Chapter 8
Transgenesis and Genome Manipulations

8.1 Introduction

In the early 1980s, the expression of *transgenic animals* was proposed to define animals having *foreign DNA sequences stably and deliberately inserted into their genome through human intermediaries*. With time and the advent of new techniques, this concept has progressively evolved, and nowadays, it is probably more appropriate to consider that transgenic animals are animals *whose genetic characteristics have been altered using one of the techniques of genetic engineering*. Whatever the definition, transgenic animals belong to the category of genetically modified or genetically engineered organisms (GMOs).

Transgenic mice can be created by using a variety of experimental procedures depending upon the aim of the experiment. Among these procedures, the microinjection of foreign DNA fragments directly into one of the pronuclei of embryos at the one-cell stage has been, and still is, widely used. Another popular technology, which was developed almost concomitantly, makes use of pluripotent stem cell lines derived from mouse embryos [embryonic stem (ES) cells], which can be cultivated and manipulated in vitro just like somatic cells and subsequently inserted into a blastocyst to participate in the formation of the germline of a chimeric organism. Transgenic animals have also been created by lentiviral infection of early embryos, by transposable elements, and by a few other techniques such as those recently reported that make use of specially designed site-specific nucleases.

Transgenic mice are produced routinely in an ever-increasing number of laboratories. They are also made to order by several private companies. All these transgenic animals have been invaluable for answering biological questions related to gene function and regulation. They are instrumental in the analysis of tissue differentiation and ontogeny, for example, by allowing the tracking of cell lineages. Finally, they allow the development of refined animal models of human genetic diseases.

In the previous chapter we concluded that the discovery of the mutagen ethyl-nitrosourea (ENU) could be considered a milestone in the history of mouse genetics, essentially because it made possible the creation of a virtually

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unlimited number of new mutant alleles. Similarly, the advent of transgenic technology has been a true revolution, eliciting unprecedented changes in mammalian genetics and related fields. This chapter focuses on the production and use of transgenic mice.

8.2 Transgenesis Resulting from Pronuclear Injection of Cloned DNAs

The stable insertion of foreign DNAs into the germ line through microinjection into the pronuclei of fertilized mouse eggs was reported in the early 1980s in simultaneously several laboratories using the same technique but with different DNA molecules (Brinster et al. 1981; Costantini and Lacy 1981; Gordon and Ruddle 1981; Harbers et al. 1981; Wagner et al. 1981a, b). It was not until 1982 that the first transgenic mouse with a clear phenotype was developed by Palmiter, Brinster, and colleagues: a "giant" mouse carrying (and overexpressing) a rat growth hormone gene (Palmiter et al. 1982). Since these first descriptions, the technique has been improved and a variety of protocols for the efficient generation of transgenic mice has been published. Among the most popular "cookbooks" dealing with the subject, we recommend those by Hogan et al. (1994), and more recently by Hammes and Schedl (2000), Jackson and Abbott (2000), Houdebine (2003), Nagy et al. (2003), and Koentgen et al. (2010). We also recommend visiting the webpage of the International Society for Transgenic Technologies (ISTT) at http://www.transtechsociety.org/.

8.2.1 The Basic Experimental Protocol

The production of transgenic mice is achieved by injection, generally with a sharpened glass micropipette, of a few picoliters of a DNA solution (concentration ~2 ng/μl) directly into one of the pronuclei, while the egg proper (the zygote) is held, by suction, to another glass micropipette (the holding pipette). In most instances, the foreign DNA is injected into the male pronucleus because it is a little bigger and closer to the egg membrane than the female pronucleus.[1] In skilled hands, around 10–20 % of the microinjected eggs develop to term into a transgenic animal. Identification of the transgenic status is achieved by PCR amplification of DNA samples prepared from the presumptive transgenic animals with specific primers, and confirmation is obtained by using Southern blotting (Fig. 8.1).

[1] For this reason, the technique is sometimes designated "*pronuclear transgenesis.*"

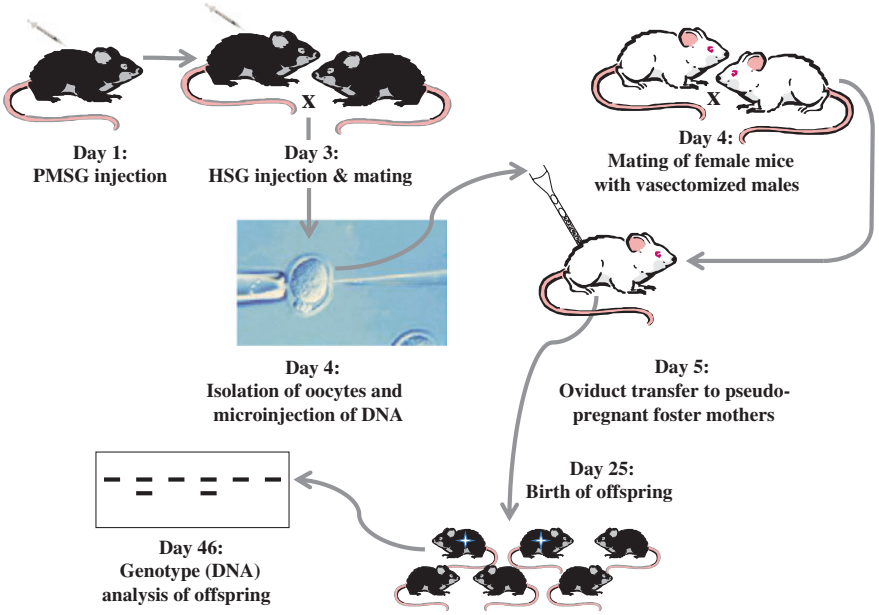

Fig. 8.1 *Producing transgenic mice by pronuclear injection*. The chart represents the different steps for the production of transgenic mice by pronuclear injection. Eggs are flushed out of the oviduct immediately after fertilization and then the transgene is microinjected in vitro with a glass micropipette. Once injected, the eggs are kept in vitro for a few hours and then transplanted into pseudo-pregnant females. Genotyping of the G0 (presumptive) transgenic mice can be achieved at any time from birth onwards. Every pup genotyped as positive by PCR (i.e., hemizygous Tg/0 carrier) should be considered a "founder," and independent lines should be developed from each founder

The DNA that is injected into the pronucleus can be either an unmodified or a natural copy of a gene cloned in its native genomic configuration, with its natural promoter, all its introns and other 5′or 3′ regulatory sequences, plus a few tenths of kb upstream and downstream of the sequences of interest. In most instances, however, the DNA that is used for transgenesis (the "transgene" proper) is artificial and designed in the laboratory according to the purpose of the experiment. It generally consists of several elements gathered in vitro, one piece at a time, then assembled using the most appropriate recombinant DNA technology. Finally, the transgene is cloned into a plasmid for amplification, mass production, and storage. When constructing such a fusion or chimeric gene for expression in transgenic mice, it is often easier to use a cDNA clone incorporating the coding sequences rather than the genomic DNA. This is especially true when the coding sequences in question stretch over a very long DNA segment or when they comprise many exons. Unfortunately, the levels of gene expression obtained with cDNA-based constructs are often lower than those obtained when genomic sequences are used.

Among the many explanations that can account for this observation, the existence of enhancers in the introns is the most likely (see Chap. 5).

Once selected, the relevant cDNA is placed under the control of a promoter, whose choice depends upon where and when it is desired that the transgene be expressed. When using cDNA (rather than genomic DNA) as a source of coding sequences, it is important to make sure that there is a translational start codon (AUG) within an upstream Kozak sequence (A/GCC**Pu**CC**AUG**G), which lies within the short 5′ untranslated region and directs translation of mRNA, and that there is an in-frame stop codon (UGA, UAG, UAA) for translational termination. Finally, it is also recommended to add an intron at the 5′ or 3′ end of the transgene because this allows the production of a more stable mRNA transcript and, finally, better transgenic expression (Brinster et al. 1988).

Experience teaches that the integration of the foreign DNA into the chromosome of the host probably occurs at random. In most instances, DNA integration occurs at the one-cell stage and at a single site but this is not a rule, and in 10–20 % of cases, the integration is delayed and occurs later during development. The mechanism of stable integration into the host genome is not precisely known, but it likely requires a double break (a nick) in the host (or recipient) DNA that is promptly repaired. Some scientists have suggested that this break might be the consequence of a trauma caused by the glass micropipette or by the injection of the DNA suspension. Even if this suggestion makes sense, it is probably not the only way for a transgene to integrate into a genome since delayed integrations, which are observed occasionally, are obviously not trauma dependent. When the foreign DNA does not integrate and stays isolated (as an *episome*, for example) in the nucleus for a few hours and integrates only at a later stage of development (2-cell; 4-cell), the organism develops as a mosaic. In this case, the detection of the transgene is more difficult and its transmission is unpredictable. In the case where the foreign DNA is present in all cells of the founder transgenic animal (noted F0, sometimes G0), it is then transmitted generation after generation as a new dominant "Mendelian" character.

The generic symbolic designation for a transgenic insertion is Tg. When the structure of the transgene is known, which is generally the case, a more precise designation applies. In this regard, we encourage the readers to refer to the guidelines for the standardized genetic nomenclature of transgenes in mice and rats at: http://www.informatics.jax.org/mgihome/nomen/gene.shtml#transg.

In contrast to gene and allele symbols, transgene symbols must not be italicized when they result from insertions of foreign DNA because they are not part of the native mouse genome.

The founder transgenic animals are hemizygous for the DNA segment (the symbol should be Tg/0, not Tg/–), and accordingly, the establishment of a "transgenic strain," in which the transgene is propagated by sexual reproduction, requires genotyping at each generation to avoid losing the transgenic DNA, unless the carriers have an obvious phenotype.

A method of safely maintaining a transgene in a mouse strain is to put it in the homozygous state, but this is difficult to achieve in practice. One reliable way

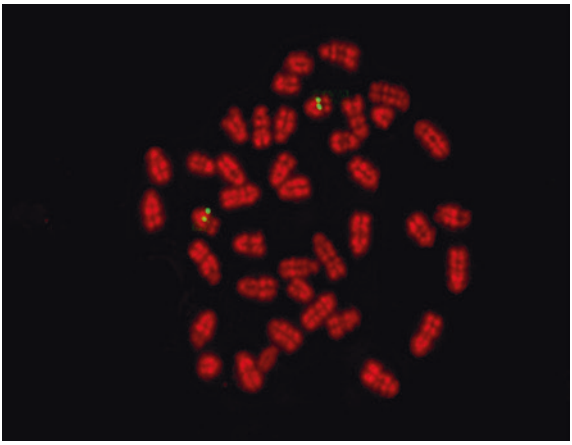

Fig. 8.2 *Fluorescence in situ hybridization*. Fluorescence in situ hybridization (*FISH*) with a transgene-specific probe indicates the localization of the transgene (*green dots*) in the karyotype (duplicated metaphase chromosomes). In this case, the transgenic insertion is homozygous (two copies). Using a chromosome or gene-specific probe with a different fluorescent staining allows for localization of the transgene on a specific chromosome (see Chap. 3)

of sorting out homozygous (Tg/Tg) from hemizygous (Tg/0) mice relies on the statistical analysis of their progeny when mated with a wild-type (WT or non-transgenic) partner (i.e., a progeny testing). A male mouse, identified as a carrier of the transgenic insertion based on a DNA test, producing only Tg/0 transgenic offspring in a progeny of 10 pups, when crossed with a non-transgenic partner has a greater than 90 % chance of being homozygous for the transgene (Tg/Tg). When the progeny size increases to 15, with only Tg/0 offspring, the probability increases to 99 %. Other possible means of identifying homozygous Tg/Tg mice are by quantitative real-time PCR (qRT-PCR) to determine zygosity and to distinguish hemizygous from homozygous transgenic mice (Ballester et al. 2004), or by cloning a segment of the DNA, flanking the transgene by inverse PCR and using it as a chromosomal marker for transgene localization. The transgenic insertion can also be visualized by in situ hybridization with a fluorescent dye (FISH) and accordingly located on a specific chromosome (see Chap. 3) (Fig. 8.2).

8.2.2 Factors Influencing Transgenic Expression

The number of copies of the transgene that integrates into the host genome is not controlled and ranges from one to several tens or even hundreds. Because sticky ends are generated when the foreign DNA is processed for injection, the cloned DNA copies are generally arranged in head-to-tail arrays in the transgenic insertion with frequent, and sometimes extensive, rearrangements generated in the

flanking regions. Using quantitative PCR technology, it is possible to roughly estimate the number of copies of the transgenic DNA; however, this is neither accurate nor reliable and, as we shall see, it sometimes changes with time.

As we already mentioned, investigators have no way of choosing the location where the foreign DNA will integrate. However, the integration site can seriously influence the transcription, and accordingly the expression, of the transgene. This is the case, for example, when the insertion site is in a heterochromatic or untranscribed (hypermethylated) region of the genome or when it is strongly influenced by a silencer sequence operating in its close vicinity. In these two cases, the transgene is weakly expressed or not expressed at all. Conversely, the sequences surrounding the transgene may contain regulatory elements acting on its promoter as enhancers of transcription. These enhancer sequences sometimes lead to an ectopic expression of the transgene; in other words, to an expression pattern that does not match with the spatial or temporal expression normally expected from the transcriptional regulatory elements the transgene contains.

These unexpected and somewhat erratic variations in expression are the consequences of a phenomenon known as the *position effect* and represent one of the main weaknesses of pronuclear transgenesis. The position effect and variations in copy numbers are two serious drawbacks, because both can affect transgenic expression. For this reason, in all cases, it is absolutely essential to make sure that the transgene is indeed fully expressed by checking whether all of the expected transcription products are present. One should also verify, as thoroughly as possible, that the structure of the transgene has not been affected by the mechanical handling of the DNA during the process of injection. This recommendation is especially important for large transgenes such as those made from yeast artificial chromosomes (YACs) or bacterial artificial chromosomes (BACs).

Since it is impossible to predict the effects of the integration site (i.e., the genomic environment) and of the number of copies on transgenic expression, it is highly recommended, when developing a transgenic strain for experimental purposes, to compare the offspring of several different founder transgenic mice and to consider only the features common to at least two independent strains as reliably attributable to the transgenic DNA. For this reason, it is not recommended to intercross mice originating from different founders but, on the contrary, to develop independent Tg lines from each founder.

Another classical observation when breeding transgenic animals is that 7–10 % of the transgenic insertions appear to be lethal when homozygous, presumably because a recessive lethal mutation (most probably a gene disruption) was mechanically generated at the time of integration in the recipient genome (insertional mutagenesis).

Finally, one must keep in mind that transgenic insertions are not always stable over time, and many investigators have reported the spontaneous and unexpected loss of the transgene from their favorite transgenic line. When a transgenic line is considered optimal and reliable, it is wise to preserve it as frozen sperm or embryos.

