Application of Molecular Genetics

16

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16.1 Biotechnology to Study Human Gene

Biotechnology plays an important role in the study of human genes. Techniques from molecular biology can be used to identify genes associated with diseases and the mutations involved in them. These techniques can be used for prenatal testing as well. A sample can be taken from the amniotic fluid or fetal tissue to obtain DNA which can be screened for mutations using recombinant DNA technology. This is especially useful in cases where the affected protein cannot be detected in the early stages because it is either produced in lower quantities or not expressed in fetal stages. This allows us to predict if the newborn will be affected by certain diseases that it may be susceptible to on the basis of family history.

16.1.1 Huntington's Disease

Huntington's disease (HD) is a neurodegenerative disorder which involves loss of motor control, jerky movements, and decline in cognition accompanied by change in personality and psychiatric symptoms. It is a rare disease, and symptoms of the disease appear later in life between 35 and 40 years of age. It is inherited in an autosomal-dominant manner. George Huntington was the first to describe this disorder in detail in 1872. Since the symptoms appear later, many affected individuals already have children and have passed on the genes to their offspring.

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There is a 50% chance of the offspring getting affected with the disorder as it shows autosomal-dominant inheritance.

Huntington (*HTT*) was the first disease-associated gene to be mapped to the human chromosome in 1983. It started with a group of scientists who worked on finding a DNA probe that showed a specific restriction fragment length polymorphism (RFLP) pattern for HD. They tested 12 probes on Southern blot of chromosomal DNA digested with HindIII. One of the probes showed a specific RFLP pattern for DNA from two families which had a history of HD. A large amount of effort over 20 years was devoted to identifying these families and obtaining their pedigree and medical histories. To identify where in the human DNA this HD-specific probe was binding, researchers made use of a series of mouse cell lines called human-mouse somatic cell hybrids. These cell lines were engineered to contain a specific subset of human chromosomes. On hybridizing the probe to a number of these cell lines, it was found that the probe recognized a region on the fourth chromosome. They therefore concluded that the gene responsible for HD was present on the human chromosome 4 and in the region to which the probe was binding.

Over the next 10 years, efforts were put in to identify this gene and the nature of its mutations. It was found that the gene had a trinucleotide repeat of CAG at the beginning of the gene. In normal individuals, number of this repeat varied from 6 to 21. In affected individuals, this number was found to be greater than 40, even up to 100. The trinucleotide expansion was identified as the cause of the disease. Today, molecular genetic approaches can be used to detect the number of CAG repeats in the *HTT* gene. We can therefore predict if an individual will suffer from the disease later in life and the chances of passing on the disease to their offspring.

16.1.2 Cystic Fibrosis

Cystic fibrosis is caused by a mutation in the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene. Cystic fibrosis affects cells that produce mucous, sweat, and digestive juices. It causes the fluids to become thick and blocks ducts and passageways adversely affecting the lung and digestive system. It may cause difficulty in breathing and persistent lung infections. It is inherited in an autosomal-recessive pattern.

One of the most common mutations in CFTR is a 3 bp DNA deletion known as 508. To detect mutations in *CFTR* for the purpose of diagnosis, allele-specific oligonucleotides (ASO) are used. These are probes constructed as an exact match against the mutated allele or the normal allele. This probe will only bind to the allele which is an exact match and will not bind even if there a single nucleotide mismatch. To do the test, DNA is isolated from the white blood cells of the individual to be tested. This is spotted on a nylon membrane and allowed to hybridize with both the ASOs against normal and mutated allele under specific conditions which do not allow a mismatch. In unaffected homozygotes, only the ASO against the normal allele will hybridize. In case of carriers, both ASO against normal as well as mutated

