroacetate (DOSPA) and dioleylphosphatidylethanolamine (DOPE) [11].

26.3.2.3 Polymers

Polymers used for gene therapy are generally classified in two categories: natural and synthetic. Polymer size is determined by the amine-to-phosphate (N/P) ratio. Higher molecular weight polymers are more stable, while lower molecular weight ones have a higher transfection efficiency. Some examples of polymer vector backbones are poly(ethyleneimine) (PEI), poly (β-aminoesters), β-cyclodextrin, poly(amidoamine) dendrimers (PAMAM), poly(2-diethylaminoethyl methacrylate) (PDEAEMA), and polyphosphoesters [11].

26.4 Factors Affecting the Success of Gene Therapy

From diagnosis to clinical treatment, gene therapy requires the involvement of a wide range of expertise. In order to achieve life-long correction of genetic diseases, we need to take many different factors into consideration so that the therapy can be safe and effective.

26.4.1 Choice of Gene Delivery Vector

Careful selection of a delivery vector is crucial for each clinical case, since every vector has its own strengths and weaknesses. The advantages of using nonviral vectors are the low production costs with less complicated procedures and their ability to accommodate any size of gene expression cassette. However, the transfection efficiency of nonviral vectors is significantly lower than that of most of the viral vectors. In addition, the plasmid DNA packaged in the nonviral vectors is readily degraded and the CpG island sequence in the bacterial backbone can initiate immune responses [26, 42]. On the other hand, viral vectors may involve the risk of insertional mutagenesis and of acute immune responses. Nevertheless, the broad tissue tropism, ability to establish long-term gene expression by episomal persistence or chromosomal integration, and capability of infecting dividing and nondividing cells have attracted many investigators in utilizing viral vectors [41].

26.4.2 Route of Administration

The route of administration is another key variable in gene delivery, because the biodistribution of the vectors can directly affect the efficiency of vector delivery. For systemic delivery, intravascular injection is the route most commonly used. Direct muscle injection can be used in muscle-related disease (e.g., muscular dystrophy) or to serve as the tissue to generate secretable protein to be released into the bloodstream (e.g., a-antitrypsin) [5]. Hepatic artery injection is used in targeting the vector to the liver. This route is especially important for many enzyme-dependent therapies, since these molecules are modified in the liver to establish full enzymatic activities [5]. Alternative direct injection protocols have been developed to target other organs. Besides direct introduction of vectors to the patient (in vivo targeting), ex vivo administration is also widely used, particularly when retroviruses are used as the delivery vector. This method is performed by infecting (viral) or transfecting (nonviral) gene delivery vectors to cells that are removed from the patient's body. The cells containing the vector genomes are then reintroduced into the patient.

26.4.3 Integrating or Nonintegrating Vectors

Integration of a gene therapy vector cassette into the target cells chromosome allows long-term correction in both dividing (e.g., bone marrow) and nondividing or slowly dividing (e.g., muscle) cells. In particular, retroviral vectors have been widely used for ex vivo gene therapy based on genome integration into rapidly growing cells [36, 45, 50]. However, random insertional mutagenesis can be dangerous, as it can cause activation of oncogenes or disruption of functional genes. Site-directed integration, which is a more challenging method, has been proposed to minimize the manipulations or changes caused to the host genome by the introduction of a gene therapy vector. For nondividing or slowly dividing cells, vector genomes that can persist episomally in the nucleus of the cell can also establish long-term gene expression. For example, long-term gene expression has been demonstrated with AAV vectors in animals, such as mice, dogs, and primates [43, 55, 59] owing to viral genome persistence as episomal circles and/or concatemers [6, 47, 48, 59]. Preliminary clinical trial data suggested that transgene expression is established in human for at least 4 years [23]. The premise of long-term gene expression without potential insertional mutagenesis into patients' genomes that would cause secondary mutations is optimistic.

26.4.4 Therapeutic Means

Recent advances in molecular biology and cell biology provide new insights on the types of therapeutic means applicable to gene therapy.

26.4.4.1 Gene Complementation

A typical gene therapy vector carries a transgene expression cassette including, but not limited to, the following basic elements: a promoter, a cDNA of the transgene of interest, and a poly-A signal. This cassette supplements the patient with the correct gene and utilizes the protein production mechanism in the patient's body to produce the therapeutic protein.

26.4.4.2 Gene Knockdown

Alternatively, when reducing protein level is therapeutically desirable, siRNA can be used to knock down protein levels by degrading specific mRNAs [13, 20, 62]. Since the specific siRNA sequences are only 21–23 nucleotides, it can easily fit into all vectors that are limited by smaller packaging capacity. Gene knockdown strategy has been mostly used in cancer gene therapy because overexpression of oncogenes is one of the leading causes of tumorigenesis.

26.4.4.3 Gene Correction

Another potential therapy utilizes zinc fingers, a class of DNA-binding proteins with specific recognition sequences. Zinc fingers recognizing a specific DNA sequence can be delivered by a gene therapy vector, and this element targets the repair of the mutated gene in the chromosome [24, 53], so that the patient can produce the therapeutic protein from the endogenous gene.

26.4.5 Regulation of Gene Expression

It is important to design the cassette with regulatory elements so that the expression of the transgene can be controlled in very specific ways. Hyper- or hypotransgene expression would lead to over- or underdosage.

Therefore, a regulation system should be included in the vector DNA cassette. For example, tetracycline-dependent regulable gene expression, which relies on the drug doxycyclin, controls the expression of the transgene through transcriptional regulation [19]. Other approaches include dimerizer, ecdysone-responsive, quorum-sensing and zinc-finger-based regulatory systems [52].

26.5 Safety Issues of Gene Therapy

As for any type of disease treatments, patient safety is critical. For the gene therapy field, the importance of the safety issue has been exemplified by multiple clinical cases, most notably the Gelsinger and the *LMO2* insertional mutagenesis cases.

In 1999, patient Jesse Gelsinger, who suffered from ornithine transcarbamylase deficiency, was treated with a replication-defective Ad vector, which was injected through his hepatic artery into the liver. Four days later, Jesse died of a cascade of organ failures triggered by the immune response to the Ad vector [29, 54]. The three main contributions to his death were identified as his poor liver function, other complications of his health prior to the trial, and the incomplete disclosure of the potential risks of Jesse's treatment to his family [51]. More stringent gene therapy regulations have been established since this case to further protect the safety of patients.

In fall 2002, the Necker Hospital in Paris announced that the two youngest boys among 11 patients enrolled in a gene therapy trial for the treatment of X-linked severe combined immunodeficiency (X-SCID) had developed leukemia [21]. The retroviral vector used in this trial inserted the vector genome close to the promoter of the *LMO2* gene in the host genome, which encodes a transcription factor. The LMO2 protein was then continuously expressed, leading to the development of T-cell acute lymphoblastic leukemia. The trial is ongoing, and all participating patients are closely monitored. Even though it has been speculated that the insertional mutagenesis is due to the retroviral vector used, a recent report suggested that the choice of transgene is equally important. The one used in this trial, *IL2RG*, was demonstrated to be more highly oncogenic than expected [58]. This result illustrates that the safety of transgenes used in other trials should also be assessed.

Despite the setback of several incidences, successful cases of gene therapy have been demonstrated. For example, two patients with SCID are able to live outside of a protective environment 10 years after gene therapy treatment [51]. Similarly, the trial performed in the United Kingdom for four young SCID boys was successful [17]. In addition, researchers in the U.S. started a hemophilia B and a Canavan disease trial in which patients are treated with viral vectors expressing factor IX and aspartoacylase enzyme, respectively. The trials are still ongoing with no signs of toxicity [23, 35]. Several current trials have also demonstrated that gene therapy is a safe treatment.

The impact of the deaths of patients in gene therapy trials led to a meeting of the health recombinant DNA advisory committee (RAC) at the National Institutes of Health (NIH) to assess the safety of gene therapy [1]. This meeting included a comprehensive discussion of the experimental protocols, preclinical data (including large animal experimental data), and ethical issues (including the informed consent document) [1]. The committee released a report suggesting that better communication of scientific findings among the scientific community is needed (safety and toxicity data, purity and integrity of vector preparation, dosage, and route of administration to be used). In addition, clear and in-depth information making informed consent possible should be provided to the patients and their families, with all potential risks and benefits related to the trial detailed, and the health condition of the patient before and after the trial should be monitored carefully.

26.6 Difficulties in Achieving Successful Gene Therapy

1. *Expensive Large-Scale Production of Vectors*. Gene therapy has been considered to be an expensive therapy because the cost of manufacturing high-titer vectors sufficient for clinical uses is extremely high. It is particularly true for generating viral vectors. For example, the traditional triple-transfection method [60] used in generating AAV vectors in mammalian cells has been a valuable method for generating enough high-titer and high-quality AAV vectors for research and small-scale clinical trials. However, in order to generate enough vectors for a large number of patients, alternative methods must

be developed (e.g., an insect cell packaging system) [27]. In addition, the generation of retroviral vectors has been improved by the development of vector-producing cell lines so that higher yield production of vectors can be achieved [12].

2. *Uniqueness of Every Disease*. One of the biggest challenges of gene therapy is that every single disease requires a unique vector for the therapy, and it is therefore impossible to generate a "universal vector" for gene delivery. However, these genetic diseases share one common element – i.e., a mutated gene. As the general principle of gene therapy is to introduce the correct gene to the patient's body, this strategy can be widely used in treating most, if not all, genetic diseases.
3. *Ethical Issues of Gene Therapy*. One of the biggest and most debated topics in gene therapy is the possibility of performing germline or in utero gene correction or modification. One would discern that this is morally incorrect if it created an unfair advantage to those who can afford or have access to the technology in creating "designer babies," leading to segregation of humans into classes. However, if law enforcement can oversee the legal issues of germline correction, it would be possible to prevent the mutated gene(s) from being passed on to subsequent generations and thus eliminate the social and economic tribulations that arise out of genetic diseases. Alternative strategies to prevent or cure diseases should be our priority, but the issue of germline correction should merit continuous discussion both in the government and in the wider community (including patients and scientists) [37, 56].

26.7 Conclusions

In conclusion, we have summarized the basic principle of gene therapy and discussed the types of gene delivery vectors and other criteria in leading to successful gene therapy. This is a newly emerging field, and further basic research and clinical trials would provide us with valuable knowledge that would assist us in making gene therapy a more effective and a safer method of treating diseases. It is certainly a complex process for gene therapy to go from benchtops to clinics, but with all the lessons we have learnt from the past, gene therapy is destined to develop into a successful therapeutic option, leading to better lives for mankind.

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Cloning in Research and Treatment of Human Genetic Disease

27

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27.1 Introduction

Revolutionary new opportunities to study human disease were heralded by the development of two new cell-based techniques. These are the methods able to obtain normal development after somatic cell nuclear transfer (SCNT) [45] and to derive stem cells from human embryos [38]. Use of these two new techniques together will provide opportunities to study inherited human disease that are not available in any other way. Such cells would provide new opportunities to study the pathophysiology of inherited human diseases and in turn make it possible to use high-throughput screens to identify drugs able to prevent symptoms of the disease.

In the longer term, cells derived from cloned human embryos may be used in treatment of disease. They would offer the potential advantage of being immunologically matched to the patient who donated the nuclear donor cell and so could be used for therapy without the need for immunosuppression to prevent rejection. Cell lines that are homozygous at the major histocompatibility antigens are expected to be particularly valuable for transplantation. Calculations suggest that if these genotypes could be selected a comparatively small number of lines would provide an immune match to a very large proportion of the population [36]. Such a bank of cell lines could be established by somatic cell nuclear transfer (SCNT). None of these objectives have yet been realized, and it is clear that a great deal remains to be learned before these new approaches become available for either research or therapy.

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This chapter will describe the potential benefits that may arise from the production of embryo stem cells by SCNT and then describe the present procedures for and outcomes of nuclear transfer, before considering new approaches to the development of effective procedures.

27.2 The Value of Cell Lines of Specific Genotype

27.2.1 Studies of Inherited Disease

There are many different approaches to the isolation of affected cells for the study of inherited disease, and it is unlikely that any one will be optimal for all diseases. In the case of conditions that affect an accessible tissue, such as the skin or the hematopoetic system, then cells may be obtained directly from the patient. Full benefit from this approach will depend upon the isolation of progenitor cells that can be multiplied extensively in culture to allow detailed and repeated experimentation with the same population of cells over a considerable period of time. These methods have been established for some tissues and used for research and therapy [37, 57].

Several other strategies involve derivation of the affected cell type from embryo stem cells that have the causative genotype. If dominant mutations have been identified and the affected cell types can be derived from human embryo stem cells then the introduction of the mutation into an existing stem cell line makes possible a direct comparison between the same cell population with or without the mutated gene.

Alternatively, preimplantation genetic diagnosis may be used by prospective parents to ensure the birth of a child who has not inherited the disease by selection of embryos that do not carry the causative mutation. By contrast, with the consent of the parents those embryos that carry the mutation may be used for research, rather than being destroyed. In this case there is no unaffected control population of cells.

The specific circumstances in which nuclear transfer would provide a unique opportunity apply when the causative allele(s) has/have not been identified and the affected tissue cannot be recovered from the patient. The family of diseases that are variously known as motor neuron disease (MND), amyotrophic lateral sclerosis (ALS), or Lou Gehrig's disease is one such case.

ALS is a relentlessly progressive muscle-wasting disease [8]. Degeneration of motor neurons is the common cause of this fatal condition, but the molecular mechanisms leading to degeneration are not well understood. Several genetic and environmental factors contribute to the pathogenesis of ALS. The great majority of cases are sporadic with no evidence of inheritance in the family, but in a small proportion of cases (approximately 10%) the disease does occur frequently within a family. Penetrance is variable, but in the great majority of cases those with mutations in superoxide dismutase (SOD1) develop the symptoms of ALS. Mutations in SOD1 account for approximately 20% of inherited cases, and genetic analysis has identified other dominant and recessive familial ALS loci on chromosomes 2, 9, 15, 18, and 20 [17].

It was at first assumed that ALS caused by mutations in SOD1 reflected a loss of function of the damaged gene, but this seems not to be the case. No symptoms of ALS were evident in mice in which SOD1 had been deleted [9]. By contrast, mice do develop symptoms of ALS if mutated human genes are overexpressed, suggesting that the effect of the mutation is through a toxic effect of the abnormal protein, rather than a loss of function [9].

While the symptoms of the disease reflect loss of motor neurons, until recently it was not known whether these cells were the site of the primary lesion. New studies in mice carrying mutant SOD1 genes that could be removed from motor neurons or microglia in a tissue-specific manner identified two phases in the development of the disease. Removal of the transgene from microglia had little effect on the development of first symptoms, but dramatically slowed progression through later stages [2]. Conversely, removal of the gene from progenitors of motor neurons delayed early stages of the disease. These observations suggest that the first symptoms reflect changes in the motor neurons, but that later in the development of the disease changes in microglia exacerbate the damage in the motor neurons. The observation that ALS is not cell autonomous has profound implications for cell therapy, because it suggests that healthy glial cells may be able to provide protection for failing motor neurons. In addition, they also show that both glia and neurons should be studied in search of an understanding of the causes of ALS.

Cells suitable for these analyses could be obtained from mouse or human embryo stem cell lines. Cell lines derived from mice carrying a mutant human SOD1 transgene could be studied to identify changes in gene expression and other aspects of cell function. There are also several new ways by which suitable cells derived from human embryo stem cells may be obtained. For those cases in which the mutation has been identified, the mutation can be introduced into existing embryo stem cells or pre-implantation genetic diagnosis used to identify embryos that have the mutation.

To complement this approach, nuclear transfer will make it possible to study familial cases in which the mutation has not yet been identified, which means some 8% of all cases. As resources allow, it will also be possible to study neural lineages from sporadic cases (90%) in search of the factors that have made the patients unusually sensitive to environmental factors.

The aim of all these approaches would be to identify the molecular changes in the cells with a view to establishing a high-throughput screen for small molecules able to prevent degeneration of motor neurons or glial cells. Differences associated with the disease would be sought by profiling gene expression at RNA and protein levels and by observation of protein distribution within cells. At present, candidate drugs may be assessed for their ability to delay development of the disease in mice in which transgenes over express mutant forms of human SOD1. A handful of compounds can be compared each year, at considerable cost. By contrast, a high-throughput screen based upon tissue culture would be able to compare thousands of compounds in the same time and at a similar cost. Candidates identified in this way would be then be evaluated in animal studies before being considered for clinical trials.

Many other human genetic diseases could be studied by the same approach. Other candidate conditions for study include other neurodegenerative diseases, psychiatric conditions, cardiomyopathy, and some forms of cancer. The advantage of using nuclear transfer is greatest if the mutation that causes the disease is not known. It is also essential that the affected cell types can be produced from embryo stem cells in the laboratory. Finally, it must be possible to make observations on the cells in search of the molecular mechanism that causes the disease. These studies need not necessarily concern the physiological function of the cells. In the case of ALS, the demonstration of a harmful effect of corrupted protein in transgenic animals strongly suggests that the fate of the protein in the cells should be an initial subject of study.

27.2.2 Cells for Therapy

When considering cell therapy, the ideal would be to be able to offer a perfect immunological match for every patient. This might be achieved by having a very large network of banks or by using SCNT to produce "patient-specific cells." It is implied that SCNT could be used to produce a cell for each person as required. In terms of the requirement for skilled personnel, high-quality facilities and the supply of oocytes, the sheer scale of that task is almost beyond imagination. In reality it seems probable that SCNT might be used to provide cells for very specific purposes, such as gene therapy or to produce cell lines that could be used to treat large numbers of patients, rather than individuals (see below).

Similarly, the idea of holding a universal bank of cell lines collected at random from donated embryos is also impracticable on several practical grounds, which include cost. The first estimates have been made of the effect of only being able to hold a limited number of lines on the probability of being able to provide cells with an acceptable match for the population of the United Kingdom [36]. To estimate the likely genotype of cell lines provided by donors available at random, the databases of the genotype of 10,000 consecutive organ donors was used. Patient populations were simulated by use of genotype information on 6,577 patients waiting for organ transplantation. The authors considered only HLA-A, HLA-B, and HLA-DR, and calculated the best possible match. A bank of 150 lines was estimated to provide a perfect match for nearly 20% of the population. The probability of having either a perfect match or a mismatch at HLA-A or HLA-B approached 40% (37.9%). A panel of this size was expected to provide at least a match for HLA-DR for 84.9% of the patient population. As the size of the bank was increased beyond 150 lines there was a diminishing return for the additional resource.

By contrast, it was predicted that there would be great advantage in obtaining cells lines that are homozygous for common HLA types [36]. A bank of just 10 lines homozygous for common HLA types was expected to provide a perfect mach for 37.7% and a mismatch only at HLA-A or HLA-B for 67.7% of the patient population. These calculations provide a robust indication of the potential benefit of using SCNT to obtain cell lines of selected homozygous genotypes.

27.3 Somatic Cell Nuclear Transfer

SCNT is just one of a number of methods of producing cells of specific genotype. They may be produced by SCNT using a cell from a person with the desired genotype as the nuclear donor. Alternatively, progress is being made toward the establishment of methods for treating somatic cells in such a way that they acquire many of the characteristics of ES cells. The alternatives will be considered later in the chapter.

27.3.1 Procedure for Nuclear Transfer

Two cells are required for SCNT, an oocyte and a nuclear donor cell [5]. Typically the oocytes are at the second metaphase of meiosis, but a recent publication has demonstrated a benefit following the use of early zygotes as recipient cells [27]. In most procedures, the oocyte is enucleated before the nucleus of a donor cell is introduced. However, in some cases the nucleus is introduced before the same pipette is used to enucleate the oocytes [26]. Nuclear transfer may occur by fusing the entire donor cell to the enucleated oocytes or by rupturing the donor cell and injecting the nucleus and some cytoplasm directly into the oocyte.

If an oocyte has been used as the recipient cell, the reconstructed embryo must then be induced to resume meiosis by parthenogenetic activation. A variety of procedures have been established, and there appear to be differences between species in the specific procedure that is most effective. In addition, there are differences between species in the optimum interval between introduction of the donor nucleus and the activation stimulus, which may be either simultaneous or delayed. In sheep, there was no evidence of an effect of varying this interval [4] and in cattle there was an advantage in delaying activation [42], whereas in the mouse cloned pups were only produced if activation was delayed [40].

Typically, the embryos are cultured for a period before transfer to recipient females. This step provides information on the early development of the embryos, and because only those embryos that are developing normally are transferred to surrogate females it also reduces the number of recipient females that are required.

27.3.2 Present Successes and Limitations

The present procedures for SCNT are widely used, and in that sense repeatable, but they are very inefficient. Viable offspring have been produced after nuclear transfer in a number of species, including mouse, cow, pig, rabbit, rat, horse, cat, and dog. Although these species have been cloned there are no cloned primates at present, despite a considerable research effort.

Similarly, offspring have been obtained after transfer of a variety of different adult somatic cell types (reviewed in [24]). In general, the experiments have not been carried out on a large enough scale to permit meaningful statistical comparisons; this would require several hundred, perhaps thousands, of nuclear transfers for each cell type. There is a suggestion that as cells are taken from later stages of development the efficiency decreases [47], as had been shown previously in amphibians [12], but there are reports suggesting exceptions [32]. This confusion may reflect the fact that in some experiments the procedures being used had not been optimized for each cell type.

The overall inefficiency reflects losses at all stages of development and after birth. The general experience is that offspring derived by SCNT require additional assistance at birth. The birth of calves is often induced to facilitate provision of assistance [43]. Typically, cloned mice are delivered by caesarean section and the pups cross fostered [40]. A variety of different abnormalities have been reported in cloned offspring. These include difficulties in breathing and increased birth weight [43].

At the present time the molecular mechanisms that lead to the birth of cloned offspring are not understood. Following transfer of the nucleus into the cytoplasm of the recipient oocytes or zygote, unknown factors in the oocyte cytoplasm must act to modify the epigenetic mechanisms that regulate gene expression of the transferred nucleus so that the embryo is able to develop. It is possible that offspring are derived from donor cells that are more amenable to reprogramming. Alternatively, the inefficiency may reflect a requirement for a number of key epigenetic changes that may be independent events such that failure for them all to occur appropriately leaves the resulting embryo with some, but not all, of the required changes. In this case it would be expected that embryos produced by nuclear transfer a variable and unusual pattern of gene expression and chromatin organisation. This is indeed the case.

In some of the resulting embryos the pattern is similar to that in embryos produced by fertilization; however, in others the pattern of DNA methylation and histone modification is more like that of a donor nucleus than of an embryo produced by fertilization (reviewed in [50]). Improvements to the efficiency of SCNT probably depend upon methods of assisting in the reprogramming of the nucleus. Techniques to reprogram cells directly may well contribute to attainment of this objective (see Sect. 27.5).

27.4 Cells from Cloned Embryos

Several independent groups have now demonstrated that ES cells can be derived from cloned mouse embryos and that these lines are able to contribute to chimeras with normal efficiency [18, 22, 41]. An intriguing point is that it is possible to derive ES cell lines from many more cloned embryos than would have become offspring (16% vs 2%) had the embryos been transferred to recipients [39].

27.4.1 Cells from Cloned Mouse Embryos

A recent study analyzed a sample from a collection of more than 150 cell lines and demonstrated equivalence of embryo stem cells derived from mouse blastocysts produced by nuclear transfer and fertilization [39]. This was found to be so in regard to ability to form all lineages in vivo, patterns of gene expression as determined by microarray analysis, expression of markers of pluripotency, and presence of tissue-specific patterns of DNA methylation. These studies lend great encouragement to plans to use cells from cloned human embryos in research. However, it should be appreciated that only a very small sample of the lines was assessed.

27.4.2 Derivation of Cells from Cloned Human Embryos

By contrast, progress in the development of methods for the derivation of cells from cloned human embryos has been very limited, and this area of research marred by fraudulent claims. Two groups have reported the production of blastocysts after transfer of nuclei to human oocytes [15, 31]. Neither of these reports led to the derivation of embryo stem cell lines. In both of those studies fresh oocytes were available and results were less encouraging when oocytes that had not been fertilized during an IVF protocol were then used as recipient oocytes.

It is noticeable that despite considerable research effort there are no reported clones of adult primates. Several groups have described development to the blastocyst stage of embryos produced by SCNT, and in some cases pregnancy was established, but these failed to develop beyond 60 days in any case [23]. This limited success may reflect practical difficulties in carrying out research in these species, but it has also been reported that spindle formation is different in primates and evidence has been presented to suggest that critical factors are removed during the nuclear transfer process in primates [28]. As development appeared to be normal after fertilization of oocytes from which the spindle was removed temporarily and then replaced, it seems that the effect is not due to the physical trauma of micromanipulation or exposure to drugs during the procedure, but to actual loss of as yet unknown critical factors during enucleation [28].

The fact that variations in protocol were required for success in nuclear transfer in different species suggests that we should not be surprised to find that further modifications are required to establish effective procedures in a new species.

27.4.3 Interspecies Nuclear Transfer

In an alternative approach to the production of cells from cloned embryos, cells with many of the characteristics of stem cells were derived following transfer of human nuclei into rabbit oocytes [7]. At present this is the only approach that has produced "human" cloned cells in culture.

When it was published this report was a cause of great controversy. Previous experiments had provided very little evidence to suggest that normal development was possible after interspecies nuclear transfer unless the two species were extremely closely related. However, since the initial report, several groups have shown that it is possible to obtain at least early development after transfer between two divergent species. Embryos have developed to the blastocyst stage after transfer of primate nuclei into rabbit [48, 49] and bovine [16] oocytes. Two factors that may limit the value of interspecies SCNT concern the source of mitochondria in the cells derived from these embryos and the existence of differences between species in the epigenetic mechanisms that regulate early development.

A number of studies have monitored the fate of mitochondria after interspecies nuclear transfer and demonstrated that mitochondria derived from both the oocyte and donor cell were present during early cleavage, although the oocyte contributed a greater number [14, 20, 21].

The limited information that is available has revealed differences between species in some of the epigenetic mechanisms that regulate development, despite the fact that in a general sense they are conserved. Demethylation of DNA in the transferred nucleus was compared when nuclei were transferred from pig cells into rabbit oocytes or rabbit cells into pig oocytes [6]. These were associated with the oocytes rather than the transferred nucleus. Similarly, between-species sperm injections revealed differences between the oocytes in demethylation of the male pronucleus, although in this case the origin of the sperm also had an effect [1].

It seems likely that procedures for the derivation of ES cells of a specific genotype through nuclear transfer can be significantly improved. Many factors might be expected to influence development of embryos produced in this way. Is the organization of mitochondrial genomes in the two species compatible? Should the protocols used for production and culture of such embryos be those for the species from which the oocytes were recovered or the nuclear donors? Moreover, a great deal remains to be learned about embryos and cells produced by interspecies nuclear transfer. Are the cells capable of being passaged many times, essentially indefinitely, as is expected of embryo stem cells?

Several reasons are put forward for using of animal oocytes. First, if there are indeed differences in early development between primates and other groups of mammals it may be possible to identify these by comparison of development between embryos produced by transfer of nuclei from the same primate donor population into primate and nonprimate oocytes. Additionally, it may be possible to derive cell lines in this way. If they are obtained, it will be important to discover whether the cells in fact have all of the characteristics of human embryo stem cells. If not, do they have sufficient similarity to allow them to be used in research to study disease? Clearly there would be great benefit in being able to use animal oocytes, most clearly in the numbers available. In fact it will only be possible to pursue this approach to the study of many diseases if such a source of oocytes is identified.

27.5 Direct Reprogramming of Somatic Cells

The birth of offspring following transfer of nuclei from adult somatic cells into enucleated oocytes was the first observation to demonstrate that under appropriate circumstances gene function in such nuclei can be reprogrammed to allow them to control normal development [45]. Since that time several approaches have been used to investigate reprogramming, and more particularly to assess the ability of other methods to achieve similar ends. Most strikingly, the introduction of transcription factors into skin fibroblasts gave a small proportion of the cells many of the characteristics of embryo stem cells (see below).

In the first studies seeking factors that may change epigenetics during SCNT the small volume of cytoplasm in mammalian oocytes imposes strict limits on biochemical analyses, but this has been overcome by use of amphibian oocytes [3, 13, 29, 30] or embryo stem cells (see below).

It is many years since it was first demonstrated that amphibian oocytes are able to reprogram gene expression in mammalian nuclei (reviewed in [13]), providing an opportunity to define the molecular events involved and perhaps in the longer term to identify the active factors. The first change in the nuclei is a pronounced increase in volume of the transferred nucleus [30], and a further 2 days' incubation are required for the initiation of expression of key genes, such as Oct4. However, the oocytes are incubated at 18°C and the authors argue that this is equivalent to 12 h at 37°C [3]. Gene expression is accompanied by demethylation of promoter regions, and key binding sites have been identified [30]. Two proteins have been shown to have a role in reprogramming [19, 35] The identity of other active factors remains to be defined, but in the future this approach may provide another means of obtaining cells of a chosen phenotype from a specific patient.

When adult somatic cells were fused to embryo stem cells it was found that the somatic nucleus was reprogrammed toward ES cell phenotype [33], provided that the nucleus of the embryo stem cell was present [11]. Since that initial experiment a number of groups have studied the phenomenon, with a view either to developing a means of direct reprogramming or to using this approach to identify the active factors responsible for the reprogramming activity [10]. Despite a number of ingenious approaches, so far nobody has devised a method for the effective specific removal of the chromosomes of the embryo stem cell from the heterokaryon. While cell fusion seems unlikely to yield cells suitable for therapy, any new understanding of the nature of the key reprogramming factors would be extremely valuable.

Recently a radically different approach has provided another means of producing cells of specific genotype. The authors speculated that the introduction of key transcription factors would be able to modify cells in such a way that they acquired the characteristics associated with the specific panel of transcription factors. They identified in total 22 factors that are known either to be essential for embryo stem cell maintenance or were at least known to be expressed in embryo stem cells. Retroviral vectors were used to introduce this group of candidate transcription factors into fibroblast cultures [34]. After careful analysis it was found that just four factors were able to make some murine fibroblasts pluripotent. These factors were Oct3/4, Sox2, c-Myc, and Klf4. The cells were able to form tissues of all the major lineages in culture and in chimeras, although in the first study none of the chimeras survived to term. Subsequently, it was shown that cells selected on the basis of their expression of Nanog or Oct4 after introduction of these four factors produced cells that are able to contribute to germline chimeras [25, 44].

This most remarkable demonstration has created an entirely new means of obtaining cells of specific genotype, although it remains to be shown whether an effective procedure can be established for human somatic cells.

27.6 Looking to the Future

It is essential in the development of these methods that a precise assessment is made of the effect of the reprogramming procedure upon the cell lines that are derived. A number of strategies may be considered to clarify the situation. A great deal has been made in both scientific and popular press of the potential value of cells from cloned embryos in therapy, but in their haste to consider this use many people have overlooked the considerable benefits that may be gained by the use of such cells in research into inherited diseases. Such studies would provide opportunities to study diseases that are not available in any other way at present and to perform high-throughput screening of potential therapeutics. This laboratory-based research will also enable us to learn whether or not cells obtained by reprogramming function normally might indeed be suitable for use in therapy.