8.2.3 Using Transgenic Mice for Studying Gene Function and Regulation

A virtually unlimited number of transgenes can be engineered in vitro by the association of any coding sequence—normal or mutant—taken from any gene of any species, including plants and bacteria, and controlled by any regulatory elements. The use of transgenic mice is then a very convenient and efficient way to assess the function of genes. We will consider a few cases that have been selected as informative examples.

8.2.3.1 The Use of Transgenic Mice to Define the Function of Genes

Examples of this approach are provided by the homeogenes and the oncogenes, both of which are important actors in mammalian development. Homeobox-containing genes, the homeogenes, are transcriptional regulators with a remote ancestral origin, which are present in mammalian genomes and arranged in four paralogous clusters (*Hoxa*, *Hoxb*, *Hoxc,* and *Hoxd*). Because their structures are very similar, it was impossible to decide a priori whether each of these genes had a specific function, whether they had an effect because of the copy number (additive effect) or whether some of the copies were simple "backup" copies, preserved by evolution for unknown purposes. Transgenic mice were then made for some of these homeogenes with an intact coding sequence driven by a regulatory sequence different from the native one (driving ubiquitous expression, for example). In most instances, the embryos born with such extra transgenic insertions exhibited severe "homeotic" transformations indicating that indeed, most of the homeogenes in the *Hox* clusters had a specific function in the developmental patterning of the mouse embryo, a patterning reminiscent of their function in *Drosophila*, where they were initially discovered (Duboule 1998).

Transgenic mice have also been created with the coding sequence of (intact or mutated) oncogenes, or the sequence of genes whose function were not completely understood, downstream of a variety of regulatory sequences. Among these genes are the oncogenes *Abl1*, *Jun*, *Mos*, *Nras*, and *Myc*, as well as the tumor suppressor genes *Trp53* and *Rb*. Transgenic mice overexpressing oncogenes develop neoplasias in different tissues, depending on the promoter selected for the construct. For example, mice overexpressing the oncogene *Myc* driven by immunoglobulin enhancers develop lymphoid malignancies (Adams et al. 1985). The famous *OncoMouse*™ (the name is a trademark) is another example, but in this case, it carries the activated oncogene *v-Ha-ras* under the control of the MMTV promoter and, hence, produces mammary tumors (Hanahan et al. 2007). The subsequent analysis of these transgenic animals has provided an enormous amount of information concerning the role of these oncogenes in the regulation of several basic cellular functions and during the process of malignant transformation. The unique advantage of transgenesis in the case of homeogenes, oncogenes, and tumor

suppressor genes is to make the analysis of gene function(s) possible at the level of the whole organism.

8.2.3.2 Using Transgenic Mice to Identify and Characterize the Regulatory Sequences of Genes

While many mammalian genes are constantly and ubiquitously expressed, others are expressed in a tissue-specific manner, or only during embryonic life or only in the adult organism. Such variations in expression patterns occur because the genes are controlled by regulatory sequences that are in many cases, although not always, located in *cis* and upstream of the coding regions.[2] A good example of such tissue-specific regulation was reported for the gene encoding the cytokine leptin, which is expressed almost exclusively in adipocytes. After positional cloning of the mouse mutant gene *obese* (*Lep*^{ob}-Chr 6) (Zhang et al. 1994), it was demonstrated that the obese phenotype was a consequence of a nonsense mutation in codon 105 of the gene encoding the 16 kDa leptin protein. Researchers also learned that the highly tissue-specific expression of the *Lep* gene is controlled by a *cis*-acting regulatory sequence 161 bp long located upstream of exon 1 (He et al. 1995). For many genes, unfortunately, the regulatory sequences are not yet characterized and geneticists must design experiments to identify them accurately (see Chap. 5). This is important for a better understanding of gene regulation, of course, but it is also important if we consider that accumulating such data will certainly help in the future in silico identification of the regulatory elements based on sequence analogies.[3]

Transgenic mice are helpful for the identification of these regulatory sequences because experience teaches us that genes cloned in their native genomic configuration and introduced into the mouse germ line by transgenesis retain, in most instances, their tissue-specific and stage-specific patterns of expression, despite their integration at random sites. A popular strategy is to design in the laboratory a series of transgenes whose coding sequence encodes an easy-to-detect product which is not normally encoded in a mammalian genome (such a sequence is called a *reporter gene*), and to associate it by genetic engineering with a variety of regulatory DNA sequences, either upstream of the coding region, at the 5′ end or, less frequently, downstream of the 3′ end.

The gene encoding chloramphenicol acetyltransferase (CAT), from a transposon of *Escherichia coli*, has been extensively used to characterize the specific expression

[2] The genetic elements regulating gene expression are sometimes numerous and not always located in the close vicinity of structural genes. This explains (at least in part) why cloned structural genes, when used as transgenes, are sometimes regulated differently from the same genes in their natural, native environment (see Chap. 5). This point is inherent to transgenesis by in ovo injection and must always be kept in mind.

[3] In situ hybridization with labeled cDNAs is another way of analyzing the expression profile of a given gene.

associated with regulatory sequences because CAT activity can be assayed thanks to a very sensitive enzymatic test that has no background in eukaryotic cells (Overbeek et al. 1985). CAT has been progressively replaced by the gene encoding luciferase in the firefly (*Photinus pyralis*), largely because the assay to measure it is easier (Lira et al. 1990). *lac*Z, the historical gene encoding β-galactosidase of *Escherichia coli* (Goring et al. 1987), has been the cellular marker of choice to track cells in embryos and adults because of the ease of its detection and high cellular resolution in fixed embryos and tissues. The *lac*Z gene appeared to be particularly useful for studies of tissue- or position-specific gene expression. However, a major limitation is that *lac*Z cannot be used to mark cells in living tissues because the protocol to detect its expression requires tissue fixation. Fluorescent proteins offer advantages over enzyme-based reporters (e.g., *lac*Z, CAT) in the sense that their visualization does not require tissue fixation and is both quantitative and noninvasive. Indeed, fluorescent proteins make it possible to mark specific cells in living organisms, and also to follow such cells using fluorescence-imaging techniques (Fig. 8.3).

A classical reporter gene has been developed that consists of the sequence of the green fluorescent protein (GFP) of the jellyfish *Aequora victoria* (Misteli and Spector 1997). The product of this gene emits a green fluorescence elicited by direct illumination with blue light, and the analysis of the expression pattern requires neither fixation of the tissue nor cofactor or specific substrate, only UV light. Several variants of the wild-type GFP have been produced that emit in the blue (BFP), cyan (CFP), and yellow (YFP) regions. A series of variants derived from the red fluorescent protein (RFP) of the sea anemone *Discosoma sp.* are increasingly used because they emit a range of wavelengths in the red region, from the dark red of cherry to the yellow of banana. Interestingly, these different reporter genes can be combined allowing multiplexing and co-visualization

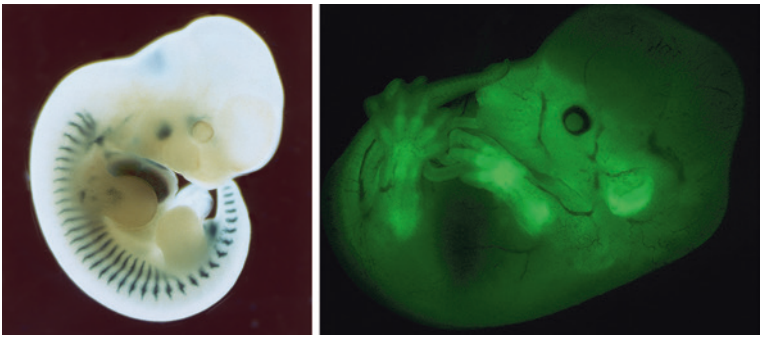

Fig. 8.3 *Analysis of gene expression with a reporter gene*. *Left* expression of the structural gene encoding *Lac*Z with regulation by the *Desmin* promoter. Observation of this embryo allows for detection of the tissues in which *Desmin*, a type III intermediate filament, is expressed (Courtesy C. Babinet). *Right* the embryo (recovered 13 days post-fertilization) is heterozygous for a knock-in allele in which the H2B-GFP coding sequence has been inserted in-frame into the gene encoding the platelet-derived growth factor receptor, alpha polypeptide ($Pdgfra^{+/H2B\text{-}GFP}$) (Courtesy J. Artus)

of several fluorescent proteins expressed in different tissues of a mouse (Passamaneck et al. 2006). Transgenic mice with reporter genes have been, and still are, extensively used by developmental geneticists (Lichtman et al. 2008). They have also greatly contributed to the annotation of the noncoding sequences.

8.2.4 The Use of Transgenic Technology to Generate Tissue- or Cell-Specific Ablations

Transgenic animals have been designed using tissue-specific regulatory sequences associated with sequences encoding cytotoxic proteins, with the aim of programming the genetic ablation of specific cell types either in the developing embryo or in the adult (Breitman et al. 1989). The most common strategy makes use of sequences encoding toxic proteins such as the A chains of the diphtheria toxin (DT-A) or of ricin (R-A), both of which block protein synthesis. In this case, the cytotoxic effect takes place as soon as the transgene is expressed. These studies indicate that programmed ablation of specific cell types can be stably transmitted generation after generation through the germ line (Breitman et al. 1987).

Another strategy has been developed that relies on the induced intracellular expression of the enzyme thymidine kinase (tk) of the herpes simplex virus (HSV). This enzyme is not directly toxic to animal cells but, unlike the mammalian thymidine kinase, it can phosphorylate certain nucleoside analogs such as acyclovir or ganciclovir, converting them into drugs that are toxic to dividing cells. In this particular case, the cell-killing effect becomes conditional since it depends both on the expression of the gene coding for viral thymidine kinase and on the administration of nucleoside analogs.

These methods of genetic ablation can be used to confirm the tissue specificity of a promoter; from this point of view, they appear complementary to the methods described above. Unfortunately, these methods, particularly the one using the highly toxic DT-A or R-A toxins as cell-killing agents, have a major drawback—the consequence of the extreme sensitivity of eukaryotic cells to these toxins. If the regulatory elements used in transgenic construction are not specific enough, a background expression of the transgene in cells that are not targeted results in misleading pathological conditions. This is mostly why, nowadays, this strategy of cell- or tissue-specific ablation has been abandoned for more specific approaches (see further).

8.2.5 Transgenic Complementation of a Mutant Allele Identified by Positional Cloning

As we already mentioned in the previous chapters, positional cloning of mouse mutations is an efficient approach for assessing the function of genes because the strategy directly associates a mutant phenotype with a specific gene. For example,

cloning a gene that is responsible for a leukodystrophy, once mutated, will point by definition to a gene involved in the development and organization of the white matter of the nervous system. However, when the candidate gene has only two alleles—one normal and one mutant—with the mutant being, for example, the consequence of a missense mutation (which occurs in about 75 % of cases), it is risky to conclude that the mutant allele is indeed responsible for the phenotype because there is always a chance, even if small, that the two observations (the phenotype and the mutation) are independent. In this case, it is generally necessary to prove that the missense allele is indeed causative of the pathology, and this can be achieved either by generating other alleles by mutagenesis (see Chap. 7 and later in this chapter) or by attempting to rescue the mutant phenotype by transgenic complementation. In this case, an appropriate breeding protocol is used to obtain genotypes that are certainly homozygous for the recessive mutation in question (*mut/mut*), normally leading to the deleterious phenotype, plus an additional (normal), functional transgenic copy of the candidate gene. The observation of a normal or nearly normal phenotype for this genotype validates the candidacy of the gene cloned by a positional approach. An example of transgenic rescue was reported endorsing the suspicion that a missense mutation in the gene encoding tubulin-specific chaperone E (*Tbce*^{pmn}-Chr 13) was indeed responsible for the deleterious phenotype of the mouse mutation *progressive motor neuronopathy* (Martin et al. 2002).

8.2.6 Using Transgenic Mice for Modeling Human Diseases

Different types of transgenic mice have been designed either to allow scientists to conduct experiments that were not possible with normal mice or to model a pathological condition that exists only in humans. We will provide a few examples to demonstrate the versatility of this transgenic technology.

8.2.6.1 Making Transgenic Mice Susceptible to Human Infectious Diseases

Poliovirus, the causative agent of poliomyelitis, infects primates but cannot spontaneously infect mice except for some type 2 virulent strains. Transgenic animals susceptible to all three poliovirus serotypes have been produced by pronuclear injection of the cloned human gene encoding the cellular receptor for the virus (Koike et al. 1991). These transgenic mice, when inoculated with poliovirus, mimic some of the clinical symptoms observed in humans and monkeys and are good models for studying the molecular mechanisms of pathogenesis of the virus as well as for testing vaccines against poliovirus infections.

Another example is the bacteria *Listeria monocytogenes*. These bacteria, once ingested by humans, can produce severe and sometimes fatal infections. The mechanisms by which the bacteria passes through the human intestinal barrier

is well known: It requires the intervention of a surface protein called *internalin*, which interacts with a host receptor, E-cadherin, to promote entry into intestinal epithelial cells. Murine E-cadherin, in contrast to human or guinea pig E-cadherins, does not interact with internalin, excluding the mouse as a model for experimental oral infection with *L. monocytogenes*. In contrast, in transgenic mice expressing human E-cadherin, internalin was found to mediate invasion of enterocytes and crossing of the intestinal barrier (Lecuit et al. 2001). These results illustrate well the value of transgenesis for understanding the physiopathology of human infections.

Models of the kind we have just described are, of course, of greatest interest for the study of infectious pathology, in particular for the development of efficient therapies and vaccines. Unfortunately, they illustrate rather exceptional situations and, in many cases where transgenesis was used to make animals susceptible to human pathogens, the situation has been discouraging. The determinism of susceptibility to infectious agents is sometimes complex and is rarely determined by the presence or absence of a single, species-specific cellular receptor. Progress in this area certainly awaits the discovery of genes whose products facilitate viral integration into the cell and full development of the replicative cycle. For example, scientists at the Rockefeller University and at the Scripps Research Institute demonstrated that the genes encoding CD81 and occludin were required for Hepatitis C virus (HCV) to enter human cells, and they demonstrated that making mice transgenic for these human genes made it possible to infect these transgenic animals with HCV (Dorner et al. 2011).

Even if perfect and faithful models cannot be made available simply by the mere addition of a few DNA segments, transgenic technology remains an interesting strategy to make progress in some aspects of infectious pathology. Transgenic technology, for example, allowed scientists to clarify the role of the complex cluster of genes encoding oligo-adenylate synthetase 1 (*Oas1*) in mouse susceptibility to flaviviruses (Scherbik et al. 2007; Simon-Chazottes et al. 2011) and has already provided insights into the pathogenesis of HIV-1.