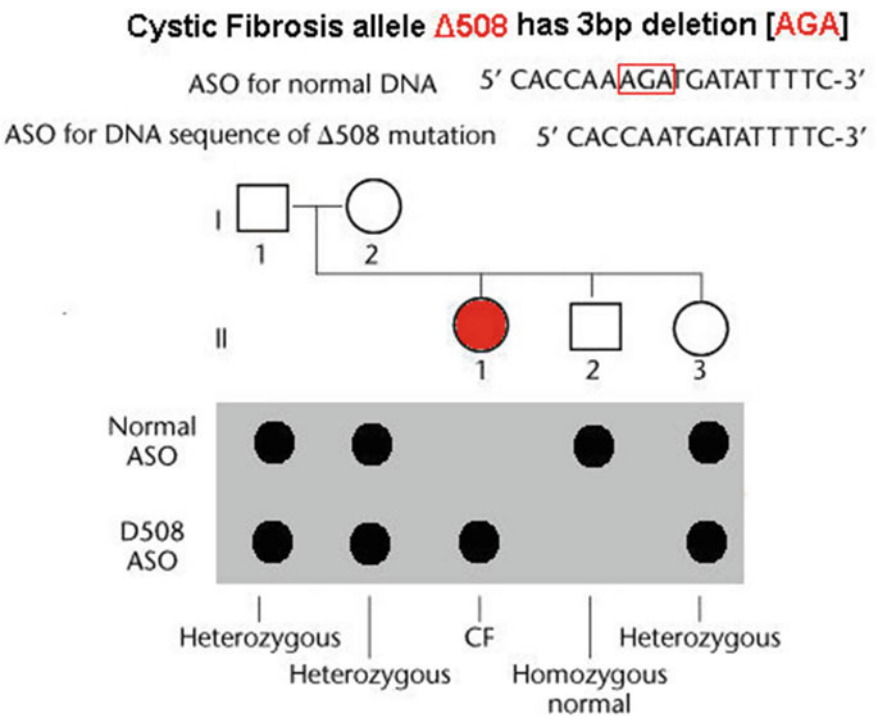

Fig. 16.1 Genetic diagnosis of cystic fibrosis. Allele-specific oligonucleotides are prepared for both normal allele (normal ASO) and allele having Δ508 deletion (D508 ASO). DNA is extracted and blotted for parents and three children. Both ASOs hybridize for parents both of whom are heterozygous. The affected child (child 1) shows hybridization only with D508 ASO. Child 2 is homozygous for the normal allele and shows hybridization with only normal ASO. Child 3 is a carrier and shows hybridization with both ASOs

allele will hybridize. In case of affected individuals, only the ASO against mutant allele will hybridize. This is illustrated in Fig. 16.1. Although a powerful technique, it suffers from an obvious limitation. The *CFTR* gene may have mutations other than 508, which will not be detected by this technique. Thus, a negative result on this does not necessarily mean that the individual has no mutations in this gene. However, as more mutations are identified and more genomic data is available, we will be able to identify most if not all mutations that are present in the population and provide a screening that has a better coverage.

16.1.3 Sickle Cell Anemia

Sickle cell anemia is a disorder in which the shape of erythrocytes is affected due to a substitution mutation in the β globin gene. The abnormal erythrocytes become elongated and curved resembling a sickle due to polymerization at low oxygen

tension. The normal erythrocytes are disc shaped. Aggregation of red blood cells leads to oxygen deprivation to many tissues which might severely damage them.

The mutation in β globin gene eliminates restriction site for the enzymes *Mst*II and *Cvn*I. These differences lead to a different restriction pattern seen on the Southern blot for mutated allele and normal allele. This distinguishing feature can be used to diagnose individuals having the mutated allele. The MstII restriction enzyme has three sites in the normal β globin gene cleaving the gene into two fragments. In the mutated allele, the site in the middle is lost cleaving the gene into a single fragment. DNA can be extracted from an individual exposed to *MstII* digestion, and the fragments can be separated by gel electrophoresis. This can be transferred to a nylon membrane and visualized by Southern hybridization using probes that recognize the fragments of β globin gene. Two small fragments indicate that the individual is homozygous for the normal allele. A single large fragment denotes that the individual is homozygous for the abnormal allele. A large fragment and two small fragments indicate that the individual is heterozygous, having one normal and one abnormal allele.

The difference in pattern of restriction digestion fragments produced can be utilized here to diagnose individuals having sickle cell anemia. It can be used to perform prenatal screening to test the genotype of the fetus and determine if he/she will suffer from the disease. However, not all mutations will eliminate or create a restriction site. The use of this technique is therefore limited.

16.2 Biotechnology to Study Plants

Plant breeders often select plants having favorable characteristics and use their seeds for further breeding. This is nothing but manual selection of traits to ensure a more robust crop with desirable traits. Biotechnology can be used for the same. Genes that confer the desirable trait from different organisms can be isolated and introduced in the genome of the plant of interest. We can increase nutritional value of crops, obtain insect- and herbicide-resistant crops, and also increase their yield.