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Genetic Medicine and Global Health

28

David J. Weatherall

Abstract Recent estimates suggest that nearly 8 million children are born each year with a serious birth defect of genetic or partial genetic origin, and over 3 million children under the age of 5 years die from birth defects each year. As poorer countries go through the epidemiological transition following improvements in social conditions and health care, many babies with serious genetic diseases who would have died in early life with these conditions unrecognized are now surviving for diagnosis and management. The reasons for the particularly high frequency of some genetic diseases in poorer countries are complex; undoubtedly natural selection, consanguinity, and increased parental age are important factors. Common monogenic diseases such as the hemoglobinopathies are presenting an increasingly severe health burden. DNA diagnostics and modern genomic technologies have an increasingly important part to play in the control of many common communicable diseases, and as countries go through the epidemiological transition many of them are encountering epidemics of diseases of westernization, including cardiac disease, hypertension, and type 2 diabetes, almost certainly reflecting changes in environment associated with variable genetic susceptibility. The introduction of clinical genetics and genetic technology into the developing countries raises many complex ethical and social issues, not to mention the high costs of this technology. Recent reports from the World Health Organization have stressed the potential value of evolving North/South partnerships between centers in genetics in the richer countries and the developing countries; these might be followed by South/South partnerships between emerging countries with expertise gained in this area and adjacent countries where no such skills exist. But none of these developments will occur without a greater recognition of the importance of genomics on the part of the major international health agencies.

Contents

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28.1 Introduction

During the second half of the twentieth century the development of clinical genetics was restricted largely to the richer, developed countries. This is not surprising, considering that the developing countries were still suffering from high levels of childhood mortality that were due to malnutrition, poor sanitation, and the ravages of communicable disease. In the face of these problems international health agencies have tended to disregard the burden of disease caused by genetic disorders and congenital malformation; even in a recent authoritative review of priorities for research in the developing countries, with the exception of the hemoglobinopathies they are not mentioned [20].

On the other hand, the partial completion of the human genome project in 2001 and subsequent successes in sequencing the genomes of a variety of pathogens and disease vectors suggested that genomics might have an increasing place to play in global health issues. In 2002 the World Health Organization (WHO) published a report entitled *Genomics and World Health* [48], which concluded that DNA technology should be slowly introduced into some of the developing countries, particularly for the control of common monogenic diseases and communicable diseases.

The current state of clinical genetics and the availability of genetic technology varies widely, particularly among the developing countries. Furthermore, their application in these countries raises social and ethical problems that are completely different from those encountered in the developed world. This chapter summarizes a few of the more important aspects of this complex scene.

28.2 Environmental Factors and Genetic Disease

28.2.1 *Poverty and the Epidemiological Transition*

During the last decade of the twentieth century gross domestic product per head in the developing countries grew by 1.6% a year, and the proportion of people living on less than $1 a day fell from 29 to 23%. Most of this progress was made in Asia, however, and in other parts of the world, notably sub-Saharan Africa, the number of poor people increased. Furthermore, 150 million children living in low- and middle- income economies are still suffering from malnutrition and, unless the situation improves, a similar number are expected to be underweight by 2020. And the plight of the developing countries has become even more difficult because of an inability to control their major killers. About 70% of the 40 million people affected by HIV/AIDS are concentrated in countries with dysfunctional healthcare systems. Tuberculosis, often drug resistant, has re-emerged, with 9 million new cases and 2 million deaths each year, and death rates from malaria are similar [47, 52].

Yet despite this depressing scenario, the latter half of the twentieth century did see some significant improvements in the overall health of many populations in the developing countries. Largely as the result of, improvements in hygiene and social conditions the childhood mortality rate, i.e., the number of children who died in the first 5 years of life, started to decline, in some countries quite dramatically [10]. These improvements in population health were most marked in some of the middle-income countries, notably in South America and the Caribbean, East Asia and the Pacific, the Mediterranean region, the Middle East, and parts of North Africa.

The consequences of this epidemiological transition for the recognition of the importance of genetic disease in many developing countries were quite remarkable. The high frequency of thalassemia in Cyprus is a good example [39]. This condition was not known to occur on the island until 1944, when the clinical findings in 20 patients were reported. This paper highlights the difficulty in identifying this disease in an island population against the background of chronic malaria and other infections; it was published at the end of an extremely successful 3-year program to control anopheline breeding, at which time it was found that anemia secondary to malaria had almost disappeared. Hence, within this remarkably short period it became clear that there was a high frequency of genetic anemia with features of thalassemia in the island population. By the early 1970s it was estimated that, if no steps were taken to control the disease, in about 40 "years" time the blood required to treat all the children with thalassemia would amount to 78,000 units per annum, 40% of the population would need to be donors, and the total cost to the health services would equal or exceed the island's health budget [39]. The same pattern of the increasing realization that many populations of the developing countries have relatively high frequencies of genetic disease has slowly emerged throughout the world over the last 50 years.

In short, the epidemiological transition has improved children's health to the extent that many of those who were born with genetic disease at an earlier time, and whose condition would not have been recognized against a background of severe malnutrition and infection, have begun to survive long enough to present for diagnosis and treatment.

28.2.2 Why Are Genetic Disease and Congenital Malformation Commoner in the Developing Countries?

As discussed in the next section, there is considerable evidence showing that genetic disease and congenital malformation occur more frequently in countries with a low gross national income (GNI) per capita. There are several reasons why this might be the case [10].

28.2.2.1 Selection

As discussed later in this chapter, there is strong evidence to show that the very high frequency of the thalassemias and structural hemoglobin variants in many tropical countries is the result of heterozygote selection by relative resistance to infection by *P. falciparum* malaria. A similar mechanism is responsible for the very high frequency of glucose-6-phosphate dehydrogenase (G6PD) deficiency in parts of the world where malaria has been very common in the past or still is a major health hazard. Although, as discussed below, many problems remain to be clarified, there is no doubt that natural selection working through malaria has been largely responsible for the extraordinarily high gene frequencies for these disorders in many countries of the world [39].

28.2.2.2 Consanguineous Marriage

Although it has been extremely difficult to obtain accurate data, it is clear that consanguineous marriage is still practiced in many parts of the world and may be acceptable to a minimum of 20% of the world's population [10]. This practice is especially common throughout the eastern Mediterranean, North Africa and the Indian subcontinent and, to a lesser extent, parts of South America and sub-Saharan Africa [6, 7, 25]. As well as increasing the birth prevalence of autosomal recessive diseases, the risk of neonatal and childhood death, intellectual disability, and serious birth defects appears to be significantly increased in first-cousin marriages.

28.2.2.3 Parental Age

The percentage of women over the age of 35 years delivering infants is high in middle- and low-income countries, many of which do not have screening, prenatal diagnosis, or related services. For example, the birth prevalence of Down syndrome can reach 2–3 per 1,000 live births in these populations, a range currently double that seen in high-income countries [44, 49].

28.2.2.4 Population Migration

Large-scale population movements from areas of high frequency for single gene defects may introduce these disorders into new populations. As evidenced by the spread of the hemoglobin disorders, notably sickle cell disease, to the Americas, the Caribbean, and Europe by the slave trade and later migrations, this mechanism has led to a particularly high frequency of sickle cell anemia in many developed or developing countries. Other examples include the introduction of Huntington disease to Venezuela, spinocerebellar atrophy to Cuba, and porphyria to South Africa [10].

28.2.2.5 Poverty and Dysfunctional Healthcare Systems

There is an increased rate of birth defects in poorer countries. The reasons are not clear but may reflect maternal malnutrition and increased exposure to alcohol and infection. And since many of these countries have very limited facilities for the correction of structural birth defects, their frequency increases steadily in these communities. Similarly, the frequency of neural defects, fetal alcohol syndrome, congenital syphilis and rubella, and related disorders reflects the lack of prenatal care in many of these populations [11].

28.3 Global Burden of Genetic Disease and Congenital Malformation

For a variety of reasons, including limited diagnostic facilities in many of the developing countries and the extraordinary heterogeneity in the gene frequency of monogenic disorders even within small geographic distances, it has been extremely difficult to obtain accurate data about the global frequency of genetic disease and congenital malformation [45]. The most ambitious attempt to date to produce data of this kind was reported in the recent study supported by March of Dimes [10].

Overall global figures for the frequency of genetic disease and congenital malformation are summarized in Table 28.1. Data relating to individual diseases will be found in the appropriate chapters of this book and in the March of Dimes report [10]. A word of caution about these figures is necessary, however. While reasonably accurate data are available in many developed countries, this is not the case for many parts of the developing world. In countries with high infant and childhood mortalities resulting from malnutrition and infection, congenital malformation or genetic disease may not be recognized and reporting is limited. Furthermore, genetic disease that has reached a high frequency by natural selection tends to be very unevenly distributed even within short geographic distances, a subject to which we will return when we consider the genetic disorders of hemoglobin. Hence, analyzing populations in a few centers does not make it possible to obtain a figure for the overall frequency in a particular country. Hence, much of the data shown in Table 28.1 reflects information obtained from developed countries, augmented with what little is known about these frequencies in the developing world.

Even more serious problems face attempts to determine the economic burden of genetic disease and congenital malformation in many countries. Although there are some reasonable data on the costs of caring

Table 28.1 Minimum estimates for the birth prevalence of infants with serious congenital disorders, by WHO region [46]

WHO region	Population, millions (1996)	Births/ yarrmillions (1996)	Congenital malformations /1,000	Chromosomal disorders /1,000	Single gene disorders /1,000	Total congenital disorders /1,000	Annual affected live births
Eastern Mediterranean	506	18.1	35.7	4.3	27.3	69	1,237,225
Africa	540	23.0	30.8	4.4	25.0	61	1,412,427
SE Asian	1,401	38.2	31.0	3.9	14.7	51	1,946,606
Europe	867	10.8	31.3	3.7	12.4	49	522,832
Americas	782	16.2	30.9	3.8	11.9	48	774,235
Western Pacific	1,650	31.3	30.6	3.5	11.4	47	1,464,067
Total	5,746	137.6	31.5	3.9	16.8	53	7,357,392

for patients with genetic disorders and cost-benefit analyses of different forms of management for these conditions in the developed countries, the position regarding the developing world is much more complex and uncertain. There are widespread differences in the ways these conditions are managed, if they are managed at all, and it has been difficult to obtain accurate information about costs or cost-benefit ratios. A start has been made in this important field in the case of the hemoglobinopathies, and current progress is discussed later in this chapter.

Currently, the economic burden of disease is assessed by converting conditions to Disability Adjusted Life Years (DALYs), a measurement that attempts to include not only mortality but the burden and effectiveness of supporting patients with particular diseases [27]. Using this approach, health economists attempt to compare the economic burden of different diseases with one another. Overall, most work in this field has been directed at the major infectious diseases and the important diseases of the richer countries: heart disease, cancer, stroke, and similar disorders. Genetic disease has received little attention from health economists. However, if cases are to be made to governments and international health organizations about the importance of genetic disease it will be vital to attempt to analyze these conditions in terms of DALYs in the future. We return to this question below, when we consider the genetic disorders of hemoglobin.

28.4 Monogenic Disease

Although monogenic diseases occur in every ethnic group, and owing to founder effects and other mechanisms may occur at unusually high frequencies in localized regions, only the inherited disorders of hemoglobin occur at sufficiently high frequencies to present a major public health problem for a large number of the developing countries. These conditions are described in detail elsewhere in this book. Here, issues relating to their impact on global health are discussed, with particular reference to how limited successes in their control in at least some developing countries offer a model on which programs for the control and management of genetic diseases might be based in these difficult environments.

28.4.1 Inherited Disorders of Hemoglobin

Although there are many different forms of thalassemia, and over 700 structural hemoglobin (Hb) variants, $\alpha-$and $\beta-$thalassemia together with three structural variants, Hbs S, C and E, are the only hemoglobinopathies that reach high enough frequencies to cause a major burden on the healthcare services of the developing countries.

28.4.1.1 Global Distribution

The global distribution of the important hemoglobin disorders [24, 38, 39] is summarized in Figs. 28.1 and 28.2. The β-thalassemias are distributed at varying frequencies right across the tropical belt, ranging from sub-Saharan Africa through the Mediterranean region, the Middle East, the Indian subcontinent, and East and Southeast Asia. The $\alpha-$thalassemias have a different pattern of distribution, a fact of considerable global health significance. There are two main forms, α^+-and α^o-thalassemia. α^+-Thalassemia is caused by deletion or inactivation by mutation of one of the pairs of $\alpha-$globin genes (-α/αα), while α^o-thalassemia is due to the deletion of both pairs of $\alpha-$genes (--/αα). The α^+-thalassemias, the commonest monogenic diseases, are spread at varying and sometimes extremely high frequencies across the region shown in Fig. 28.1, while the α^o-thalassemias are restricted to parts of Southeast Asia and the Mediterranean islands. The result is that the severe, symptomatic forms of $\alpha-$thalassemia are restricted to the latter regions.

The world distribution of the Hb S and E genes, shown in Fig. 28.2, is quite remarkable [24, 39, 40]. The sickle cell gene is found in populations stretching from sub-Saharan Africa through the oasis regions of the Middle East to localized regions of the Indian subcontinent. It is not observed further east. On the other hand, Hb E is found on the eastern side of the Indian subcontinent and stretches through Burma (Myanmar) to Southeast Asia. In short, the Old World has extremely high frequencies of two $\beta-$globin-chain variants, which are separated by a line that runs North-South through the eastern part of the Indian subcontinent.

Hemoglobin C, which is of less clinical importance, occurs only in localized areas of West and North Africa.

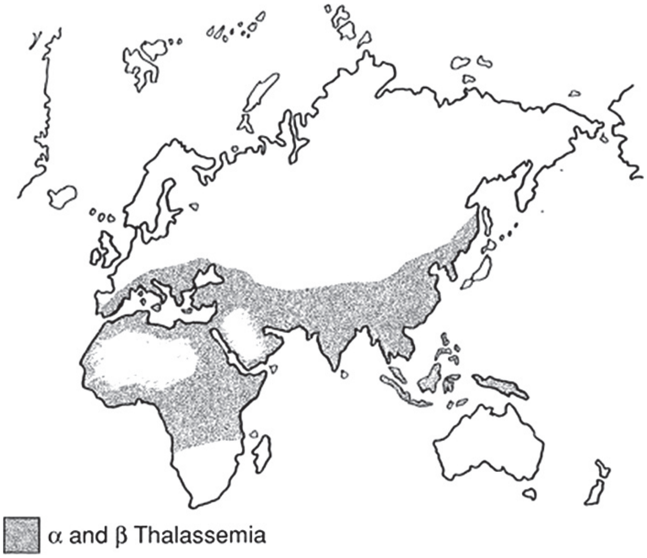

Fig. 28.1 World distribution of α- and β-thalassemia [39]

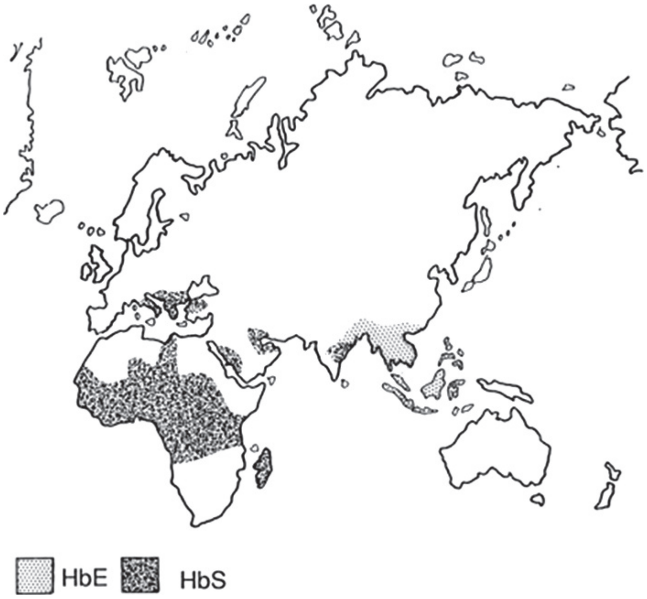

Fig. 28.2 World distribution of hemoglobin S and E [38]

28.4.1.2 Frequency

Estimated frequencies for the different inherited disorders of hemoglobin are discussed in several reviews [10, 24, 39, 40]. Frequencies for each of the designated regions of the World Health Organization are given in Table 28.2. As explained earlier, these are very approximate data, simply because of the extraordinary heterogeneity in the frequency of these gene frequencies within short geographic distances of one another. Even

Table 28.2 Percent carrier frequencies for common hemoglobin disorders by WHO region [24, 38, 39]

Region	Hb S	Hb C	Hb E	β-Thalassemia	α^{o}-Thalassemia	α^{+}-Thalassemia
Americas	1–20	0–10	0–20	0–3	0–5	0–40
Eastern Mediterranean	0–60	0–3	0–2	2–18	0–2	1–80
Europe	0–30	0–5	0–20	0–19	1–2	0–12
Southeast Asia	0–40	0	0–70	0–11	1–30	3–40
Sub-Saharan Africa	1–38	0–21	0	0–12	0	10–50
Western Pacific	0	0	0	0–13	0	2–60

Note: many of these data are derived from small population samples

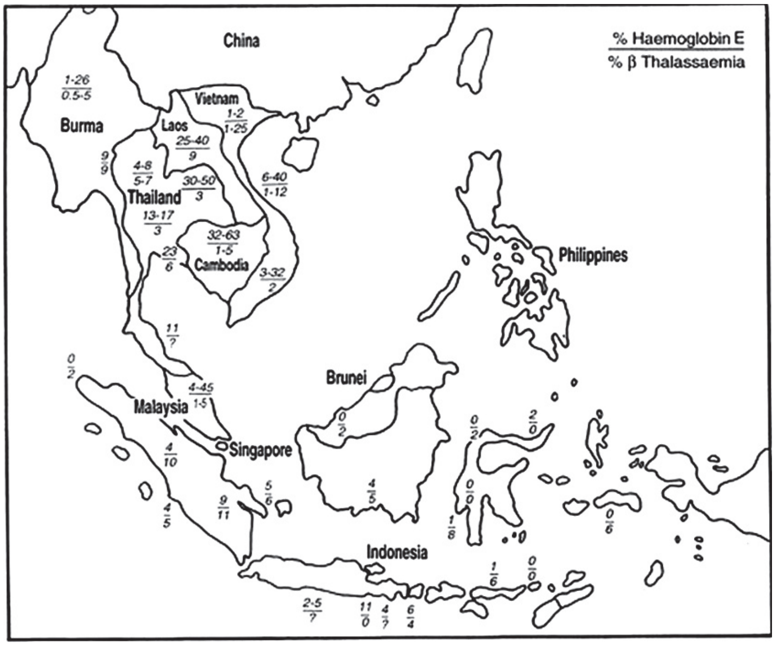

Fig. 28.3 Distribution of Hb E and β thalassemia in SE Asia [13, 39]

in a relatively small island population such as Sri Lanka there are considerable differences between populations resident only a few hundred miles apart [13].

Despite these uncertainties, certain generalizations can be made. The sickle cell and β thalassemia genes, though they occur at varying frequencies in different populations, rarely occur in above 20% of the population; there are a few exceptions, including the particularly high frequencies of the sickle cell gene in some of the oasis populations of the Middle East and localized pockets in India, and the very high frequency of β-thalassemia in the Maldives. On the other hand, the α^{+}-thalassemias occur at much higher frequencies, affecting up to 70 or 80% of the population in some regions. The α^{o}–thalassemias occur at similar frequencies to the β-thalassemias. As in the α^{+}-thalassemias, the carrier rates for Hb E greatly exceed those of Hb S; in many Asian populations they reach as high as 70%, notably in parts of northern Thailand and Cambodia.

The remarkable diversity of these gene frequencies within populations presents a major problem for developing rational public health programs. The distribution of β-thalassemia and Hb E in the Indonesian islands is shown in Fig. 28.3, and the estimated calculation of new births with serious forms of thalassemia in this population is shown in Table 28.3. Although the latter gives some indication of the likely health burden of these conditions for the future, even after a study carried out at this level of detail the estimated number of new births is beset with considerable uncertainty. Approximate figures for new births with serious hemoglobin disorders for Thailand, a country in which frequencies have been mapped in more detail than most, is given in Table 28.4.

Table 28.3 An estimated population burden of the β-thalassemias in Indonesia

Population 200 million		
Annual births		(750–2,850)
β-Thalassemia major	1,600	(1,000–4,750)
Hb E thalassemia	2,500	
Total annual units of blood for treatment	1.25 million	

From A.S.M. Sofro, J.B. Clegg, D.J. Weatherall, unpublished data

Table 28.4 The public health burden produced by thalassemia in Thailand in 1999

Population 61.5 million (68% rural)	
Infant mortality 25/1.000	
Total patients with thalassemia	523,750
Births of new cases per year	
β-Thalassemia major	625 (6,250)
Hb E/β-Thalassemia	3,250 (97,500)
Hb H Disease	7,000 (420,000)

Data supplied by the Thai Thalassemia Association and Dr. S. Fucharoen; bracketed figures are estimates of current numbers of patients in the community

28.4.1.3 Population Genetics and Dynamics

Although from association studies and other sources of evidence it has been suspected for a long time that the high gene frequencies for the common hemoglobin disorders are attributable to the effects of natural selection mediated through carrier protection against *P. falciparum* malaria, it is only recently that this mechanism has been confirmed directly by case control studies, at least in the case of the sickle cell, Hb C, and α-thalassemia genes [22, 38, 40, 50]. Taking strict World Health Organization criteria for the severity of malaria, particularly the occurrence of coma or profound anemia, the sickle cell trait seems to offer approximately 60–70% protection, while the homozygous state for α^+-thalassemia offers 30–60% protection. Similarly, high protection rates have been found in the case of the homozygous state for Hb C and, although the appropriate case control studies have not been done, there is a considerable body of evidence suggesting that the high carrier states for β-thalassemia and Hb E also reflect protection against malaria.

The eradication of *P. falciparum* malaria would undoubtedly result in a slow decline in the frequency of the important inherited hemoglobin disorders, but this would take many generations before its effect became apparent in clinical practice [38]. Furthermore, there is widespread drug resistance to *P. falciparum* and clear evidence of a recrudescence of the disease in some parts of Africa and Asia. Thus, for public health planning there is no reason to believe that the hemoglobin disorders will show any decline in their current frequency for the foreseeable future. These observations provide further evidence that as countries pass through an epidemiological transition the hemoglobin disorders will present an increasingly serious public health problem. Current estimates for the annual numbers of births of babies with these diseases [10] suggest that the figure is approximately 300,000, though this may well be a considerable underestimate owing to lack of accurate population data in many high-frequency countries.

28.4.1.4 Clinical Load and Cost–Benefit Issues of Control and Management Posed by the Inherited Hemoglobin Disorders

Like most of the inherited disorders of hemoglobin, the common sickle cell disorders, i.e., sickle cell anemia, Hb SC disease, and HbS β thalassemia, all show considerable clinical heterogeneity. The fact that the form of sickle cell anemia which arose independently in Asia is, overall, milder than that seen in Africa suggests that genetic factors play an important part in this clinical heterogeneity. However, while the ability to produce Hb F and the co-inheritance of α-thalassemia have been identified as playing a part, from such twin data as are available it is clear that there must be other genetic factors, and in particular that the environment is a major determinant of the course of the disease [43].

There are limited mortality data for the sickling disorders. Early studies in Africa suggested that very few children with sickle cell anemia survived beyond the first few years of life [15]; however, in some parts of Africa, following the introduction of antimalarial and other public health measures the situation seems to have improved although no accurate mortality data are available [1, 26]. The position is different in some of the developing countries. Recent US data suggest that the median age of death is 42 years for men and 48 years for women [14]. A cohort study in Jamaica has shown how improved social conditions and treatment have increased survival; 70% of those enrolled starting in 1973 survived to age 20 years, as did 80% of those enrolled 3–6 years later [42].

Preliminary attempts at assessing the burden of sickle cell disease in terms of DALYs have been reported recently [42]. Based on birth and scanty survival data it appears that in Africa this disorder may account for between 0.5 and 4.5 million DALYs, or less than 1%, to approximately 2% of the burden for children under 5. Again, although data are extremely limited, it appears that sickle cell anemia may account for at least 1–2.2 million DALYs in low- and middle-income countries. Clearly, we have only the flimsiest of ideas about the total global health burden imposed by the sickle cell disorders. Such as are available probably reflect a major underestimate, and the situation will undoubtedly change as developing countries, particularly those in sub-Saharan Africa and parts of Asia, pass through the epidemiological transition.

Extensive data on the economic aspects of sickle cell anemia have been obtained from the USA [12]. An estimated 75,000 hospital admissions of both children and adults occurred each year from 1989 through 1993. In 1993 the direct costs of management were $ 575 million. Although it has been reported that the development of centers with expertise in the treatment of sickle cell anemia may significantly lower health,care costs [28, 53], these advantages may have been reduced by the more intensive care of certain subsets of patients over recent years, e.g., the use of chronic transfusion. A pilot study in Benin found that the development of a similar program reduced the frequency and severity of acute complications, with an annual cost per family of $ 40 and an annual cost for each hospitalization of $ 100 [33].

To what extent are current approaches to the management of the sickle disorders cost effective? From the limited data that are available it appears that specialized treatment centers and, in particular, neonatal screening programs are effective approaches to the control of sickle cell anemia. It is now clear that neonatal screening followed by the use of appropriate prophylactic treatment with penicillin prevents deaths [37], and in a recent survey of studies of this kind it became quite clear that this approach was highly cost effective [42]. However, although other recent advances in therapy, e.g., the control of strokes by monitoring and transfusion, have been shown to be clinically effective, none of them have yet been exposed to more detailed cost-benefit studies.

Except in a limited number of cases in which the phenotype of the homozygous or compound heterozygous state for β-thalassemia is modified by either a mild β – thalassemia allele or some other genetic modifier/s, these conditions are almost always transfusion dependent, with resulting costs for procuring and processing blood and ensuring it is free from infection, chelating agents to prevent iron overload, and treatment for the many complications that arise. The life expectancy varies widely between different populations. While there has been a major improvement in many of the developed countries, such data as there are from the developing countries suggests that the situation is much worse. In many, there are no government programs for the control and management of thalassemia and, although transfusions may be available, only those who can afford them are able to obtain drugs for removal of iron and treatment of complications. Hence, the median survival for these children in Thailand is approximately 10 years; in many developing countries it is shorter and may not exceed 1–2 years.

Several detailed analyses of the cost of treatment have been reported [3, 42]. Remarkably, the basic costs were similar in Thailand, east European countries, and Canada. Recently, an attempt has been made to convert these data into DALYs [42]. Considering deaths alone, it appears that severe β – thalassemia contributes about 1.5–3 million DALYs to the world burden. Since there are no accurate global estimates of the number of treated survivors it is difficult to assess the true number of DALYs caused by this disease, but from limited regional data it appears that the number of DALYs in parts of Asia may be comparable with that for some of the common communicable diseases [42].

There is another neglected aspect of the global health problem caused by the β-thalassemias. In populations east of the Indian subcontinent there is a very high frequency of both Hb E and β thalassemia, and hence Hb E β-thalassemia is very common. Indeed, in parts of India and in Bangladesh, Burma (Myanmar), Thailand, and Indonesia, the frequency of Hb E β – thalassemia greatly exceeds that of homozygous or compound heterozygous β – thalassemia. Hb E β – thalassemia is remarkable for its clinical heterogeneity; different individuals who received the same β – thalassemia mutations may have phenotypes ranging from those identical to thalassemia major to a mild condition which does not require transfusion and is associated with long survival. Although some of the genetic factors responsible for this heterogeneity have been determined [32, 34, 51] it is clear that many genetic and environmental factors remain to be discovered before the wide phenotypic variation of this disease can be understood. This is a particular challenge for public health measures directed at the prevention and management of the β – thalassemias in many Asian countries. For example, the current inability to predict the phenotype poses considerable ethical and counseling problems for developing prenatal diagnosis programmes and presents equally complex problems for determining the optimal approach to management.

Because of their more localized occurrence and the fact that babies homozygous for α^{o}-thalassemia are stillborn or die shortly after delivery, this condition produces less of a health burden although considerable distress to families. The delivery of these babies is often associated with severe obstetric complications, including postpartum hemorrhage. Although some of these babies have been rescued for life-long transfusion by intrauterine transfusion, this practice has not become widespread, notably because of the high frequency of the associated congenital abnormalities.

While Hb H disease has been thought to be a fairly innocuous condition, there is increasing evidence from such high-frequency countries as Thailand that a significant proportion of the patients may be more severely aemic and become transfusion dependent. So far, it has not been possible to assess the burden of the α thalassemias in terms of DALYs.

As regards cost-benefit analyses of different approaches to managing the β-thalassemias, although, as mentioned earlier, there are some data on costs of standard therapy there is very little published data on the cost-benefit ratios of particular approaches [42]. If matching donors are available, bone marrow transplantation does appear to be cost effective as a treatment for β-thalassemia [5]. There are no comparable data for the α-thalassemias. There is even less information about the costs of managing the thalassemias relative to national health budgets; it has been calculated that, in Sri Lanka, management of the disease will consume approximately 5–8% of the country's health budget, based on 1999 figures [13].

Control by Screening and Prenatal Diagnosis. Screening and prenatal diagnosis programe for the control of β-thalassemia have been established in many countries [8, 38]. In Europe, 80–90% of counseled at-risk couples now request prenatal diagnosis, and in the late 1970s and 1980s in Cyprus and Sardinia the birth rate of new cases of severe β-thalassemia fell by almost 90%; a comparable fall occurred in Greece and Italy and is now occurring in many other countries. There is no doubt that this is the most cost-effective approach to the control of β-thalassemia.

Screening programs that incorporate both α – and β – thalassemia allow the detection of couples who are homozygous for α^{o}-thalassemia and hence risk having a baby with the Hb Bart's hydrops syndrome. While these babies die in utero or at term, screening and termination of pregnancy is still considered to be important because pregnancies associated with this condition show a very high maternal morbidity.

Hitherto, premarital screening and prenatal diagnosis have been much less widely applied for the control of sickle cell anemia. This is probably because, if adequately counseled, couples realize the extraordinary clinical diversity of this condition and many are loth to undergo termination of pregnancy. A much higher uptake is reported from some European countries; whether this reflects more directed counseling or a different parental perception of the level of severity of a disease for which they would be willing to undergo termination, is not known. The same questions apply for Hb H disease and Hb E β thalassemia, both of which are similarly associated with extremely variable phenotypes.

28.4.2 Other Monogenic Diseases in the Developing Countries

Although, because of their high frequency and the fact that they have acted as model diseases for developing clinical genetic programs in the developing countries, this section has focused on the hemoglobinopathies, there are other single-gene disorders that have important implications for developing countries.

The relationship between the high frequency of G6PD deficiency and malaria has been discussed earlier. The increased sensitivity of those with this enzyme deficiency to antimalarial drugs, notably primaquine, is imposing increasingly important problems for the control of *P. vivax* malaria in many Asian countries. Primaquine is currently the only drug that is available to destroy *P. vivax* in the hepatic stage of its life cycle, which gives rise to recurrent attacks of the disease. Although full primaquine resistance has not yet been encountered, there are signs that it is on the way, and already much longer courses of primaquine are being administered for the eradication of *P. vivax* infections. There is an urgent need for a much simpler, cheaper method of identifying those who are G6PD deficient in high-frequency countries. G6PD deficiency is also associated with a high frequency of neonatal jaundice and kernicterus in many countries [10]. Although the precise mechanisms of the high bilirubin levels and the reasons why this phenomenon is restricted to certain countries remain to be determined, neonatal screening for G6PD deficiency has become absolutely essential in many countries.