8.2.6.2 Transgenic Models of Human Genetic Diseases

A mouse model of the human disease *osteogenesis imperfecta* type II (OMIM 166210) has been produced by injecting *in ovo* an abnormal mouse pro-α1 (I) collagen gene (*Col1a1*), orthologous to the abnormal human gene (Stacey et al. 1988; Pereira et al. 1993). The animals carrying such a transgene appeared very sick soon after birth, because of the modification of the extracellular matrix by the abnormal collagen fibers. In this case, the transgene had a dominant deleterious effect, the affected animals were almost impossible to breed, and the model proved to be of limited value. Nowadays, much better models can be generated using advanced techniques of transgenesis, as we will describe later.

A transgenic mouse strain has been created by pronuclear injection of both the normal human α-globin and the abnormal β^s-globin gene characteristic of sickle-cell anemia (Ryan et al. 1990). These animals were bred to β-thalassemic mice

to reduce endogenous mouse globin levels. When erythrocytes from these mice were deoxygenated, greater than 90 % of the cells displayed the same characteristic sickle shapes as erythrocytes from humans with sickle-cell disease. Compared to controls, the mice had decreased hematocrits, elevated reticulocyte counts, reduced hemoglobin concentrations, and splenomegaly, which are all indications of human sickle-cell disease. Such models are also of great help in the understanding of the pathophysiology of this debilitating disease as well as in the development of new drugs and therapies.

8.2.7 Transgenic Animals with Large DNA Inserts

Several techniques have been used to create mice transgenic for large DNA fragments. Among these techniques, the direct pronuclear microinjection of purified YACs or BACs has been the most popular (Jakobovits et al. 1993; Schedl et al. 1993; Lee and Jaenisch 1996; Van Keuren et al. 2009; Rossant et al. 2011). Such transgenic mice, when available, are very helpful for understanding the mechanisms operating when, for example, the genetic defect results from an unknown alteration occurring in a relatively large genetic region, or simply when the molecular origin of the defect is not completely clear. Several examples documenting the ability of wild-type alleles carried in YACs to complement mutations have been reported. The first one was the simple, complete rescue of the classical mouse albino mutation after injection into the germ line of albino (Tyr^{c}/Tyr^{c}) mice of a 250 kb YAC encompassing the wild-type mouse tyrosinase (*Tyr*) gene with all its introns and 155 kb of the 5′ flanking region (Schedl et al. 1992).

Original animal models of human genetic diseases have also been created using YAC transgenes. Among these, we must cite a model for Charcot–Marie–Tooth disease type 1A (Huxley et al. 1996) and a model for Huntington disease in which large intergenerational trinucleotide repeat expansions could be recreated, endorsing the use of these transgenic mouse models to refine the understanding of triplet repeat expansion and the resulting pathogenesis (Gomes-Pereira et al. 2011).

The possibility of inserting large-sized DNA fragments into the mouse genome will certainly be very useful for a better understanding of the phenotypic impact of the variations in genomic copy number (CNVs) (discussed in Chap. 5), as well as for the production of better models of Down syndrome (discussed in Chap. 3). Many fragments cloned from human chromosome 21 have been added to the mouse genome by *in ovo* transgenesis, producing phenotypes more or less reminiscent of those of human trisomy 21 (Smith et al. 1995; O'Doherty et al. 2005; Yu et al. 2010; Herault et al. 2012; Rueda et al. 2013). None of these models is perfect because of the complexity of the phenotype when several genes on different mouse chromosomes are used, but good progress is being made and transgenesis appears to be a technique of choice in this matter.

Many transgenic models of Alzheimer disease have been developed over the past several years. Most of these models replicate some of the pathological

features of the disease, such as plaque-like amyloid accumulations and astrocytic inflammation, but not all phenotypic aspects. In particular, the behavioral deficits are not faithfully modeled (Lithner et al. 2011).

Transgenesis with BACs or other large chromosomal segments is bound to become a very popular technology, with the foreseeable development of quantitative genetics in the years to come. The reason is that, unlike in the case of single Mendelian mutations, the genomic regions that have a quantitative effect on the phenotype are mostly unknown and, in this case, BACs containing the DNA segment where a quantitative trait locus (QTL) has been localized can be transferred into zygotes and the resulting mice tested for the quantitative trait in question. However, for this system to be applicable, BAC libraries must be available that contain the appropriate alternative alleles at the QTL in question (Heintz 2001; Abiola et al. 2003). Such libraries are now being prepared for different mouse species and strains.

8.2.8 Transgenic Knockdowns

In Chap. 5, when describing the different sorts of RNAs that are encoded in the mouse genome, we discussed the case of siRNAs and their possible use for gene silencing. Experiments of that kind have been undertaken several years ago by Katsuki et al. (1988) to assess the possibility of controlling gene expression by inducing the production of antisense RNAs in the genome. For their experiment, the Japanese scientists constructed a plasmid containing the promoter of the gene encoding the mouse myelin basic protein (MBP), followed by a portion of the rabbit β-globin gene associated with the mouse MBP-cDNA in the antisense orientation and a polyadenylation site. They observed that several transgenic mice for this transgenic construction had a phenotype similar to that of the mutant mouse *shiverer* (*Mbp*^{shi}-Chr 18). Antisense MBP messenger RNA was transcribed at high level in these mice, while the endogenous messenger RNA was reduced. The researchers concluded that the mice with an abnormal phenotype were *constitutive knockdowns* and that the transgene expression in vivo resulted in RNA interference (RNAi).

Since this first (successful) experiment, several other attempts at production of knockdown have been undertaken; some have been successful but most have failed. The reason is that, unlike in plants or invertebrates, double-stranded RNAs (dsRNAs) elicit an interferon response in mammals, resulting in global inhibition of protein synthesis and non-specific mRNA degradation. For this reason, short synthetic dsRNAs, whose length is below 30 bp, have been used to trigger the specific knockdown of mRNAs in mammalian cells without interferon induction. In the best experimental conditions, the efficiency of target knockdown can be as high as 90 % or greater, with permanent gene silencing in transgenic organisms indicating that the production of transgenic antisense RNA is an interesting approach to assessing gene function in vivo (Hitz et al. 2009).

8.2.9 Assessing the Mutagenic Activity of Chemicals with Transgenic Mice

As briefly mentioned in Chap. 7, at least two independent mice transgenic strains have been developed to assess in vivo the mutagenic activity of chemical substances of the environment: These strains are commercially available under the names of Big Blue® and Muta™Mouse (Wahnschaffe et al. 2005a, b). Transgenic mice of the Big Blue® strain have ~30–40 copies of the lambda LIZα shuttle phage vector integrated into their genome and the target for mutagenesis is the *lac*I gene. Muta™Mouse mice have ~80 copies of the lambda-gt10-*lac*Z shuttle vector integrated into their genome and the target is the entire *lac*Z gene.

Whatever the transgenic strain, the chemical compound to be tested is administered to the transgenic mice under several forms and at different doses. After a few days, DNA samples are then extracted from several tissues of the tested mice. The targeted genes are excised and packaged into lambda phage heads by using specially designed molecular kits, and the phages are transfected into bacteria. Finally, the transfected bacteria are plated on indicator plates containing selected chromogenic substances. Under these conditions, the phage-transfected bacteria with mutations in the targeted genes form plaques of a different color from those of bacteria with a non-mutated target gene, and the ratio of colored plaques to colorless plaques is a reliable measurement of the mutagenicity of the compound tested.[4] These transgenic strains have been extensively used and have provided fast and reliable estimations.

8.2.10 Mutations Induced by Pronuclear Transgenesis

As mentioned earlier, approximately 8–10 % of transgenic insertions result in recessive lethal mutations, and a much lower percentage in recessive viable. Good examples of the situation are two independent alleles at the *Formin* locus[5] (*Fmn1*-Chr 2) and a mutation described as *cryptorchidism with white spotting* (*crsp*-Chr 5) (Woychik et al. 1985; Messing et al. 1990; Overbeek et al. 2001). Such mutations by insertions would appear a priori to be interesting situations, considering that the inserted DNA (whose sequence is known) could be used as a tag for the identification of the mutated gene and accordingly for facilitating its positional cloning (for reviews, see (Jaenisch 1988; Gridley et al. 1990; Meisler 1992). In practice, however, the cloning of DNA flanking the insertion loci has often proved difficult as a consequence of the structural changes generated by the insertion.

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[4] The phage-transfected bacteria with mutations in the *lac*I gene form blue plaques, whereas bacteria with a non-mutated *lac*I form colorless plaques in tests with the Big Blue® strain. With the Muta™Mouse strain, the basic principle is similar but the color of the plaques depends upon the experimental conditions.

[5] The first of the two alleles resulting from a transgenic insertion at the *Formin* locus (*Fmn*-Chr 5) has been known for a long time under the name of *limb deformity* (*ld*).

8.3 Generating Alterations in the Mouse Genome Using Embryonic Stem Cells

The technique of transgenesis by pronuclear injection of exogenous DNA sequences has been a true revolution in mammalian genetics. It has enabled hundreds of experiments that have provided answers to fundamental questions regarding the organization and functioning of the mammalian genome and has permitted the creation of many useful and original animal models for biomedical research. Unfortunately, the technique has some important limitations. One is that it allows the addition but not the deletion or substitution of genomic material meaning that, except in some rare situations, it is not possible to produce alterations with a recessive phenotypic expression. Another limitation is that the injected DNA inserts randomly in the genome of the host, and for this reason, the expression of the transgene often varies from one founder transgenic mouse to the next due to unique interactions with other genomic sequences in the background and disconnection of the transgene from its natural regulatory elements (see Chap. 5). Such limitations do not apply to the genetic alterations produced by using the techniques of genetic engineering in embryo-derived stem cells (ES cells). These techniques have been developed over the last 30 years and are still extensively used in the mouse for the production of a variety of targeted alterations. We will review the most commonly used.

8.3.1 Embryonic Stem Cells and their Advantages

ES cells were developed in the early 1980s (Evans and Kaufman 1981; Martin et al. 1981). They were derived from cells dissected from the inner cell mass (ICM) of blastocysts that were cultured in vitro, generally on feeder layers of fibroblasts, in tissue culture media supplemented with a few percent of fetal calf serum, with a high concentration of glucose, with glutamine and β-mercaptoethanol. To prevent these cells from differentiating in vitro, low concentrations of leukemia inhibitory factor (LIF) were added to the medium and the cells were re-plated at a relatively rapid pace.

ES cells represent a material of choice for geneticists because they can be manipulated (almost) like ordinary somatic cells, as long as they are maintained in vitro, while retaining all their developmental potentialities, in particular their capacity to differentiate into derivatives of all three embryonic germ layers (pluripotency). In addition, and most importantly, when merged with the cells of the ICM of a recipient blastocyst, many ES cells are capable of participating in the formation of chimeric embryos, and provided that these ES cells are euploid (i.e., with 2n chromosomes, a normal XY or XX complement, and no deletions or other types of chromosomal rearrangements), they are often capable of participating in the formation of the germ-cell lineage of the embryos in question. It is then

possible to apply to ES cells the classical techniques used in somatic cell genetics while they are in vitro (e.g., selection based on resistance or susceptibility to a specific drug), to isolate clones of cells with a pre-defined genetic characteristic, to "shuttle" them back into the germ line of a chimeric mouse, and finally to breed a strain of mice that have integrated into their genome an alteration engineered in vitro. The first experiments on genetic engineering with this type of cells were carried out by Gossler et al. (1986) and by Robertson et al. (1986). They were real breakthroughs,[6, 7] when these experiments were performed, most of the ES cell lines available for the purpose of scientific research were derived either from embryos of the 129/SvPas inbred strain (new nomenclature 129S2) or from the 129/J strain (new nomenclature 129P3/J). Nowadays, taking advantage of technological progress, especially in terms of culture conditions, many other ES cell lines have been derived from a variety of strains and most of them are stable and reliable, producing a high percentage of chimeric animals and a good germ line transmission ratio. The ES cell lines derived from strain C57BL/6N have become popular and have been selected in many transnational projects. This was a wise choice given that the reference sequence of the mouse genome is also from the C57BL/6 inbred strain.[8] ES cell lines derived from NOD, BALB/c, and some immunodeficient strains (such as NSG) are also available or under development. On the other hand, in the laboratory rat, the development of germ line-competent ES cells was only possible very recently (Ping et al. 2008).

Chimeras resulting from the fusion of an engineered ES cell with cells of the ICM of a recipient embryo can be identified, a few days after birth, for example, on the basis of their dappled coat color. This is very obvious when, for example,

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[6] Well before the development of ES cells, another kind of cell, the *embryonal carcinoma* or EC cells, was used by oncologists and geneticists for investigating the genetics of cell–tissue differentiation. These cells were derived from spontaneous or experimentally induced testicular or ovarian teratocarcinomas (Stevens 1960). They were cultured in vitro, in the form of stable undifferentiated cell lines and then transplanted into mice of the same strain (syngeneic transplantation). Most of these cell lines, once engrafted, were able to differentiate into a variety of tissue (nervous tissue, bone, fat tissue, muscle, etc.), and some even proved able to participate in the formation of a chimeric organism (Papaioannou et al. 1975). They had, however, major drawbacks for the study of tissue differentiation: They were malignant and became rapidly aneuploid, and accordingly, they could not be used for the production of chimeric mice with germ line transmission.

[7] *Induced pluripotent stem cells* (iPSCs) are pluripotent cells derived from adult somatic cells after forced re-expression of some specific genes that are normally inactive. Such cells have been established in many species including human and mice. These iPSCs have many characteristics in common with ES cells and are being used in many experiments (for example, in the area of regenerative medicine). However, they have no obvious advantages over the long-established ES cells for the production of transgenic mice, and accordingly, they will not be considered in this chapter.

[8] The two strains C57BL/6N (ES cells) and C57BL/6J (genome sequence) are not completely identical, and recent estimates indicate a difference of ~1–2 % (SNPs) at the genome level (see Chap. 9).