16.2.1 Transgenic Crops

Plants whose genome have been modified by genetic engineering techniques to introduce a desirable trait are called transgenic plants. Vitamin A deficiency is a major health concern especially in Africa and Southeast Asia. Vitamin A deficiency may lead to blindness and also weakens the immune system. In these countries, vitamin A-rich foods like milk, eggs, and fish are generally expensive and not within the reach of the poorer sectors. Rice is relatively cheaper and is a staple diet in these parts. Though rice is a good source of carbohydrates, it lacks micronutrients like vitamins and minerals. Golden rice was created in 1999 with an intention to fortify rice crop with vitamin A. This is called biofortification. The technology was

provided free of cost by its investors for use in the public sector rice varieties without any limitations on use of recombinant crops and seeds.

Genetic engineering in rice plant allows it to biosynthesize β carotene which is a precursor of vitamin A. β carotene is produced in the endosperm, giving the rice its characteristic golden-yellow color. For this, two genes were introduced in the rice plant, Phytoene synthase (*psy*) from daffodil and phytoene desaturase (*crtl*) from soil bacterium *Erwinia uredovora.* These genes are placed under endosperm-specific promoter. The introduction of these gene leads to the synthesis of lycopene which is naturally converted to β carotene by the plant's endogenous enzymes. In 2005, golden rice 2 was announced which produced significantly higher amounts of β carotene than the original golden rice. Golden rice received its first approval in 2018 from Australia, New Zealand, Canada, and the USA. Golden rice is a cost-effective way to provide essential nutrients to a large population. It is also safe for consumption.

16.2.2 Herbicide Resistance

A large portion of the crop yield is destroyed due to weed infestation. These unwanted weeds can be removed by herbicides. The herbicides, however, may also affect the crop plants. They may get washed into the water and deposited in the soil which also adversely affects the environment. Herbicide-resistant plants will protect plants from herbicides while allowing surrounding weeds to be destroyed.

Glyphosate is an herbicide which has the ability to kill plants by inhibiting an enzyme called EPSP synthase which is present in the chloroplast. This enzyme is important for the synthesis of essential amino acids, and without this enzyme, plants are unable to survive. This herbicide does not affect humans and is effective at low concentrations. It is also rapidly destroyed by soil microorganisms. EPSP synthase is also present in bacteria and essential for their survival. There is a strain of *E. coli* that is however resistant to glyphosate. The *EPSP synthase* gene of this resistant strain can be used to confer resistance against glyphosate in crop plants. To do this, the *EPSP synthase* gene from glyphosate-resistant *E. coli* was cloned into a vector under a plant viral promoter sequence and upstream of the plant's transcription termination sequence. This recombinant vector was introduced into the bacterium *Agrobacterium tumefaciens*.

Discs were cut out from plant leaves and infected with *Agrobacterium tumefaciens* carrying the vector. Due to infection with bacteria, the plant tissue developed calluses which consists of unorganized plant parenchymal cells. These callus cells were tested for their ability to resist glyphosate. The calluses which were able to survive were further cultured and grown into transgenic plants. These plants were exposed to high concentration of glyphosate, and only those plants which were able to produce the resistant EPSP synthase gene in high quantities were able to survive and the others died.

Glyphosate-resistant corn and soybean have been created in this manner and are available in the USA and other countries since its introduction in 1996.

Unfortunately, persistent use of herbicides due to resistant crops have now led to the evolution of herbicide-resistant weeds. Weeds are developing resistance mechanisms for a large number of herbicides. We therefore need more studies pertaining to understanding evolution of resistance and sustainable solutions addressing these issues.

16.2.3 Insect Resistance

Just like weeds, insects also impact crop production to a large extent. With growing human population, it is essential to optimize our food production to meet the demand. Creating insect-resistant crops have led to an increase in overall yield of crops like corn, potato, and cotton. It has also cut down on the use of insecticides which may have harmful effects on humans.