There is now considerable evidence showing that the 7/7 promoter genotype of *UGT1A1*, which is common in many countries and is associated with Gilbert's disease, is an important hazard for thousands of individuals, particularly in the Indian subcontinent and Africa, with chronic hemolytic disorders such as Hb E β-thalassemia and sickle cell anemia [18, 31]. This genotype is associated with particularly high bilirubin levels in individuals with these conditions and a very marked increase in the likelihood of developing gallstones.

Examples of the growing importance of pharmacogenetics for the developing countries are given in a later section; a brief account of other monogenic disorders in the developing countries, together with common birth defects, is given elsewhere [10].

28.5 Communicable Disease

Progress towards the application of genomics for the control and management of communicable disease has involved studies of the genetic constitution of infectious organisms and of the vectors that transmit them. In addition, much is being learnt about the genetic basis for individual variation in susceptibility to infectious diseases, work that also promises to have important practical implications in the future [41, 48].

28.5.1 Infectious Agents

Since the first establishment of the complete DNA sequence of the genome of a virus in 1977, the sequences of many viruses and bacteria have been determined and a start has been made on obtaining similar information about the much more complex genomes of common parasites. As well as providing invaluable information about some of the basic mechanisms of pathogenicity and the ways in which infectious agents are able to evade the immune system, these studies have provided a wide range of new diagnostic agents and vaccine candidates [48].

DNA technology has also been of great value in subtyping different strains of viruses and in identifying new infectious agents with the potential to produce major epidemics. In practice, DNA diagnostics have to be carefully compared with more standard culture methods for the diagnosis of infectious disease and for the identification of antibiotic-resistant organisms.

DNA-diagnostic agents are turning out to be of particular value for the identification of organisms that are difficult to grow in culture, an approach which is already proving cost effective in some developing countries. By establishing simplified PCR methods and by the use of inventive approaches, including simplification of protocols and bulk preparation of reagents from crude ingredients, combined with recycling, it has been possible to develop routine diagnostic procedures for such diseases as leishmaniasis, dengue, and leptospirosis in several South American countries [17, 48].

Knowledge of the pathogen genome is also being applied to the development of new therapeutic agents and, in particular, of vaccines [23]. The pathogen genome has already yielded vaccine candidates against *Neisseria meningitidis*, group B, and *Mycobacterium tuberculosis* [48]. And although progress has been extremely slow toward developing a vaccine against HIV/AIDS, current drug therapy for this condition is based almost entirely on studies of the virus at the molecular level. Progress toward the development of vaccines against viruses, bacteria, and malarial parasites is the subject of several extensive reviews [21, 36].

In 2002 the complete genome sequence of the human malaria parasite *P. falciparum* was established, and considerable progress is being made towards sequencing the genome of the other important human malaria parasite, *P. vivax* [9, 16]. Apart from providing important information about the biology of these parasites, this work is already leading to some practical applications. Drug resistance has become a major problem in the control and treatment of severe malaria caused by *P. falciparum*; chloroquine resistance is now almost universal, for example. It has been found that mutations in the chloroquine resistance transporter of *P. falciparum*, encoded by the gene *pfcrt*, confer chloroquine resistance in laboratory strains of the parasite. The identification of these mutations has the potential to be of considerable value for public health surveillance of antimalarial resistance [48]. Information collected from the genome of *P. falciparum* has also led to the development of at least one new antimalarial drug [48] and has become an integral part of efforts to produce vaccines against the different stages of the life cycle of the parasite.

28.5.2 Vectors

The complete genome sequence of the malaria-transmitting mosquito *Anopheles gambiae* was reported in 2002 [19]. This has led to a considerable increase in knowledge about the biology of this vector and has also raised the possibility of novel approaches to vector control. For example, the discovery that transposons can be used to introduce genes into the genome of mosquitoes, and hence reduce their ability to transmit malaria, suggests that, despite the inherent difficulties and potential dangers involved, genomics will provide new approaches to the control of malaria transmission [2]. Similar avenues are being explored for the potential modification of other communicable disease vectors.

28.5.3 Varying Susceptibility

As discussed above, there is now strong evidence to indicate that some of the important hemoglobin disorders have risen to their highest frequencies because of relative resistance of heterozygotes to *P. falciparum* malaria. However, many other genes have now been identified that have the same effect. As shown in Table 28.5, as well as blood group antigens and other red cell proteins, they include those that regulate the HLA-DR system and a variety of cytokines. Similarly, progress is also being made to identify genes that modify susceptibility to other infections, including tuberculosis, HIV/AIDS, leprosy, and hepatitis B.

Table 28.5 Genetic variation and malarial susceptibility

Red cell
α- and β-Thalassemia. Hb S, C and E
Membrane: ovalocytosis (band 3). Others
Metabolism: G6P-D. PK (murine)
Blood groups: Duffy. GYPA. GYPB (S-s-U)
GYPC (Gerbich). P. Se
Receptors: CR1
Immune genes
HLA-DR, IFN-G, IL1A/IL1B, IL10, IL12B, MBL2, NOS2A, TNF, CD40 Lig
Other host receptors
ICAM1. CD36. CD31

GYP Glycophorin; *CR* Complement receptor; *IFN*-G Interferon gamma; *IL* Interleukin; *MBL* Mannose binding lectin; *NOS* Nitric oxide synthase; *TNF* Tumor necrosis factor; *ICAM* Intercellular adhesion molecule, *pk* Pyruvate kinase

This rapidly moving field may have important practical indications for both prevention and management of the important communicable diseases, particularly those that affect the developing countries. For example, the discovery that individuals who are negative for Duffy blood group are resistant to *P. vivax* malaria, and the subsequent finding that the Duffy chemokine receptor is vital for entry of the parasite into red cells has led to efforts to develop a vaccine directed at this protein [9]. Furthermore, if vaccines are to be tested in field studies in tropical countries, and particularly if they are attenuating vaccines, it may be extremely important to know the frequency of genetic polymorphisms, such as α – thalassemia and the sickle cell gene which, themselves, produce a considerable degree of protection, when designing appropriate protection studies.

28.5.4 Pharmacogenetics and Treatment

The problems of managing *P. vivax* malaria in populations with a high frequency of G6PD deficiency and the identification of chloroquine-resistant malaria parasites have been discussed above. However, there is increasing evidence suggesting that there may be other polymorphisms that may be of significance in the treatment of common infectious diseases. For example, polymorphisms of *MDR1*, a gene that regulates the expression *p*-glycoprotein and which may hence be important in defense mechanisms against potentially toxic agents ingested in the diet, have been shown to be much more common in West African and African-American populations than in those of European or Japanese backgrounds. It has been suggested that a particular variant of *MDR1* is common in Africa because it offers a selective advantage against gastrointestinal-tract infections. However, there is now clear evidence that this variant reduces the efficacy of the protease inhibitors and related agents that are now widely used for the treatment of HIV-1 infections [35].

The application of pharmacogenetic studies as an adjuvant to drug therapy in the developing world will clearly add to the costs of treatment; each case will have to be examined for its cost-effectiveness on an individual basis.

28.6 Genetic Components of Other Common Diseases: A Global View

As shown in Table 28.1, congenital malformations, including heart defects, neural tube defects, and cleft lip with or without cleft palette, constitute an important proportion of children born with genetic disease or congenital malformation. These disorders almost certainly reflect interactions between environmental factors and genetic susceptibility mediated through multiple genes. Some of these conditions are discussed elsewhere in this book, and some of the environmental factors involved have been reviewed recently [10].

Approaches to determining the genetic component of major noncommunicable adult diseases, cardiovascular disease, diabetes, and neurological disease, for example, are also described elsewhere in this book. Here, we review very briefly a few of the global aspects of this growing and particularly complex field, many aspects of which are covered in more detail elsewhere [20, 48].

As countries pass through the epidemiological transition, the burden of disease attributable to malnutrition and infection declines and the pattern of illness starts to assume that of the developed countries [20]. For example, by 2001 cardiovascular disease was responsible for about 30% of deaths worldwide. It is now predicted that it will be the leading cause of death and disability worldwide by 2020, largely because of its increasing frequency in low- and middle-income countries. In 2003 about 194 million people worldwide had diabetes, and it is estimated that by 2025 this figure will increase to 333 million. In 2003 the developing countries accounted for 141 million of those with diabetes. It is believed that the number of people with diabetes will double in three of the six developing regions of the world by 2025; these extraordinary epidemiological changes must reflect a major environmental component in the causation of these conditions.

The major goal in attempting to define the particular susceptibility genes involved in these multigenic disorders is to learn more about their underlying pathophysiology and hence to develop more logical approaches to their treatment. A longer term goal is to define individuals at particularly high risk for these conditions so that public health measures may be focused on these particular groups with the more effective use of man power and facilities.

Several messages are emerging from global epidemiological studies that may have important implications for the search for susceptibility genes for these conditions. First, it is becoming clear that many of these diseases, type 2 diabetes, the metabolic syndrome, and asthma, for example, are not single entities but reflect several different conditions, which may have different genetic and environmental causes. Hence, when whole-genome searches or association studies are launched in attempts to identify the genes involved, the importance of accurate phenotyping and associated epidemiological data cannot be overestimated. Furthermore, there are indications that the pattern of response to environmental factors may vary considerably from race to race. For example, African races seem to be particularly prone to hypertension and cardiovascular disease, whereas Asian populations and some population isolates are more prone to the development of obesity and type 2 diabetes. Again, this raises the possibility of the environmental agents of western life acting in different genetic backgrounds.

Similar types of information relating to differences in age-specific incidence rates of Alzheimer's disease, together with low incidence rates reported from parts of India and Africa, raise the possibility of variation in environmental factors or gene-environment interactions in the causation of this condition [20]. These possibilities point to the importance of ensuring ethnic homogeneity of studies directed at identifying genes involved in susceptibility to common diseases and, equally importantly, to the value of developing partnerships in research of this type between workers in different parts of the world, a topic that we shall return to in the next section.

28.7 Global Control of Genetic Disease

The development of approaches to the global control and management of genetic disease is much too large a topic to be covered fully. It has been reviewed in detail in several reports [4, 10, 48]. Here, a few key ethical and organizational issues are summarized briefly.

28.7.1 Transcultural, Ethical, and Counseling Issues

Ethical issues in genetic research, screening, and testing in developing countries are discussed in detail in a report by the World Health Organization [48] and in reports by the Nuffield Council on Bioethics [29, 30].

While the general principles that have evolved for good ethical and regulatory practice in clinical genetics in the developing countries apply equally to the poorer countries of the world, their application in different social backgrounds raises many problems, a field that is in urgent need of further investigation. A few of these issues are highlighted here.

28.7.1.1 Ethnic Differences in Interpreting the Nature of Disease

It is important to realize that even the best-intentioned efforts to introduce the medical practices of developed countries, such as genetic counseling, into developing countries may fail simply because of the completely different perceptions of the nature of disease. Disease is often viewed as the action of evil spirits or other external forces, a belief that may be confirmed by visits to local healers. In these circumstances even the simplest explanations of the nature of genetic disease may be extremely difficult to communicate; a great deal of time and patience is required to evolve more appropriate approaches and methods to confirm what has been understood.

28.7.1.2 Gender Issues

Many developing countries still have strongly patriarchal societies, and this raises particular problems for women who are carriers of genetic disease or who have had affected children. Despite careful explanation of the mode of inheritance of recessive disorders, husbands very frequently blame their wives for having a child with a serious genetic disease. This often has the effect of breaking up the family, and in some communities there is a high rate of suicide in these families.

Genetic information can also be used to discriminate or stigmatize in the context of other social practices. For example, in countries in which arranged marriages are still common, the fact that a screening program has shown that a woman is a carrier for a genetic disease can make her unmarriageable, or it can subject her to physical and other harms if she gives birth to children with diseases for which she is deemed responsible. In such countries as India, for example, the use of sex selection to avoid the birth of female babies is still practiced and has had a substantial effect on the sex ratio of the population in some regions. As pointed out in the report of the World Health Organization [48], sex selection is the result of deep-seated, entrenched beliefs and values in societies that have long histories of subordinating and devaluing women; long-term public education strategies are needed to combat these beliefs and values effectively.

28.7.1.3 Patient Discrimination

Observations in a number of developing countries have shown that children with severe genetic disease may be stigmatized and even ostracized by their communities. For example, in Sri Lanka children with thalassemia who require regular blood transfusions have been ostracized by their peers, been classified as “blood suckers,” and have found difficulties in continuing their education or obtaining employment when they are older.

28.7.1.4 Informed Consent

Although the principles of informed consent for research in the developing countries are similar to those in developed countries, their application may be much more difficult in societies with a limited understanding of the nature of disease. This is particularly relevant to genetic disease. The principles to be applied in research in developing countries have been defined in a discussion paper [30], and these difficult issues are discussed in detail in a further report [48]. Major problems include: explaining how some genetic tests cannot be necessarily followed up by adequate therapeutic interventions; difficulties in communicating the nature of the tests or associated research in populations with limited education; the danger of patients agreeing to being part of a research project as the only way of obtaining medical care; and, in particular, the fact that those performing genetic research in developing countries are often scientists from developed countries or large multinational pharmaceutical or biotechnology companies with research needs that are not directly relevant to the needs of developing countries.

28.7.1.5 Lack of Regulatory or Ethical Bodies

Another problem for the establishment of clinical genetic programs or research in developing countries results from the lack of established ethical or regulatory bodies. While at least some developing countries are now establishing governmental or institutional ethical review bodies, through lack of knowledge it is very difficult for them to assess or regulate the establishment of genetic services or genetic research, particularly if the latter is being carried out by scientists from outside the country. Again, these problems will only be solved by extensive education programs.

28.7.1.6 Biobanks and Biopiracy

Some developing countries offer desirable opportunities for the development of genetic databases, particularly when the population is genetically homogeneous. Although in some cases these are established by public health authorities within the country, in others they are set up by private corporations. These databases raise a number of ethical issues, including profit sharing with the community from which the data are gathered, questions of informed consent, and the participation of individuals in these programs. These important issues are considered in detail in reports from the World Health Organization [48] and the Nuffield Council for Bioethics [29].

28.7.2 Genetic Services in Developing Countries

There have been several reviews of approaches to developing genetic services in general [3, 10, 48] and with particular respect to the requirements of the developing countries [4, 42, 48].

Although the basic principles are similar irrespective of the environments in which genetic services are established, there are problems particular to the developing countries. First, it is essential to try to persuade the particular government involved to accept that there is a clinical need and to appoint a reasonably senior person in government to take responsibility for developing the service. Trained medical personnel must be available to lead the development, and this often requires a period of training in a genetic center in a developed country. Establishing programs for the hemoglobinopathies is different, because this field, probably for historical reasons, has never come under the auspices of clinical genetics and usually requires special training in centers with experience of the field for appropriate pediatricians and hematologists.

Once the basic personnel are in place a prioritized program should be developed, which includes the establishment of the frequency of particular genetic disorders in the community, the training of appropriate counselors, and the development of a major public education program. Next, decisions about whether community control of serious genetic disease will be achieved by prenatal diagnosis or community education alone should be addressed. The final stage involves the development of premarital or prenatal screening programs and the establishment of one or more center with expertise in both the laboratory diagnosis and clinical management of genetic disorders.

While much of this may be self-evident to geneticists in the developed countries there are problems to be overcome at each stage in many developing countries. Their governments often do not think that genetic disease is a high priority, given the many other more pressing problems of communicable disease and the increase in frequency of noncommunicable disease that most of them are experiencing. It may be difficult for them to obtain adequate training for their staff overseas, and even more difficult to provide facilities for them after training. Unless there is strong leadership from government or clinicians it is extremely difficult to organize and synchronize the development of programs of this kind. And, last but by no means least, there are major problems in funding both preventative and management programs.

Currently, it seems unlikely that many developing countries will be able to evolve clinical genetic programs without at least some international help. How can this best be organized?

28.7.3 Organizing International Help for Developing Genetic Programs in Poorer Countries

Although the World Health Organization has accepted that genetic disease and congenital malformation are becoming increasingly serious international health problems, the same is not true of other large international public health agencies or funding organizations. What, therefore, is the best way forward?

In a report published by the World Health Organization [48] and in a follow-up article [41], suggestions were made about how the international medical and scientific community might best help the developing countries in establishing programs such as clinical genetics. These approaches were based in part on the successes in the hemoglobin field over the previous 20 years in developing programs for the control of the thalassemias in many developing countries. They were built on the concept of North/South partnerships, i.e., partnerships between workers in centers with expertise in the developed countries and those in whom these skills were lacking in the developing world. At best, they were sustainable and led to both the training of staff and the transfer of both clinical and laboratory technology to the developing countries. Programs of this type led to the successful application of carrier screening and prenatal diagnosis for the thalassemias in many developing countries, and in some cases were later integrated into the control of other genetic diseases.

The natural successor to a North/South partnership would be the development of a South/South partnership in which those developing countries that have gained skills in the diagnosis and treatment of genetic disorders formed partnerships with countries in which these skills were lacking. Several South/South partnerships along these lines are being planned as this book is being prepared.

There is of course a major problem that will have to be solved for the development of further partnerships of this type. Many North/South partnerships were funded on a research basis by the developed countries and, when successful, at least in some cases were then taken over by governments of the developing countries. But this is not always possible, and for a process of this type to be developed further more sources of funding will have to be obtained, either as part of the aid programs of the Northern partners or from large international agencies and charities, or both.

Another major issue that will have to be faced is the high priority that must be given to determining more accurately the frequency of genetic disease and hence the future health burden that it will present to the governments of developing countries as they go through their epidemiological transition. The more accurate the assessment of this load and its conversion into DALYs, the only language which the international public health community understands, will be a major priority for North/South and South/South partnerships in the immediate future if the true problem of genetic disease for the global community is to be appreciated. In this context announced recently that the next Global Burden of Diseases, Injuries and Risk Factors programme, an extremely influential series of studies that attempt to assess the health burden of all the most important diseases, and which does much to influence the governmental planning of priorities for health care, is to include the hemoglobinopathies and an assessment of genetic risk factors for both communicable and noncommunicable diseases. If successful, these new additions to the program should do much to increase the awareness of the importance of genetic disease and DNA technology among the international health community.

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Genetic Databases

29

Introductory Note by Stylianos E. Antonarakis

The establishment and maintenance of databases are absolutely essential for access to and exploration of the genome and understanding of genomic variability and how it relates to human phenotypes. It is no longer possible to carry out genetic research or practice clinical genetics without the use of databases. Scientific progress in genetics/genomics virtually always requires the use of databases.

The editors of the new edition of this book felt a compelling need to include chapters on many of the existing important databases. Not all valid databases, however, have been included. The rationale for inclusion of databases was to cover knowledge and information on (a) the human genome and (b) human genetic pathogenic and polymorphic variability and phenotypic, including clinical, variability. For the first goal, we include the genome browsers of UCSC (chapter 29.1) and ENSEMBL (chapter 29.2). The information included in these databases is complementary and partly redundant, but each of the two has specific advantages/particular features, making them attractive to different users. In addition, these two genome browsers collaborate and exchange information, which adds to their value.

We have included several examples of the more medically (phenotype) oriented databases: the historical "OMIM" (the first gene and phenotype database, initiated by the late Victor McKusick), "Gene Tests," the Locus Specific Databases, DECIPHER, the database of copy number variants, "EntrezGene," "dbGAP," a genotype and phenotype database that includes information on genome-wide association studies, and "HGMD," the human genome mutation database. Goals, infrastructure, funding, and likely users of each of these databases are described and differ considerably, but all are extremely useful for our understanding of human genetic phenotypes and clinical manifestations as well as for planning diagnostic and presymptomatic laboratory studies.

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Databases and Genome Browsers 29.1

Rachel A. Harte, Donna Karolchik, Robert M. Kuhn, W. James Kent, and David Haussler

Abstract The advent of the human genome project and subsequent projects to sequence genomes of other species and multiple individuals has driven the need for tools that can visualize vast amounts of genomics data. Software for genome browsing has had a vast impact in the arenas of human medical and genetics research, enabling researchers to process and integrate different data types from a large variety of sources. Three major genome browsers are freely accessible online – the University of California, Santa Cruz (UCSC) Genome Browser, the Wellcome Trust Sanger Institute (WTSI)/European Bioinformatics Institute (EBI) Ensembl browser and the National Center for Biotechnology Information (NCBI) MapViewer. The UCSC Genome Browser is a key part of the UCSC Genome Bioinformatics suite of integrated tools that facilitate data mining, together with allowing users to visualize and query their own data in the context of the existing Genome Browser annotations. The chapter provides an overview of the types of annotation data displayed by the Genome Browser, as well as step-by-step examples illustrating how to create custom tracks and query both the Genome Browser and Table Browser. The Genome Browser offers links to several programs: BLAT for performing fast sequence alignment to genomes; the In Silico PCR tool for aligning primers to the genome, and liftOver for converting genomic coordinates from one assembly to another. Other tools in the suite include the Gene Sorter for sorting genes based on their relationships such as expression profiles and genomic proximity; the Proteome Browser, which shows protein-related information in graphical form and links out to external protein-related sites; and Genome Graphs, which allows the user to display genome-wide datasets such as those from SNP association studies, linkage studies and homozygosity mapping. The suite of UCSC Genome Bioinformatics tools, data downloads, extensive documentation and links to further training materials can be found at http://genome.ucsc.edu.

Contents

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29.1.1 Historical Background

The past 30 years have brought many exciting developments to the field of genomics, culminating in the full sequencing of many organisms and the development of methodologies to further the functional annotation of the genomes. As the vast amount of sequence data accumulated, a major question arose: how should it be visualized together with the available annotations?

This question has been answered by the development of several programs that allow researchers to view genomic data for model organisms on the web. The Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org/) [11,39] was the first such program, designed for the *Saccharomyces cerevisiae* genome assembly. ACEDB [12,26] was adopted by both the *Caenorhabditis elegans* sequencing project [53] and the WormBase project (http://www.wormbase.org/) [44,50], which hosts data on *C. elegans* and related worm species.

The sequencing of the human genome demanded a more robust method of viewing the genome and its annotations, and the ability to link to other relevant biological databases, integrating data from many different sources. The Genome Browser at the University of California, Santa Cruz (UCSC) (http://genome.ucsc.edu/) [19,23,25,30,33,34] is one of three tools that was developed – along with Ensembl (http://www.ensembl.org) [22] and the NCBI MapViewer (http://www.ncbi.nlm.nih.gov/mapview/) [45] – to provide a web-based method of interacting with the human genome. The annotations combined with various data mining tools facilitate many aspects of medical and human genetics research (see Box 29.1.1). All of these browsers have since expanded to include the genomes of a number of model organisms. The UCSC Genome Browser includes genome assemblies from human, chimp, mouse, rat, zebrafish, and other selected vertebrates, *C. elegans*, *S. cerevisiae*, *Drosophila melanogaster*, and a large collection of other flies and insects.

Box 29.1.1. Importance of genome browsers in medical and human genetics research

- Visualization of the results of genome-wide scans such as SNP association studies, linkage studies, and homozygosity studies.
- Convenient definition of candidate regions and candidate genes.
- Browsing a wealth of information on features in a candidate region including protein-coding and non-protein-coding genes, alternative splicing, conserved regulatory regions, and known polymorphisms.
- In-depth information on genes, including Online Mendelian Inheritance in Man (OMIM) [37], Gene Ontology (GO) [54], RefSeq [43], and Swiss-Prot summary information from UniProt [56], expression level in various tissues, and links to orthologs in model organism databases.
- Methods for large-scale data mining to define sets of genes meeting various criteria.
- Facilities for viewing personal data sets side-by-side with public data and combining personal data with public data in the data mining tools.
- Display of simple nucleotide polymorphisms (SNPs) and copy number polymorphisms (CNPs), including deletions of various sizes in the human population and in cancerous cells.
- Access to the evolutionary history of each base from multiple genome alignments and plots of conservation levels.

The UCSC Genome Browser is a web-based CGI (Common Gateway Interface) application adapted from a program implemented in the C programming language to view alternative splice forms of a gene prediction in *C. elegans* [23,31,32]. Extensive changes were made to adapt this program for the display of the human genome, which is 30 times larger than that of the worm. The resulting Genome Browser is fast, easy to use, and freely available through the Internet. The Genome Browser database is extensible to accommodate new genomes and annotation data as they become available.

29.1.2 Database Organization

As a sequencing project for an organism matures, genomic assembly updates are released, each containing improvements in the sequence coverage and/or assembly. The sequence and annotation data for each genome assembly are stored in their own database in the Genome Browser [23], using the publicly available software, My Structured Query Language (MySQL) (http://dev.mysql.com/doc/). As more assemblies are added, selected older assemblies are moved to the archive server (http://genome-archive.cse.ucsc.edu). Each of the web servers for the Genome Browser has its own local copy of the databases to enable rapid access to the data.

Within each assembly database, there are tables containing data sets anchored to the assembly by coordinate positions. These data tables are the basis for the annotation tracks in the Genome Browser. Additionally, a data set may have secondary or tertiary tables containing nonpositional data such as identifiers that link the data to other annotation databases. Programs have been specially developed to facilitate the batch loading of various types of data, including BED (Browser Extensible Data), PSL (Pattern Space Layout), WIG (wiggle format for continuously variable data), microarray and gene prediction data in GFF (Gene-Finding Format) or GTF (Gene Transfer Format). Supplemental databases, such as UniProt [56] or Gene Ontology (GO) [54], are obtained from external sources. The hgFixed database contains data that do not change with time, such as microarray data, and that may be used by several different assemblies as well as by the Gene Sorter tool [29] (see Sect. 29.1.8.4). The Proteome Browser [19,21] (see Sect. 29.1.8.5) and the VisiGene tool [33] also have their own separate databases.

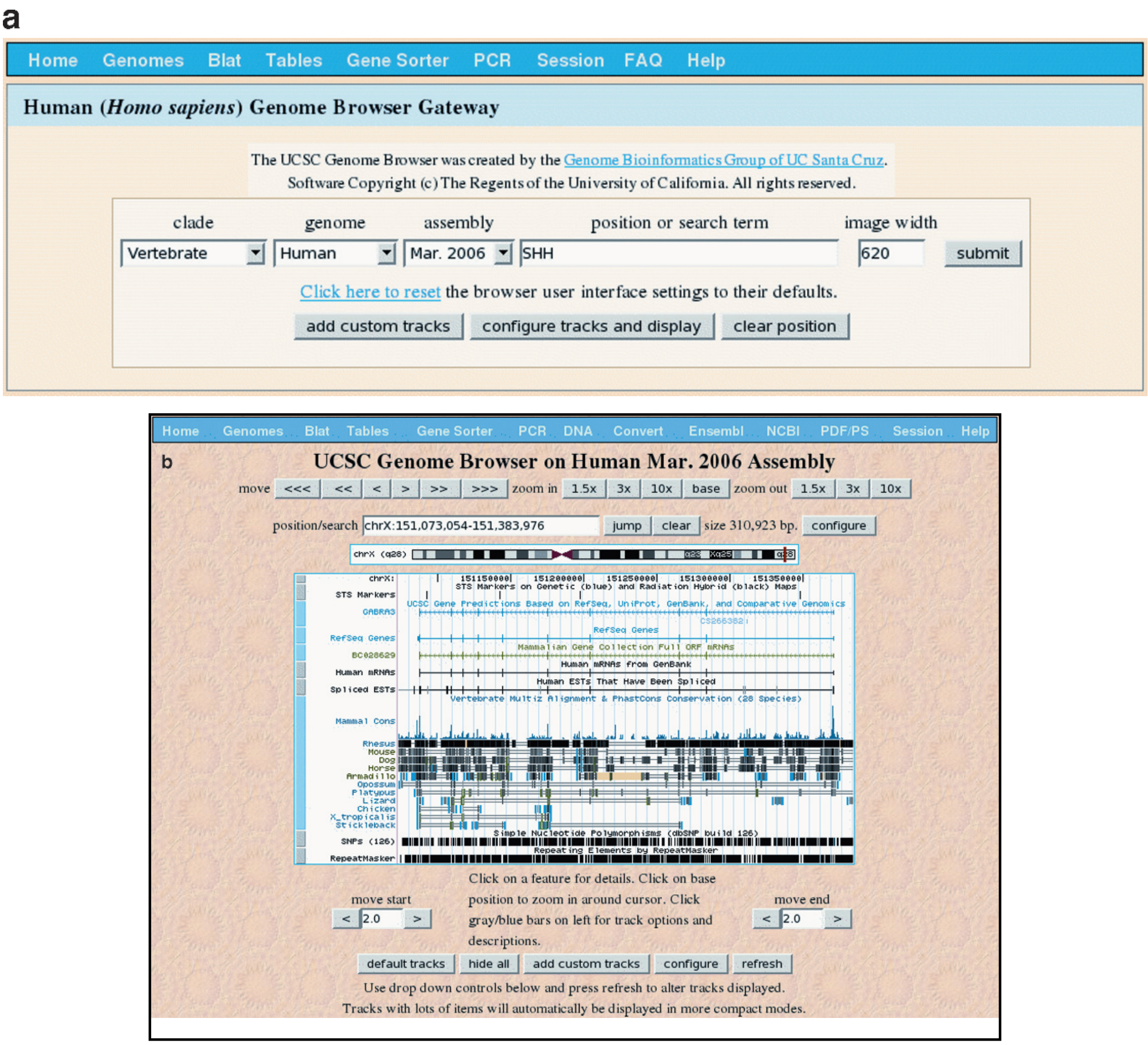
Database tables are optimized to allow rapid display in the Browser graphic. This is achieved by table indexing; some tables also use a binning scheme to speed up table lookups [30]. For very large data sets, such as mRNAs, ESTs and alignments, rapid data lookup and display are improved by creating a data table for each chromosome. Data are stored in Indexed Sequential Access Method (ISAM) tables that may be swapped in and out separately, which allows continuous updating of databases. The ability to modify tables is of particular importance for frequently updated tables such as those tables relating to mRNAs, which are updated nightly, and EST tables, which are updated weekly.

29.1.3 Genome Annotations

To access annotations, open the URL http://genome.ucsc.edu in an Internet browser and then click on the Genomes link, which leads to the Gateway page (Fig. 29.1a). Here, several of the most recent assemblies are available for each genome. Upon selection of an assembly, the "position or search term" text box displays the default position of entry. A new location can be selected by typing in a different genomic position or a search term, such as a gene name, description, accession number or other annotation identifier. The Gateway page presents a set of example search terms supported for the selected assembly. To open the Genome Browser at the requested location, press the "submit" button. If a search term is ambiguous, the user is taken to an intermediate page displaying information about the various results. The search result will be highlighted in the Browser display. The same query method may also be used in the position/search box above the Browser image (Fig. 29.1b).

29.1.3.1 Overview of Tracks in the Genome Browser

The Genome Browser displays annotations as horizontal "tracks" using the genome sequence as the display coordinates. Most features are displayed horizontally, with the exception of "wiggle" tracks (such as the Conservation track), which show a score associated with features on a vertical axis (Fig. 29.1b). There are


a
Home Genomes Blat Tables Gene Sorter PCR Session FAQ Help
clade
genome
assembly
position or search term
image width
Vertebrate
Human
Mar. 2006
SHH
620
submit
add custom tracks
configure tracks and display
clear position
b
Home Genomes Blat Tables Gene Sorter PCR DNA Convert Ensembl NCBI PDF/PS Session Help
move <<< << < > >> >>> zoom in 1.5x 3x 10x base zoom out 1.5x 3x 10x
position/search
chrX:151,073,054-151,383,976
jump clear configure
chrX (q28)
STS Markers
GABRA3
RefSeq Genes
BC028629
Human mRNAs
Spliced ESTs
Mammal Cons
Rhesus
Mouse
Dog
Horse
Armadillo
Opossum
Platypus
Lizard
Chicken
X_tropicalis
Stickleback
SNPs (126)
RepeatMasker
STS Markers on Genetic (blue) and Radiation Hybrid (black) Maps
UCSC Gene Predictions Based on RefSeq, UniProt, GenBank, and Comparative Genomics
RefSeq Genes
Mammalian Gene Collection Full ORF mRNAs
Human mRNAs from GenBank
Human ESTs That Have Been Spliced
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Simple Nucleotide Polymorphisms (dbSNP build 126)
Repeating Elements by RepeatMasker
move start
< 2.0 >
move end
< 2.0 >
default tracks hide all add custom tracks configure refresh

Human (*Homo sapiens*) Genome Browser Gateway

The UCSC Genome Browser was created by the Genome Bioinformatics Group of UC Santa Cruz.
Software Copyright (c) The Regents of the University of California. All rights reserved.