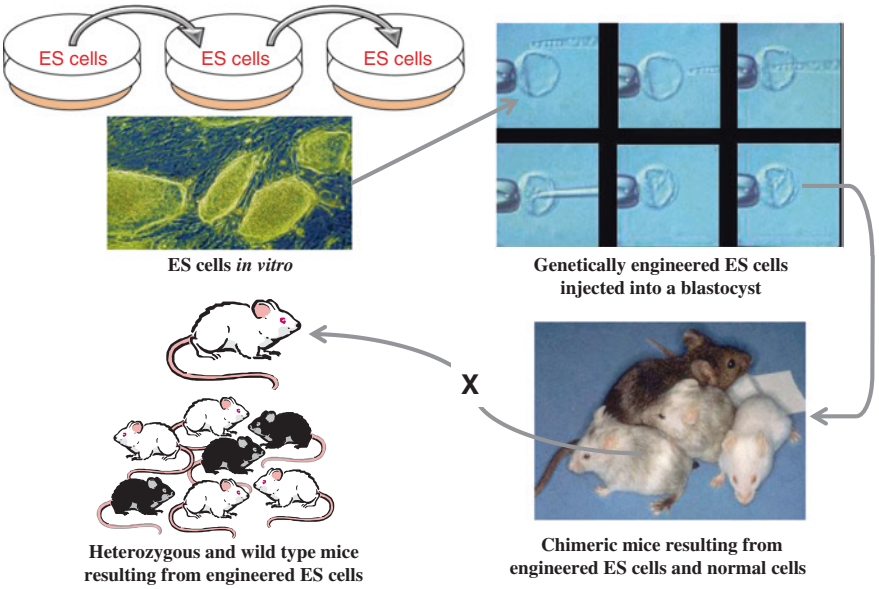

Fig. 8.4 *Targeted mutagenesis in the mouse using engineered ES cells*. The chart represents the different steps for the production of transgenic mice from genetically modified ES cells. ES cells can be cultured in vitro for several generations, remaining in an undifferentiated status. While in vitro, the ES cells can be manipulated like ordinary somatic cell lines and, in particular, can then be selected on the basis of specific criteria. ES cells can also be placed inside full-grown blastocysts where they spontaneously merge with the inner cell mass. Provided that the ES cells are still pluripotent and euploid, fertile chimeric mice can result from these reconstructed blastocysts. Mice with a dappled coat color in the figure are chimeras derived from blastocysts of (albino) hybrid mice (CSJF1) into which ES cells derived from a pigmented strain (129/Sv) were injected after several generations of in vitro culture. The size of the spots may vary according to the experimental conditions, but this does not faithfully reflect the percentage of chimerism in the germline. All of the other pigmented offspring of the chimeric mice are heterozygous for the genetic alteration(s) that may have been engineered in the ES cells. Two more generations are then necessary to observe the alteration in the homozygous state, and selection of the progenitors requires DNA genotyping

the ES cells are derived from the C57BL/6N inbred strain (which is non-agouti *a/a*—i.e., solid black) and the recipient blastocyst from either a wild-type (agouti *A/A*) or albino (Tyr^c/Tyr^c) strain. In these conditions, the chimeras exhibit a mixture of black and agouti (or albino) spots (Fig. 8.4).

Using coat color as a reference, one can estimate the percentage of chimerism, but a high level of chimerism does not necessarily correspond to a high rate of germ line transmission. Although chimeras can be from either sex, males are generally the only sex with germ line transmission because the majority of ES cell lines are XY. When grown in vitro for several generations, many (male) ES cells have a tendency to lose their Y chromosome and become XO.

8.3.2 Targeted Mutagenesis in ES Cells

The basic principle that characterizes targeted mutagenesis consists of applying a selection pressure on ES cells cultured in vitro that confers an advantage to the cells that may have lost (or acquired), spontaneously or after experimental manipulation, a characteristic encoded by a specific gene. The loss of a specific characteristic may result from a mutation, a deletion or any other kind of alteration, impairing the function of a given gene. The acquisition of a new heritable characteristic generally results from the transfection of foreign DNA molecules into the ES cells, followed by selection of the transfected cells based on a selective advantage conferred by the exogenous DNA. Once selected, the mutant or genetically modified ES cells are used for the production of chimeras, with the hope that a substantial proportion of the modified ES cells will still participate in the formation of the gametes. This will allow the production of transgenic mice with a targeted alteration in their genome.

8.3.2.1 The In Vitro Production of Mouse Models of Lesch–Nyhan Syndrome

Lesch–Nyhan syndrome (OMIM 308000) is a rare and severe X-linked metabolic disease in humans. The defect is characterized by the absence or inactivity of the enzyme hypoxanthine phosphoribosyl transferase (HPRT), an essential enzyme for the catabolism of purines. No animal model for this disease was available up to the mid-1980s, when two independent teams published the isolation of *Hprt*$^-$ clones of ES cells resulting from mutations in the *Hprt* gene. This was achieved after in vitro selection of *Hprt*$^-$/Y mutant cells that occurred spontaneously or after mutagenic treatment and became resistant to the toxic effect of the purine analog 6-thioguanine (6TG) when added to the culture medium.

Hooper et al. (1987) isolated a few *Hprt*$^-$ clones that occurred spontaneously and were selected with 6TG, injected them into blastocysts, bred chimeric mice and finally succeeded in establishing an *Hprt*$^-$ mutant strain. The isolation of clones of mutant ES cells by the mere in vitro selection on a particular phenotype (and genotype) was proved successful, although with a very low yield.

A technical improvement came from the use of mouse retroviruses as mutagenic agents and was a consequence of the early observations by Jaenisch and colleagues (1981). Two major conclusions of these pioneering experiments were that (i) retroviral vectors can be efficiently used as mutagenic agents for mammalian embryonic cells; and (ii) these vectors insert into the genome without generating extensive chromosomal rearrangements. Based on these observations, ES cells infected with the Moloney murine leukemia virus (M-MuLV) and mutant (null) alleles were recovered after selection with 6TG, at the same *Hprt* locus (*Hprt*$^-$), but this time, at a higher frequency (Kuehn et al. 1987).

The experiments reported above by Hooper and colleagues and Kuehn and colleagues were published simultaneously. They were the first experiments reporting the generation of a mutant strain in vitro, in ES cells, after selection of a particular phenotype. Surprisingly, however, the mutant mice, supposed to be a model of Lesch–Nyhan syndrome, did not exhibit any symptoms reminiscent of the human syndrome.[9, 10] From the genetic point of view, the result was somewhat disappointing but was nevertheless a great technical achievement, opening the way to many other technical refinements.

Considering the relatively high efficiency of the technique in terms of proviral integration numbers, massive infections of ES cells have been achieved from which embryos heterozygous for random insertions have been bred. These mutations by insertion have been put into the homozygous state using the classical two-generation micro-pedigrees (cross, backcross), and mutant phenotypes have been observed on some rare occasions. An interesting example is the recessive lethal mutation *Nodal*$^{tm1.1Mku}$ (Chr 10), with a block at the gastrula stage, which was found to be the consequence of a proviral insertion causing the loss of function of *Nodal*, a TGFβ-related gene (Lowe et al. 2001). Another mutation of the same kind (*Lrp4*^{dan}-Chr 2) was found to cause a syndrome of polysyndactyly as a consequence of the insertion of the proviral copy into the gene encoding MEGF7/LRP4, a member of the low-density lipoprotein receptor family (Simon-Chazottes et al. 2006) (Fig. 8.5).

The strategy that consists of infecting ES cells with M-MuLV, or any other kind of retrovirus, followed by the breeding of mice derived from the infected ES cells, allowed the identification of a few genes with effects on development. The retroviruses are mutagenic when they integrate into an exon or when they insert into an intron and disorganize the splicing process of the transcript encoded in the neighboring exons. An advantage in this case is that the retroviral insertion can also be used as a tag to identify DNA clones containing the mutated gene. Unfortunately, the yield of the strategy is low because, in most instances, retroviral insertions occur in noncoding regions and accordingly they have no direct or mechanical mutagenic effects. Another major drawback is that, for most autosomal genes in the mammalian genome, there is no efficient way to select in vitro the cells heterozygous for a recessive allele. In these conditions, it is necessary to breed mice homozygous for each proviral insertion and to unambiguously associate homozygosity for the proviral insertion with a specific phenotype, in general by the observation of tight linkage. This, however, is a tedious, risky and time-consuming enterprise.

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[9] Mutations at the mouse *Hprt* locus probably occurred spontaneously in the past but were not recorded due to the complete absence of symptoms in the affected mice. We will never know for sure.

[10] The observation of differences (sometimes dramatic) in the symptomatology associated with a human syndrome and those observed in mice affected by mutations in the same orthologous gene is common. This, however, does not affect the value of the model.

Fig. 8.5 *Proviral insertional mutagenesis*. After experimental infection of ES cells with a defective Moloney retrovirus, a proviral copy was inserted, by chance, into the first intron of the gene encoding the low-density lipoprotein receptor related protein 4 (*Lrp4*-Chr 2). This insertional mutation disorganized the splicing process of the gene in question, making it virtually inactive. This resulted in the production of a fully penetrant, autosomal recessive mutation characterized by severe polysyndactyly (allele $Lrp4^{dan}$). The images on the *left* show normal paws from wild-type mice. On the *right*, the images depict paws from homozygous mutant mice with malformed digits and syndactyly

8.3.2.2 Another Model of Lesch–Nyhan Syndrome Resulting from Gene Targeting

In addition to the drawbacks mentioned above, one must also remember that one cannot target the integration of retroviruses at a specific site in the genome. In these conditions, the mutations generated are random and unpredictable. From this point of view, homologous recombination of extrinsic DNA molecules in ES cells resulting in the replacement of an endogenous gene by a different allele, in most cases non-functional, has been another breakthrough due to its potential applications. This technique is generally referred to as *gene targeting*.

The principle for the production of targeted mutations by homologous recombination is based on the observation that DNA fragments, once introduced into ES cells by an appropriate experimental procedure (e.g., electroporation or transfection), can recombine with the DNA of the host cells to become part of their genome. In most instances, the recombination occurs at non-homologous (or illegitimate) sites, but in some rare instances, it occurs at the homologous site. As a consequence, and provided that the transfected DNA molecules have been previously adequately modified by genetic engineering in vitro, a homologous recombination event can result in the replacement of an active and functional gene by an inactive one.

The idea that homologous recombination could occur in mammalian cells, and in particular in ES cells, originated from observations made in other eukaryotic organisms, in particular in the yeast *Saccharomyces cerevisiae*, where similar experiments had been successfully achieved. The detailed molecular mechanisms at work in the recombination process are not yet fully understood. It is likely that the mechanisms of homologous recombination overlap with those of illegitimate recombination, but a number of experiments indicate that they are not completely identical (for review, see Hooper 1992). Homologous recombination, of course, occurs at a much lower frequency than random integration (Smithies et al. 1985; Wong and Capecchi 1986). At this point, it should be noted that the idea of developing such a strategy was quite audacious if one compares the relatively small size of a cloned DNA that can be handled experimentally, to the gigantic dimensions of a mammalian genome!

To increase the yield of homologous recombination events, experience teaches us that the DNA molecule transfected into the ES cells must be linear, as large as technically possible, for instance up to 10 kb and more if possible, and should have the greatest possible length of sequence homology with the targeted DNA in the ES cell.

The first endogenous mouse gene that was modified by homologous recombination in ES cells was again the one encoding hypoxanthine-guanine phosphoribosyl transferase (*Hprt*-Chr X) (Thomas and Capecchi 1987). The experiment consisted of three steps. In the first step, a DNA molecule cloned from the *Hprt* targeted region and containing a few exons, the intervening introns and some flanking DNA sequences was cloned. In the second step, one exon in the cloned *Hprt*-DNA molecule was replaced by a piece of DNA of roughly the same size but with a different origin. Finally, the engineered cloned DNA was transfected into normal ES cells by electroporation. The idea underlying this manipulation was that, in the event of successful homologous recombination, the substitution of an exon by a segment of exogenous DNA would make the modified *Hprt* gene unable to transcribe a functional mRNA, thus generating a null allele.

While designing these "faked" or "counterfeit" DNA constructs to replace the targeted gene, scientists, instead of using segments of noncoding DNA as a foreign sequence, had the clever idea to use a minigene of bacterial origin encoding the enzyme neomycin phosphotransferase (neo^r) and capable of conferring to the transfected cells the capacity to resist to the toxic effect of neomycin. In these conditions, when plated in a culture medium with the antibiotic neomycin or, more precisely, with one of its amino glycoside analogs, G418, the normal ES cells were all killed while the cells synthesizing neomycin phosphotransferase (neo^r) resisted the cytotoxic effect of the drug. In other words, only those ES cells having stably integrated an engineered DNA molecule into their chromosomes, either at the targeted locus site or anywhere else in the genome, could survive. The rare ES cells clones where a strictly homologous recombination occurred would likely have reciprocally exchanged a functional copy of the *Hprt* gene for a non-functional one, and at the same time, they would also have acquired the property to resist the toxic effects of 6TG just like the $Hprt^-$ mutant cells reported above.

The advantages of this technique are twofold. The first is that, after selection with G418 (eliminating all cells with no stable DNA integration) and selection with 6TG (eliminating all cells with a functional *Hprt* gene), the only ES cells that would still grow in vitro are those where a homologous recombination event occurred. In other words, only the cells where the gene actually targeted has been effectively inactivated, or "knocked-out," would survive. The second advantage is that the mutation frequency by homologous recombination is higher than with any other technique. In the case reported above, for example, one stably transfected ES cell clone out of 150 was found to be a knockout (Capecchi 1989). This frequency of recombination events was considered high enough to adapt the technique to all cases where it was suitable for generating a null allele, even though the sorting out of the homologous recombinant ES cells from the non-homologous recombinant cells could not be achieved by the same, in vitro selection as in the case, we just reported for *Hprt*$^-$ cells.

Since these early experiments, thousands of genes have been inactivated using the gene-targeting strategy.[11] Genes inactivated by homologous recombination in ES cells are now collectively designated by the name of "*knockout*" or "*knock-out"* (KO). The in vitro engineered DNA molecule used for targeting the homologous native counterpart in the chromosome of the ES cells is designated the "*recombination vector*" Nowadays, in all experiments of this kind, confirmation that the expected event of homologous recombination actually occurred in the manipulated ES cells is sought by PCR amplification of critical DNA fragments with an appropriate set of primers followed by sequencing and confirmation by Southern blotting. The ES cells in question are then placed into a recipient blastocyst for the production of a chimera. The genetically engineered ES cells, once confirmed "reliable" and capable of participating in the germ line of the chimeric mouse, are stored deep-frozen for future use or distribution to the community.

8.3.2.3 Generating a Variety of Knockout Alleles by Homologous Recombination

Many of the knockout mutations that have been generated in mouse ES cells over the past several years have resulted from the use of replacement vectors as described above. In this case, after homologous recombination, the targeted gene is deleted by one of its specific coding sequences, which is replaced by a heterologous DNA that is, in many cases, a *selection cassette*. As a consequence of this substitution, the gene is inactivated and, at the same time, the manipulated ES cells acquire a selective advantage over a drug and can be positively selected. Several variations on this basic scenario have been used, and it is impossible to describe them all in this chapter. However, we can say that most of these strategies

[11] For their discoveries of the *principles for introducing specific gene modifications in mice by the use of embryonic stem cells* Drs. Mario Capecchi, Martin Evans, and Oliver Smithies were awarded the Nobel Prize in Medicine or Physiology in 2007.

consist of using a variety of selection cassettes, making use of bacterial genes encoding either resistance to hygromycin *B* or puromycin as alternatives to the *neo*^{r} cassette.