Genetically modified crops containing δ-endotoxin also known as Cry proteins from *Bacillus thuringiensis* (Bt) were introduced in the mid-1990s. Most of the recently produced insect-resistant strains contain multiple Cry proteins which are toxic to Lepidoptera and Coleoptera species. The Bt toxin when ingested by the insect is solubilized in the midgut where it gets proteolytically cleaved at the N terminal to an active form. The active molecules bind to a receptor in the epithelial cells of the midgut. This induces formation of pores in the membrane resulting in osmotic lysis and cell death which eventually kills the insect. In the transgenic plants, Bt toxin is expressed directly in its active form. Transgenic insect-resistant crops have had a major beneficial impact on agriculture by improving crop yield and reduction of pests.

16.2.4 Production of Biofuels

With rapidly depleting sources of fossil fuels and its negative impact on the environment, it is essential to look for alternate sources of energy. Biofuels are one such source which can fully or partially replace the use of fossil fuels. The first generation of biofuel utilized sugarcane or sugar beet for production of ethanol through fermentation. Different alcohols, for example, butanol, can be produced by applying different fermentation process. The first generation of biofuels utilized crops which can also be used as food or feed, thereby increasing their demand and creating shortage of food crops. With the advancements in biotechnology, second generation of biofuels were developed which reduced reliability on food crops. Lignocellulosic biofeedstock was used for this which was a less expensive biomass. The cellulose and hemicellulose present in this biomass is broken down into its constituent simple sugars through enzyme-catalyzed hydrolysis. These are then fermented with the use of microorganisms to produce alcohol. These fuels are also known as cellulosic ethanol or cellulosic biobutanol. Newer biofuels now referred to as the third generation of biofuels are produced by using algae biomass with microbial enzymes. Microalgae offer many advantages because they are able to

rapidly double their biomass. They also are rich in oil, and some of them have oil content about 80% of their dry weight. They can also be grown in waste or non-potable water. This solution also has its own problems. It requires a high density of algae culture over large surfaces which is not very optimum. Apart from algae, other prokaryotes or eukaryotes which show high accumulation of oils are also being considered for production of biofuels.

There are multiple stages at which use of biotechnology can increase efficiency of production of biofuels. Microbial enzyme activity can be enhanced and improved via biotechnology to achieve better microbial digestion and fermentation of biomass. The use of genetically modified organisms for pretreatment or conversion to ethanol can boost productivity. The cell wall and composition of lignocellulose in plants being used for production of biofuels can be modified using biotechnology to increase the yield of ethanol. Using herbicide- and insecticide-resistant plants can improve the biomass produced. Creating plants that can survive and grow in harsh soil or weather conditions will allow plants meant for biofuel production to be grown on nonarable land. This will allow arable land to be used only for crop production.

16.3 Biotechnology to Study Pharma Product

Biotechnology can make use of the fact that all organisms contain the same nucleotides and follow the same code for its conversion to proteins. This allows a human gene to be inserted into a plant or a bacteria to produce the protein of interest. The bacteria or plants can then be cultured on a large scale, and protein of interest can be isolated and purified. Thus, the bacteria are acting as molecular factories producing the protein of interest on a large scale.

16.3.1 Recombinant Insulin

Insulin became one of the first human protein to be produced using recombinant DNA technology and licensed for therapeutic use in 1982. Insulin is required in the body for glucose metabolism, and lack of insulin leads to diabetes. Insulin can be given to diabetics externally which allows them to maintain their blood glucose levels. Insulin is produced by cells in the pancreas in the form of a precursor peptide known as preproinsulin. This gets cleaved, and some amino acids are removed from its center and at its end. This leads to formation of two polypeptide chains called A and B which are held together by disulfide bonds.

Before the use of rDNA technology, insulin was produced by extracting it from porcine or bovine pancreas. There were two issues associated with this method. One was that although animal insulin was chemically similar to human insulin, it is not identical. This difference led to immune reaction from patients causing inactivation of insulin and inflammation in many patients. Secondly, production of insulin from animals was very expensive and difficult to obtain in large quantities. Both these issues were prevented by using bacteria to produce human insulin. Since

posttranslational modifications differ between bacteria and humans, the insulin gene was not inserted as is in *E. coli.* Instead, the polypeptides were synthesized separately using two different plasmids. Polypeptide chain A has 21 amino acids, and polypeptide chain B has 30 amino acids. The genes for these two chains were constructed using oligonucleotide synthesis (Fig 16.2). The genes were inserted in the vector adjacent to a lacZ gene to produce fusion proteins. The fusion proteins consisted of polypeptide A or B fused to β galactosidase (product of *lacZ* gene). The vector also contained a gene for antibiotic resistance which was useful in selection of bacteria containing the vectors. These recombinant bacteria were cultured in large-scale fermenters. From the bacterial extracts, fusion proteins were isolated and treated with cyanogen bromide to remove the β galactosidase (Fig 16.2). The insulin chains were then purified and mixed. The chains were able to spontaneously unite to form the active molecule. This insulin was capable of being purified, packaged, and used in therapy.