Click here to reset the browser user interface settings to their defaults.

UCSC Genome Browser on Human Mar. 2006 Assembly

size 310,923 bp.

Click on a feature for details. Click on base position to zoom in around cursor. Click gray/blue bars on left for track options and descriptions.

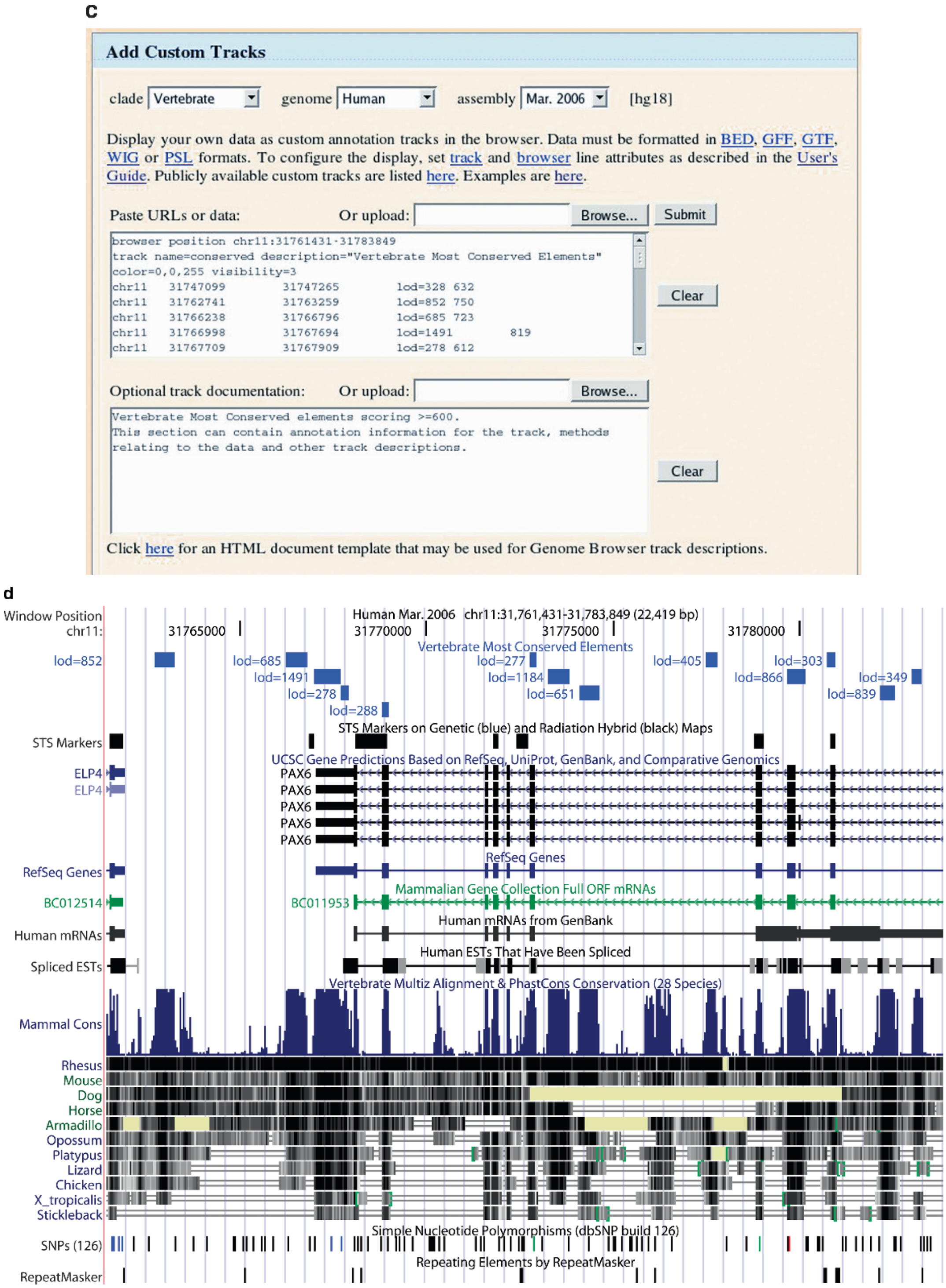
Use drop down controls below and press refresh to alter tracks displayed.
Tracks with lots of items will automatically be displayed in more compact modes.

Fig. 29.1 (**a–d**) Browser features. (**a**) Genome Browser Gateway page showing SHH in the search/position box. (**b**) Human hg18 (NCBI Build 36) Genome Browser default view. (**c**) Adding a custom track to the human (hg18) Genome Browser. This interface may be reached via the "add custom tracks" button on the Gateway page (**a**) or underneath the Browser image (b).

a wide range of tracks particularly for human, mouse and other model organisms. Many of these tracks are computed at UCSC using public software developed internally or by other institutions; the remaining tracks are contributed by external collaborators. Annotations are organized into groups based on data type (Table 29.1). Track data may be hidden, compressed, or displayed in full using the set of track controls – organized by group – found below the Browser image. The compression feature is useful if there is a large amount of data in the displayed region or if a large number of tracks are visible.

Each track has an associated description page that may be accessed by clicking on its track control or the mini-button to the left of the track in the Browser graphic. Any color-coding conventions or configuration options used in the track are explained on this page. Click on an item in a track to view a details page giving further information related to that item and links to external databases, if appropriate.

Some tracks are organism specific, e.g., the Consensus CoDing Sequence (CCDS) (http://www.ncbi.nlm.nih.gov/projects/CCDS/) [42] annotation for the human and mouse genomes. Newer human



c

Add Custom Tracks

clade Vertebrate ▾ genome Human ▾ assembly Mar. 2006 ▾ [hg18]

Display your own data as custom annotation tracks in the browser. Data must be formatted in BED, GFF, GTF, WIG or PSL formats. To configure the display, set track and browser line attributes as described in the User's Guide. Publicly available custom tracks are listed here. Examples are here.

Paste URLs or data: Or upload: [Browse...] [Submit]

```
browser position chr11:31761431-31783849
track name=conserved description="Vertebrate Most Conserved Elements"
color=0,0,255 visibility=3
chr11   31747099        31747265        lod=328 632
chr11   31762741        31763259        lod=852 750
chr11   31766238        31766796        lod=685 723
chr11   31766998        31767694        lod=1491        819
chr11   31767709        31767909        lod=278 612
```

[Clear]

Optional track documentation: Or upload: [Browse...]

```
Vertebrate Most Conserved elements scoring >=600.
This section can contain annotation information for the track, methods
relating to the data and other track descriptions.
```

[Clear]

Click here for an HTML document template that may be used for Genome Browser track descriptions.

d

Window Position
chr11:
31765000
Human Mar. 2006 chr11:31,761,431-31,783,849 (22,419 bp)
31770000
31775000
31780000
Vertebrate Most Conserved Elements
lod=852
lod=685
lod=1491
lod=278
lod=288
lod=277
lod=1184
lod=651
lod=405
lod=303
lod=866
lod=349
lod=839
STS Markers on Genetic (blue) and Radiation Hybrid (black) Maps
STS Markers
UCSC Gene Predictions Based on RefSeq, UniProt, GenBank, and Comparative Genomics
ELP4
ELP4
PAX6
PAX6
PAX6
PAX6
PAX6
RefSeq Genes
RefSeq Genes
Mammalian Gene Collection Full ORF mRNAs
BC012514
BC011953
Human mRNAs from GenBank
Human mRNAs
Human ESTs That Have Been Spliced
Spliced ESTs
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Mammal Cons
Rhesus
Mouse
Dog
Horse
Armadillo
Opossum
Platypus
Lizard
Chicken
X_tropicalis
Stickleback
Simple Nucleotide Polymorphisms (dbSNP build 126)
SNPs (126)
Repeating Elements by RepeatMasker
RepeatMasker

Fig. 29.1 (continued) The human March 2006 assembly is selected. Data are pasted into the box and a description can also be added where indicated. In this example, the custom track specifies the default Browser position, the color of the track features and the initial display visibility (*full*). Data shown are in BED format and they represent highly conserved regions from a multiple alignment of genomes of 28 species. (**d**) Viewing the custom track of highly conserved elements in the human (hg18) Genome Browser. The default position for the track shows part of the *PAX6* gene locus. Data displayed in the custom track represent regions of the Most Conserved track with a score of ≥600. The Most Conserved track is based on data from the multiple alignment of the genomes of 28 vertebrate species displayed in the Vertebrate Multiz Alignment and PhastCons Conservation track. Conservation in this track is displayed in the graph-like "wiggle" format

Table 29.1 Genome Browser annotation track groups

Track group	Example track types
Mapping and sequencing tracks	Genome sequence, contig and scaffold positions, GC percent, bacterial artificial chromosome (BAC), and fosmid ends, restriction enzymes, short match (motifs)
Phenotype and disease associations	GAD View [5], OMIM Genes [2], Quantitative trait loci (QTL)
Gene and gene prediction tracks	Known genes set (organism specific), Consensus Coding DNA Sequencing Project (CCDS) gene annotations [42], RefSeq mRNAs [43] for both reference and other species, Vertebrate Genome Annotation (VEGA) genes [61], Mammalian Gene Collection (MGC) genes [55], Ensembl genes [22], TransMap [34], non-coding RNAs, pseudogenes, gene predictions such as N-SCAN [17], Acembly [57] and Geneid [18]
mRNA and EST Tracks	GenBank [6] mRNAs and ESTs for reference and other species, EST clusters such as UniGene [45] and TIGR Gene Index [35], alternative splice forms
Expression and regulation	In situ hybridization and microarray probes, expression data, *cis*-regulatory regions
Comparative genomics	Multiple alignments, conservation, pairwise alignments with the genomes or proteins of other organisms
Variation and repeats	SNPs and other variation data [59], recombination, repeats, microsatellites

assemblies have additional tracks of data generated by the Encyclopedia of DNA elements (ENCODE) Consortium [13], for which UCSC hosts the official sequence-related data repository [ENCODE Data Coordination Center (DCC)]; microarray data are hosted by GEO [4] and ArrayExpress [40]. The ENCODE project groups provide the Genome Browser with a rich source of data on DNA replication, chromatin regulation, promoter function, gene models, transcription (tiling arrays and RNA-seq), variation, and multiple species comparisons to aid researchers in understanding the functional elements of the genome. Tracks from the pilot phase [14] of the ENCODE project are presented in separate ENCODE track groups; tracks displaying production phase data are integrated directly into the track groups listed in Table 29.1 and are denoted by a National Human Genome Research Institute (NHGRI) icon displayed to the left of the track control label. To organize large amounts of data from similar experiments with different cell lines and/or conditions, many of the ENCODE tracks are composite tracks that include subtracks. For example, the ChIP-chip and the ChIP-seq data include experiments that determine the binding sites of DNA-binding proteins such as transcription factors. These data are often based on experiments in multiple cell lines, which are grouped together into one track. The UCSC ENCODE DCC portal page (http://genome.ucsc.edu/ENCODE/index.html), which is accessed through the ENCODE link on the Genome Browser home page, displays recent project announcements and links to information on the cell types used in the project, a log of recently released tracks, data downloads, contributors, publications, the ENCODE Consortium data policy and an alternative ENCODE pilot project browser portal [58].

29.1.3.1.1 Mapping and Sequencing Tracks

Several tracks contain data pertaining to the genome assembly. Contig and scaffold locations in the genome assembly can be displayed. A Gap track shows the unsequenced gaps between these elements, and the local GC percent in the genome sequence is shown as a graph. The genome sequence itself may be viewed when zoomed in to base level.

Fluorescent in situ hybridization (FISH) clones annotations show the location of FISH-mapped bacterial artificial chromosome (BAC) clones from the BAC Resource Consortium. The Chromosome Bands track shows the approximate locations of Giemsa-stained chromosome bands. The Sequence-Tagged Site (STS) Markers track shows STS markers including markers from NCBI's UniSTS database [45]. Many of these markers are used in the construction of genome-wide genetic and physical maps. Pairs of sequences that form the 5′ and 3′ ends of single BAC clones are aligned using BLAT (BLAST-like alignment tool) [27] (see also Sect. 29.1.6) and displayed in the BAC End Pairs track. Two dynamically created tracks, which have no underlying tables, are Short Match and Restriction Enzymes. The Short Match track indicates the position of short nucleotide motifs of the user's choice in the display; Restriction Enzymes shows the location of the cutting sites of enzymes and may be user limited to a subset of restriction enzymes.

29.1.3.1.2 Phenotype and Disease Associations

This group, which is focused primarily on human assemblies, contains data relating to mutations associated with human diseases and their phenotypes. Data from the Genetic Association Database (GAD) (http://www.grc.nia.nih.gov/branches/rrb/dna/association) [5] is hosted in the GAD View track. GAD is a curated archive for published human genetic linkage data and genetic association studies of complex diseases and disorders. The OMIM Genes track displays genes associated with the Online Mendelian Inheritance in Man (OMIM) database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim) [2], a collection of human genes and genetic phenotypes. Quantitative trait loci (QTL) tracks provide both human and rat data from the Rat Genome Database (RGD) (http://rgd.mcw.edu/) and mouse data from Mouse Genome Informatics (MGI) (http://www.informatics.jax.org/). Each locus is linked back to its RGD or MGI report, which includes information such as the trait, associated disease, phenotypes, population and markers. More genetic association tracks will be added as research grows in this field.

29.1.3.1.3 Gene and Gene Prediction Tracks and mRNA and EST Tracks

The mRNA, EST, and gene and gene prediction tracks show physical evidence supporting the positions of genes. GenBank mRNAs and ESTs [6] are aligned to the genome sequence using BLAT [27] (see Sect. 29.1.6). The spliced EST track is created by examining the resulting EST alignments for evidence of splicing. In a similar manner, the RefSeq Genes track is created from RefSeq mRNAs [43]. The Institute for Genomic Research (TIGR) Gene Index [35] is the alignment of clusters of ESTs; UniGene [45] mRNA/EST clusters are aligned by BLAT. The human and mouse UCSC Genes and rat Known Genes tracks consolidate gene prediction information from various lines of evidence and are supported by rich details pages with links to resources at other locations [20] (see Sect. 29.1.3.3).

Vertebrate Genome Annotation (VEGA) genes (from the Wellcome Trust Sanger Institute) [61] provide a set of high-quality manually curated genome annotations. Non-protein-coding RNAs, RNAs with secondary structure such as those predicted by EvoFold [41], pseudogenes, alternative splice form predictions and Ensembl protein superfamilies are also represented in tracks for some organisms.

Tracks for gene prediction annotations are obtained mainly from external sources. The Ensembl [22] and AceView (Acembly) [57] tracks predict genes based on genomic, mRNA and EST evidence. The Exoniphy program finds exons conserved in multiple species based on a phylogenetic hidden Markov model (HMM) [49]. The N-SCAN [17] tool produces gene predictions using information from the genome and from genomic multiple sequence alignments. Geneid [18] is an example of *ab initio* gene predictions.

29.1.3.1.4 Expression and Regulation

This group contains tracks that display experimental data relating to genome elements such as mRNA transcripts, BLAT alignments [27] (see Sect. 29.1.6) of sequences used for microarray probe selection such as those for Affymetrix GeneChip arrays and probes used for *in situ* hybridization experiments. Microarray data tracks exhibit red and green coloring in the probe sequence alignments to show the level of expression of a gene relative to expression over a wide range of tissues as for the Gene Atlas data from the Genomics Institute of the Novartis Research Foundation (GNF) [51,52]. Clicking on an alignment in this type of display leads to a detailed view of the expression for individual probes in that region.

29.1.3.1.5 Variation and Repeats

Repeats are found by RepeatMasker (http://www.repeatmasker.org) (software courtesy of Arian Smit, Institute for Systems Biology) and Tandem Repeat Finder (TRF) [7] (Simple Repeats track). The Microsatellite track shows di- and trinucleotide repeats that have a highly polymorphic tendency. Segmental Duplications track shows regions that are likely to have been duplicated in the genome. Simple Nucleotide Polymorphisms (SNPs) from NCBI's dbSNP (http://www.ncbi.nlm.nih.gov/projects/SNP/) [45,47] contain single nucleotide polymorphisms and small insertions and deletions. Other SNP-related tracks show commercially available genotyping SNP arrays, recombination rates and hotspots [59].

29.1.3.2 Comparative Genomics Tracks

The UCSC Genome Browser is noted for its wide selection of comparative genomics tracks showing homology with other species. These annotations include pairwise and multiple alignments of genomes, sequence conservation and alignments of proteins from another species. Homology with other species is important for locating putative genes or regions of regulation, for assigning possible functions to genes, and for studying evolutionary aspects of genome development.

29.1.3.2.1 Chains, Nets, and Conservation

Pairwise cross-species whole genome alignments are created using the BLASTZ [46] program, which is very sensitive for alignments on a genome-wide scale. Related alignment fragments are linked together to form larger structures called "chains" [28]. Chains contain homologous genes. From these chains, best-in-genome alignments are selected to form the net tracks. Net tracks are useful for visualizing long-range synteny between organisms.

Multiple alignments are also generated from the best-in-genome pairwise alignments using multiz [9]. Conservation scores and regions of high conservation between genomes of organisms in the multiple alignment are computed using phastCons [48]. The Browser displays these scores in a continuously variable format that provides a visually intuitive view of conserved regions. The comparative alignments and conservation scores may be obtained from the UCSC downloads server.

29.1.3.2.2 Cross-Species Protein Alignments

Amino acid sequences diverge more slowly than nucleic acid sequences, and alignments of proteins from one species to another are therefore particularly useful for finding genes in distantly related or incompletely sequenced organisms and for annotating genes in other organisms for which a rich experimental annotation has not been established. This is of particular importance for a recently sequenced genome that does not have extensive gene annotations of its own. Human Proteins tracks [19] have been created for most such organisms. These human homologs are found by first aligning human known gene proteins (see Sect. 29.1.3.3) to the genome using BLAT [27] (see Sect. 29.1.6) to find the exon boundaries relative to the proteins. The peptide sequences of the putative exons are then aligned to the genome using tBlastn [1], which is a more sensitive alignment program than BLAT. Exon alignments are chained together, and the single best chain is retained after filtering on certain criteria. In a similar manner, the *D. melanogaster* proteins are aligned to other insect genomes. The UCSC Genes details pages (see Sect. 29.1.3.3) show the best Blastp [1] homologs in the gene sets for a number of model organisms.

29.1.3.3 UCSC Genes Set

The UCSC Known Genes protein-coding genes set [20] was produced for the human, mouse and rat assemblies by a fully automated process that combines both mRNA and protein evidence for the existence of protein-coding genes. Through improvements in the Known Genes process, this annotation evolved into the UCSC Genes set for the human and mouse genomes, which includes both protein-coding and putative noncoding genes. A moderately conservative prediction set, UCSC Genes contains 99.9% of the RefSeq Genes and is based on sequence data from GenBank [6], RefSeq [43] and UniProt [56]. To be incorporated into the UCSC Genes set, a transcript must have at least one supporting GenBank mRNA and another line of evidence. RefSeq genes require no additional evidence. If possible, a UniProt protein is found to represent the protein encoded by a transcript. Some transcripts that are annotated as non-coding may actually be protein-coding, but the evidence for the associated protein is weaker. The UCSC Genes set is more inclusive than RefSeq, containing more protein-coding genes, greater coverage of splice variants, and more non-coding genes. UCSC Genes have their own accessions, which remain stable between releases with only a change of the suffix to indicate a change of version. An additional track, Alt Events, is created as part of the UCSC Genes method. This track depicts various types of alternative splicing, alternate promoter usage, and other events that result in the production of multiple transcripts from a gene.

Each UCSC Genes or Known Genes annotation is accompanied by a details page rich in collected information and references from many sources. A gene symbol is assigned to the gene from Entrez Gene at NCBI [31] if it is a RefSeq mRNA; otherwise the Human Gene Organization (HUGO) (http://www.hugo-international.org) [10] gene name is used. Isoforms and variant names are shown, together with a gene description. The Quick Links section connects to views of the gene in UCSC tools and external websites. The sequence section links to the genomic, mRNA and protein sequences. If available, relevant microarray expression data are displayed. Homologs in other species are identified by the best Blastp alignment [1]. For closely related species such as human, mouse and rat, the non-syntenic alignments are removed. For more evolutionarily distant pairs of species, reciprocal-best Blastp is used for ortholog identification. Links to internal and external sites show related homolog information. Links to external pathway databases, protein structures, GO [54] annotations, and further descriptions from various sources such as GenBank [6] mRNAs and UniProt [56] are also displayed if available. The VisiGene tool [33], which provides a virtual microscope for viewing in situ hybridization images, is fully integrated with both the UCSC Genes and with the Gene Sorter (see Sect. 29.1.8.4).

29.1.4 Displaying and Sharing Data Using Custom Annotation Tracks

It is possible to temporarily display personal data alongside the existing genome annotations in the Genome Browser using the custom track feature [25,33,34]. Custom tracks ensure data privacy because they can be viewed only on the machine on which the data reside or via a custom URL that can be shared with collaborators. Data may be uploaded in a number of different formats: BED, GFF, GTF, PSL alignment, and WIG (graph-like display) formats. Data in custom tracks may be used to query the underlying database tables of the Genome Browser (see Table Browser, Sect. 29.1.7). The Genome Browser User's Guide contains instructions for creating and uploading a custom track. To load a custom track, click the "add custom tracks" button on the Gateway page (Fig. 29.1a) or below the Browser image (Fig. 29.1b). Links to several custom tracks submitted by Genome Browser users may be accessed via the "Custom Tracks" link on the Genome Browser home page. See Fig. 29.1c and 29.1d for an example of custom track loading.

29.1.5 Example Analysis Using the Genome Browser

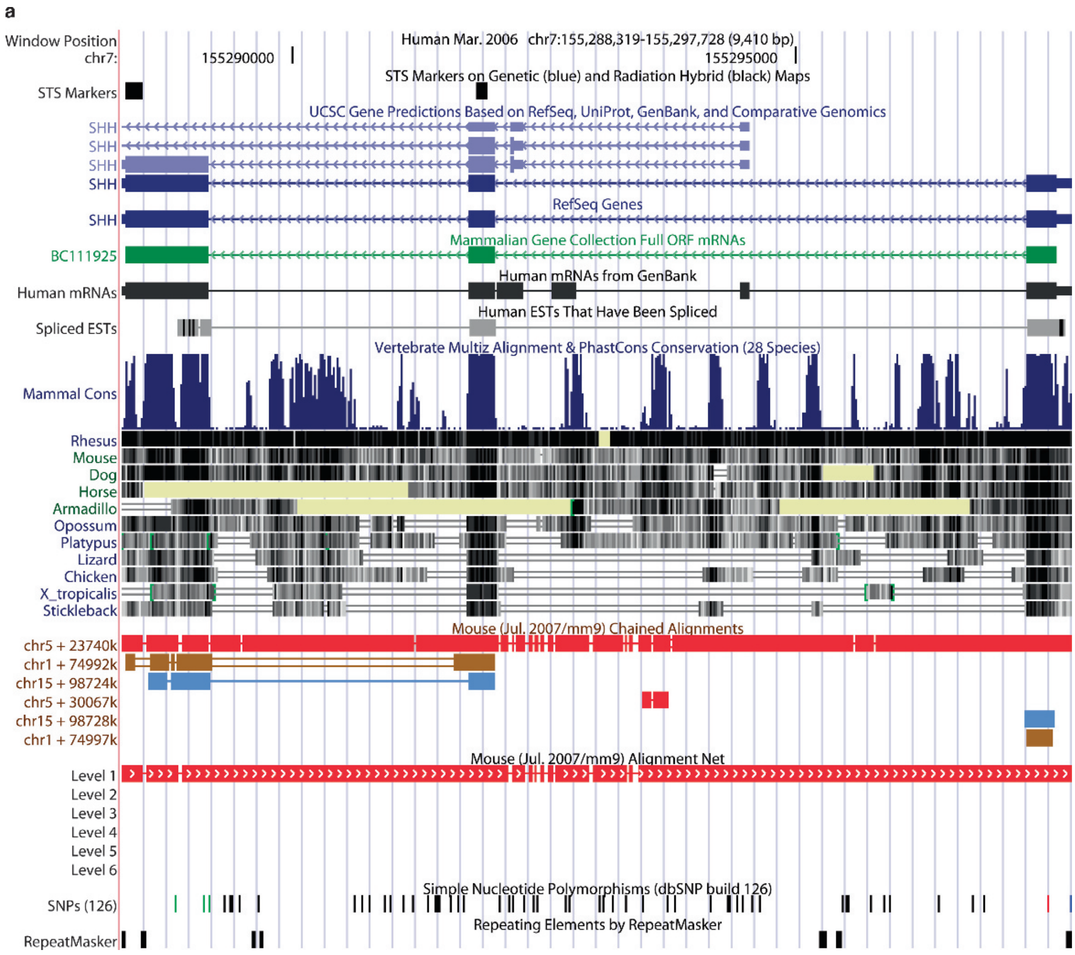
Example: Search for homologs for a human gene in another organism

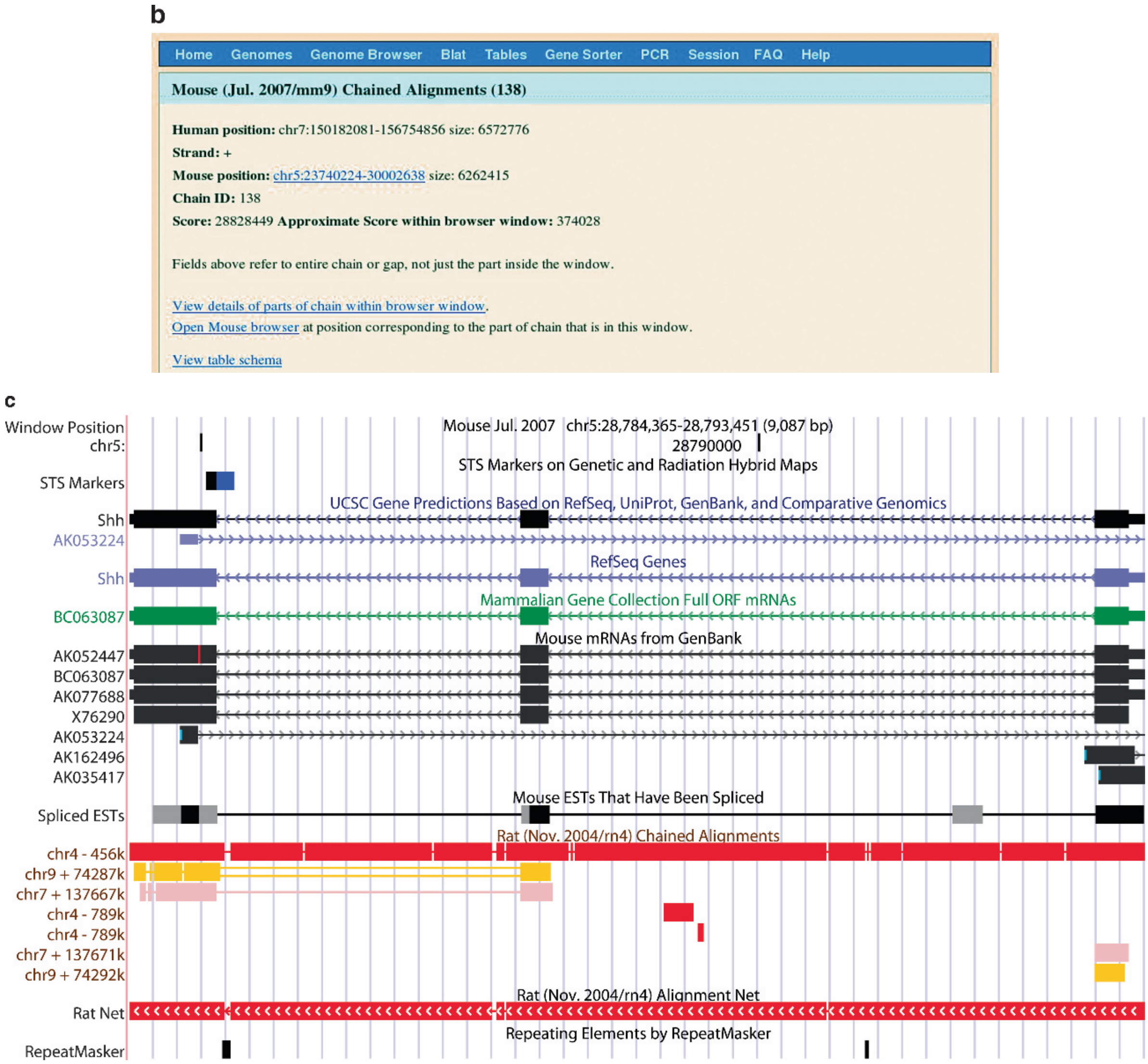
Suppose you are interested in finding genes belonging to the hedgehog family of signaling proteins. Here are the steps that one would take to find homologs in another species using the human March 2006 assembly (NCBI Build 36, known as hg18 at UCSC) on the UCSC Genome Browser:

1. Go to the Genome Browser home page (http://genome.ucsc.edu) and click on the "Genomes" or "Genome Browser" link on the top or side menu.
2. On the Gateway page, select "Mammal" as the clade, "Human" as the genome, and "March 2006" as the assembly (Fig. 29.1a).
3. Enter SHH – the HUGO gene name for the human sonic hedgehog gene – into the "position or search term" box and press the "submit" button.
4. Several search results are returned. Click on the SHH gene in the RefSeq Genes section. The Browser will display the region of the genome to which this mRNA aligns (Fig. 29.2a).
5. If the Conservation track is not displayed, scroll down to the Comparative Genomics section below the Browser image and open the track by selecting

the "pack" display mode from the Conservation track control and clicking the "refresh" button. This track shows multiple alignments of other species to the human genome and a measure of evolutionary conservation among the species. The exons are in regions of high conservation; conservation levels tend to drop off at the exon/intron boundary. Other regions of conservation may play as yet undiscovered, regulatory roles. In our example, the mouse appears to have high conservation in the coding region of the SHH gene; thus, it would be a good organism to use to look for homologs of SHH.