The design of the selection cassettes in the replacement vectors for homologous recombination depends on the nature of the targeted gene. If the gene in question is transcriptionally active in the ES cells, then the selection cassette is transcribed and positive selection with a drug can operate. However, if the gene in question is not expressed in the ES cells or if its expression pattern is unknown, it is then necessary to design a vector that incorporates a promoter active in ES cells, allowing the gene to be "switched on" when requested.

Replacement vectors allowing positive/negative selection have also been designed by inserting a *neo*^{r} mini-gene between two regions of homology, and inserting a gene encoding herpes simplex virus thymidine kinase (HSV*tk*) outside of the regions of homology. ES cells that are transfected in vitro with such a replacement vector are then subjected to a double selection: (i) the first one with G418, inducing the destruction of all ES cells that had not integrated at least one copy of the vector (and accordingly the *neo*^{r} mini-gene); and (ii) the second selection with the guanosine analog ganciclovir (GANC, sometimes spelled gancyclovir), killing the cells containing a functional thymidine kinase (*tk*) gene. This second level of selection eliminates the ES cells, in which non-homologous recombination occurred because in this case the HSV*tk* component of the vector is generally retained, while it is deleted after homologous recombination. In these conditions, only the very few cells where homologous recombination occurred can survive (Figs. 8.6 and 8.7).

The techniques for gene inactivation which we just mentioned have been described in detail in several review papers and book chapters (Hooper 1992; Hasty et al. 2000; Babinet and Cohen-Tannoudji 2001; DeChiara 2001; Koentgen et al. 2010). The use of replacement vectors with a selectable marker has been and still is very popular for the generation of null alleles because it is relatively

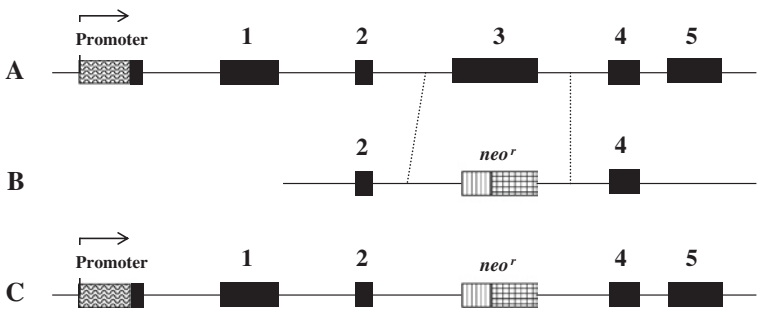

Fig. 8.6 *Gene targeting with a replacement vector 1.* Recombination events occurring in the regions flanking the *neo*^{r} cassette result in the deletion of exon 3 and its replacement by the *neo*^{r} cassette. The *neo*^{r} cassette confers a selective advantage on the recombined ES cells. The longer the sequence homology between the replacement vector and the host DNA, the better

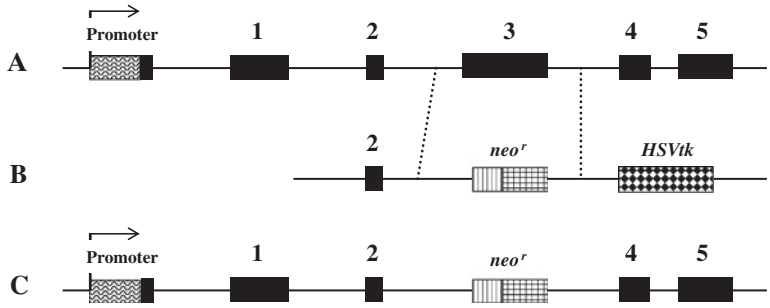

Fig. 8.7 *Gene targeting with a replacement vector 2*. Gene targeting with a replacement vector engineered with a positive/negative selection cassette. After homologous recombination, the *HSVtk* cassette is deleted while the *neo*^{r} cassette replaces exon 3. This recombination confers to the recombinant ES cells a selective advantage to *G418* and a selective disadvantage to *Ganciclovir*

straightforward and produces stable, permanent alterations. Unfortunately, cases have been reported where the selection cassette alters to some extent the expression of neighboring genes.

8.3.2.4 Generating Point Mutations by Homologous Recombination in ES Cells

The strategies described above, which make use of replacement vectors, require the introduction of extrinsic DNA sequences of various sizes into the genome of ES cells. Although mostly unknown, the consequences of this manipulation may have some possible adverse effects. This is why scientists have developed an alternative strategy, in two steps, leading to the creation of specific base-pair changes (missense or nonsense) in a specific DNA sequence, allowing the generation of so-called *knock-in* (KI) animals.[12]

The strategy in question is based on two successive steps of homologous recombination, with positive and negative selection, and makes use of mutant *Hprt*$^-$ ES cells similar to those resulting from the experiments reported above (Hooper et al. 1987; Kuehn et al. 1987) and two replacement vectors. The first replacement vector is designed to replace an exon of the targeted gene in HPRT-deficient (*Hprt*$^-$) cells with a functional *Hprt* minigene after the first homologous recombination (Selfridge et al. 1992).[13] After this first replacement, the recombinant ES cells are no longer resistant to the toxic effect of 6-thioguanine (6TG) and can grow

[12] The definition of *knock-in* also applies to the targeted insertion (and substitution) of any coding sequence at a particular locus of an organism. In these conditions, and in most instances, the inserted coding sequence is controlled by the regulatory regions of the targeted gene.

[13] The HPRT mini-gene is a selection cassette that is unique, since selection may be applied for its presence or absence.

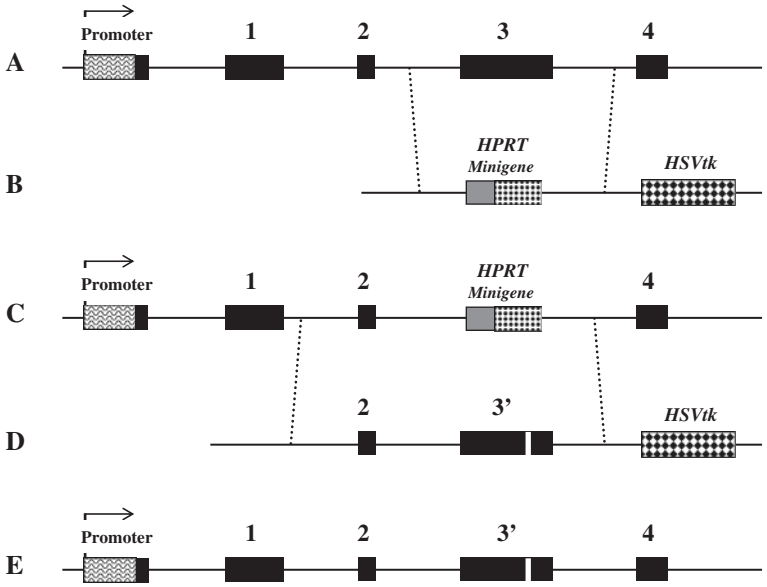

Fig. 8.8 *Induction of point mutations*. Induction of point mutations with two replacement vectors in *Hprt*$^-$ mutant ES cells. The first replacement vector substitutes an *Hprt* (functional) mini-gene for exon 3 and confers resistance to HAT (hypoxanthine, aminopterine, thymidine). The second recombination replaces the *Hprt* mini-gene by a mutated exon 3 (exon 3′) engineered in vitro. The ES cell then becomes sensitive to HAT but insensitive to 6-thioguanine. This homologous recombination is a *knock-in* (*KI*) because the original gene is replaced by a modified version, even if the gene is merely a mutant allele with only a point mutation

normally in so-called Littlefield's hypoxanthine, aminopterin, and thymidine (HAT) culture medium because they have a functional HPRT.[14] In these recombinant *Hprt*$^+$ cells, the targeted gene is deleted by one exon after replacement by the *Hprt* mini-gene. A second replacement vector is then designed that corresponds perfectly to the sequence of the original targeted gene, with the exception of a single base pair difference (an SNP) in the targeted exon.[15] This vector is synthesized in vitro, using a PCR technique of directed mutagenesis that is now routine in most laboratories. After this second replacement, homologously recombined ES cells are killed in HAT medium but survive selection by 6-thioguanine (6TG), as in the case of the original *Hprt*$^-$ deficient ES cells (Stacey et al. 1994) (Fig. 8.8).

Finally, and although it has no deleterious effects in the mouse, if the *Hprt*$^-$ mutation is considered undesirable, it can be easily eliminated by two rounds of sexual reproduction once the "offspring" of the mutated ES cells are born.

[14] *Hprt*$^-$ cells cannot grow in HAT medium because aminopterin blocks the endogenous synthesis of both purines and pyrimidines.

[15] Mice of this type are not transgenic animals sensu stricto because they do not have any exogenous DNA sequences "stably inserted into their genome." However, they are still GMOs.

This sophisticated technique of double replacement has potential applications, among which is the generation of a series of co-isogenic strains of mice (see Chap. 9). This can be achieved by using the same *Hprt*$^-$ ES cells, which are derived from a highly inbred strain, then by targeting these cells with a variety of second-set vectors from different inbred strains (replacing the *Hprt* mini-gene). One can then generate a variety of different point mutations in the same genetic background (the same inbred strains).

Alternative techniques using an insertion vector instead of a replacement vector, and known as the *hit-and-run* or *in-and-out* techniques, have been used for the generation of point mutations in targeted genes (Hasty et al. 1991; Valancius and Smithies 1991). These techniques required two rare intra-chromosomal recombination events to occur, and accordingly, they appeared to be less efficient than the technique making use of two replacement vectors. For this reason, they have been abandoned.

8.3.2.5 Knock-Ins Are Sometimes Sophisticated Knockouts

An interesting variation of the technique used for obtaining knockout mutations has been designed by introducing, via the replacement vector, the coding sequence of reporter genes in-frame with the promoter of the targeted gene. To give an example of the high degree of sophistication of this method, we refer to an experiment designed to assess the function of the genes encoding *connexins* (Filippov et al. 2003). Connexins are expressed in the various cell types of the central nervous system and are thought to regulate some of the functional properties exhibited by immature and mature cells. Understanding the specific role of each connexin in these processes required an unambiguous characterization of their spatial and temporal pattern of expression. To achieve this aim with connexin 26 (CX26) (gene symbol *Gjb2*, for gap junction membrane channel protein beta 2), scientists generated a reporter allele ($Gjb2^{lacZ}$) in which the pattern of expression of the gene encoding β-galactosidase was controlled by the endogenous *Gjb2* promoter. Then, by observing $+/Gjb2^{lacZ}$ heterozygous mice, the researchers could easily identify the tissues expressing CX26 (i.e., liver, kidney, skin, cochlea, small intestine, placenta, and thyroid gland) and demonstrated that the expression of CX26/*Gjb2* is restricted to the meninges both in embryonic and adult brain. The same researchers also noted that homozygous $Gjb2^{lacZ}/Gjb2^{lacZ}$ knockout embryos died early in utero, indicating that at least one intact copy of the *Gjb2* gene is necessary for normal embryonic development.

Such a mutation, where a gene is inactivated by the insertion of a foreign coding sequence driven by the same promoter, is also designated as a *knock-in*.[16] The knock-in strategy is universal and can be applied to any gene to inactivate it and, at

[16] In short, the main difference between a *knock-out* and a *knock-in* allele is that, in the case of a *knock-in,* the gene product is different from the normal allele but still has a function, even if the function in question is totally unrelated to the function of the original allele. In the case of a *knock-out*, the gene has simply been made inoperative.

the same time, visualize its expression pattern in the developing embryo or in the adult. The knocked-in genes are in general more faithfully expressed than the transgenes produced by pronuclear injection.

8.3.2.6 Engineering Conditional Knockout Mutations—The Cre-*lox*P Strategy

When produced by using one of the techniques described above, knockout mutations affect all the cells of the developing embryo in which the gene is normally expressed, starting from the early stages of development. For this reason, the mutations in question are often designated *constitutive knockouts*. Since most of the knockout alleles behave as recessives, the situation is in general well tolerated as long as the allele stays heterozygous. However, when the knockout allele is homozygous, the gene is permanently switched off in all cells, and the situation may become problematic. This is the case, for example, when the knockout allele results in early embryonic lethality because this hinders the analysis of the gene function(s) in later developmental stages or in the adult. It is also a drawback when the inactivation of the targeted gene results in the deregulation or misregulation of the expression of other genes.

To bypass these drawbacks, gene-targeting strategies have been developed that allow the (knockout) mutations to be made conditional (*conditional knockout* or *cko* mice). With conditional mutations, both the timing of gene inactivation and the cells or tissues in which the gene is to be "switched off" can be controlled. The discovery and development of these techniques has been another fundamental achievement in transgenesis.

The strategies used for the production of conditional knockouts make use of two transgenic strains: one in which the targeted gene is modified in a way that ensures its future inactivation and the other where the time- or tissue-specific expression of the mutation is programmed. Each of the two strains is normal and fully viable, but when intercrossed, all the ingredients necessary for inactivation are merged into the genome of their offspring.

The most popular strategy is known as the Cre-*lox*P strategy and makes use of Cre recombinase (from cyclization recombinase), a 38 kDa enzyme derived from the bacteriophage P1 (Utomo et al. 1999; Nagy 2000). Cre recombinase cuts and recombines the DNA strand at specific sites called *lox*P sites (short for locus of X-ing over P1) (Sauer 1993). These *lox*P sites consist of two 13 bp inverted (palindromic) repeats separated by an 8-bp asymmetric spacer region that defines the orientation of the site. Such sites do not exist in the mammalian genome (Fig. 8.9). When the *lox*P sites are in the same orientation and on the same strand (or chromosome), the intervening stretch of DNA is excised as a circular loop. When two *lox*P sites are in opposite orientations and on the same chromosome, the intervening DNA segment is inverted. Finally, when the *lox*P sites are on two different chromosomes, the recombinase generates a reciprocal translocation. When there are more than two *lox*P sites in the same genome, a variety of recombinations can occur.