16.3.2 Recombinant Growth Hormone

Human growth hormone is produced in the body by the pituitary gland and released into the blood. It performs a host of biological functions like metabolism of proteins, carbohydrates, and lipids as well as cell proliferation and immune regulation. Growth hormone is essential to ensure proper growth and stature, and its deficiency may lead to dwarfism. Dwarfism can be treated by administering growth hormone. It is also used in the treatment of burns, bone fractures and disintegration, and gastric burns. Till mid-1980s, the only source of human growth hormone was human cadaver tissue. The supply for this hormone was therefore limited. There were also reports that associated pituitary-derived growth hormone with Creutzfeldt-Jakob disease. Recombinant DNA technology provided a means of safely producing abundant amounts of human growth hormone (hGH).

hGH is produced by the pituitary as a prehormone. It contains a hydrophobic leader peptide of 20 amino acids. During secretion, this leader peptide is removed to produce the mature hormone of 191 amino acids in length. To facilitate the direct expression of mature hormone, cDNA coding for the leader peptide was removed. The cDNA for growth hormone was partially chemically synthesized and partially derived from the actual mRNA of human pituitary. This was significant because unlike insulin, which is only 51 amino acids long, chemically synthesizing entire mRNA for such a large protein like hGH would have been difficult. This cDNA was cloned into a plasmid which was introduced into a strain of *E. coli.* Since nonglycosylated form of the hormone was active, prokaryotic system was preferred for its production. The recombinant bacteria is grown in large quantities, and growth hormone produced was isolated and purified.