6. Next, display the Mouse Chain and Mouse Net tracks by setting their track controls (also in the Comparative Genomics section) to "full" visibility. The Mouse Chain track shows regions that are homologous to the human genome; the Mouse Net track can be used to determine which homologs are in the same syntenic location in the two species, and hence likely to be orthologs. Each alignment is color coded to indicate the chromosome on which the aligning region resides in the mouse genome. For example, the first alignment in the chain and the net track is red which, from the color key below the Browser graphic, signifies chromosome 5. The net track shows the best aligning sequence from mouse to this region of the human genome; thus, the best long-range homology to mouse for this region lies on mouse chromosome 5.
7. The top alignment in a Chain track is typically the best long-range alignment. Select the top chain in the Mouse Chain track to view more details about the alignment, including a link to the corresponding sequence in the mouse Genome Browser (Fig. 29.2b). Note that both the UCSC Genes and the RefSeq Genes tracks in this region of the mouse genome show a mouse homolog of the SHH gene (Fig. 29.2c).


a
Window Position
chr7:
155290000
Human Mar. 2006 chr7:155,288,319-155,297,728 (9,410 bp)
155295000
STS Markers on Genetic (blue) and Radiation Hybrid (black) Maps
STS Markers
UCSC Gene Predictions Based on RefSeq, UniProt, GenBank, and Comparative Genomics
SHH
SHH
SHH
SHH
RefSeq Genes
SHH
Mammalian Gene Collection Full ORF mRNAs
BC111925
Human mRNAs from GenBank
Human mRNAs
Human ESTs That Have Been Spliced
Spliced ESTs
Vertebrate Multiz Alignment & PhastCons Conservation (28 Species)
Mammal Cons
Rhesus
Mouse
Dog
Horse
Armadillo
Opossum
Platypus
Lizard
Chicken
X_tropicalis
Stickleback
Mouse (Jul. 2007/mm9) Chained Alignments
chr5 + 23740k
chr1 + 74992k
chr15 + 98724k
chr5 + 30067k
chr15 + 98728k
chr1 + 74997k
Mouse (Jul. 2007/mm9) Alignment Net
Level 1
Level 2
Level 3
Level 4
Level 5
Level 6
Simple Nucleotide Polymorphisms (dbSNP build 126)
SNPs (126)
Repeating Elements by RepeatMasker
RepeatMasker



Mouse (Jul. 2007/mm9) Chained Alignments (138)

Human position: chr7:150182081-156754856 size: 6572776
Strand: +
Mouse position: chr5:23740224-30002638 size: 6262415
Chain ID: 138
Score: 28828449 **Approximate Score within browser window:** 374028

Fields above refer to entire chain or gap, not just the part inside the window.

View details of parts of chain within browser window.
Open Mouse browser at position corresponding to the part of chain that is in this window.

View table schema

c
Window Position
chr5:
Mouse Jul. 2007 chr5:28,784,365-28,793,451 (9,087 bp)
28790000
STS Markers on Genetic and Radiation Hybrid Maps
STS Markers
UCSC Gene Predictions Based on RefSeq, UniProt, GenBank, and Comparative Genomics
Shh
AK053224
RefSeq Genes
Shh
Mammalian Gene Collection Full ORF mRNAs
BC063087
Mouse mRNAs from GenBank
AK052447
BC063087
AK077688
X76290
AK053224
AK162496
AK035417
Mouse ESTs That Have Been Spliced
Spliced ESTs
Rat (Nov. 2004/rn4) Chained Alignments
chr4 - 456k
chr9 + 74287k
chr7 + 137667k
chr4 - 789k
chr4 - 789k
chr7 + 137671k
chr9 + 74292k
Rat (Nov. 2004/rn4) Alignment Net
Rat Net
Repeating Elements by RepeatMasker
RepeatMasker

Fig. 29.2 Finding homologs using the Genome Browser. (**a**) Human hg18 (NCBI Build 36) Genome Browser showing the SHH gene with Mouse (mm9, NCBI Build 37) Chain and Mouse Net tracks visible. In the Conservation track (shown at "pack" visibility), scores and pairwise alignments are displayed as a gray-scale density plot. Regions of conservation between genomes of different species are seen for the exonic regions of SHH in this track. The first mouse chain (*red*) aligns to the mouse *Shh* ortholog gene locus; the second mouse chain (*brown*) is an alignment of the mouse genome region containing the Ihh (Indian hedgehog) gene and the third mouse chain (*blue*) is an alignment to the mouse genome region containing the Dhh (Desert hedgehog) gene. Ihh and Dhh are paralogs of the sonic hedgehog gene. Clicking on the first mouse chain (*red*) leads to the view shown in (**b**). (**b**) Details page for the first mouse chain and link to the mouse Genome Browser for the region of the chain in this window. (**c**) Mouse Genome Browser for the mm9 assembly, showing the region of the mouse genome that was aligned in the mouse chain in (**b**). The gene in both the UCSC Genes and the RefSeq Genes tracks is Shh, which is the mouse ortholog of human SHH. Rat Chain and Rat Net tracks are displayed to show that there is a Shh homolog on chromosome 4 of the rat genome (rn4, Baylor v3.4 assembly)

8. Repeating this for the other alignments in the chain track shows that the alignment from mouse chromosome 1 is for Ihh (Indian hedgehog). Similarly, the sequence aligning from mouse chromosome 15 contains the Dhh (desert hedgehog). Dhh and Ihh are members of the same protein family as sonic hedgehog. The chain track therefore identifies homologs of SHH in mouse.

29.1.6 Using BLAT for Genome-Wide Alignments

BLAT [27] is an extremely fast alignment tool that is useful for aligning DNA, mRNA, or translated protein sequences within species or highly conserved regions. Aligning sequences to a genome requires the slow process of building an index of the entire genome. The BLAT server builds an index of the genome, which it holds in memory; this index is then queried with each sequence presented for alignment. For DNA, this consists of all nonoverlapping 11mers, except those from regions that are heavily involved in repeats. For proteins, the index is created using tetramers.

DNA BLAT is designed to find aligning regions of 95% identity or more and of at least 40 bases in length. It can find perfect matches of 30 bp and sometimes down to 20 bp, although it may miss more divergent or shorter sequences. By default, web-based BLAT can find perfect matches as short as 21 bp in nonrepeat regions. Protein BLAT works optimally for alignments of at least 80% identity and of at least 20 amino acids in length.

Many of the Genome Browser pages contain links to a web interface for BLAT. Sequences can be pasted into the text box provided on the BLAT web page or uploaded from a file. A genome-wide search for the sequence typically takes just seconds.

The BLAT program executables and source code are available for download for use on the command line. BLAT is freely obtainable for academic, personal, and nonprofit use, but a license must be sought for commercial purposes. Command-line BLAT settings may be optimized to adjust the sensitivity. If several nearby regions of homology are returned between two sequences as separate alignments, BLAT will stitch these together into a single alignment. It is also able to correctly position splice sites. Therefore, BLAT is used to create mRNA and EST alignments to genomes for display in the Genome Browsers.

29.1.7 Table Browser

29.1.7.1 Overview

The Table Browser [19,24] provides a web-based means of extracting data from the underlying MySQL databases using filtering and free-form query options. Queries can be made on a selected table as well as on related tables in the database. Query results also may be passed to Galaxy (http://galaxy.psu.edu/) [16] – a web-based tool that facilitates queries on multiple data sources, including the UCSC Genome Browser and Ensembl, and allows the user to combine results or do further calculations. The "describe table schema" button shows the schema of the selected table, together with table field descriptions and examples of data in each field. When creating a filter for a database query, if there are related tables, then the option is given to add fields of the related tables into the filter. One of the output formats also allows the selection of fields from the selected and related tables. Other output formats include BED, GTF, custom track, and sequence. In addition, data points can be downloaded for wiggle (graph-like) format tracks, or multiple alignment format (MAF) may be used to obtain alignments from the multiple alignment tracks. Subregions of features can also be specified for the output. For example, the BED format output allows selection of upstream or downstream regions, introns, coding exons, or whole genes.

Certain identifiers or accessions may be provided to allow batch searching of tables. Otherwise, tables can be queried to obtain data on a genome-wide scale, restricted to the ENCODE regions, or for a specified gene, accession, or position.

Custom tracks can be created from Table Browser queries and then viewed in the Genome Browser or loaded as a track into the Table Browser. The custom track data are then available for further Table Browser queries and for intersections with other tracks, thereby allowing compound queries. Data uploaded into the Genome Browser to form a custom track are also available in the Table Browser, making it an excellent tool for comparing one's own data with the Browser's annotations.

Another useful feature is the ability to do correlations. The position/score vectors of two selected tables are then intersected. Scores are retained only if both tables have a score. A linear regression is performed on the two vectors, resulting in a report with several statistics, including the Pearson's correlation coefficient (r), scatterplots of the two data vectors and residuals versus fitted, and value histograms.

29.1.7.2 Example Using the Table Browser

Problem: Find the human UCSC Genes on chromosome one that overlap with genes represented by Affymetrix probe sets in the Affymetrix HG-U133 Plus 2.0 GeneChip.

1. Open the Table Browser by clicking on the Tables link on the Genome Browser home page.
2. Select the genome and assembly of interest, e.g., human (March 2006) (hg18) (Fig. 29.3).
3. Select “Genes and Gene Predictions” as the group, “UCSC Genes” as the track, and “knownGene” as the table.
4. Select “genome” as the region.
5. Press the filter button and type “chr1” into the box adjacent to “chrom does match” then press “submit.”
6. Press the intersection button. Select “Expression and Regulation” as the group, “Affy U133Plus2” as the track and “AffyU133Plus2” as the table, then press “submit.”
7. Select “BED” as the output format, then press “get output.”
8. Select “Whole Gene” and then press “get BED.”

The output will list the positions of UCSC Genes that overlap with the sequences used for the Affymetrix HG-U133 Plus 2.0 GeneChip probe design.

29.1.8 Tools

29.1.8.1 Introduction

Several web-based tools are available to supplement the effectiveness of the Genome Browser as a data mining tool. All of the tools mentioned in this section are accessible from either the side menu on the home page or the top menu on the application pages. Some of these tools can be downloaded as executables; the source is available in the Genome Browser source tree, which may also be downloaded. As with BLAT, the source is free for academic, nonprofit, and personal use, but commercial use requires a license.

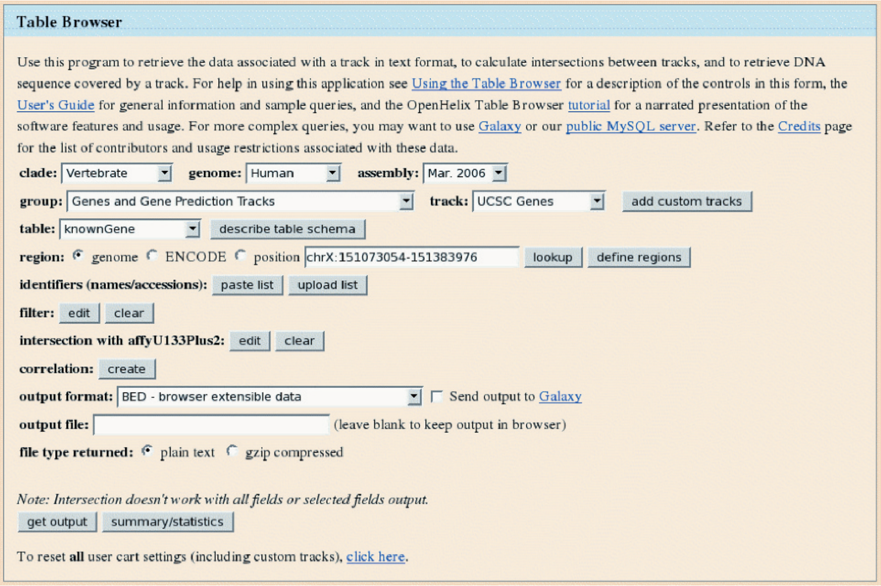


Table Browser

Use this program to retrieve the data associated with a track in text format, to calculate intersections between tracks, and to retrieve DNA sequence covered by a track. For help in using this application see Using the Table Browser for a description of the controls in this form, the User's Guide for general information and sample queries, and the OpenHelix Table Browser tutorial for a narrated presentation of the software features and usage. For more complex queries, you may want to use Galaxy or our public MySQL server. Refer to the Credits page for the list of contributors and usage restrictions associated with these data.

clade: Vertebrate **genome:** Human **assembly:** Mar. 2006

group: Genes and Gene Prediction Tracks **track:** UCSC Genes add custom tracks

table: knownGene describe table schema

region: ● genome ○ ENCODE ○ position chrX:151073054-151383976 lookup define regions

identifiers (names/accessions): paste list upload list

filter: edit clear

intersection with affyU133Plus2: edit clear

correlation: create

output format: BED - browser extensible data □ Send output to Galaxy

output file: (leave blank to keep output in browser)

file type returned: ● plain text ○ gzip compressed

Note: Intersection doesn't work with all fields or selected fields output.

get output summary/statistics

To reset **all** user cart settings (including custom tracks), click here.

Fig. 29.3 Using the Table Browser. Table Browser interface with human hg18 (NCBI Build 36) chosen as the assembly, and the knownGene table from the UCSC Genes track also selected. The “genome” option is selected for the region setting to search the entire genome. Filtering is set up and an intersection with the Affymetrix U133Plus2 track is created. BED is selected as the output format.

29.1.8.2 In Silico PCR

The in silico PCR tool (isPcr) [19] is available through a web interface, and also as a download for use on a local machine. It indexes the genome for fast sequence searching of an exact match of primer pairs to a genome or to UCSC Genes transcripts on selected human and mouse assemblies. If successful, it returns the sequence that lies between and includes the primer sequences. ExonPrimer (courtesy of Tim Strom), which is accessible through a link on UCSC Genes or Known Genes details pages (see Sect. 29.1.3.3), is a useful tool that can be used in conjunction with isPcr. It is a third-party program that aids in the design of intronic primers with certain criteria for different assemblies.

29.1.8.3 Lifting Coordinates Between Assemblies

The liftOver program [19] has a web interface, reached via the Utilities link on the home page, that allows the conversion of genomic position coordinates or annotations from one assembly version to another. It also retrieves putative homologous regions in other species by using the chained and netted alignments. Input regions, which can be uploaded as a file, can be defined using either position or BED format. The liftOver program and input files can be downloaded for local use. In addition to the liftOver utility, a Convert link on the top menu bar of most Genome Browser web pages allows conversion of the current genome position to that of another assembly. Same-species assembly conversions are based on BLAT alignments [27] (see Sect. 29.1.6); conversions between species use the pairwise chains and nets produced by BLASTZ [46], which is more sensitive than BLAT for cross-species genome alignments.

29.1.8.4 Gene Sorter

Genes evolve together and perform functions together, so that it is important to be able to view their relationships to each other. This is the principle behind the Gene Sorter [29,34], which presents a gene-based view of a genome rather than the chromosome-based view shown in the Genome Browser. The Gene Sorter is available for most of the model organism assemblies. For the human, mouse and rat genomes, the gene set produced for the UCSC Genes or Known Genes track (see Sect. 29.1.3.3) is shown. The gene sets of other organisms are obtained from third-party annotations (Ensembl [22], FlyBase [22,31], WormBase [8], SGD [11,39]). To examine the relationship of a set of genes, enter a gene name or accession in the "search" text box; the Gene Sorter will return a list of related genes sorted by the criteria specified in the "sort by" list. For each gene displayed, the Gene Sorter by default displays the gene name, selected microarray expression data, a Blastp [1] E-value from the alignment of each gene to the selected gene, the genomic position and the mRNA description. The configuration controls allow selection of other types of data for display.

By default, genes are sorted by distance based on expression data. In the Gene Sorter, the expression data are gene-specific and not transcript-specific as in the Genome Browser. For each gene, the best representative probe set representing a single transcript has been selected for that gene. For expression data, the distance between genes is calculated as a weighted sum of the log expression ratio values. Genes may also be sorted by proximity within the genome, protein similarity, name, and annotation terms. The protein similarity measures include Blastp similarity, Pfam domains [15] and protein–protein interactions.

29.1.8.5 Proteome Browser

The Proteome Browser [19,21] is a protein-centric view of selected model organism genomes with links to a number of external sites providing extensive information about proteins. Accessions, keywords, or gene names may be entered in the query text box; the view displayed is for a single protein. The Proteome Browser tracks show exon structure, polarity, hydrophobicity, cysteine and predicted glycosylation locations, Superfamily domains, and amino acids that exhibit deviation from normal abundance. The FAST-All (FASTA) protein sequence is also provided. Histograms show where the protein falls on the genome-wide distribution for protein properties such as isoelectric point, molecular weight, exon number, InterPro domains [38], cysteine locations, hydrophobicity and other characteristics. The Proteome Browser provides links to protein domain information from a wide variety of resources at other locations.

29.1.8.6 Genome Graphs

Genome Graphs [25] is a tool that was developed to support whole-genome linkage, association and homozygosity studies. It allows visualization of these data types in a genome-wide view that also supports user-generated data as custom tracks. Linkage and association studies facilitate the identification of regions of the genome that carry variants associated with disease. Genome Graphs may be used to upload several sets of genome-wide data so that they can be viewed together. The display can be restricted to regions that pass a desired significance threshold. Genes in the regions that pass the threshold can be displayed in the Gene Sorter (see Sect. 29.1.8.4), an area of interest can be viewed in the Genome Browser, and the correlation coefficient (*r*) among the data sets may also be calculated.

29.1.9 Further Information

An extensive online documentation set accompanies the Genome Browser, which can be accessed via the Help and FAQ links on the top menu bars of most web pages. Further training can be accessed via the Training link of the left blue menu bar of the home page. Data downloads are available through the downloads server (http://hgdownload.cse.ucsc.edu/). A mailing list, genome@soe.ucsc.edu, provides a forum to which users can post questions regarding Genome Browser usage and search previously answered questions. A low-volume mailing list, genome-announce@soe.ucsc.edu, broadcasts important announcements about new software and data releases and problems with the website. Several full and partial mirrors of the Genome Browser are listed on the mirrors page, which is accessible via a link on the side menu bar of the home page; these are useful when the UCSC site is unavailable. Some mirrors do not have all the organism assemblies found on the UCSC site, but they keep at least the two latest assemblies for most of the main organisms. Questions regarding Genome Browser mirrors or the setup of mirrors may be directed to the mailing list, genome-mirror@soe.ucsc.edu.

29.1.10 Future Directions

In future years, the Genome Browser database will continually expand to include newly sequenced and updated vertebrate genomes, as well as selected genomes from other clades, as they become available. The quality of the UCSC Genes set (see Sect. 29.1.3.3) will be further refined. In addition to the existing multiple alignments found in the Comparative Genomics group, protein multiple alignments that mirror these will be explored. Support for high-throughput data and large-scale sequence alignments will be added. Data from published genome-wide association studies will be made available as tracks for Genome Graphs (see Sect. 29.1.8.6). To identify specific disease-associated variants in patients, DNA sequencing of entire regions is becoming increasingly common; medical sequencing and human variation data will become more widely available in future versions of the Genome Browser, along with support for custom tracks that display medical sequencing data and highlight the variations found among different patients. Color-coding will be used to differentiate potentially deleterious mutations from those that may be harmless. Because the UCSC Genome Browser is continually evolving, the software and data may have changed in the interim since this chapter was written.

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Ensembl Genome Browser

29.2

Xosé M. Fernández and Ewan Birney

Abstract Recent years have seen the release of huge amounts of sequence data from genome sequencing centers. However, this raw sequence data is most valuable to the laboratory biologist when provided along with quality annotation of the genomic sequence.

Ensembl provides access to genomic information with a number of visualization tools, becoming one of the world's primary resources for genomic research, a resource through which scientists can access the human genome as well as the genomes of other model organisms. Thus, researchers can download data directly, whether it is the DNA sequence of a genomic contig, or positions of SNPs in a given gene. The key Ensembl web pages are highlighted in this chapter.

Because of the complexity of the genome and the many different ways in which scientists want to use it, Ensembl provides many levels of access with a high degree of flexibility. Through the Ensembl website a wet-lab researcher with a simple web browser can for example perform BLAST searches against the assembly of a genome, download a genomic sequence, or search for all members of a determined protein family. But Ensembl is also an all-round software and database system that can be installed locally to serve the needs of a genomic center or a bioinformatics division in a pharmaceutical company, enabling complex data mining of the genome or large-scale sequence annotation.

Contents

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29.2.1 Genomes Galore

The number of species sequenced is continuously increasing[1] owing to the efforts of genome-sequencing centers worldwide. What used to be a trickle is now a flood, as more and more data is obtained with new sequencing technologies [7, 12, 13, 76, 80, 99, 100]. These high-throughput whole-genome shotgun sequencing technologies deliver genomes several orders of magnitude faster than "traditional" electrophoretic methods, but we should remember that any raw sequence is only valuable to most scientists when provided along with quality annotation of the underlying genomic sequence [8].

Nowadays, the generally accepted "gold standard" for annotation of eukaryotic genomes is that made by a human being [5]. Manual annotation is based on information derived from sequence homology searches and the results of various *ab initio* gene prediction methods [14, 15], and also from literature reviews. Annotation of large genomes, such as mouse and human, in this way is slow and labor intensive, taking large teams of annotators years to complete. As a result, the annotation can almost never be entirely up to date [73] and free of inconsistencies (the annotation process usually begins before the sequencing process is complete). Hence, an automated annotation system is desirable, since it provides a relatively rapid way of delivering annotation at genome level and can be updated as new data becomes available from newer (and more complete) assemblies. To address this need, the Ensembl annotation system has been developed by observing how annotators build gene structures and condensing this process into an algorithm that codes this set of rules.

Ensembl provides an automatically annotated gene set alongside a graphical web-based interface for visualization of genomes [39]. Ensembl gene models are based on experimental evidence [9], which is imported from the manually curated protein set provided by UniProt/Swiss-Prot [117], the manually curated NCBI RefSeq [91], and the automatically annotated UniProt/TrEMBL protein records. UTR sequences are annotated to the extent supported by EMBL [67, 112] mRNA records.

29.2.1.1 Genomes in Context

Looking back in time to before Ensembl was available, we can trace the need for such an approach. After the sequencing of the first microbial genome, that of bacteriophage ΦX174 [104], many more genome sequences followed. The first bacterial genome was fully sequenced in 1995, when the sequence of the *Haemophilus influenzae* strain *Rd* was completed [38]; since then hundreds of genomes have been sequenced.

Moving to the human arena, the first human gene was cloned in 1977, but we had to wait 22 years before the first human chromosome was completely sequenced [32]. The pace accelerated [22, 26, 27, 31, 51, 52, 58, 82, 101] toward the ultimate goal: completion of the sequencing of the entire human genome within the Human Genome Project (HGP). When we refer to the HGP we are talking about the international effort that formally started in October 1990 and which delivered the draft sequence of the human genome in 2003 [59, 60]. In addition to determining the complete sequence of the 3 billion DNA base pairs in the human genome, the HGP carried out a number of parallel, preliminary studies, mainly on *Escherichia coli* [11] and mouse [81], in order to develop new approaches to optimize the tools used to handle the amount of information generated for human. This process helped to extend our understanding of human gene function (for a more detailed account see [21]).

The following landmarks highlight the effort undertaken: by February 2001, when the draft human sequence was published, the yeast *Saccharomyces cerevisiae* [46], the microscopic soil worm

[1] At the time of writing, the following completed eukaryotic genomes were deposited at EMBL: *Anopheles gambiae, Arabidopsis thaliana, Ashbya gossypii, Aspergillus fumigatus, Aspergillus niger, Bos taurus, Caenorhabditis briggsae, Caenorhabditis elegans, Candida albicans, Candida glabrata, Canis familiaris, Cryptococcus neoformans, Cryptosporidium parvum, Cyanidioschyzon merolae, Danio rerio, Debaryomyces hansenii, Dictyostelium discoideum, Drosophila melanogaster, Drosophila pseudoobscura, Drosophila simulans, Drosophila yakuba, Encephalitozoon cuniculi, Equus caballus, Gallus gallus, Guillardia theta, Hemiselmis andersenii*, *Homo sapiens, Kluyveromyces lactis, Leishmania braziliensis, Leishmania infantum, Leishmania major, Macaca mulatta, Monodelphis domestica, Mus musculus, Ornithorhynchus anatinus, Oryza sativa, Oryzias latipes, Ostreococcus lucimarinus, Ostreococcus tauri, Pan troglodytes, Paramecium tetraurelia, Pichia stipitis, Plasmodium falciparum, Rattus norvegicus, Saccharomyces cerevisiae, Schizosaccharomyces pombe, Spizellomyces punctatus, Toxoplasma gondii, Trypanosoma brucei*, *Trypanosoma cruzi*, *Vitis vinifera*, and *Yarrowia lipolytica.*

Caenorhabditis elegans [115], and the fruit fly *Drosophila melanogaster* [1, 85] genomes were finished. Drafts were available for the plant *Arabidopsis thaliana* [4] and for one of the chromosomes of the malaria parasite *Plasmodium falciparum* [40]. In addition, complete sequences had been obtained for over 30 microorganisms (including the bacteria *E. coli* [11] and *Mycobacterium tuberculosis* [20]; Table 29.2.1). Work was progressing on many other genomes, some mammalian species (chimpanzee [116], opossum [78], rat [43], mouse [81], rhesus macaque [95]), chicken [53], *Danio rerio* [108], *Takifugu rubripes* [3], *Tetraodon nigroviridis* [98], *Caenorhabditis briggsae* [110], and *Drosophila pseudoobscura* [96], but also plants such as rice [45, 127], amongst others (see Table 29.2.2).

Both human and mouse genomes are assembled by The Genome Reference Consortium (The Wellcome Trust Sanger Institute, The Genome Center at Washington University and The National Centre for Biotechnology Information). The same genomic assembly can be browsed using different genome browsers: UCSC Genome Browser [68] (featured in Chap. 29.1), NCBI Map Viewer [124], and Ensembl [39]. Since different algorithms are used to annotate genomes this can lead to different gene sets, a less than ideal scenario. This is why the Consensus CDS (CCDS) project has been established; members include groups from the European Bioinformatics Institute (EBI), NCBI, Wellcome Trust Sanger Institute, and University of California, Santa Cruz (UCSC), all working toward a unique set of human and mouse protein coding regions consistently annotated by different projects.

Table 29.2.1 A selection of genomes with complete or draft sequence published

Species	Size (Mb)	Divergence (My)[a]	Gene count
Homo sapiens	3,272	–	~25,000
Pan troglodytes (chimpanzee)	2,734	6–14	~25,000
Mus musculus (mouse)	2,932	75	~28,000
Oryctolagus cuniculus (rabbit)	3,400	40[b]	–
Cavia porcellus (guinea pig)	3,400	51[c]	–
Loxodonta africana (African elephant)	3,000	60[d]	–
Echinops telfairi (tenrec)	3,000	70[d]	–
Felis catus (domestic cat)	3,000	75[d]	–
Sorex araneus (common shrew)	3,000	75[d]	–
Erinaceus europeaus (hedgehog)	3,600	80[d]	–
Dasypus novemcinctus (armadillo)	3,000	95[d]	–
Caenorhabditis elegans (soil roundworm)	100	97[e]	21,249
Monodelphis domestica (opossum)	1,571	173 [69]	~21,000
Takifugu rubripes (tiger pufferfish)	329	450[f] [70]	~22,000
Tetraodon nigroviridis (green spotted pufferfish)	402	450[e] [70]	~28,000
Danio rerio (zebrafish)	1,600	360[e] [70]	~25,000
Drosophila melanogaster (fruit fly)	180[g]	250[h] [41]	14,752
Anopheles gambiae PEST	278	250[g] [41]	~13,000
Arabidopsis thaliana (mouse-ear cress)	120	150[i] [17]	~26,000
Oryza sativa (rice)	450	150[h] [17]	~43,000
Saccharomyces cerevisiae (yeast)	12.1	–	7,122
Escherichia coli K12	4.64	–	4,289 [11]
Mycobacterium tuberculosis H37Rv	4.41	–	3,918 [20]
Haemophylus influenzae KW20 Rd	1.83	–	1,737 [38]
ΦX174	5,386 bp	–	12 [104]

[a] Estimated divergence from the last common ancestor (compared with human, unless specified differently)

[b] Although there is some debate in the phylogeny of rodents, the placement of lagomorphs is uncontroversial [57, 83]

[c] Rodent phylogeny still debated, *Caviomorpha* placed according to [57]

[d] Phylogeny based on molecular data [83, 109]

[e] Estimated divergence time between *C. elegans* and *C. briggsae* [18]

[f] Divergence between zebrafish and pufferfish lineages 280 Mya. *Tetraodon* and *Takifugu* are 20–30 Mya apart [98]

[g] *Drosophila*'s genome is made up of 120 Mb euchromatic plus 60 Mb heterochromatic DNA

[h] Divergence between *Drosophila* and *Anopheles* lineages

[i] Divergence between *Arabidopsis* and rice lineages

Table 29.2.2 A selection of genomes currently being sequenced

Species	Size (Mb)	Divergence (Mya)[a]
Homo sapiens neanderthalensis (Neanderthal)	3,000	0.3[b]
Gorilla gorilla (gorilla)	4,000	7[b]
Pongo pygmaeus albelii (orang-utan)	3,000	14[c]
Callithrix jacchus (marmoset)	3,000	35
Tachyglossus aculeatus (echidna)	3,500	180[d]
Macropus eugenii (wallaby)	3,600	80 [83]
Petromyzon marinus (sea lamprey)	2,070	460[e]
Saccoglossus kowalevskii (acorn worm)	1,100	545

[a] Phylogenies are supported either by morphology or molecular data when available

[b] Based on preliminary analysis of 62,250 bp [88]

[c] Estimation based on morphological [47], fossil [44] and molecular data [111]

[d] Phylogeny based on recent molecular, morphological and fossil data [84]

[e] Suggested by molecular maximum likelihood trees [114]

29.2.1.2 ENCODE: Shifting the Paradigm

The ENCyclopedia Of DNA Elements (ENCODE) [34] is an international consortium organized by the National Human Genome Research Institute (NHGRI). NHGRI is an institute within the National Institutes of Health (NIH) that has approached the exploratory study of 1% of the human genome using different techniques in order to assess which ones will be best extended to the rest of the genome later. Initial findings [35] support a reformulation of the traditional view of the genome as a collection of independent genes separated by "junk" DNA [42]. Unexpectedly, it appears that most human DNA is transcribed. Many mRNA transcripts bridge so-called noncoding regions and join established protein-coding genes, and these transcripts are extensively overlapping [118].

29.2.2 Ensembl Annotation

Ensembl has developed a complex algorithm that delivers automatically annotated gene sets [25, 36] based on biological evidence. Ensembl uses biological evidence from various protein sequence and mRNA sequence sources (e.g., UniProt and RefSeq) to deliver transcript models placed on the genome assembly. In addition to protein coding genes, Ensembl also annotates noncoding RNA genes (ncRNAs include siRNA, miRNA, tRNA); in this case the primary source of information is Rfam [48].

Ensembl has developed a comparative approach to annotate these coverage genomes (e.g., bushbaby), where the alignment of complete proteins and cDNAs would not deliver a complete gene set. The approach is based on aligning whole genomes [106] using a reference genome (i.e., projecting a dataset from a well-annotated species, such as human, onto the genome of a less well-understood species, such as bushbaby). In this way, Ensembl can overcome fragmentation, gaps, missing sequence and misassemblies in the underlying genomic assembly.

The comparative method also identifies regions of sequence similarity across species that have persisted through evolution (at least for the last 100 My). These conserved sequences are indicative of some function: we could include here genes and smaller regulatory elements [77] (such as promoters, enhancers, transcription factor-binding sites, and other key players in the determination of activation of genes and pathways in the cell).

At the time of writing, Ensembl incorporates over 50 genomes (mostly vertebrates) into its databases and browser. At the time of its conception, only the human genome, the first vertebrate sequence fully determined, was displayed. Now, using comparative methods and the draft sequences currently available[2] we can estimate gene number (see Table 29.2.1) and locate regulatory elements even of species for which we only have a limited (i.e., low-coverage) assembly.