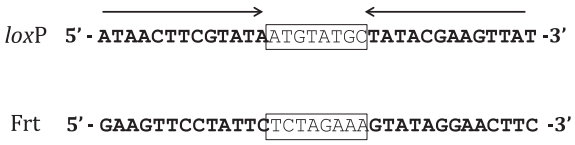

Fig. 8.9 *loxP and Frt sites*. A *lox*P site (*top*) consists of two 13-bp palindromic sequences (*arrowed*) flanking an 8-bp spacer region (*boxed*). These 8-bp define the directionality of the *lox*P site. When two *lox*P sites are placed on the same strand and in the same orientation, the Cre recombinase deletes the intervening sequence plus one *lox*P site. When the sites are in opposite orientations, Cre generates an inversion of the intervening sequence and both *lox*P sites are retained. When the *lox*P sites are on different chromosomes, the Cre-recombinase generates a reciprocal translocation. Nucleotide sequence of the 34-bp-long *FRT* site (*below*). The palindromic sequences bind the recombinase, whereas the spacer is the site of DNA break, exchange, and ligation

To illustrate the basic principle of the method, we will take a historical example: the case of T-lymphocyte-specific inactivation of the gene encoding the DNA-directed βpolymerase (*Polb*-Chr 8)(Gu et al. 1994). In this experiment, a strain of mice (strain A) had its *Polb* gene specifically modified by targeted homologous recombination with a replacement vector. The replacement vector was designed in such a way that an essential sequence of the *Polb* gene, actually the promoter and the first exon, became flanked by two *lox*P sites. The replacement vector was also designed in such a way that it contained two selection cassettes: a *neo*^{r} cassette and a thymidine kinase (HSV*tk*) cassette, themselves flanked by a third *lox*P site as indicated in Fig. 8.10. After homologous recombination, the targeted gene, *Polb,* ended up with three *lox*P sites inserted in the same orientation: the first one upstream of the promoter and exon 1, a second one in intron 1 upstream of the selection cassettes, and a third site downstream of the cassettes but upstream of exon 2. As geneticists say, the gene was then *floxed* (flanked by *lox*P sites) but, at this point, it was still functional and normally transcribed, and the mutation was only cryptic, or "premeditated", so to speak. The *neo*^{r} and HSV*tk* cassettes were useful for positive/negative selection with the classical drugs G418 or ganciclovir, should it be necessary.

Concurrently, another strain of mice (strain B) transgenic for a gene encoding Cre-recombinase was produced by classical pronuclear microinjection. The Cre-encoding transgene in this case was driven by a lymphocyte creatine kinase (*lck*) promoter, which is specific for T cells. When strains A and B were intercrossed, generating double transgenic (*bigenic*) mice, the product of the Lck-Cre transgene triggered deletion of the floxed segment in one or both chromosomes according to the genetic constitution (heterozygous or homozygous) of strain A, but in T cells exclusively. The consequences of the mutation (symbolized *Polb*$^-$)[17] on T cells could then be analyzed because mutant mice were viable, whereas they would have died if the mutation had been expressed ubiquitously during development.

[17] According to the official nomenclature rules, the symbol for this mutation should be *Polb*$^{tm1.1Rsky}$. This was the first targeted mutagenesis at this locus in Rajewsky's laboratory.

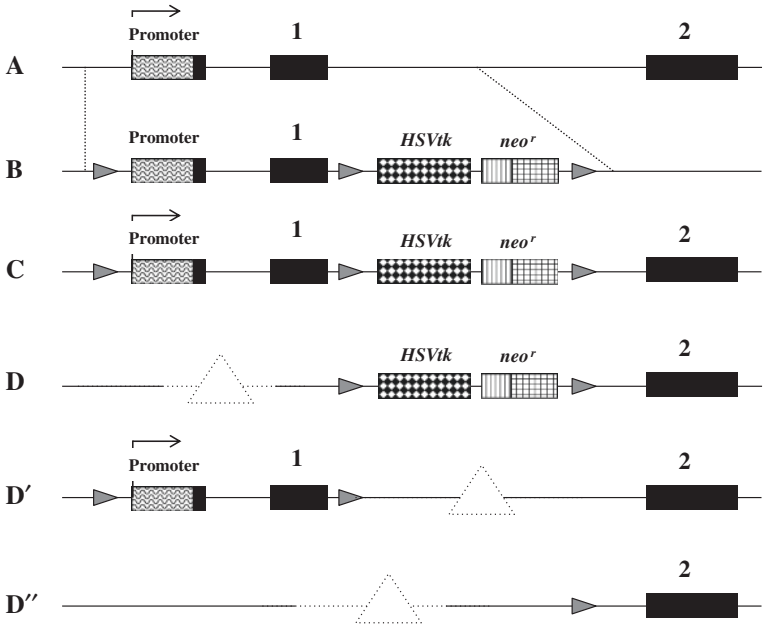

Fig. 8.10 *Inducing gene-targeted deletions with the Cre-loxP system*. In this experiment, the replacement vector (*B*) was designed in such a way that the *Polb* targeted region ended up with three *lox*P sites inserted in the same orientation: the first one upstream of the promoter and exon 1, a second one in intron 1, upstream of the selection cassettes, and a third one downstream of the cassettes but upstream of exon 2 (*C*). When Cre is synthesized, the segments flanked by two *loxP* sites (the *floxed* regions) are deleted, producing three different types of ES cells (*D*, *D′*, *D″*). The ES cells in which the targeted gene is deleted (and permanently inactivated—*D* & *D″*) are the most interesting. The *neo*^{r} and HSV*tk* cassettes were useful for positive/negative selection with the classical drugs G418 and *Ganciclovir*

Hundreds of experiments of the type described above, leading to tissue- or cell-specific gene inactivation, have been performed in recent years using either the Cre-*lox*P system or a similar system known as FLP-*Frt* (FLP for Flippase recombination enzyme—*Frt* for Flippase Recognition target). The FLP-*Frt* system is very similar to the Cre-*lox*P system but makes use of a yeast recombinase with another specific restriction site.

With these systems, an unlimited number of mutations may be designed, keeping in mind that Cre (or FLP) deletes any DNA segment once the latter is flanked by two *lox*P (or *Frt*) sites, provided these sites are oriented in the same direction. When there are more than two *lox*P sites in the same cell, as is the case in Fig. 8.10, Cre cuts at each site and, under specific conditions, generates a variety of deletions or translocations. Selection can then be applied to retain one cell type and not the others if selection cassettes have been judiciously inserted in critical regions (Gu et al. 1994).[18]

[18] This explains why, with such molecular tools, any kind of chromosomal rearrangement can be engineered in vitro. In the past, these chromosomal rearrangements were occasionally collected in the progenies of mice after irradiation in the post-meiotic stages (see Chap. 3).

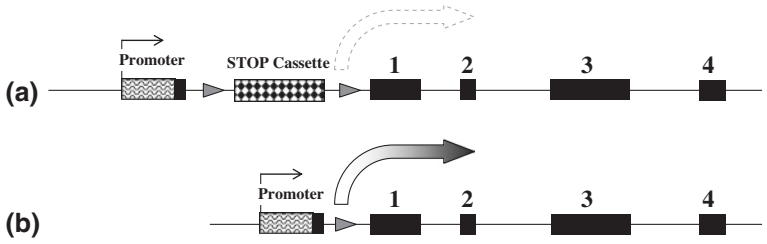

Fig. 8.11 *Cre-loxP regulation of transcription*. **a** A floxed "stop" cassette hampers transcription of the gene downstream. **b** When the "stop" sequence is deleted by the action of the Cre-recombinase, transcription resumes

A similar strategy has been employed using the same strain A (with floxed *Polb*) and another strain (strain C) with the interferon-inducible promoter of the gene *Mx1* to regulate Cre expression. After crossing strain A with strain C, *Polb* inactivation was induced in adult animals after interferon treatment. In this case, inactivation was complete in liver, spleen, and bone marrow while it was incomplete in other tissues (Kuhn et al. 1995). These experimental results demonstrated that Cre-mediated recombination could also be effectively induced in nondividing cells. The expression of the Cre transgene can be made inducible, adding more sophistication to the system. The tamoxifen-inducible Cre^{ERT2}, which can be activated by administration of tamoxifen to the transgenic mice, is very popular (Feil et al. 2009). Nowadays, many Cre-expressing lines are being produced as knock-in mice that incorporate the Cre sequence into the gene of interest (instead of creating transgenic lines using pronuclear microinjection).

The Cre-*lox*P strategy can also be used to regulate the expression of a specific protein in a tissue- or cell-specific way using a strategy that is schematically outlined in Fig. 8.11. In this example, the *lac*Z gene is a reporter gene driven by a ubiquitous promoter (e.g., *Rosa 26*) with a floxed "stop" sequence inserted between the promoter and the *lac*Z coding sequence. The "stop" sequence is a short segment of DNA with several terminator codons that impede translation of the protein. When the floxed "stop" sequence is deleted by the action of Cre in some specific cells or tissues, then the *lac*Z gene is transcribed following the same pattern of cell/tissue specificity (Lakso et al. 1992; Pichel et al. 1993) (Fig. 8.12).

To add versatility to the method, it must be kept in mind that both the Cre and FLP recombinases can be used, simultaneously or successively, in the same experiment.

Since experiments on conditional targeting all entail the use of mouse strains that synthesize Cre (these strains are designated Cre-deleters), either ubiquitously or in specific tissues or cell types (strain B or C, in the case of *Polb*, reported above), geneticists have agreed to establish a specific database listing all the Cre strains available (The Cre-X-Mice database at http://nagy.mshri.on.ca/cre_new/search/Search.php and The Jackson Laboratory Cre Resources at http://www.creportal.org/). These strains are, in general, freely available on the basis of a material

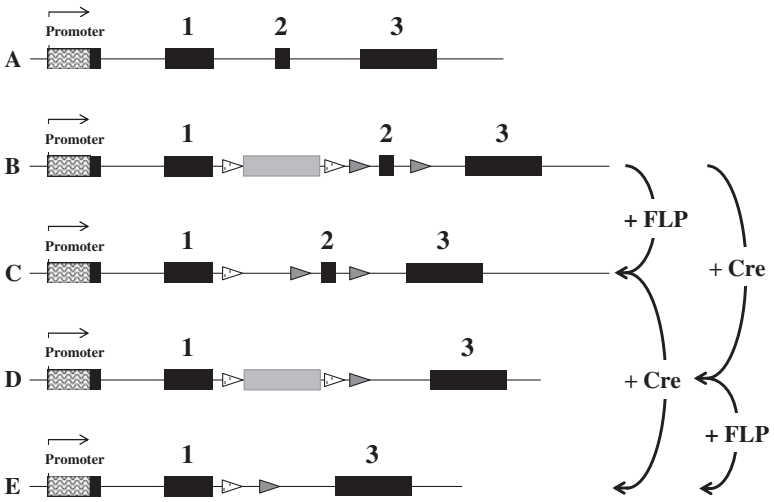

Fig. 8.12 *Inducing targeted deletions with the Cre-loxP and FLP-Frt systems*. The Cre and FLP recombinases can be used successively in the same experiment. In the case presented here, when FLP is used first, the selection cassette (*shaded box*) is deleted ($B \rightarrow C$). Alternatively, if Cre is used first, exon 2 is deleted ($B \rightarrow D$). Finally, when Cre and Frt are used successively, the selection cassette and exon 2 are both deleted ($B \rightarrow E$)

transfer agreement (MTA). This attitude, which is more and more common in the community of mouse geneticists, has saved and still saves a lot of research money. It has been made simpler every day with the use of the internet.

8.3.2.7 Gene Trapping and Targeted Trapping in ES Cells

In an earlier section of this chapter (Sect. 8.3.2.1), we reported experiments in which retroviruses were successfully used for producing insertional mutations in ES cells. These experiments revealed that, unlike the cloned DNA molecules injected into the pronucleus, retroviruses integrate into the genome of ES cells without generating extensive chromosomal rearrangements. In these conditions, when mutations were induced, the proviral copy could be used as a tag for cloning the flanking sequences and finally for identifying the mutated genes. Unfortunately, other than these advantages, the retroviruses have two major drawbacks: first, they insert randomly in the genome and infrequently in exons; second, using a proviral insertion for "harpooning" the flanking sequences is sometimes misleading, especially when there are many proviral insertions in the same ES cells.

In order to improve the efficiency of recovering mutations that are likely to have a phenotypic expression, an original strategy known as *gene trapping* was developed in several laboratories (Gossler et al. 1989; Friedrich and Soriano 1991; Skarnes et al. 1992; Evans et al. 1997; Cecconi and Meyer 2000; Stanford

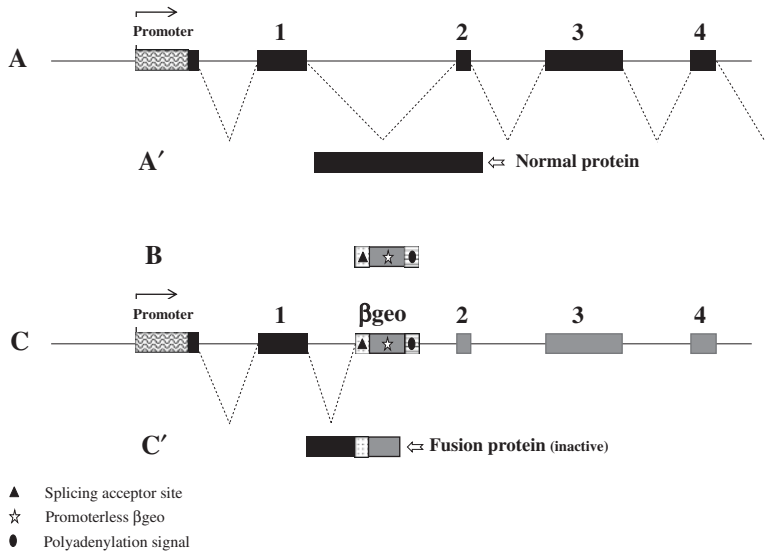

Fig. 8.13 *Gene trapping*. When a promoterless synthetic reporter gene, such as βgeo, sandwiched between a splice acceptor site and a polyadenylation signal (*B*) inserts, by chance, into one of the introns of an expressed gene ($A \rightarrow C$), the reporter gene is transcribed as if it were an exon of the gene. This generates a fusion mRNA, which is (sometimes) translated into a non-functional fusion protein C' (the trapped gene is inactivated). (This figure is redrawn from Skarnes et al. 1992)

et al. 2001; Hansen et al. 2003; Stryke et al. 2003). The principle of this strategy consisted of transfecting ES cells with a promoterless reporter gene and/or a selectable genetic marker flanked, upstream, by a 3′ splice acceptor (SA) site, and downstream by a polyadenylation signal (pA) (Fig. 8.13).

In early experiments, a popular promoterless gene was engineered by fusion of a β-galactosidase moiety (acting as a reporter) with a neomycin-resistant moiety (acting as a selectable marker) and was designated βgeo (contraction of *β-gal* with *neo*). When such a cassette was inserted in an intron, the gene was said to be "trapped." Nowadays, a variety of promoterless artificial genes have been designed with different reporter sequences, making the method more efficient and more versatile.