D. melanogaster, *C. elegans*, and *S. cerevisiae* are manually annotated by FlyBase [30], WormBase [18], and SGD [19], respectively. Ensembl imports these

[2] At the time of writing, Ensembl incorporates annotation for human, chimpanzee, orang-utan, macaque, marmoset, gorilla, mouse, rat, dog, cow, and chicken; opossum, platypus, and the frog *Xenopus tropicalis*; fishes such as zebrafish, *Takifugu rubripes*, *Tetraodon nigroviridis*, stickleback, and *Medaka*; *Drosophila melanogaster*, *Aedes* and *Anopheles* mosquitoes; the tunicates *Ciona intestinalis* and *C. savignyi; Caenorhabditis elegans* and the yeast *Saccharomyces cerevisiae*. Several organisms were sequenced with low-coverage assemblies: alpaca, armadillo, bushbaby, cat, dolphin, elephant, guinea pig, hedgehog, horse, hyrax, kangaroo rat, lesser hedgehog tenrec, megabat, microbat, mouse lemur, pika, rabbit, shrew, squirrel, tarsier, tree shrew.

datasets to incorporate them into the comparative analysis, but no gene build is run for these genomes.

29.2.3 Region in Detail: Introduction

In order to visualize features on a genome, Ensembl has condensed the annotated assembly into a customizable view. The "Region in detail" page provides a graphical representation of a region, where different panels allow visualization of varying windows of the chromosomal region at different resolutions (Fig. 29.2.1). Gene predictions (e.g., Ensembl genes, EST genes, *ab initio* models) and annotations, such as variations (e.g., SNPs), are displayed along clones, microarray probesets and other genomic features.

Features displayed above the DNA genome assembly (blue bar) are in the forward strand, while features below the DNA are on the reverse strand. Nonstranded features are shown at the bottom of the panel (e.g., SNPs). In Fig. 29.2.1, IL12B_HUMAN (ENSG00000113302) is on the reverse strand [as the arrowhead before the gene ID ("<") indicates], while ENSG0000022160 (a snRNA in the ncRNA track) is on the forward strand. The chromosome in this region is also covered by contig AC011418 (the CTB-9P16 clone from the human tilepath covers this region).

In order to navigate in this view, the zooming ladder allows you to focus on a particular region or to expand the field of view. There is also the possibility of rubberbanding ("click and drag" your mouse) around a region in the "Chromosome" or "Top panel" panel to change the view.

Region in detail allows you to see up to 1 Mb; if you want to zoom out and see a wider region, use Region overview. Syntenic blocks (conserved gene order across species) are available; however, some higher resolution annotations, such as SNPs, are not available in this page. (From the "Configure this page" window go to the "Top panel" tab and select the species for which you want to compare conserved gene order.)

29.2.3.1 Region in Detail: Features

Ensembl provides gene sets (Ensembl genes, Ensembl EST gene models based on EST evidence, along with imports such as tRNA and miRNA genes, manually annotated genes from VEGA, and *ab initio* gene models such as GENSCAN), but is not limited to gene models. Several microarray probe sets (*GeneChip*® from *Affymetrix*, *BeadChip*™ from *Illumina*, *Agilent* and *BioArrays* from *CodeLink*™) are mapped to the assembly along with regulatory elements (CpG islands, models from predictive programs such as Eponine [29] and FirstEF [93], and elements in the *CisRED* [97] database), variations (SNPs, insertions, deletions) and other sequences (from NCBI RefSeq, UniGene [124], and other databases of ESTs, mRNAs, and/or proteins). These annotations can be displayed using the options available in the "Configure this page" menu ("Main panel") of "Region in detail."

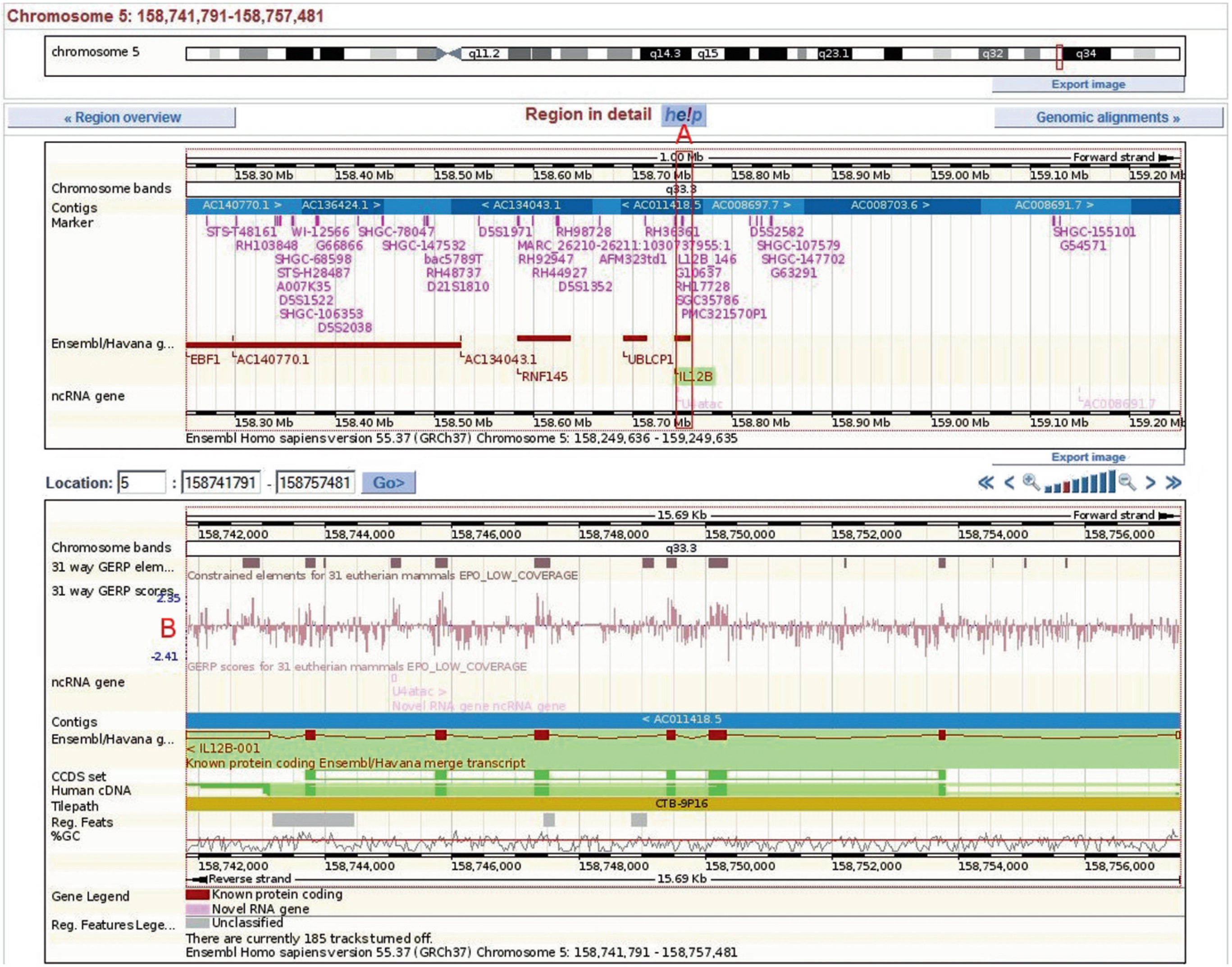
29.2.3.2 Region in Detail: Repeats, Decorations, and Export

"Region in detail" is highly customizable: there are over 100 tracks available. Images can be exported in different editable formats (e.g., PDF, SVG). Context menus yield more information about individual features and tracks.

Repeat sequences are also available (as *Repeats* under the Main panel tab on the Configuration page). Genome size does not correlate with organism complexity: our genome is 200 times as large as that of *Saccharomyces cerevisiae*, but that of *Amoeba dubia* is 200 times the size of ours, largely because of different quantities of repetitive sequences. In human we find that the genome is 50% repeat sequences and only 5% coding sequence. In "Region in detail," retrotransposons such as long terminal repeats (LTR), long interspersed elements (LINE), short interspersed elements (SINE, the most thoroughly characterized as belonging to the Alu family [55]), tandem repeats, and other repeats are displayed.

29.2.4 DAS Sources

Ensembl can enrich its annotation by displaying third-party data external to Ensembl databases by means of the Distributed Annotation System (DAS) [28]. DAS is a communication protocol that allows the exchange


Chromosome 5: 158,741,791-158,757,481

Location: 5 : 158741791 - 158757481 Go>

Ensembl Homo sapiens version 55.37 (GRCh37) Chromosome 5: 158,249,636 - 159,249,635

There are currently 185 tracks turned off.

Ensembl Homo sapiens version 55.37 (GRCh37) Chromosome 5: 158,741,791 - 158,757,481

Fig. 29.2.1 'Region in detail' is structured in *three panels* representing different zoom levels of the genome. An ideogram of chromosome 5 appears at the *top*, with chromosomal bands labeled (5q33.3). The following *panel* (*top panel*) shows a region of 1 Mb corresponding to the *red box* shown on the chromosome (labeled *A*). Gene models and markers are displayed. A *scale bar* depicts the physical map coordinates for the region, just above the contig track where the individual contig sequences that form the genome sequence assembly are shown, in different shades of blue. The *third panel* (*main panel*) is very rich in features and can be customized (see text) of biological sequence annotation, removing the need to store it in a single database; in this way resources can be spread over multiple servers in different geographic locations. Furthermore, it provides a way to display data across different databases, enhancing ease of access for the user.

Ensembl acts as a DAS client, gathering and integrating sequence annotation from multiple remote servers and displaying it in "Region in detail" as additional tracks. Note that shortly after a new genome assembly is released some DAS sources might not be available, as it can take some time to map their information to the new reference (Ensembl is not responsible for these remote servers).

The DAS registry (www.dasregistry.org) is a directory of DAS services available. It provides statistics for every source and keeps track of every service (contacting the owner in case the service was down).

Examples of DAS sources are clones (CpG island clones), alternative annotations (NCBI Gnomon, IMGT Genes), new features (CNV, CAGE), data from research projects (DECIPHER), BAC libraries, etc. The DAS sources available vary with species and are accessible for viewing in the configuration panel.

29.2.5 Comparative Genomics

The study of evolution has moved to the genomic era with the availability of whole-genome sequences from several species [102, 119]. Nowadays, we can track how the pieces of the genomic jigsaw have been shuffled during evolution, outlining the importance of gene order conservation, going beyond individual gene and protein comparison across species.

Function is often inferred from coding sequences, but it is not well understood how to decipher functional properties from noncoding sequences. Hence, there is a strong bias toward the analysis and description of events in coding regions [125], such as gene duplications and protein sequence evolution, while noncoding, regulatory sequences often go unnoticed. Since most of the genome is noncoding we are reaching the limit of what can be expected from evolutionary genetics on the sole basis of coding sequences [121]. We should move forward, taking full advantage of the range of organisms sequenced, understanding regulatory sequences as well as the coding sequences.

Cross-species comparison of closely related genomes seems the obvious way to identify functional elements in the human genome (both coding and noncoding) [90]. Evolution has conserved many of the DNA sequences behind coding genes or in the elements that regulate gene expression, making comparisons of genome sequences between species an effective and efficient means of finding new genes and functional sequences [72]. A number of eutherian mammals have been sequenced (Tables 29.2.1 and 29.2.2), providing us with a blueprint or characteristic gene set for the mammalian clade. The next step is to model the effect of mutation on functional elements of the genome, before investigating noncoding regions in order to understand gene expression control [90].

Conserved regions are identified using whole-genome comparisons on the nucleic acid level between two species (e.g., human/mouse) determined with BLASTZ [106]. When comparing more distant species, the translated BLAT [64] algorithm is used to compare genomes at the amino acid level; thus, there would be a bias toward protein-coding regions, although conserved noncoding regions are also detected.

Global multiple alignments are calculated with the Pecan algorithm (B. Paten, unpublished work) and can be displayed in "Region in detail" ("Multiple alignments" in the main panel tab). Conservation scores are calculated using the multiple alignments, and "constrained elements" are determined using GERP [23]. These can be displayed in "Region in detail" ("Conservation" track, labeled B in Fig. 29.2.1).

Ensembl also identifies large regions of conserved sequence (synteny), as previously mentioned in Sect. 29.2.3. These syntenic regions are determined using BLASTZ-net analysis, and they can be viewed using the configuration panel ("Synteny") in "Region overview." Syntenic blocks are also available in the Synteny page (Location | Synteny); conserved regions are displayed by means of a diagram of chromosomes, along with a table of homologous genes between two species for a region.

Gene Trees. Ensembl performs multiple transcript comparisons to identify genes that are paralogous (homology follows a gene duplication; i.e., two genes are descended from one ancestral gene and may have evolved to have different functions) and orthologous (functional equivalent genes diverged in two genomes further to a speciation event). Lack of selection on paralogous genes can result in pseudogenes, i.e., one copy loses its promoter and is not transcribed, so that it can mutate over time with few negative consequences. The transcript set in Ensembl is scanned for signs of processed pseudogenes (genes that have been reverse-transcribed from mRNA and reintegrated at random into the genome) and annotated as such. However, pseudogenes resulting from gene duplications in the genome (complete with introns) are much harder to annotate.

Orthology and paralogy can help us understand genome rearrangements and the evolution of function. These relationships are displayed using phylogenetic gene trees, as shown in Fig. 29.2.2. Maximum-likelihood phylogenetic trees are generated with TreeBeST (Li Heng et al., unpublished work) in order to provide a representation of the evolutionary history of gene families. Duplication and speciation nodes are inferred from the reconciliation with their species tree. The Gene Tree page (Fig. 29.2.2) can be accessed from specific gene pages using the link at the left.

29.2.6 GeneView

Once a gene has been selected, the Gene tab shows annotation focused on a gene (Fig. 29.2.3). At the top of the view there is a summary for the entry with links to external databases (HGNC, CCDS) and to other pages within Ensembl (e.g., "Region in detail" shown in Fig. 29.2.1).

There are several shortcuts to more pages:

- *Splice Variants.* All Ensembl transcripts for the gene. Protein domains and motifs are aligned in the graphical display.
- *Supporting Evidence.* Where the initial proteins and mRNAs aligned to the genome in the Ensembl annotation are shown.
- *Sequence.* Features in the gene structure such as exons or SNPs can be highlighted using the options available in the "Configure this page" menu.
- *External References.* Where external records, or identifiers and information in databases external to Ensembl, are specifically attached to the Ensembl gene.
- *Regulation.* Species-specific regulatory feature information if available. In the case of human, this includes predicted promoters and enhancers from the Ensembl Regulatory Build, which represent a single best guess of regulatory elements and annotations based on statistically significant association with genomics features.
- *Comparative Genomics.* A number of options are available within this category: Gene Trees, orthologues, paralogues, and protein families.
- *Genetic Variation.* Provides information about SNPs and genetic variation in general.
- *ID History.* Ensembl keeps its identifiers (for genes, transcripts, and proteins) stable throughout releases, and can therefore be tracked in case an identifier was retired and a new one assigned (or two identifiers may be merged). This page provides an *ID History Map* showing the release number on the x-axis and stable IDs on the y-axis. Small squares or nodes correspond to the ID shown on the left and represent an update in the version of the ID. Versions are updated if there has been a change in the gene, transcript, or protein model. Nodes (squares) are connected by a line if the versions are related. This line reflects the score of how well the versions match, for recent releases. If a score is not calculated, the line will be gray (unknown score).

There is a cartoon depicting the structure of the gene (in this case on the reverse strand, drawn under the assembly; this means that this gene's 5′ end is on the right and its 3′ end, on the left; Fig. 29.2.3). In this particular instance Ensembl only annotates one unique transcript; if there were alternative splicing forms, they would be seen here.

UTRs are displayed as unfilled boxes, and coding regions as filled boxes. Manually annotated transcripts (VEGA) are displayed in blue, while Ensembl transcripts are displayed in red; EST-based models are displayed in magenta. In Fig. 29.2.3, only the Ensembl-known protein-coding transcript is displayed. For options to view other annotations, such as VEGA transcripts, choose the option in the "Configuration panel."

When a transcript is selected, there will be a "Transcript" tab, which also offers several shortcuts on the left.

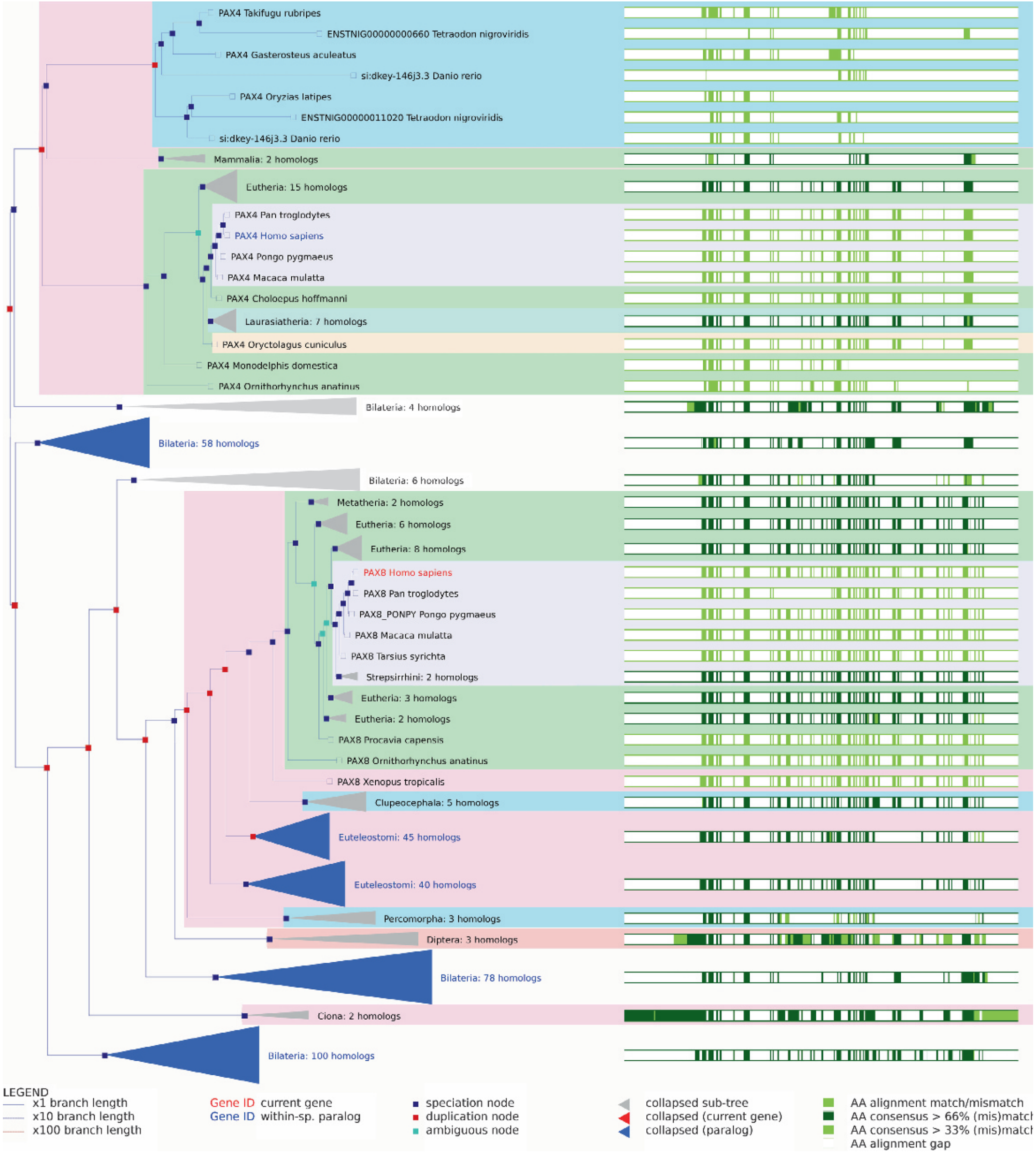
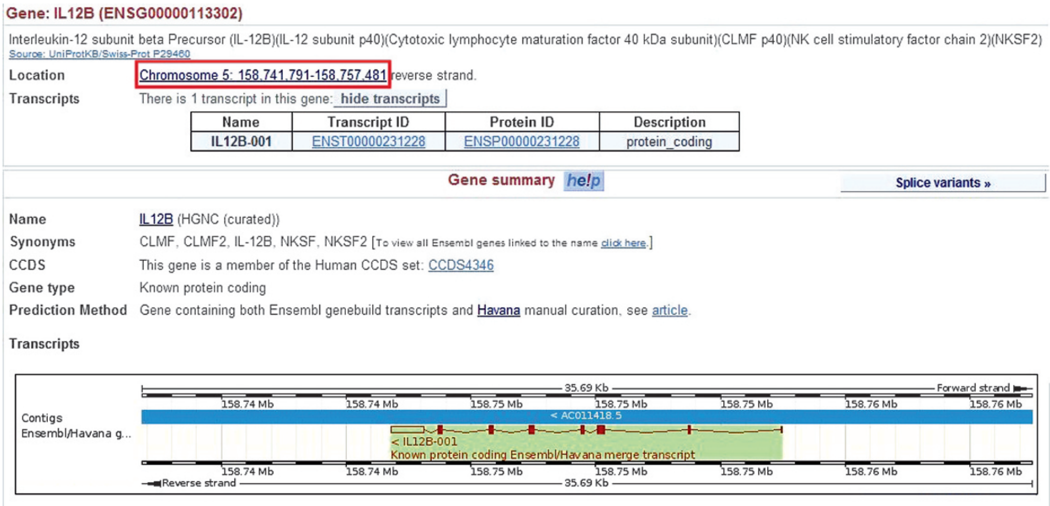


Fig. 29.2.2 Detail from *Gene Trees*, displaying the phylogenetic tree and schematic representation of multiple alignment (in *green*) for the PAX2/PAX5/PAX8 cluster centered on the human PAX8 gene (in *red*). Distinct branches are visible with the fish clade at the top displaying the teleost ancestral duplication [62], and the orthologue gene PAX4 (from chicken to human). Some nodes (associated to orthologues PAX2 and PAX5) appear collapsed (*blue* and *gray* triangles). The *green bars* at the *right* of the tree provide a schematic representation of the multiple alignment of the peptides (*full boxes* indicate matches/mismatches; *open boxes* indicate gaps in the alignment)



Gene: IL12B (ENSG00000113302)

Interleukin-12 subunit beta Precursor (IL-12B)(IL-12 subunit p40)(Cytotoxic lymphocyte maturation factor 40 kDa subunit)(CLMF p40)(NK cell stimulatory factor chain 2)(NKSF2)
Source: UniProtKB/Swiss-Prot P29460

Location	Chromosome 5: 158,741,791-158,757,481 reverse strand.
Transcripts	There is 1 transcript in this gene: hide transcripts

Name	Transcript ID	Protein ID	Description
IL12B-001	ENST00000231228	ENSP00000231228	protein_coding

Gene summary he!p Splice variants »

Name	IL12B (HGNC (curated))
Synonyms	CLMF, CLMF2, IL-12B, NKSF, NKSF2 [To view all Ensembl genes linked to the name click here.]
CCDS	This gene is a member of the Human CCDS set: CCDS4346
Gene type	Known protein coding
Prediction Method	Gene containing both Ensembl genebuild transcripts and Havana manual curation, see article.

Transcripts

Fig. 29.2.3 The Gene summary incorporates links to external sources mapped to this transcript. This gene IL12B (ENSG00000113302) is a member of the human CCDS set (see text). A transcript has been annotated by Ensembl (ENST0000231228). The chromosomal location is highlighted

29.2.7 Variation

29.2.7.1 Variation Image

Most variations in Ensembl are imported from NCBI dbSNP, though a minority are from other sources and projects, such as resequencing efforts. The source of a SNP (single nucleotide polymorphism) is available on the “Variation summary” page, obtainable for any SNP displayed in Ensembl. A variety of views are available for exploring variations. For SNP variation within a gene, one option is the “Variation image,” which provides a graphical display, while a table of all SNPs in one gene is available in “Variation Table.” The user may filter information on the basis of the validation method of the SNPs displayed (either by NCBI dbSNP terms: frequency, cluster, or by genotype data from HapMap, etc.). SNPs may also be filtered by class [insertion-deletions (indels), microsatellite repeats, multinucleotide polymorphisms (MNPs) etc.], or by type (intronic, upstream, synonymous or silent mutation, frameshift, etc.).

29.2.7.2 Comparison Image

Comparison image provides a display of the SNPs and variations within the nucleic acid and/or protein sequence in one view. Any variation can then be clicked on to find the Variation summary page for more information. Ensembl incorporates SNPs from the resequencing effort undertaken for multiple inbred mouse strains [24]. The comparison image allows users to explore the catalogue of genetic variants for a particular mouse strain, for example, and compare it with the same region in other strains (or individuals in the case of humans, breeds in the case of dogs).

SNP data can be exported from this page, filtered by SNP type (synonymous/nonsynonymous, coding/intronic, upstream/downstream, etc.), by individual, strain or breed, and class (indels, MNP, heterozygous variation, etc.).

29.2.8 Ensembl: An Example

We will now explore Ensembl, starting with some basic information about the Interleukin-12 beta chain precursor (*IL12B*). This gene encodes a subunit of this cytokine that acts on natural killer cells. Searching for “human gene IL12B” in the text search box found on the home page (www.ensembl.org) takes us to the results page, where we have a link to the Gene summary page for this entry.

On the Gene summary page we find information about orthologues and paralogues across species, whole-genome alignments, splice variants, matches in other databases, and more. For example, in release 55, human IL12B is ENSG00000113302, has no splice variants, and is located on chromosome 5, bp 158,741,791–158,757,481 (as shown in the Location tab). Alignments are shown with other mammals and vertebrates, and it has good correspondence (100% match) to the IL12B_HUMAN entry in UniProtKB/Swiss-Prot. Find these facts on the Gene summary page in the stable archive site:

http://Jul2009.archive.ensembl.org/Homo_sapiens/Gene/Summary?db=core;g=ENSG00000113302

If we follow the link to "Region in detail" from the search results or from the "Location" tab of the Gene summary page (alternatively, click the base pair numbers indicating the location of the gene), we see this gene in its genomic context:

http://Jul2009.archive.ensembl.org/Homo_sapiens/Location/View?db=core;g=ENSG00000113302

At the top of "Region in detail," STS markers (sequence tagged sites) from several databases (thus, there may be synonyms) are displayed along the assembly. They have been mapped to the genome using e-PCR [105], and following any marker link brings up the Marker page, showing the PCR primers that define this unique segment (as well as the expected size of the amplicon).

We can see the IL12B gene is located in chromosome 5 band q33.3, and more specifically, in contig AC011418. If we select clone sets from "Misc regions" menu in the "Configuration panel" we can see clones for this region on this display.

Variations in the form of SNPs can be selected in the "Variation features" menu, but they are only shown when the region displayed is less than 50 kb long. Selectable subsets of these variations are SNPs which have been genotyped, and two GeneChip® Human mapping array sets (100K and 500K) from Affymetrix.

For a more detailed walk-through of the website and information about the pages, please see [37].

29.2.9 BioMart Overview

BioMart is a data "warehouse" originally developed for Ensembl [63], which now has become a joint project between the European Bioinformatics Institute (EBI) and the Ontario Institute for Cancer Research (OiCR), providing a query-oriented data management system to interact with different datasets (Ensembl is just one of many).

The initial query system has now shifted toward a federated approach that has been deployed for several biological databases and therefore provides a gateway to Ensembl from numerous databases, with no need to store those sources locally.

BioMart retrieves information from databases without users having to become familiar with their schema or having any programming expertise. It also provides interactive access to information stored in the Ensembl databases, as well as allowing integration of in-house data (for those ready to set up a local BioMart).

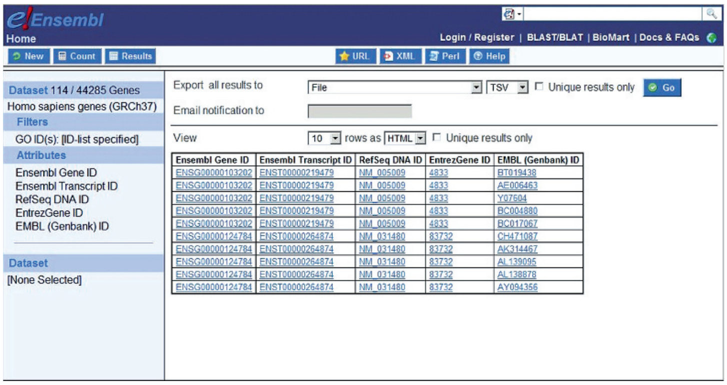
Example Using BioMart. BioMart can be used to convert Entrez Gene IDs [74] to Ensembl identifiers (e.g., ENSG00000113302) and/or RefSeq [91], Entrez Genes or EMBL [67] (GenBank) identifiers.

Select database (Ensembl 51) and dataset (*Homo sapiens* genes), limit your query using "Filters," and in the "Gene" panel enter your ID. Choose "External references" from the "Attributes" in the "External" panel (RefSeq, Entrez Gene ID, and EMBL), and get your "Results" (Fig. 29.2.4).

This tool can also export gene, peptide, UTR (untranslated region), and flanking sequences in FASTA format for a given list of genes, or even all the genes in a genome. Names of clones spanning a particular region can also be quickly obtained.

A powerful capacity of BioMart is the possibility of joining queries from different databases using the same web interface (select the second "Dataset" option in the web interface, Fig. 29.2.4). In this way, for example, information from UniProt can be displayed alongside Ensembl annotation.

In the next example we will retrieve annotation associated to *kinases*. For this purpose we will use the Gene Ontology (GO) [16] term associated with *kinase activity* (GO: 0016301). GO is functional clustering based on a hierarchical vocabulary, providing a description of a gene (molecular function, cellular component, and biological process). From the BioMart Central Server (www.biomart.org) select "Ensembl Genes" and "*Homo sapiens* genes" as your database and dataset, respectively. Amongst the "Attributes" include RefSeq, EMBL (GenBank), and EntrezGene ID. We can filter the results of this query with information retrieved from UniProt by choosing this secondary dataset (Fig. 29.2.5).



Dataset 114 / 44285 Genes
Homo sapiens genes (GRCh37)
Filters
GO ID(s): [ID-list specified]
Attributes
Ensembl Gene ID
Ensembl Transcript ID
RefSeq DNA ID
EntrezGene ID
EMBL (Genbank) ID
Dataset
[None Selected]

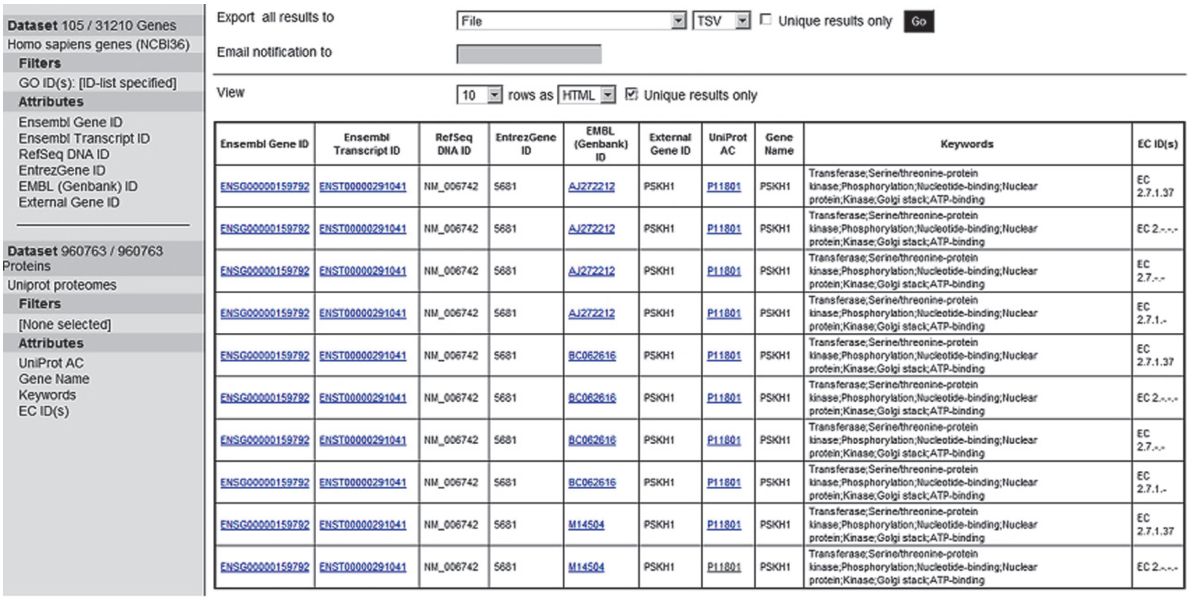
Export all results to File TSV ☐ Unique results only

Email notification to

View 10 rows as HTML ☐ Unique results only

Ensembl Gene ID	Ensembl Transcript ID	RefSeq DNA ID	EntrezGene ID	EMBL (Genbank) ID
ENSG00000103202	ENST00000219479	NM_005009	4833	BT019438
ENSG00000103202	ENST00000219479	NM_005009	4833	AE006463
ENSG00000103202	ENST00000219479	NM_005009	4833	Y07604
ENSG00000103202	ENST00000219479	NM_005009	4833	BC004880
ENSG00000103202	ENST00000219479	NM_005009	4833	BC017067
ENSG00000124784	ENST00000264874	NM_031480	83732	CH471087
ENSG00000124784	ENST00000264874	NM_031480	83732	AK314467
ENSG00000124784	ENST00000264874	NM_031480	83732	AL139095
ENSG00000124784	ENST00000264874	NM_031480	83732	AL138878
ENSG00000124784	ENST00000264874	NM_031480	83732	AY094356

Fig. 29.2.4 The BioMart result preview window showing gene IDs for a specified gene list. For the full table, the view can be changed to 'all' rows, or the file exported using the '*Go*' button. In this example, Ensembl Transcript IDs are mapped to NCBI RefSeq entries, Entrez Gene IDs, and mRNAs in EMBL-Bank



Dataset 105 / 31210 Genes
Homo sapiens genes (NCBI36)
Filters
GO ID(s): [ID-list specified]
Attributes
Ensembl Gene ID
Ensembl Transcript ID
RefSeq DNA ID
EntrezGene ID
EMBL (Genbank) ID
External Gene ID

Dataset 960763 / 960763 Proteins
Uniprot proteomes
Filters
[None selected]
Attributes
UniProt AC
Gene Name
Keywords
EC ID(s)

Export all results to File TSV ☐ Unique results only

Email notification to

View 10 rows as HTML ☑ Unique results only

Ensembl Gene ID	Ensembl Transcript ID	RefSeq DNA ID	EntrezGene ID	EMBL (Genbank) ID	External Gene ID	UniProt AC	Gene Name	Keywords	EC ID(s)
ENSG00000159792	ENST00000291041	NM_006742	5681	AJ272212	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.7.1.37
ENSG00000159792	ENST00000291041	NM_006742	5681	AJ272212	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.-.-.-
ENSG00000159792	ENST00000291041	NM_006742	5681	AJ272212	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.7.-.-
ENSG00000159792	ENST00000291041	NM_006742	5681	AJ272212	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.7.1.-
ENSG00000159792	ENST00000291041	NM_006742	5681	BC062616	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.7.1.37
ENSG00000159792	ENST00000291041	NM_006742	5681	BC062616	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.-.-.-
ENSG00000159792	ENST00000291041	NM_006742	5681	BC062616	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.7.-.-
ENSG00000159792	ENST00000291041	NM_006742	5681	BC062616	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.7.1.-
ENSG00000159792	ENST00000291041	NM_006742	5681	M14504	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.7.1.37
ENSG00000159792	ENST00000291041	NM_006742	5681	M14504	PSKH1	P11801	PSKH1	Transferase;Serine/threonine-protein kinase;Phosphorylation;Nucleotide-binding;Nuclear protein;Kinase;Golgi stack;ATP-binding	EC 2.-.-.-

Fig. 29.2.5 Export table from BioMart using the linked Dataset option. As summarized on the *left*, Ensembl *Homo sapiens* genes are chosen as the first dataset and are linked to UniProt proteomes. Attributes (*column headers*) are shown for both datasets in one table

Amongst the "Attributes" available from UniProt, we will select "Gene Name," "Keywords," and "EC (Enzyme Commission) [61] ID" (the last assigned by the International Union of Biochemistry and Molecular Biology, IUMIB).

More tutorials and documentation about BioMart can be found at www.biomart.org, and also in the help and information sections of the Ensembl browser.

29.2.10 Customizing Ensembl

User Accounts. Ensembl has introduced free user accounts, opening up new scope for customization. Normally, customized settings are stored locally as cookies, but when users move to a different computer those settings do not transfer to the new machine. When the user has an account, any customized settings are stored centrally in Ensembl, and when the user logs on these will be taken into account no matter what computer is being used.

An additional advantage of having an account in Ensembl is the possibility of creating user groups (e.g., for a research group or for collaborating consortia). Thus, annotation could be added (e.g., relevant publications/notes about a particular gene) and shared within the group. Furthermore, BLAST results may be stored for an extended period when using one's account.

29.2.10.1 Displaying User Data on Ensembl

Ensembl offers the option of uploading your own information, either with DAS or using a URL-based upload. This data can be temporarily displayed in "Region in detail" and "Region overview" along Ensembl's annotation without the need to set up your own server. Upload of data formatted in the following formats is supported: GFF, GTF, BED, and PSL (see Sect. 29.2.4). Our help documentation (in the "Custom Annotation" section of the Help pages) includes a step-by-step explanation of the procedure to follow so as to visualize your own data in the Ensembl framework.

29.2.11 Archive

Ensembl releases a new version of the browser bimonthly and updates gene builds whenever new assemblies become available. Ensembl has implemented an archive site where researchers can access older versions of Ensembl. This archive (http://archive.ensembl.org) is the appropriate way of referring to a particular region or link in publications, ensuring that other investigators can visualize exactly the same genomic landscape and annotation as is described in the publication. Similarly, old releases of BioMart can be accessed through these archive sites. An archive site was used in Sect. 29.2.6.

29.2.12 *Pre!* Site

Although Ensembl updates gene sets whenever a new genome assembly is made public, this process can take several months. A skeleton site is put together shortly after we can access the new assembly at (http://pre.ensembl.org), so that investigators can peek at the new genomic landscape. These *Pre!* sites include some basic annotation, such as CpG islands, mRNAs and proteins, and GENSCAN transcripts, but no Ensembl gene models.

29.2.13 Further Information

Ensembl links to the EBI, providing cross links to the wealth of information hosted there (e.g., literature, patent information from the European Patent Office, microarray data from ArrayExpress [89], macromolecular structures from MSD [113], and molecular interactions from IntAct [65], amongst many other databases). A sister project, Ensembl Genomes, has extended Ensembl across the taxonomic space providing annotation to bacteria, plants, fungi, protists and metazoa.

Ensembl believes in open source, and therefore all the data generated is freely available [56, 103]. The browser can be installed locally (although this is not a trivial task, as the hardware requirements might exceed those available to the average user), and help is provided through a dedicated helpdesk that can be reached at helpdesk@ensembl.org.

Ensembl provides access to RDBMS systems[3] that can be used for high-level access to our data via direct SQL or via APIs that the Ensembl project provides, as well as via any third-party software that can directly communicate with these RDBMS instances.

29.2.14 Outlook

New species will be incorporated into Ensembl as their genome assemblies are established following the sequencing effort. The analysis of the great ape clade will come into the spotlight as a valuable tool to increase our understanding of human evolution. Genomic sequences from orang-utan (*Pongo pygmaeus albelii*), chimpanzee *(Pan troglodytes),* rhesus macaque *(Macacca mulata)*, and marmoset (*Callithrix jacchus*, a New World monkey extensively used in the

[3] At ensembldb.ensembl.org (with username "anonymous"), note that this is not an URL but the location of a MySQL instance.

laboratory) should allow comparative analysis, providing more resolution to the picture and helping to identify those features in the human genome that differ among primates [75], with the ultimate goal of defining and better understanding the unique DNA sequences that set primates apart from other mammals and, moreover, humans [50] apart from other primates.

Exciting advances in sequencing and gene detection are revealing more and more about genomes. New "parallel" high-throughput low-cost sequencing platforms deliver short reads, which are the perfect match for ancient DNA, which is fragmented and not amenable to sequencing by traditional methods. New sequencing techniques sacrifice read length for much higher throughput, resulting in high coverage with short reads. Thus, the 454 single-molecule sequencing method could deliver an assembled genome from millions of reads.

Next-generation sequencing paves the way to new information that was unobtainable or prohibitively expensive with traditional methods, e.g., experimental platforms such as microarrays. Epigenome sequencing and genome-wide profiles of DNA-protein interactions become reality with these technologies [79]. Similar methods based on ditag sequencing [87] are being used to profile the methylation status of cancer genomes [122].

Genome sequences of individual humans are already available [71, 120],[4] and there are more to come. More individual genomes will provide additional haplotypes, and Ensembl is committed to providing tools for this future data.

Genome-wide association (GWA) studies [2, 33, 49, 107, 122, 123, 126] should provide new tools for unraveling the genetic basis of many common causes of human morbidity and mortality [54]. The Cancer Genome Atlas is an effort coordinated by the NCI and NHGRI to map the genomic changes involved in cancer, while ClinSeq, a pilot study from the NHGRI, attempts large-scale medical sequencing focusing on common diseases (such as coronary heart disease, with around 200–400 genes associated) and plans to analyze 1,000 individuals over 2 years. Other projects will focus on whole-genome resequencing of hundreds of individuals.

Gains and losses of large segments of DNA sequence (from 10,000 to 5 million base pairs) are known as copy number variation (CNV) [94]. New technologies allow high-resolution characterization of CNV within the framework of GWA studies. Genome browsers such as Ensembl will adapt and expand their scope to incorporate these new types of data in the exciting future ahead.

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[4] Available http://jimwatsonsequence.cshl.edu, here users can explore the Nobel Prize winner's genome.

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Databases in Human and Medical Genetics 29.3

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Abstract This chapter provides an introduction to the major, freely available, Internet-accessible databases in human and medical genetics used by healthcare providers in the diagnosis, management, and genetic counseling of persons with inherited disorders and their families, as well as by researchers for gene discovery, recording allelic variants, and cataloging genotype-phenotype relationships. Databases discussed include: GeneTests (view: www.genetests.org); Online Mendelian Inheritance in Man (view: www.ncbi.nlm.nih.gov/Omim); locus specific databases (LSDBs) identified at the Human Genome Variation Society (HGVS) web site (http://www.HGVS.org/dblist.html); DatabasE of Chromosome Imbalance and Phenotype in Humans using Ensembl Resources (view: http://decipher.sanger.ac.uk); Entrez Gene (view: ncbi.nlm.nih.gov/gene); dbGap: Database of Genotype and Phenotype (view: ncbi.nlm.nih.gov/dbgap); and the Human Gene Mutation Database HGMD® (view: http://www.hgmd.org).

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Contents

29.3.1 GeneTests

29.3.1.1 Name and URL

GeneTests (view: www.genetests.org)

29.3.1.2 Background

GeneTests was initially funded in 1992 by the National Institutes of Health as “Helix: A Directory of DNA Diagnostic Laboratories,” a stand-alone desktop database accessible via telephone and fax to the data manager. Helix was released in late summer 1993 with listings for 110 diseases offered by 100 laboratories, without distinction between clinical laboratories and research-only laboratories. In 1996, Helix became Internet accessible and was joined in 1997 by an NIH-funded companion web site, "GeneClinics," comprised of full-text disease descriptions relating genetic testing to patient care. The name GeneTests replaced "Helix" in 1998. In the next 3 years, a Clinic Directory and Educational Materials including an Illustrated Glossary were added; GeneClinics was renamed GeneReviews; and, in 2001, both web sites were joined under the name GeneTests.

29.3.1.3 Purpose and Target Audiences

The purpose of GeneTests is to integrate genetic services into patient care by providing information on:

- Genetic testing for inherited disorders that is currently available in clinical laboratories worldwide (GeneTests Laboratory Directory)
- Genetics and prenatal diagnosis clinics (GeneTests Clinic Directory)
- Use of genetic testing in patient diagnosis, management, and genetic counseling (GeneReviews)

The primary intended audience is made up of all healthcare providers. Although genetic counseling and testing terms and concepts are used throughout the GeneTests web site, the companion Education Materials and Illustrated Glossary are intended to help bridge the gap for those clinicians who are not familiar with genetic terminology.

The secondary intended audience is that of researchers, who are served by the ability to list their research laboratories that are investigating a specific phenotype and/or gene(s) in order to ascertain research subjects and their relatives.

Individuals with inherited disease and their families are not an intended audience, as the language used and the required underlying medical knowledge base are too sophisticated for the average health consumer, for whom educational materials generally involve more explanation.

29.3.1.4 Location

The GeneTests and GeneReviews database and staff are located in the Department of Pediatrics at the University of Washington, Seattle, WA, USA.

GeneReviews has also been published since July 2005 on the BookShelf site developed and maintained at the National Center for Biotechnology Information (NBCI) at the National Library of Medicine (NLM) at the NIH.

29.3.1.5 Funding and Governance

GeneTests is funded by a contract from the National Institutes of Health: "Creation and Maintenance of GeneTests Database Records" (N01-LM-4-3503).

An Editorial Board that meets once a year helps decide policy. Contract performance standards are set by the NIH.

29.3.1.6 Contents

The five content areas are: *GeneReviews*, Laboratory Directory, Clinic Directory, Resources, and Educational Materials [14].

GeneReviews comprises more than 490 entries, all authored by experts and peer reviewed. One new entry is added each week; entries are updated every 2–3 years and revised as needed to keep up to date with current genetic test availability. The entries in *GeneReviews* are highly structured disease descriptions with a uniform format that allows clinicians to locate information within each entry quickly. Each entry links directly to the disease-specific clinical laboratory listings in the Laboratory Directory.

The Laboratory Directory provides information on molecular genetic testing, specialized cytogenetic testing [such as FISH (fluorescent in situ hybridization) and array CGH (comparative genome hybridization)], and biochemical genetic testing. About 615 laboratories offer testing for approximately 1,450 inherited disorders; over two-thirds of these tests are offered by clinical laboratories and about one-third, by research laboratories only. Sixty-two percent of laboratories are in the US, and 38% are non-US.

Any laboratory performing "in-house" testing may list its services. The one restriction is that any US laboratories listed as a clinical laboratory must be CLIA certified. In contrast, international laboratories do not need any official approval to be designated as clinical.

The Clinic Directory, comprised of clinics offering genetic evaluation and genetic counseling, provides contact information and information on services offered (i.e., adult genetics, biochemical genetics, cancer genetic counseling/risk assessment, pediatric genetics, preimplantation genetic diagnosis, prenatal genetics, and/or telemedicine).

The Resources database provides links to and contact information for consumer health-oriented national or international resources.

A context-sensitive Illustrated Glossary of more than 200 terms makes genetic counseling and testing terms accessible to a broad healthcare provider audience.

29.3.1.7 Search Mechanisms and Search Results

The GeneTests database is searched by selecting GeneReviews, Laboratory Directory, or Clinic Directory from the navigation bar at the top of the homepage. GeneReviews and the Laboratory Directory can be searched simultaneously by disease name, gene symbol, and protein name. In addition, GeneReviews can be searched by author and title from an alphabetic list and the Laboratory Directory by services, director's name, location, and laboratory name. The Clinic Directory is searched by state and services (US), zip code (US), and country (international). A global search function is not yet available.

Search results for GeneReviews and the Laboratory Directory may be followed by up to four buttons:

1. "Testing" (a listing of laboratories offering clinical testing)
2. "Research" (listing of laboratories conducting research)
3. "Reviews" (*GeneReview)*
4. "Resources"

The shared GeneReviews and Laboratory Directory "Disease" search function yields three possible results:

1. A result for a disorder in which alteration in one gene produces one phenotype such as Huntington disease
2. A hierarchy in which an alteration in one gene, such as *APC*, produces several phenotypes, each with a different name (familial adenomatous polyposis, Gardner syndrome, and Turcot syndrome) (See Fig. 29.3.1)
3. A hierarchy in which one phenotype, such as hereditary hemorrhagic telangiectasia, is produced by alterations in more than one gene (See Fig. 29.3.2)

In the hierarchical search result, the Testing button corresponds to a name that reflects a change in a gene. Because many disease names are affixed to a discrete phenotype and not the continuum of gene-related phenotypes, the latter names, such as "*APC*-associated polyposis conditions," typically do not exist in MeSH.

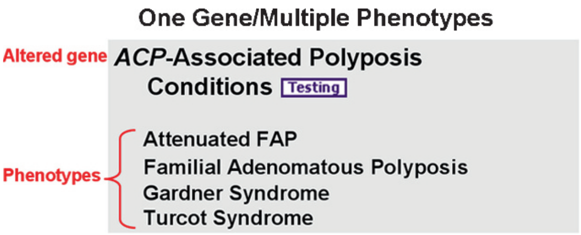

One Gene/Multiple Phenotypes
Altered gene
ACP-Associated Polyposis Conditions
Testing
Phenotypes
Attenuated FAP
Familial Adenomatous Polyposis
Gardner Syndrome
Turcot Syndrome

Fig. 29.3.1 GeneTests database hierarchical search result, in which the "parent" term is a associated with a listing of laboratories offering *APC* gene testing (accessed by selecting the "Testing" button) and the "children" are the names of phenotypes (familial adenomatous polyposis, Gardner syndrome, and Turcot syndrome) that a clinician might use as search terms

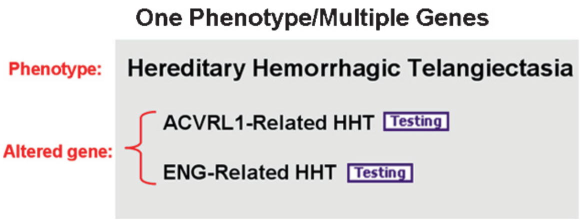


Fig. 29.3.2 GeneTests database hierarchical search result, in which the parent term is a phenotype used by clinicians as a search term and the children are the causative genes associated with a listing of laboratories offering testing for that gene (accessed by selecting the "Testing" button)

29.3.1.8 Data Maintenance

Each entry in *GeneReviews* is revised as needed to reflect currently available testing and is updated in a formal, comprehensive process every 2–3 years. Dates on each entry indicate the initial posting and either last revision or last update, whichever is more recent.

Laboratory and Clinic Directory listings are revised as needed and updated annually in a formal, online process. The date of the last update is displayed. Laboratories and clinics that fail to update are designated as "not current" for 1 year, after which they are deleted.

Resource listings are revised as needed and updated every 2 years.

29.3.1.9 Usage

GeneTests is a recognized resource in the international medical community [4, 13]. Its growth is steady; the number of new disease listings in the Laboratory Directory is about 100 per year; about 50 new expert-authored, peer-reviewed GeneReviews are added each year.

GeneTests and GeneReviews are copyrighted by the University of Washington and are made available to the general public subject to certain restrictions that are explained by the "Terms of Use" link on page footers and by selecting "About GeneTests" on the navigation bar.

As of January 2008, GeneTests had, on average, about 4,000 unique users daily, with an average of 40,000 page views per day.

29.3.1.10 Future Issues

Laboratory Directory and GeneReviews. Use of standard mutation nomenclature to improve data exchange and/or bidirectional links with mutation databases.

Laboratory Directory. Develop international collaboration(s) to exchange data on laboratory listings to reduce redundancy and ensure that only laboratories meeting the quality standards of their governing bodies are listed.

Clinic Directory. Develop a method for listing data about services provided by specialty clinics to permit patients to identify those clinics that provide the services they seek.

29.3.2 Online Mendelian Inheritance in Man

29.3.2.1 Name and URL

Online Mendelian Inheritance in Man (view: www.ncbi.nlm.nih.gov/Omim)

29.3.2.2 Background

OMIM is the online version of *Mendelian Inheritance in Man* (MIM) and is a comprehensive knowledge base of authoritative descriptions of human genes and genetic disorders. Created by Victor McKusick, MIM was first published in 1966 with 1,487 entries. This edition was subtitled "*Catalogs of Autosomal Dominant, Autosomal Recessive and X-linked Phenotypes*." The last print edition, the 12th, was published in 1998 and was subtitled "A Catalog of Human Genes and Genetic Disorders," and it contained 8,587 entries. The MIM catalogs have been maintained in a computer file since late 1963. In 1979, the National Library of Medicine adopted MIM as the experimental basis for the development of a full-text search engine called IRX (Information Retrieval experiment). IRX was made available for the authoring process in 1985 and became generally available in 1987 through funding by the Howard Hughes Medical Institute under the designation Online Mendelian Inheritance in Man or OMIM. From 1989 through 1995, OMIM was distributed along with GDB (Genome DataBase) from Johns Hopkins University. In late 1995, distribution via the World Wide Web and development of OMIM moved to the National Center for Biotechnology Information (NCBI). OMIM became fully integrated with the ENTREZ suite of programs in 2000.

29.3.2.3 Purpose and Target Audiences

OMIM is the descriptive repository of genotype-phenotype interactions. As such, OMIM catalogs all inherited traits, both simple and complex, and the genes that underlie them. As of October 2007, OMIM contained over 18,100 entries. Select mutations implicated in disease or associated with risk of disease (currently over 15,600) are cataloged as allelic variants in the relevant gene entry. OMIM also maintains a synopsis of the gene map with special emphasis on all genes and loci implicated in human disease (OMIM Morbid Map). In addition, in anticipation of phenotypic consequence of dysfunction, OMIM catalogs genes of known function.

OMIM is comprehensive and timely and is targeted at clinicians (especially clinical geneticists), researchers, and teachers. OMIM is not intended for a lay audience.

29.3.2.4 Location

Authoring and editing of OMIM are headquartered at the McKusick-Nathans Institute of Genetic Medicine of the Johns Hopkins University in Baltimore, MD, USA. Some authoring is distributed to authors from around the world. NCBI in Bethesda, MD, stores the electronic files and distributes them on the World Wide Web.

29.3.2.5 Funding and Governance

OMIM is funded by a contract from the National Institutes of Health: "Creation and Maintenance of Online Mendelian Inheritance in Man" (N01-LM43504).

OMIM has an Editorial Board comprised of subject experts and geneticists from around the world. Contract performance standards are set by the NIH.

29.3.2.6 Content

OMIM entries are divided into four main catalogs: autosomal, X-linked, Y-linked, and mitochondrial; these distinctions are made by the MIM number that defines the entry (See Table 29.3.1).

Within these catalogs, the entries are distinguished by type and defined by symbol (see Table 29.3.2):

Table 29.3.1 Numbering system for OMIM entries

MIM number	Catalog entry
1 (100,000)	Autosomal loci or phenotypes (entries created before May 15, 1994)
2 (200,000)	
3 (300,000)	X-linked loci or phenotypes
4 (400,000)	Y-linked loci or phenotypes
5 (500,000)	Mitochondrial loci or phenotypes
6 (600,000)	Autosomal loci or phenotypes (entries created after May 15, 1994)

Table 29.3.2 Symbol system for OMIM entries

Symbol	Entry type
Asterisk (*)	Indicates a gene with known sequence
Plus sign (+)	Indicates a description of a gene plus phenotype
Number sign (#)	Indicates a descriptive entry, usually of a phenotype, and does not represent a unique locus. (The reason for the use of a # sign is stated in the first paragraph of the entry)
Percent sign (%)	Indicates that the entry describes a confirmed Mendelian phenotype or phenotypic locus for which the underlying molecular basis is not yet known
Caret symbol (^)	Indicates that the entry no longer exists because it was removed from the database or moved to another entry as indicated
No symbol	Generally indicates a description of a phenotype for which the Mendelian basis, although suspected, has not been clearly established or that the separateness of this phenotype from that in another entry is unclear

Most entries have subheadings (e.g., description, cloning, gene function, gene structure, molecular genetics, clinical features, genotype-phenotype correlations, animal model). Gene entries may include allelic variants, which are a selected list of the published mutations in a gene. The selection criteria include the first six mutations, common mutations, mutations causing a different phenotype, mutations with an unusual mechanism, and missense mutations, which may give insight into structure/function relationships. OMIM now includes allelic variants that describe increased or decreased risk of a complex trait if that variant is very highly associated, replicated, and/or has demonstrated functional significance.

Phenotype entries may include a clinical synopsis, i.e., a hierarchical, structured description that begins with inheritance and ends with molecular basis (if known). Affected organ systems are included, with a semi-controlled vocabulary allowing ease of search.

The Morbid Map is a tabular listing of phenotypes and their relationship to the genome by locus or gene. The Morbid Map is included in the OMIM Synopsis of the Human Gene Map. The Morbid Map can be viewed alphabetically by disease or by chromosomal location of the disease.

The content of OMIM is greatly enhanced by copious links to other NCBI resources, including Entrez resources such as DNA and protein sequence, PubMed, and outside resources, such as GeneTests, Coriell Cell Repository, Locus Specific Mutation Databases (LSDBs), Human Gene Mutation Database (HGMD), HUGO Nomenclature, and more.

29.3.2.7 Search Mechanisms and Search Results

OMIM may be searched by MIM number, disorder, gene name/symbol (e.g., "Marfan," "FBN1," or "fibrillin"), or perhaps most conveniently by plain English (e.g., "hyponatremia and failure to thrive"). A search can be further restricted by using a "Limits" function to restrict the search to a subset of entries, field(s) within an entry, or date of update. Regardless of the method used, the search engine ranks the retrieval set so that the most relevant entries are in the top ten entries retrieved.

29.3.2.8 Data Maintenance

OMIM is updated daily. The link to the "Update log" in the blue bar on the left margin of the home page displays the number of new and changed entries by month, and within each month lists the specific entries that were added and changed.

29.3.2.9 Usage

OMIM is used extensively by researchers and clinicians around the world. As of September 2006, OMIM had, on average, 13,000 unique users daily with an average of 160,000 page views per day. In addition, data from OMIM are downloaded and displayed in many other resources around the world. OMIM is copyrighted by the Johns Hopkins University and is made available to the general public subject to certain restrictions that are explained by the "Restriction on Use" link on the home page.

29.3.2.10 Future Issues

Managing the triage and synthesis of the ever-burgeoning growth of biomedical research publications remains the greatest challenge going forward. Topics that will be challenging to render in a database include how to represent the complexities of gene and environment interaction on phenotype, the effects of genomic events, such as copy number variation, regulatory elements, and spatial and temporal changes. Specific enhancements to OMIM include collaborations with other researchers to increase links to animal model databases and to map allelic variants to the genomic sequence.

29.3.3 HGVS, Locus-Specific Databases

By definition, locus-specific databases (LSDBs) contain a listing of sequence variants in a specific gene(s) causing a Mendelian disorder or a change in phenotype, curated by (an) expert(s) in that gene [5]. Besides these listings, LSDB-connected web sites often contain a wealth of information related to the gene and the disorder/phenotype studied. Generally, LSDBs are initiated and driven by the interests of the curator, which may be research, clinical, or diagnosis in nature. The Human Genome Variation Society (HGVS) arose out of the HUGO-Mutation Database Initiative (MDI), a group of LSDB curators exchanging ideas on issues related to cataloging (pathogenic) gene variants.

29.3.3.1 Name and URL

Currently, most LSDBs are Internet based – a complete and up-to-date overview can be obtained through the HGVS web site (http://www.HGVS.org/dblist.html).

29.3.3.2 Background

LSDBs were initiated to collect all DNA variants found world-wide, both with and without pathogenic consequences, including both published variants and variants reported directly to the database. LSDBs fill the gap generated by scientific journals and central mutation databases; e.g., OMIM [8] and HGMD [20]. Journals, in general, publish only the first findings of variants in a specific gene, focused on variants that have pathogenic consequences. When more and more reports on a specific gene are published, new variants are unlikely to get published, unless they have not been reported before and/or provide new insights into the underlying disease mechanism. The central databases leave a gap here, because they do not collect all variants in all genes. OMIM generally collects the first variants described and later some with unique characteristics. HGMD collects the first report of every variant, and therefore only the clinical/laboratory phenotype associated with that particular example of the variant.

Initially, one of the most confusing aspects of databases containing information on variants in the DNA/protein sequence of genes was the way that these changes were described. Discussions regarding the uniform and unequivocal description of sequence variants were initiated in 1993 by Beaudet and Tsui [2]. These original suggestions have been widely discussed, modified, and extended, ultimately resulting in the HUGO-MDI/HGVS nomenclature recommendations [6] (http://www.HGVS.org/mutnomen/). These recommendations have now been largely accepted as a consensus and are applied world-wide, giving much more uniformity among databases.

29.3.3.3 Purpose and Target Audiences

Historically, the main purpose of an LSDB is to assist clinical diagnostics. The complete listing of all variants identified in a gene, in combination with the associated phenotype (as complete as possible), means that the LSDB should be the first resource to consult when a variant has been identified in a patient. Based on the presence of the variant and the associated phenotypic consequences as reported by others, the clinician should be helped in drawing a conclusion regarding the associated phenotype, i.e., "pathogenic or not." As such, the result of the LSDB query forms the basis for the diagnostic report. At the same time, through submission of the details of the new case to the LSDB, the clinician updates the database and increases its authority. Especially when a variant is new or has uncertain pathogenic consequences, this submission gives the clinician a unique opportunity to make these findings public immediately, giving colleagues world-wide the opportunity to assist in sorting out the potential pathogenicity that should be connected.

In addition to the list of DNA variants collected, LSDBs often contain a wealth of information on the gene, the disease, its diagnosis, and other related issues. As such, the use of LSDBs goes far beyond the LSDB itself. LSDBs attract the attention of a diverse group of users and, therefore, often provide their information in a format that can be understood by audiences ranging from scientists to interested lay persons.

29.3.3.4 Location

Since most LSDBs are initiated by interested gene experts, they are stored on many different servers world-wide (See http://www.HGVS.org/dblist.html). More recently, the HGVS has instigated initiatives to promote a more standardized LSDB structure, content, and data format, leading to databases that can be accessed from a central location. These initiatives are generally built around specific software packages for LSDB curation, including: dbRBC [3], MUTbase [18], UMD [19], and LOVD [7].

The UMD (Universal Mutation Database; http://www.UMD.be) and LOVD (the Leiden Open-source Variation Database; http://www.LOVD.nl) software are freely available. In addition, LOVD offers assistance in database set up including free-of-charge LSDB hosting.

29.3.3.5 Funding and Governance

An LSDB is generally the initiative of an interested gene expert. As a consequence, with few exceptions, LSDBs have little or no funding; and governance has not been clearly defined. The HUGO-Mutation Database Initiative and currently the HGVS have tried to organize LSDBs and attract financial support, but thus far these initiatives have not been very successful.