Transcription of the trapped genes, controlled by the endogenous promoter, resulted in a fusion (or hybrid) RNA molecule, which in turn, was translated into a non-functional protein with some sequence of the endogenous trapped gene beside some others from the sequence of the reporter/selectable marker.[19] Since the encoded fusion protein was non-functional, the trapped genes were equivalent to

[19] Trapping cassettes have also been designed with a marker gene or a selectable gene coupled to a suitable promoter but lacking a downstream polyadenylation signal. In this case, the transcript was also a hybrid molecule, utilizing the 3′ sequences of the host gene to acquire a poly (A) tail.

knockout (or loss-of-function) alleles and the sequence of the cassette could then be used as a tag for gene identification.

Although the strategy of gene-trapping works exclusively with those genes that are transcribed in ES cells, it is nevertheless a high-throughput approach for the identification of genes. It has been (and still is) widely used. Several laboratories, working in an *International Gene-Trap Consortium* (IGTC), have undertaken the establishment of large libraries of ES cells harboring gene-trap insertions. From recent estimates, over 126,500 ES cell lines, each with a trapped gene, are offered to the community on a non-collaborative basis.[20] This represents ~13,300 trapped genes (i.e., around 50 % of all the known genes in the mouse).

In the laboratories performing this type of experiment, the trapped genes are systematically identified unambiguously by using a PCR-based strategy such as 5'RACE (*rapid amplification of cDNA ends*), to generate a sequence tag unique for each insertion. By the way, this is greatly facilitated by the availability of the mouse genome sequence. Researchers who are interested can search and browse the IGTC database (www.genetrap.org) looking for the ES cell lines they are interested in, using accession numbers or IDs, keywords, sequence data, tissue expression profiles, or biological pathways.

As we already mentioned, newer gene-trap vectors have been developed, offering a variety of possibilities for post-insertional modification and the generation of a wide spectrum of alleles.

The trapped-gene libraries that exist nowadays have become an indispensable source of ready-made mutations in mice. For those readers who would like to know more about these libraries, the way they were established and their potential interest we recommend three general publications co-authored by scientists who were deeply involved in their development (Guan et al. 2010; Skarnes et al. 2011; Bradley et al. 2012). The Web site of the International Knockout mouse consortium http://www.knockoutmouse.org/ is also an important source of information, which is user-friendly and explains all the technical steps in the gene-trapping strategy.

As we explained above, gene trapping depends on the random insertion of a reporter cassette in an intron, but the cassette in question can also be inserted in a predefined position by homologous recombination. This strategy is known by the generic name of *targeted trapping* (Friedel et al. 2005). In this case, the vector (basically the same as the one used for gene trapping) is flanked by genomic sequences of the host, completely excluding the promoter. Targeted trapping in mouse ES cells is a simple though powerful tool for analysis of mammalian gene function. Provided the promoterless construct is carefully designed, most random insertions are eliminated by drug selection and the targeting frequencies can reach 50 % or even more (Fig. 8.14).

[20] With, however, some handling fees.

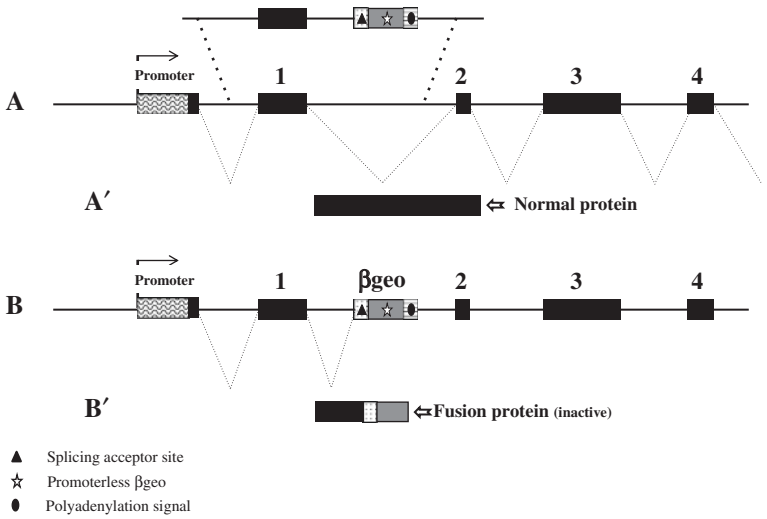

Fig. 8.14 *Targeted trapping*. In this case, insertion of the promoterless reporter gene βgeo is not random, as in the case of gene trapping, but instead results from homologous recombination with a selected region of the targeted gene ($A \rightarrow B$). As in the case of gene trapping, the promoterless gene in the cassette is activated and possibly translated into a fusion protein (B'). In this experiment, it is important that the targeted region does not contain the promoter of the gene. After characterization, the targeted or trapped ES cell clones can be deep-frozen and stored for further use. (This figure is redrawn from Skarnes et al. 1992)

8.3.3 Induction of Mutations in ES Cells with Chemical Mutagens

In Chap. 7, we explained that the induction of mutations in the mouse germ line with radiation or chemical mutagens was an efficient method for the annotation of mammalian genes because it produced all kinds of mutations (nonsense, missense, etc.) and all kinds of alleles (recessive and dominant etc.)—unlike most techniques of ES cell engineering, which produce mostly knockouts (i.e., null alleles). However, a major drawback of chemical mutagenesis is the cost of breeding and/or the time necessary to identify and characterize the new mutations. In addition, all these induced mutations are scattered throughout the whole genome, they are a mixture of different kinds, and they do not necessarily match the interest of the scientist. The genotype-based screens, which consisted of the identification, after analysis performed at the DNA level, of mice heterozygous for a mutation induced by ENU in a specific gene (as described in Chap. 7—Sect. 7.5.4), were considered more advantageous, especially when a deep-frozen sperm bank was available. Unfortunately, here again, this may still be insufficient if a series of alleles at a given locus is desired.

A genotype-based screen for ENU-induced mutations has been adapted with success for the identification of mutations induced in ES cells, in specific genes of interest (Chen et al. 2000; Munroe et al. 2000). In a series of experiments focused on two loci of importance for mouse early development, *Smad2* and *Smad4*, Vivian and colleagues (Vivian et al. 2002) mutagenized 2,060 ES cell clones by incubating the cells for 2 h in a culture medium with 0.2 mg/ml of ENU. They found a total of 29 mutations, out of which 20 were non-silent (yielding a non-silent mutation rate of 1 per 673 kb of screened DNA). This indicates that chemical mutagenesis in mouse ES cells, associated with high-throughput mutation detection, is another interesting method for the identification of mutations in non-selectable genes.

Other experiments on chemical mutagenesis of mouse ES cells have also been suggested as an alternative approach to the chemical mutagenesis of spermatogonia (Becker et al. 2006; Munroe and Schimenti 2009). This strategy has (at least) two major advantages: first, it enables the use of a variety of chemicals with different mutational spectra (different from ENU); second, it allows (at least in theory) the induction of a higher number of mutations in the mouse genome as a consequence of the possibility of performing several successive rounds of mutagenesis in vitro. In addition to these advantages in terms of efficiency, the chemical mutagenesis of ES cells has the same advantages as the gene-driven strategy described in Chap. 7 that it requires only two generations of breeding to reveal the phenotype of the induced mutations (breeding G1s, then intercrossing the G1). In addition, just like for the sperm cells in the case of gene-driven strategy, samples of successfully treated ES cells can be stored deep-frozen as long as necessary for the further detection of induced mutations. This method has not been used very much, probably because the techniques of genetic engineering were developed concurrently, but their advantages, as outlined above, are unique and should be kept in mind.

8.4 Inducible Transgenesis: The *Tet-off* and *Tet-on* Expression Systems

The Cre-*lox*P and the FLP-*Frt* strategies allow the induction of conditional gene knockout. With these strategies, researchers can inactivate virtually any gene, in any specific tissue or cell lineage, and when desired. However, once the Cre-recombinase has excised a floxed DNA segment, the situation is irreversible: the gene is permanently inactivated (or activated) in all daughter cells. Obviously, this may represent a drawback in experiments where only a transient inactivation (or activation) would be desired. It also may be desirable, in some experiments with transgenic mice, to have a transgene expressed only during a certain period but switched off the rest of the time. Unfortunately, this is not possible with the techniques described above.

The *Tet-off* and *Tet-on* inducible expression systems overcome these problems, placing the transcription of a given transgene under the control of the researcher.

In this system, the expression of a transgene is dependent on a tetracycline-controlled transactivator protein and can be regulated, both reversibly and quantitatively, by exposing the transgenic mice to the antibiotic tetracycline (Tc) or to one of its derivatives such as *doxycycline* (Dox). The technology was developed by Bujard and colleagues at the University of Heidelberg (Gossen and Bujard 1992; Baron and Bujard 2000).

The *Tet-off* system requires two critical ingredients. The first is the tetracycline-controlled transactivator protein (in short tTA). tTA is an artificial protein created

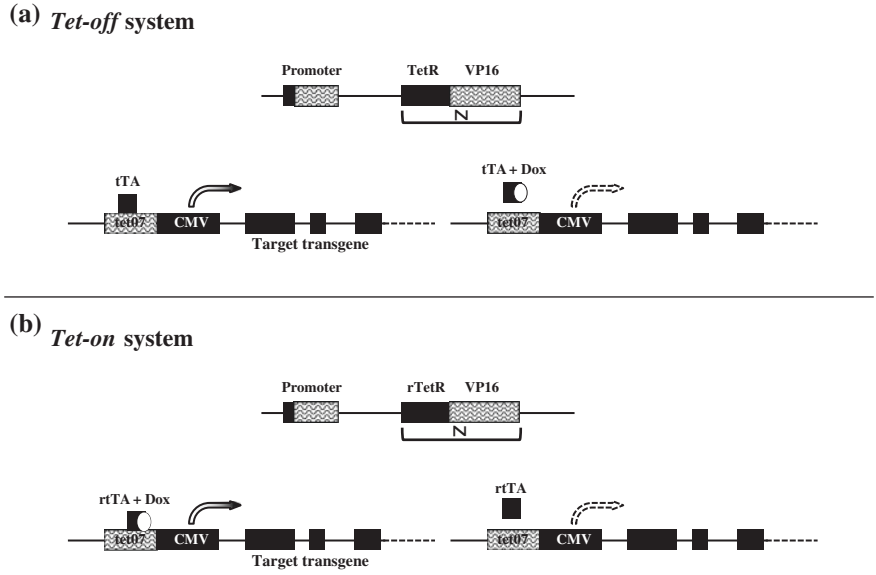

Fig. 8.15 *The "Tet-off" and "Tet-on" Expression Systems*. The *Tet-off* and *Tet-on* inducible expression systems enable transgene expression to be dependent on a tetracycline-controlled transactivator protein (tTA). Under these conditions, transgenic expression can be regulated. **a** The *Tet-off* system requires two ingredients. The first is the tTA, which is a fusion protein created with the TetR (tetracycline repressor), found in *Escherichia coli* transposon Tn10 and encoding resistance to the antibiotic tetracyclin, and a strong trans-activating domain of an herpes simplex virus protein called VP16. The second ingredient is the tetracycline-responsive promoter element (*TRE*) that is composed of a concatemer of seven *tet* operators (tetO7) fused to the minimal promoter sequences of the human cytomegalovirus immediate early gene 1 (*hCMVIE1*) promoter/enhancer. In the absence of tetracyclin (*Tc*) or doxycyclin (*Dox*), tTA binds to TRE and activates expression of the targeted gene. This induction returns to basal levels or is suppressed upon administration of Tc or Dox. The *Tet-on* system works in exactly the opposite manner. This system is based on a reverse tetracycline-controlled trans-activator (*rtTA*), which is also a fusion protein composed of the TetR and the VP16 transactivation domain. However, a four amino acid change in the TetR DNA-binding moiety alters rtTA's activity binding characteristics in such a way that it can recognize the tetO sequences in the TRE of the target transgene only in the presence of the Dox effector (delivered in the water or the food). Thus, in the *Tet-on* system, transcription of the TRE-regulated target is stimulated by rtTA only in the presence of Dox. **b** As explained in the text, both systems require the generation of double transgenic (or bigenic) mice carrying, in the same genome, the TRE-regulated target transgene and the tetracycline-controlled transactivator (*tTA* or *rtTA*)

by fusion of the TetR (tetracycline repressor), found in *Escherichia coli* transposon Tn10 and encoding resistance to Tc, with a strong transactivating domain of the herpes simplex virus protein called VP16. The second critical ingredient required for the *Tet-off* system to operate is the Tc-responsive promoter element (TRE). This promoter is composed of a concatamer of seven *tet* operators (tetO7) fused to the minimal promoter sequences of the human cytomegalovirus immediate early gene 1 (*hCMVIE1*) promoter/enhancer. In the absence of Tc or Dox, tTA binds to TRE and activates expression of the target gene. This induction returns to basal levels or is suppressed upon administration of Tc or Dox (Fig. 8.15).

The *Tet-on* system works in exactly the opposite manner. It is based on a reverse tetracycline-controlled transactivator (rtTA), which is also a fusion protein composed of TetR and the VP16 transactivation domain; however, a four-amino-acid change in the TetR DNA-binding moiety alters rtTA's activity binding characteristics such that it can recognize the tetO sequences in the TRE of the target transgene only in the presence of the Dox effector. Thus, in the *Tet-on* system, transcription of the TRE-regulated target is stimulated by rtTA only in the presence of Dox (i.e., when the drug is delivered either in the drinking water or with the food). A good example of the value of this system for cancer research is a model where an activated *Kras* oncogene is inducibly expressed in an epithelial compartment using keratin 5 (K5)-rtTA: tet-Kras bigenic mice (Vitale-Cross et al. 2004).

These *Tet-off* and *Tet-on* systems can be used, for example, to design dominant gain-of-function experiments in which temporal control of transgene expression is required (Gossen and Bujard 1992; Furth et al. 1994; Kistner et al. 1996; Schonig and Bujard 2003). The *Tet-off* expression system is more popular than the *Tet-on* system because it does not require the constant administration of a drug whose effects might be deleterious in the longterm.

8.5 Other Techniques for the Production of Transgenic Mice

Considering the efficiency of ENU mutagenesis and the potentialities of genetic engineering applied to ES cells, it is clear that mouse geneticists have at their disposition an unmatched arsenal of strategies allowing them to generate virtually any type of alteration in the genome of their favorite species. This is unfortunately not the case with other species of mammals, especially the rat, which is yet another important source of model for human diseases[21]. However, techniques have been developed to generate genomic alterations in these species and some have proved very promising. Most of these techniques have been efficiently and successfully transposed to the mouse species. We will describe the most important.

[21] Some domestic species (the rat in particular), present phenotypes that have not yet been documented in the mouse; this is why it would be important that the genetic arsenal that has been developed for the mouse be replicated in these other species.