29.3.3.6 Content

LSDB content varies widely. In its simplest form an LSDB just lists the sequence variants reported with a reference to its source only. At the other end of the spectrum, an LSDB contains, in addition to the sequence variant and its source, a wealth of data on the characterization of the variant as well as a detailed description of the phenotype of the patient. The lack of standardization among LSDBs can make their efficient use difficult. To improve the quality and overall usefulness of LSDBs, the HGVS is promoting standardization of LSDBs by regular publication of recommendations and guidelines on such topics as the description of DNA variants, LSDB content and structure, and phenotype data reporting and collection (See http://www.HGVS.org). Ultimately, these recommendations should help to achieve more standardization and allow queries across different LSDBs; e.g., identifying specific candidate genes for diagnostic testing based on a detailed phenotypic description.

29.3.3.7 Search Mechanisms and Search Results

Because LSDBs widely vary in structure and data content, the ways to query these databases vary widely. When entries are sorted simply by position in the gene, they all basically generate an overview of all variants that have been identified. In addition, many LSDBs provide static lists summarizing overall database content, including figures on the total number of variants and the type of variants (substitution, deletion, insertion, etc.) collected. Only a few LSDBs provide extensive search options (e.g., using functional or phenotypic characteristics).

More complex queries are supported using Boolean operators per column (AND, OR, NOT) and by combining queries over different columns.

Wider use of LSDBs is seriously hampered by their overall lack of standardization, as explained above. General databases such as OMIM (for phenotype) and HGMD (for DNA mutability) allow some of the more general questions to be answered.

29.3.3.8 Data Maintenance

For most LSDBs, data maintenance is performed on an ad hoc basis; i.e., when the curator has time. A publication describing a set of new variants in the gene of interest is often the trigger for such an update. Only a few larger LSDBs have an active group of curators who regularly scan the literature and/or directly contact diagnostic laboratories with a request to submit all new variants identified. LSDBs using software like LOVD allow web-based data submission by external contributors and include software tools for data curation, error checking, and automatic updating of the database.

29.3.3.9 Usage

LSDBs are widely used by those performing DNA-based diagnostics and also by lay persons searching for information on a disorder of interest to them. Usage of LSDBs largely depends on the frequency of the disorder in the population (i.e., potentially interested users) and by the amount of information available on the LSDB web site. User statistics are usually not mentioned by LSDBs; but, based on our experiences with the Leiden Muscular Dystrophy pages (http://www.DMD.nl), we anticipate that a general LSDB attracts some 500–2,000 unique visitors each month. Collectively, the 700 known LSDBs (http://www.HGVS.org) would thus attract 350,000–1,500,000 unique visitors monthly.

29.3.3.10 Future Issues

Given the myriad possible genetic variations in the estimated 24,000 genes in the human genome, it would be useful to have standard criteria for their collection and storage.

Another important issue is completeness of LSDBs. Since it is not routine practice for researchers and diagnostic laboratories to automatically submit all variants identified, LSDBs are mostly far from complete. This is unfortunate, because all users, especially diagnostic laboratories, would benefit from completeness, thus ensuring interpretation of a sequence variant ("pathogenic or not") based on existing data. It seems that a good step forward would be to make data submission an intrinsic quality control step for certified clinical laboratories.

29.3.4 Decipher

29.3.4.1 Name and URL

DatabasE of Chromosome Imbalance and Phenotype in Humans using Ensembl Resources (view: http://decipher.sanger.ac.uk)

29.3.4.2 Background

It took nearly 10 years from the first paper on subtelomeric FISH analysis for the phenotypes of some of the subtelomeric deletions to be published. Since there are 43 telomeric loci and in excess of 30,000 probes on a high-density genomic microarray, it was clear that a large-scale collaborative approach would be needed to move the process of assigning phenotype to genomic interval forward in at timely fashion.

29.3.4.3 Purpose and Target Audiences

DECIPHER is an innovative collaborative project, based at the Sanger Institute, to map molecularly defined chromosomal rearrangements onto the human genome map. The project has been developed jointly by a clinical geneticist (Helen Firth) and a molecular cytogeneticist (Nigel Carter) and members of the bioinformatics Web team at the Sanger Institute.

DECIPHER has the joint aims of:

- Making information about the gene content of deletions/duplications/translocations available to clinical geneticists to guide their management of patients
- Mapping clinical features to regions of the genome so that these observations can be used to help to define the function of genes whose role in development and disease is currently unknown

The target audiences are staff working in departments of clinical genetics world-wide and research teams studying the function of human genes.

29.3.4.4 Location

DECIPHER is located at the Wellcome Trust Sanger Institute, Hinxton, Cambridge, UK, which is on the same campus as the European Bioinformatics Institute (EBI). DECIPHER interrogates the current version of the human genome assembly displayed in the Ensembl genome browser (currently NCBI-36). Ensembl itself builds all its annotated genes and associated features onto the most recent version of the human genome assembly, which is created by the NCBI. The annotation of genes and their features are continually being reviewed, refined, and updated.

29.3.4.5 Funding and Governance

DECIPHER is funded by the Wellcome Trust Sanger Institute. The project has Multi-centre Research Ethics Committee approval in the UK (MREC 04/mre5/50 until 2014). The ethical, legal, and social implications of the project are monitored by the DECIPHER Advisory Board, which meets once or twice a year to discuss policy issues and strategy.

29.3.4.6 Contents

DECIPHER is an interactive web-based database with three main components:

1. *DECIPHER Syndromes,* a public access compendium of microdeletion/duplication disorders. The Syndromes pages within DECIPHER provide a

single, curated resource of information and web-links. For each syndrome entity DECIPHER provides:

- A brief clinical synopsis
- Information on the size and origin of a deletion/duplication
- An ideogram of the location of the deletion/duplication on the relevant chromosome
- A list of the genes contained within the aberrant interval
- An up-to-date publication reference list
- Links to support groups and other information sources (e.g., *GeneReviews*)
- A direct, clickable link to visualize the deletion/duplication in Ensembl DECIPHER Syndromes is supported by a panel of Expert Advisors.

2. *DECIPHER Projects*. These are password-protected secure domains into which centers affiliated to DECIPHER can deposit genomic and phenotypic data about individual patients in order to interpret results. As for DECIPHER Syndromes, a DECIPHER patient report generates: Information regarding the size and origin of a deletion/duplication

   - An ideogram of the location of the deletion/duplication on the relevant chromosome
   - The gene content of the aberrant interval. In DECIPHER, the clinician has a choice of gene lists: (1) HGNC genes (i.e., all genes in the interval recognized by the Human Genome Nomenclature Committee), (2) OMIM Morbid Map genes (i.e., all genes with importance in human disease that are included in the OMIM Morbid Map database), (3) OMIM genes (i.e., all genes known to the OMIM database) and (4) Imprinted genes (i.e., imprinted genes contained in the www.geneimprint.org database). With patient consent, a basic phenotype description using the restricted terminology of the London Neurogenetics database and the genomic location are passed into the publicly accessible Ensembl database. In Ensembl, they are displayed in the DECIPHER track alongside tracks displaying copy number variants identified in the normal population. This allows the clinician to see whether the chromosomal aberration overlies a known region of copy number variation or whether it coincides with a recognized syndrome and, if not, whether it has been seen before by another member of the consortium and, if so, what the phenotype of the patient(s) was.

3. *DECIPHER – Ensembl view.* From the patient report, it is possible with a single click to see the deletion/duplication/translocation in its genomic context within the Ensembl genome browser (See Fig. 29.3.3). Here known syndromes are denoted by a dark red or dark green line for syndromes caused by deletions/duplications, respectively. Single-patient entries are denoted by a pink or pale green line. If clusters of patients with the same or overlapping aberrations are seen, DECIPHER enables the clinical teams to contact each other to share information about the phenotype to facilitate the process of syndrome delineation and understanding of gene function.

29.3.4.7 Search Mechanisms and Search Results

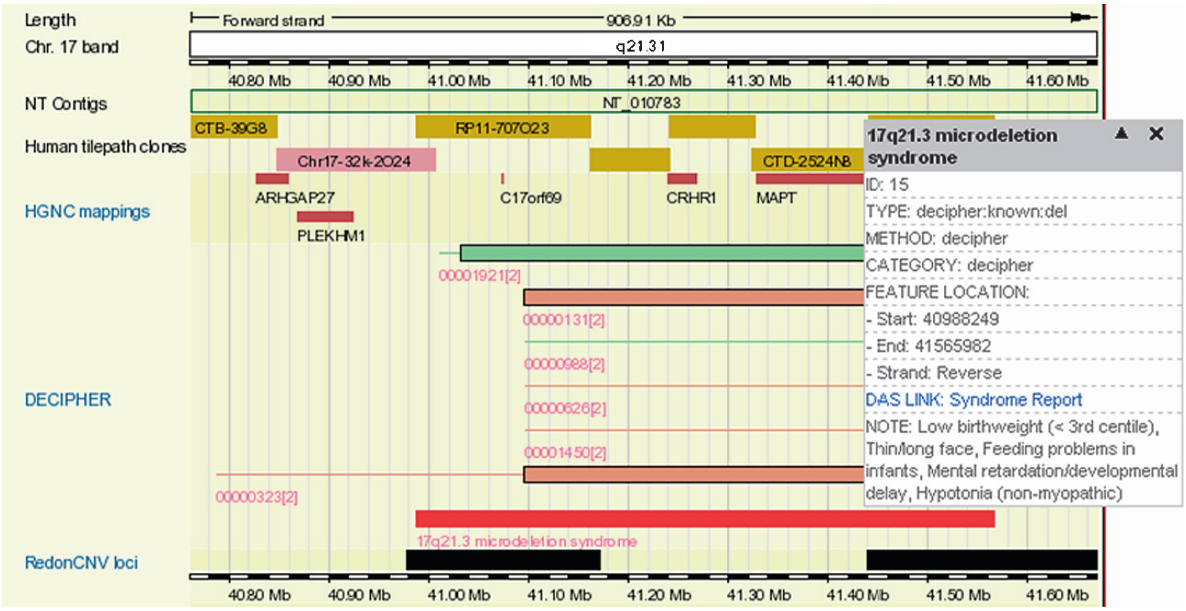
All of the consented data held within the DECIPHER database are searchable. Searches can be made by each of the following, individually or in combination:

- Chromosomal band
- Genomic position
- Chromosomes
- Phenotypes

DECIPHER members can also use this search function to interrogate nonconsented data within their own project groups. The ability to search by a phenotype term enables users to "search the genome by phenotype" and generate a display of genomic intervals associated with particular phenotypes, e.g., cleft palate. (See Fig. 29.3.4)

29.3.4.8 Data Maintenance

Each center maintains its project domain. Syndrome Reports are reviewed once a year by the DECIPHER development team, with input from the Expert Advisor for each syndrome.



17q21.3 microdeletion syndrome

ID: 15

TYPE: decipher:known:del

METHOD: decipher

CATEGORY: decipher

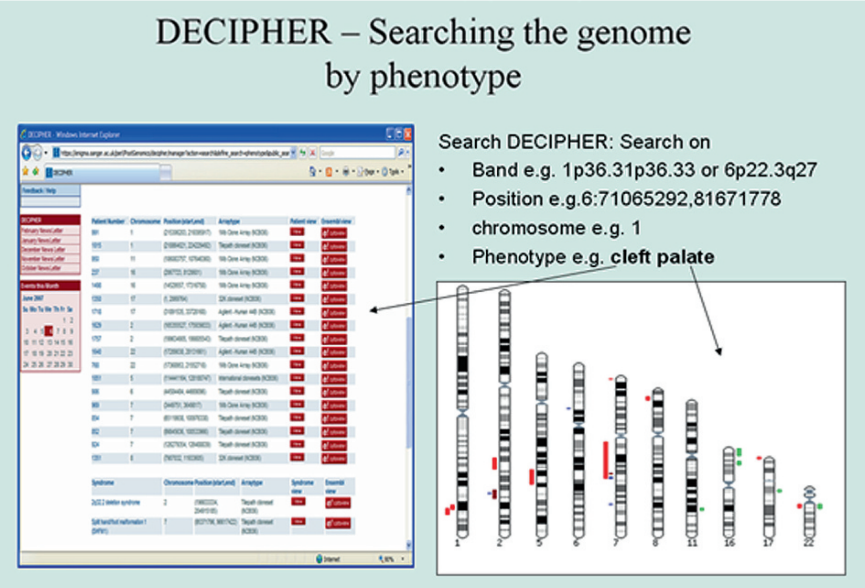
FEATURE LOCATION:

- Start: 40988249
- End: 41565982
- Strand: Reverse

DAS LINK: Syndrome Report

NOTE: Low birthweight (< 3rd centile), Thin/long face, Feeding problems in infants, Mental retardation/developmental delay, Hypotonia (non-myopathic)

Fig. 29.3.3 A *dark red* denotes a known syndrome caused by a deletion; a *dark green line* denotes a known syndrome caused by a duplication. Single-patient entries are denoted by a *pink* or *pale green line*


DECIPHER – Searching the genome by phenotype

Search DECIPHER: Search on

- Band e.g. 1p36.31p36.33 or 6p22.3q27
- Position e.g.6:71065292,81671778
- chromosome e.g. 1
- Phenotype e.g. **cleft palate**

Fig. 29.3.4 Display of genomic intervals associated with the phenotype cleft palate

29.3.4.9 Usage

As a web-based resource, DECIPHER serves its content globally. Since its inception in 2004, when it already served over 31,000 web pages, usage has increased exponentially, with over 240,000 pages viewed in the first 5 months of 2007. Data in the database have risen sharply from 30 patients in the first 6 months of its existence to over 1,000 patients by September 2007. As of September 2007 the database has 48 syndrome reports, 22 array types in use, and 84 projects derived from more than 80 centers worldwide.

29.3.4.10 Future Issues

DECIPHER interacts with the latest version of the Genome Browser, so that each time the database is queried the results are derived from the latest version of the genome assembly (currently NCBI v36). This means that gene lists and positions are always the most up to date available.

As more information about copy number variation becomes available, this will be assimilated by DECIPHER.

One of the biggest challenges for DECIPHER in the coming years will be the analysis and display of complex inheritance in which a phenotype is caused by the interaction of several loci rather than a single highly penetrant locus.

29.3.5 Entrez Gene

29.3.5.1 Name and URL

Entrez Gene (view: ncbi.nlm.nih.gov/gene)

29.3.5.2 Background

The National Center for Biotechnology Information (NCBI) at the US National Library of Medicine (NLM) started to manage gene-specific information in 1999, in conjunction with RefSeq, a project to provide reference sequence records for genomes, RNAs, and proteins [15]. The public database that resulted from that effort was called LocusLink. In 2004, LocusLink was replaced with Entrez Gene [11]. Entrez Gene is a key component in the set of the NCBI databases that supports geneticists, including PubMed, sequence databases, OMIM (X.2), dbSNP, and dbGaP (X.6) [23]. Entrez Gene is the product of multiple collaborations throughout the world.

29.3.5.3 Purpose and Target Audiences

Entrez Gene supports integration of gene-specific information both within NCBI and internationally. By assigning a unique and stable numeric identifier to a gene and connecting that identifier to names, sequences, and publications, Entrez Gene establishes a foundation that all can use to navigate to gene-specific information on the Internet. Some information is reported directly within Gene, some in other databases at NCBI, and some at other databases internationally.

Entrez Gene is intended for diverse users who want to look up information about a particular gene in one species. Key data elements that describe that gene (i.e., summary of the names and functions of a gene and its products, the pathways in which the encoded protein participates, reports of sequences and maps on which the gene is located) are provided. A table of contents facilitates navigation to subcategories of interest. More detailed information is provided via links to more specialized resources. For example, Entrez Gene does not support detailed summaries of genes or diseases; it points to OMIM (X.2) for that function. Entrez Gene does not provide detailed reports of variation known to occur in a gene; for that function it points to dbSNP and Variation Viewer. The site is likely to be of most interest to scientists, but links are provided to resources of broader interest.

29.3.5.4 Location

Entrez Gene is maintained by staff at NCBI, which is part of the National Library of Medicine at the National Institutes of Health (NIH) in Bethesda, MD, USA. Data are available without restriction; thus, many elements from Entrez Gene are integrated into multiple databases world-wide.

29.3.5.5 Funding and Governance

Entrez Gene is produced by staff of NIH and its contractors. It is overseen by a Board of Scientific Counselors to NCBI.

29.3.5.6 Contents

The contents of any Entrez Gene record vary according to the depth of available information. Data in scope for processing include names, an annotated bibliography (GeneRIF or Gene References into Function), public accessions of sequences known to be gene specific, diagrams of gene structure, descriptions of pathways or interactants for a gene's products, and names of diseases or other phenotypes associated with variants of a gene.

29.3.5.7 Search Mechanisms and Search Results

Data in Entrez Gene can be retrieved by text words, by sequence, or by genomic location. Text word searching is provided by NCBI's Entrez interface (http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=helpentrez.chapter.EntrezHelp). Searching can be as simple as entering a word or phrase into NCBI's query box and reviewing results. Specificity can be added to the query by using field restriction. Entrez and Entrez Gene provide detailed help documentation online (http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=helpbook.TOC).

Records in Entrez Gene can also be retrieved by sequence comparisons. Users of NCBI's BLAST services [1] are directed to records in Gene if the results returned by their queries are sequences associated with records in Gene (indicated by a blue icon with a letter G).

Users can also browse the genome via NCBI's Map Viewer. Clicking on the Gene symbol takes the user to the record in Entrez Gene.

Entrez Gene provides multiple views of gene-specific data. The ones used most frequently are: Full Report (most data elements displayed); Summary (name, species, and location data); and Gene Table (a summary of the sequences of products of protein-coding genes). The LinkOut display is also of particular interest, because it reports resources outside of NCBI that have registered, indicating that they have information related to a record in Gene. Connections to locus-specific databases (LSDBs) (Sect. 29.3.3) are provided by this method.

29.3.5.8 Data Maintenance

Data are maintained by a combination of automated analyses and curatorial review. Entrez Gene is updated daily with information describing each gene's structure, names, function, publications, defining sequence, disease associations, etc. It is also updated with links to gene-specific views of related information, such as variation, related genes in other species (orthologs), and expression. Entrez Gene welcomes input from the scientific community, via the RefSeq/Gene update site (http://www.ncbi.nlm.nih.gov/RefSeq/update.cgi).

In addition to the gradual, daily updates, there are periodical comprehensive revisions of the sequence and/or annotation of a genome. In that case, most records for that species will be changed. This happens about once a year for each species.

29.3.5.9 Usage

Entrez Gene is widely used, both interactively and by multiple databases world-wide that download the files Entrez Gene provides. In September 2007, the site averaged about 8,000 distinct interactive users a day.

29.3.5.10 Future Issues

Entrez Gene will continue to add functionality and content in response to advances in technology and interpretation.

The RefSeqGene project is fostering a detailed review of sequence and annotation of genes for which variants have been shown to increase risk or cause a disease.

29.3.6 Database of Genotype and Phenotype

29.3.6.1 Name and URL

dbGap: Database of Genotype and Phenotype

Public content: (view: ncbi.nlm.nih.gov/dbgap)

Authorized access content: (view: ncbi.nlm.nih.gov/dbgap controlled)

29.3.6.2 Background

dbGaP was developed in 2006 to archive and distribute the results of studies that investigate individual-level phenotype, exposure, genotype, and sequence data and the associations between them. Included are genome-wide association studies (GWAS), medical sequencing, and molecular diagnostic assays.

29.3.6.3 Purpose and Target Audiences

The advent of high-throughput and cost-effective methods for genotyping and sequencing has provided powerful tools that allow for the generation of massive amounts of genotypic data. dbGaP provides stable identifiers that make it possible for published study reports to contain discussion and citations of primary data with specificity and uniformity. dbGaP provides access to the large-scale genetic and phenotypic datasets required for GWAS designs, including public access to study documents, summaries of phenotype variables, statistical overviews of the genetic information, position of published associations on the genome, and authorized access to individual-level data.

Two levels of access are provided to dbGaP:

- Open access on the web is available for descriptions of each study, the variables being evaluated, and analyses of genotype-phenotype associations.
- Authorized access is required to download datasets of pedigrees, de-identified phenotypes, genotypes for individual study subjects, and study documents covered by copyright and having restricted terms of distribution. In addition, authorized access is required to obtain additional study data that are covered by a publication embargo and not yet public. Examples include precomputed association data, genotype QC results, cluster plots and sliding window linkage disequilibrium data provided for cases and controls.

The target audience is primarily made up of researchers. Researchers must each be identified as primary investigators in the NIH eRA system to submit requests for authorized access data.

29.3.6.4 Location

dbGaP is maintained by staff at NCBI, which is part of the National Library of Medicine at the National Institutes of Health (NIH) in Bethesda, MD, U.S.A.

29.3.6.5 Funding and Governance

dbGaP is produced by staff of NIH and its contractors. It is overseen by several advisory groups.

29.3.6.6 Contents

dbGaP archives study documents (protocols, questionnaires, data dictionaries, etc.); phenotype measures; genotypes reported from genotyping arrays or sequencing; and details of statistical associations between phenotypes and genotypes [12]. These data are organized in such a way that measurements of phenotype are linked to the study documentation. Measurements are accepted in diverse formats and are converted into a common distribution format without modification or standardization. All content elements are assigned stable, unique public accession identifiers (IDs), allowing specific data or studies to be cited in publications or to be linked to other bioinformatics resources. Variable summaries and document data dictionaries are available via anonymous FTP.

All individual-level data are de-identified by the submitter so that individuals are represented by coded IDs, to which dbGaP does not have access. Each authorized access study has a study configuration report (pdf file) available as an authorized access download component that describes the full set of available download components.

Individual level data are organized into participant sets according to any restrictions on use dictated by the study's informed consent process. Each participant set groups all study individuals who have elected the same set of restrictions. Studies that offer participants choices about the use of their data (e.g., commercial versus noncommercial uses or disease-specific only versus general research use) will possibly have individual-level data partitioned into two or more such sets.

Access to these data is granted by an NIH oversight data access committee (DAC) that reviews each request and proposed use for compliance with the restrictions on use described above.

29.3.6.7 Search Mechanisms and Search Results

Searching of the web site is provided by NCBI's Entrez system. As such, a user can query by any term, with or without field restriction. A tutorial is provided (http://www.ncbi.nlm.nih.gov/entrez/query/Gap/gap_tmpl/dbGaP_HowTo.pdf).

Results of a query are categorized by study, variables, study documents, and analyses. The user can click on any tab to view more information.

Results may be provided in multiple formats, such as .pdf for documents and a web-based browser of genomic regions with significant association to a phenotype.

29.3.6.8 Data Maintenance

Potential submissions are reviewed to ensure compliance with appropriate standards of practice [12]. NCBI staff then work with the submitters to identify and correct any errors.

When submission of phenotypic and genotypic information is complete, NCBI can calculate association data or report associations calculated by others. Links are then provided to NCBI's Map Viewer to represent regions of interest identified by a study.

A versioning system supports periodic (semiannual) updates by submitters. Additional components may be created when new technologies or methods are applied to current studies; e.g., additional genotypes on a new genotyping platform or sets of imputed genotypes derived from a new algorithm.

29.3.6.9 Usage

Because this resource is very new, usage statistics are not yet available.

29.3.6.10 Future Issues

In addition to representing information from large-scale studies, dbGaP will integrate genotype-phenotype relationships from multiple sources, including publications and OMIM.

29.3.7 The Human Gene Mutation Database

29.3.7.1 Name and URL

The Human Gene Mutation Database HGMD® (view: http://www.hgmd.org)

29.3.7.2 Background

The Human Gene Mutation Database (HGMD®) [20] constitutes a comprehensive core collection of data on germline mutations in nuclear genes underlying or associated with human inherited disease. HGMD does not include either somatic or mitochondrial mutations, because these data are covered by the Catalogue of Somatic Mutations in Cancer (COSMIC; http://www.sanger.ac.uk/genetics/CGP/cosmic/) and the Human Mitochondrial Genome Database (MitoMap; http://www.mitomap.org/), respectively. By March 2008, the database contained almost 80,000 different lesions detected in ~3,000 different genes, with new mutation entries currently accumulating at a rate exceeding 9,000 per annum. Although originally established for the scientific study of mutational mechanisms in human genes, HGMD has since acquired a much broader scope and utility.

The database was first made publicly available in April 1996. A collaborative agreement between HGMD and BIOBASE GmbH (http://www.biobase.de) was signed in January 2006. BIOBASE, based in Wolfenbüttel (Germany) and Beverly, MA, USA, is the leading content provider of biological databases, knowledge tools, and software for the life sciences. This collaborative agreement covers the exclusive world-wide marketing of HGMD to both academic and commercial subscribers.

29.3.7.3 Purpose and Target Audiences

HGMD records the first report of a disease-causing mutation or disease-associated/functional polymorphism and provides these data in a readily accessible form to all interested parties, whether they are from an academic, clinical, or commercial background. In practice, HGMD has become, de facto, the central disease-associated mutation database available to the scientific community.

The main target audiences for HGMD are academic/nonprofit institutions and commercial companies interested in utilizing mutation data. Users include: genetic counselors; clinicians; researchers; medical diagnostic laboratories; and pharmaceutical, biotechnology, and bioinformatics companies.

29.3.7.4 Location

The HGMD database curation staff is based in the Institute of Medical Genetics, part of the School of Medicine at Cardiff University, Cardiff, UK.

29.3.7.5 Funding and Governance

Because HGMD does not receive any public funding to support its upkeep, it has been necessary to develop a sustainable model for both the current and future funding of the database. The ideal model (in the opinion of the curators) would be a mixture of income from both public and private sources. This, in principle, would allow HGMD to provide free database access to academic/nonprofit users alongside a subscription-based distribution for commercial users marketed by a commercial company. With this eventual aim in mind, the HGMD curators opted to market the data in collaboration with BIOBASE GmbH, their commercial partner, while simultaneously continuing to make a core version of the database available as a free service to registered users from academic/nonprofit institutions via the Cardiff web site (http://www.hgmd.org). By insisting that commercial entities pay for access to the latest HGMD data and software tools, while providing a less up-to-date version free of charge to registered users from academic/nonprofit institutions, the HGMD curators believe that they can continue to allow free access to the bulk of their mutation data, while at the same time generating income to support HGMD from its commercial distribution.

HGMD employs a team of curators with expertise in the collection, interpretation, and editing of mutation data. The curatorial/editorial team decides which data are to be included in HGMD, and which ought to be omitted (for inclusion criteria, see Sect. 29.3.7.6).

29.3.7.6 Contents

Mutation data are obtained by means of a combination of both manual and computerized search procedures. Thus, online library screening, the PubMed database, and publicly available, locus-specific mutation databases (LSDBs; see Sect. 29.3.3) are all used to optimize (and maximize) data acquisition.

HGMD data are subdivided into ten primary categories of mutation: missense/nonsense; splicing and regulatory single nucleotide substitutions; microdeletions, micro-insertions and indels of 20 bp or less; gross deletions and gross insertions of greater than 20 bp (including copy number variations); complex rearrangements; and repeat variations. Entries are viewable on a gene-wise basis, and access to the subcategorized mutation data is available via hypertext link from each gene page. Additional links to complementary data sources are also provided here (GDB, OMIM, HUGO Nomenclature Committee, Entrez Gene, GeneCards, GenAtlas, UniGene, SwissProt, and the Human Protein Reference Database), along with access to over 2,800 annotated cDNA sequences.

Disease-causing mutations and disease-associated/functional polymorphisms are included in HGMD, if adequate evidence pointing to their pathological involvement/relevance is to be found in the published report. Exclusions occur, however, if the published data are deemed to be of insufficient quality (either by virtue of the description provided, or because of a tenuous/nonsignificant association with a clinical or laboratory phenotype). Once identified and validated, the data are entered into HGMD in a standard format.

HGMD Professional (see http://www.biobase-international.com/pages/index.php?id=hgmddatabase) contains additional information including "extended cDNA sequences" that include splice junctions, a mutation viewer/map that superimposes HGMD mutation data over cDNA sequences, links to related genes, mutation data broken down by clinical/laboratory phenotype and substituted/substituting amino acid properties and orthologous protein alignments for missense mutations. *HGMD Professional* also provides an

Table 29.3.3 Number of new records entered into HGMD (by year of entry)

Year	Number of new mutation entries[a]	Number of new gene entries[a]
2001	7,453	189
2002	5,851	197
2003	5,990	214
2004	5,480	257
2005	7,651	241
2006	9,906	287
2007	9,378	441
2008*	2,499	86

[a]Figures accurate to March 15, 2008

Advanced Search feature that has been designed to enhance mutation searching, viewing, and retrieval.

The number of new entries being logged in HGMD has been steadily increasing over the last few years (See Table 29.3.3) and now stands at over 9,000 mutations per annum. The number of new genes being entered into HGMD has also followed this upward trend, with 441 new genes being introduced in 2007 alone.

29.3.7.7 Search Mechanisms and Search Results

The public version of HGMD can be searched by gene symbol, gene name, OMIM number, GDB number, and disease/phenotype.

HGMD Professional contains an expanded search engine with full-text Boolean searching and improved gene and mutation viewing enabled. Users may additionally search for chromosomal location, literature reference, codon number, and HGMD accession number. When utilizing the Advanced Search, users may tailor their queries with more specific criteria, including amino acid change, nucleotide substitution, microdeletion/insertion/indel size and composition, motif searching (both created and abolished), dbSNP number, and article title/abstract/keywords.

Results are generally returned as a list of genes matching the search criteria. Users may then click on a gene symbol to access the gene page and, from there, the mutation data and features associated with that gene. Advanced Search results are returned as a list of mutations matching the user's search parameters and are downloadable in their entirety.

29.3.7.8 Data Maintenance

The public version of HGMD operates with a 2 1/2-year time delay, but mutation data are added automatically once this period has expired. *HGMD Professional* is released by Biobase GmbH to subscribers every 3 months, with newly added mutation data and features. The current (March 31, 2008) release is version 8.1 and contains 79,078 mutations. This is the ninth release since the beginning of 2006.

29.3.7.9 Usage

The public version of HGMD has attracted over 18,000 user registrations from over 140 different countries world-wide. On a monthly basis, there are, on average, ~14,000 queries for genes (with an equal number accessing HGMD genes via external links) from almost 6,000 users, with a total of over 160,000 pages served. *HGMD Professional* is used by both academic and commercial customers worldwide.

HGMD is a registered trademark, and the data are copyrighted by Cardiff University. Users of the public site may not download HGMD data in their entirety without permission. This is, however, generally granted if the data are to be used for noncommercial, collaborative research purposes only. Collaborators who wish to access HGMD data in full must sign a confidentiality agreement. Recent successful collaborations include the projects to sequence the genomes of *Macaca mulatta* [17] (Rhesus Macaque Genome Sequencing and Analysis Consortium 2007) and *Rattus norvegicus* [16] (Rat Genome Sequencing Consortium 2004), as well as a recent study into gains of glycosylation mutations INSERM [22]. HGMD data have also been utilized by researchers in several other recent studies [9, 10, 21].

29.3.7.10 Future Issues

The primary aim of the HGMD curators is to secure sustainable funding for HGMD via a subscription-based model. During 2008, there are plans to incorporate a fully comprehensive, functional/disease-associated polymorphism dataset into HGMD to complement the existing disease-causing mutation data. The provision of full genomic sequences for all HGMD genes and genomic coordinates for as many mutations as possible are also seen as high priorities. The provision of extra supplementary information, including additional clinical phenotypes observed with a given mutation, along with data on the in vitro characterization of specific mutations, will be added to HGMD once resources permit.

References

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