8.5.1 Transgenesis by Retroviral Infection of Early Embryos

The integration of exogenous DNA into the germ line through experimental infection of mouse embryos with retroviruses was successfully achieved a long time ago (Jaenisch 1976). Newborns and preimplantation embryos (4–8 cell stage) were infected with the Moloney murine leukemia virus (M-MuLV), and it was observed that infection of preimplantation embryos, in contrast to infection of newborns, could lead to stable integration of proviral copies into the germline. These initial experiments have yielded several mouse strains with stable germ line integrations of retroviral DNA at distinct chromosomal loci (for example, the *Mov* loci; Jaenisch 1976). One of these integrations was in the gene encoding procollagen, type I, alpha 1 ($Col1a1^{Mov13}$) (Stacey et al. 1988).

Experimental infections of preimplantation embryos have the advantage that the viral integrations are in general stable and do not generate the sort of chromosomal rearrangements that often occur with the classical pronuclear techniques. Since these integrations occur almost at random, they sometimes hit a gene (as in the case of *Col1a1*) and produce a visible mutant phenotype. Here again, the DNA of the retrovirus can be used as a "hook" to clone the DNA sequences flanking the insertion site, and this helps in the characterization of the mutant allele.

Viral infection can also be used to introduce foreign DNA into embryos or eukaryotic cells in culture, and the advantages of using mouse retroviruses as shuttles for transgenesis have been explained in detail in a review by Nicolas and Rubenstein (1988). Two of these advantages are noteworthy in the context of this chapter:

- All the sequences of the viral genome required for its replication, transcription, and integration are grouped in or adjacent to the long terminal repeat (LTR).
- All the necessary proteins for infection, reverse transcription, and integration of the viral genome can be removed from the "shuttle" virus and provided in trans by a "helper" virus, leaving space for foreign DNA inserts of up to 8–10 kb.

For transgenesis in rodents (mostly in rat), the lentiviruses derived from human HIV have been the most widely employed (Wiznerowicz and Trono 2005). The reason for this choice is that lentiviruses, unlike most other retroviruses, have the capacity to infect nondividing cells. Shuttle viruses are produced by transfection of the construct into packaging cell lines, which are engineered to provide the essential viral proteins for assembly of infectious particles. The viruses are harvested from the cell culture medium and used for microinjection into the perivitelline space of single-cell embryos (Koentgen et al. 2010). Infected embryos reverse-transcribed the lentiviral RNA into DNA (provirus) that inserts back into the genome. However, because they are defective, the viruses are capable of completing only a single infectious cycle but cannot replicate further.

Lentiviral integrations, in addition to being relatively stable and because they are less invasive than pronuclear injections, sometimes yield survival rates approaching 90 %. Another advantage is that lentiviruses integrate as single copies and are expressed more reliably than the transgenes obtained by pronuclear injections; in particular, they are less prone to epigenetic silencing (Koentgen et al. 2010). The major weakness of this technique is the limit of 8–10 kb for the transgene size.

8.5.2 *In Vivo Genome Editing: The Production of Targeted Alterations Using Engineered Nucleases*

Over the last 10 years, a totally new kind of technique has been developed for the production of gene- (or locus-) targeted mutations that make use of engineered hybrid molecules which associate sequence-specific DNA-binding domains with a non-specific DNA cleavage domain. These techniques have demonstrated significant advantages for the production of a variety of mutations at targeted sites in several species commonly used by geneticists, including *Arabidopsis thaliana*, *Caenorhabditis elegans*, the sea urchin *Echinus melo*, *Drosophila melanogater*, and *Danio rerio*, to cite just a few. Recently, the techniques in question have been successfully adapted to the production of targeted mutations (knockout and knock-in) in mammals, mainly in the rat (Geurts et al. 2009), the mouse (Carbery et al. 2010), and other domestic species (reviewed in Rémy et al. 2010; Gaj et al. 2013; Kim and Kim 2014; and Mashimo 2014). We will describe some of these techniques and discuss their possible applications for genome editing.

8.5.2.1 Zinc-Finger Nucleases and Transcription Activator-like Effector Nucleases

The molecules used in initial experiments associated zinc-finger DNA-binding motifs with the restriction endonuclease *Fok*I, and for this reason, they were called *zinc-finger nucleases* (ZFNs). For the production of mutations, two complementary ZFNs must be designed, each of them recognizing a specific DNA sequence spanning 9–18 bp on either side of a 5–6-bp sequence defining the targeted region. When injected into a cell or a pronucleus, the ZFNs assemble tightly on both sides of the targeted site, one on each strand, and *Fok*I performs double-strand breaks (DSBs).[22] Once cleaved by the endonuclease, the cellular mechanisms controlling DNA integrity are immediately triggered to repair the damage. These mechanisms are of two types. The first is known as the homology-dependent repair (HDR) mechanism, which requires a homologous (template) sequence to guide the repair: it is precise and accurate and re-establishes *ad integrum* the original sequence of the cleaved DNA strand. The second mechanism, known as non-homologous end joining (NHEJ), is more common but is more rapidly activated. NHE is much less precise and only approximately restores the damaged strands, leaving behind deletions of nucleotides and accordingly frame shift mutations that are in most instances loss-of-function mutations (Fig. 8.16).

[22] A specific ZFN binds with 3 bp at the DNA level. Since there is a great variety of such motifs, a judicious selection of 3–6 of them allows the targeting of a 9–18-bp DNA domain, which is highly specific. Libraries of ready-made ZFNs are also available which allow the targeting of virtually any sequence in the mouse genome.

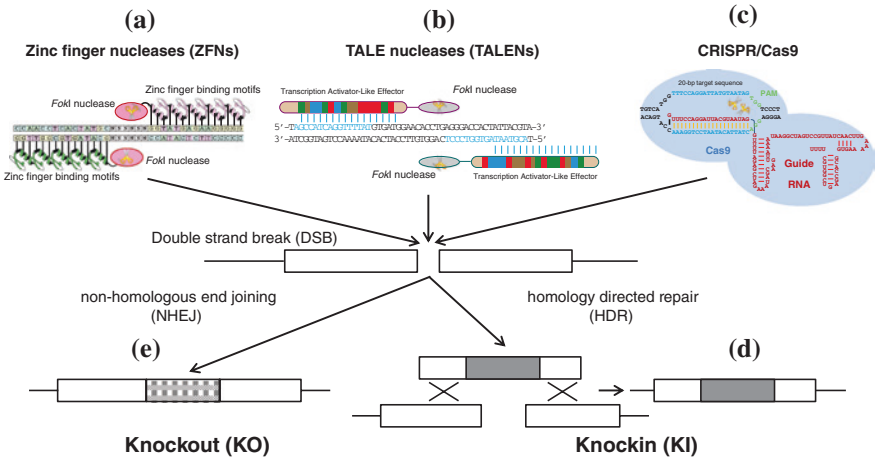

Fig. 8.16 *The production of targeted genome alterations using site-specific engineered nucleases or the CRISPR/Cas9-based RNA-guided DNA endonuclease*. The figure schematically represents three strategies used for genome editing. Zinc fingers (**a**) and TALEN modules (**b**) both bind to adjacent DNA sequences in opposite directions, leaving a small gap in between for the *Fok*I endonuclease to perform a double-stranded break (DSB). **c** With the CRISPR strategy, Cas9 unwinds the DNA duplex and performs a DSB after recognition of a specific (~20 bp) target by the gRNA, provided that the correct protospacer adjacent motif (PAM) is present. Whatever their origins, DSBs are ligated through non-homologous end joining (NHEJ) (**d**) or repaired through homology-directed repair (*HDR*) (**e**). For HDR to occur, a DNA molecule or a single-stranded synthetic DNA must be added as a template. If the sequence of the template differs from the endogenous sequence by the addition or substitution of some nucleotides, this results in a knock-in. These methods for producing mutations at specifically targeted sites are very efficient. The CRISPR/Cas9-based RNA-guided strategy permits the production of several independent point mutations in the same genome (Courtesy T. Mashimo)

The technique is simple in its practical aspects. Messenger RNAs transcribed in vitro from engineered ZFN plasmids are injected into the (male) pronuclei of mouse zygotes, exactly as in the case of pronuclear (*in ovo*) transgenesis, then the embryos are transferred into the oviduct of pseudo-pregnant females. With this technique a homozygous knockout mutation can be obtained in 4–5 months, which is much faster than with the traditional knockout strategies using ES cells.[23] Another important advantage of the technique is that it is applicable to all strains of mice, allowing for the production of a series of mutations at the same locus in different inbred backgrounds (co-isogenic strains) (Carbery et al. 2010). Finally, the technique can produce a variety of mutations, mainly deletions ranging from 1 bp to more than 1 kb, and more rarely, insertions of a few bp, but also sequence-specific mutations—which are all potential tools for the analysis of the targeted

[23] This comment concerning the time necessary to produce a knockout mutation in the mouse genome by using the ZFN strategy, although reduced, must nevertheless be compared with the time necessary to purchase, when available, an ES cell line harboring the same ready-made knockout, when the latter is available in a repository such as KOMP (https://www.komp.org/).

gene's function. Knock-in mice and rats carrying sequence-specific modifications have already been produced using ZFN technology (Cui et al. 2011).

The expression of artificial nucleases in embryonic cells at early stages of development does not seem to be toxic or to have any breakage activity outside of the targeted DNA sequence (little or no off-target events). The only drawback of the technique, which is not a major one, is that alterations may still occur at the targeted site several days after the injection, making some founder animals behave like mosaics. Another potential drawback is that, for technical reasons, the technique is probably not applicable to gene families, since the design of sequence-specific domains of the ZFNs would be difficult or even impossible in this case.

This basic technique of genome modification making use of ZFNs has undergone several improvements and developments. The first one is based on the observation, already mentioned above, that when DSBs are induced in cells by any means (for example, as a consequence of irradiation or of nuclease activity—and regardless of the nuclease) the homology-dependent repair mechanism (HDR) is activated. These mechanisms increase the potentialities of insertion of exogenous DNA that have sequence homologies at their ends with the sequence flanking the DSB. For example, adding the cloned RNA of the *lac*Z reporter gene to the mRNAs injected into the pronucleus allowed the production of knock-in transgenic mice with *lacZ* integrated between the boundaries of the DSB.

Another improvement has been the replacement of the DNA-binding components of the ZFNs by molecules from the plant bacterium *Xanthomonas* with similar DNA-binding properties. These molecules are a family of transcription activator-like effectors (TALEs) and the DNA-binding hybrid molecules are known as TALENs. TALEs have binding capacities greater than ZFNs and can match with virtually any sequence, further increasing the efficiency of the technique (Tesson et al. 2011). Over recent years, several groups have used TALENs to modify endogenous genes in a wide variety of species including insects, amphibians, fish (zebrafish), and mammals (rat, mouse, pig, and cow) (Joung and Sander 2013; Sung et al. 2013). The advantages of TALENs over ZFNs include their ease of design and assembly, their specificity, and their lower cost. Injection of the exonuclease *Exo1* in substitution for endonuclease *FokI* in the TALEN technique has been another improvement in the production of knockouts in rats (Mashimo et al. 2013).

8.5.2.2 The CRISPR/Cas9 System

The strategies that we described in the section above consisted of the production of double-strand breaks (DSBs) by the protein-guided DNA cleavage activity of engineered ZFNs or TALENs. Recently, another technique has been developed that depends on small RNAs for the production of sequence-specific cleavages (RNA-guided DNA cleavage). This strategy was developed after the identification and characterization of a defense mechanism, known as the CRISPR/Cas system,

which operates in bacteria and archaea and allows these organisms to fight infections by viruses, plasmids, or phages (Pennisi 2013).[24]

A CRISPR locus consists of a series of short direct repeats (average size 32 bp) of identical sequences, interspersed with intervening regions called *spacers*, which consist of small but variable sequences. Analysis of the sequence of these spacers indicates great similarities with the sequences of some phages and plasmids, providing a possible interpretation for the mechanism of recognition of the genome of the invaders by the CRISPR.

The CRISPR loci are transcribed into short CRISPR RNAs (crRNA). These crRNAs anneal to transactivating crRNAs (tracrRNAs) and direct sequence-specific cleavage of DNA by Cas proteins. Target recognition by the CRISPR-associated nuclease (Cas9) protein requires a *seed* sequence within the crRNA and a conserved dinucleotide-containing *protospacer adjacent motif* (PAM) sequence upstream of the crRNA-binding region (Fig. 8.16).

Engineered modifications of the CRISPR, as well as the Cas9 part, have led to an efficient way of producing DSBs at will. The CRISPR component is usually referred to as a guide RNA (gRNA). Cas9 utilizes gRNA that binds to specific DNA sequences to produce the DSBs.

The Cas9 protein consists of three more or less independent domains: one DNA-binding domain and two catalytic domains that independently cut one DNA strand. The two domains with nuclease activity can be inactivated separately by simple point mutations, and these modified versions of Cas9, with one cutting domain disabled, introduce single-strand breaks or DNA *nicks*. Even though DNA nicking is less efficient for genome editing, it dramatically reduces the chance of so-called off-target effects, since unwanted nicks are faithfully reconstructed by homology-directed repair (HDR). DSBs can be achieved at the targeted site by a pair of DNA-binding gRNAs, with sites close to each other but on opposite strands.

The RNA-guided endonucleases can be engineered to cleave virtually any DNA sequence by appropriately designing the crRNA; for example, to generate knock-in animals carrying conditional or reporter alleles (Yang et al. 2013). This technique exhibits several advantages over the methods using ZFNs or TALENs. One can, for example, generate mice carrying mutations in multiple genes across the genome in a single step by simultaneously injecting various gRNAs (Horii et al. 2014). This technique is known as multiplex gene editing and has been applied successfully not only to cells cultured in vitro but also to mouse and rat embryos (Wang et al. 2013; Wei et al. 2013). It saves a lot of breeding time when an experimental project requires the presence of several mutations in the same genome.

The genomic alterations that can be produced by using the CRISPR/Cas9 technology are not limited to the production of indels but can also consist of knock-ins. If we consider that the strategy is relatively easy to apply and somewhat faster than the other strategies using engineered nucleases, we see that CRISPR/Cas9 may well-revolutionize genomic engineering in the near future (Mashimo 2014; Zhang et al. 2014).

[24] CRISPR is an acronym for *clusters of regularly interspaced short palindromic repeats.*

8.6 Conclusion

Contemplating all the many possibilities for creating transgenic mice, one can see that geneticists now have all the tools in hand to answer virtually any questions that may arise in their analysis of gene functions. They also have at their disposition a very large collection of ready-made mutations of all kinds, waiting to be used, for example, as models of human diseases.[25] All these tools and models will be important for performing genome annotation.

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[25] To paraphrase the title of an interesting review on the subject one could say that, nowadays, geneticists have at their disposition "*a mouse for all reasons*" (International Mouse Knockout Consortium 2007).

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