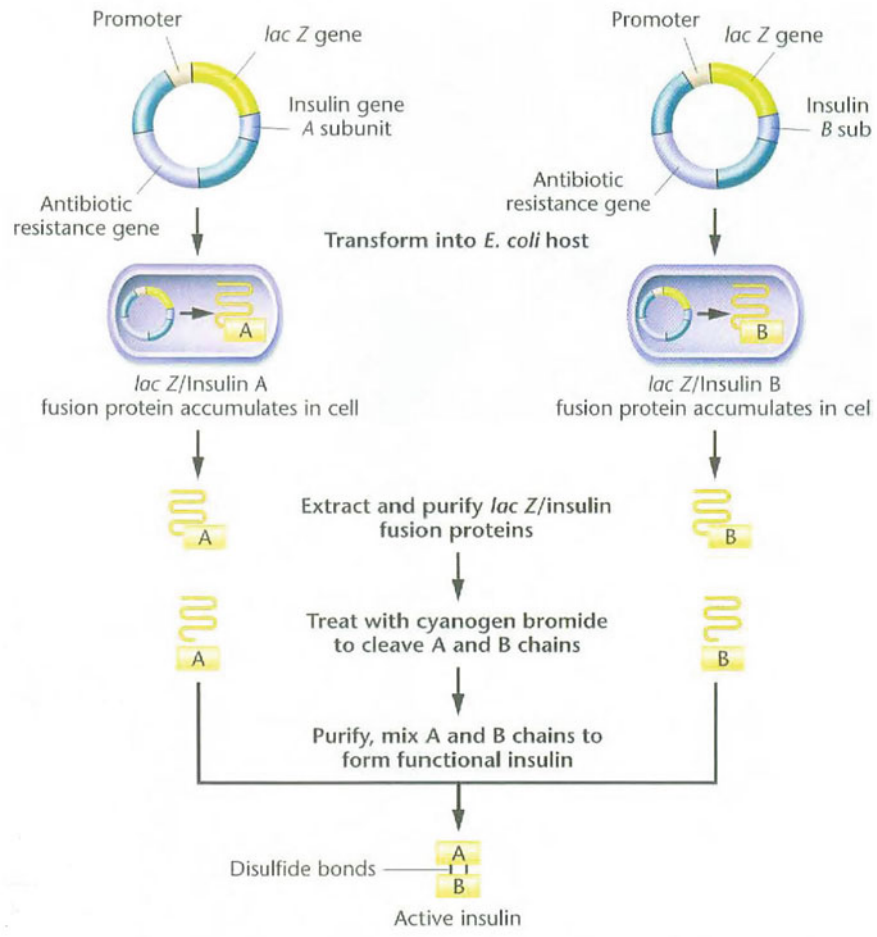

Fig. 16.2 Production of recombinant insulin. The genes coding for polypeptide chains A and B of insulin are inserted in a bacterial plasmid fused to the *lacZ* gene. The plasmid is transformed into E. coli and cultured in large-scale fermenters. The fused lacZ/insulin A or lacZ/insulin B fusion protein accumulates in the cell from where it is extracted and purified. It is further treated with cyanogen bromide to separate insulin from β galactosidase. The A and B chains are purified and mixed to form the active insulin protein

16.3.3 Recombinant Vaccine

Traditionally, vaccines have been produced by either killing or attenuating the pathogen. This inactivated pathogen is no longer able to cause the disease but stimulates the immune system to produce antibodies against it, providing protection against future infections from the pathogen. Using recombinant DNA technology, only the peptide that acts as the antigen can be produced which can elicit an immune response. These are called subunit vaccines. They provide protection against

possible risk of live or attenuated vaccines like reversal of attenuation and virulence in susceptible hosts. Additionally, the recombinant proteins can be produced in large quantities.

Recombinant protein vaccine currently in use is against hepatitis B. Hepatitis B virus (HBV) infects liver cells causing chronic infection and cirrhosis. The hepatitis B surface antigens (HBsAg) are produced in yeast expression system. Yeast cells are capable of making posttranslational modifications in proteins. Protein products that require glycosylation can therefore be produced in the eukaryotic yeast. It also secretes the HBsAg into the supernatant of the culture allowing for easier purification. The HBsAG when administered assemble into viruslike particles which are highly immunogenic and capable of eliciting an immune response. Recombinant vaccine against human papillomavirus (HPV) has also been developed which contains the L1 major capsid protein. Many subunit vaccines, however, have weak immunogenicity on their own and need to be administered with an adjuvant to promote long-lasting and strong protective immune response.

Genetic engineering can also be used to create live recombinant vaccines. The idea is to use a live recombinant vector containing heterologous antigen encoding genes. The live vector can elicit a strong immunological reaction against its own antigens as well as toward the heterologous antigens being expressed. An example is the work being done on recombinant BCG vaccine. The vector *M. bovis* BCG provides many advantages. It is safe and can elicit T-cell-mediated immunity. Recombinant BCG (rBCG) expressing foreign antigens for various diseases like malaria, tuberculosis, and HIV is being developed. For example, rBCG expressing HIV antigens has been shown to produce specific antibodies against HIV, produce interferon γ, and induce T helper and cytotoxic T cells. Efforts are also being used to utilize viral vectors for expression of heterologous antigens.

Direct injection of DNA plasmid into the muscle to induce immune response is also another approach that is being studied as a vaccine system. In a DNA vaccine system, the antigen can directly be expressed by host cells in a manner similar to viral infection. They have been shown to elicit both humoral and cell-mediated immunity. DNA vaccines avoid problems associated with producing recombinant proteins like inaccurate folding of protein and purification costs. DNA vaccines, however, have their own set of problems like low efficiency of transfection of cells in vivo, production of anti-DNA antibodies, and possible integration into host genome. Although successful in animal models, DNA vaccines have shown limited immunogenicity in primates. Ongoing efforts in increasing its effectiveness include strategies like augmenting gene expression, co-expression of cytokines and other molecules that boost immune response, and formulations to protect DNA from degradation.

Even after production of purified vaccine, there are challenges involved in administering the vaccines especially in developing countries. Absence of facilities for manufacturing, transportation, and storage pose challenges for vaccination in remote places. To circumvent these issues, creation of edible vaccines was proposed. Edible vaccines are transgenic plants or animals that express the antigen of a pathogen and, when consumed, can elicit an immune response in the body. These

vaccines would provide the advantage of being inexpensive, not require special storage conditions, as well as not require trained medical personnel for administration. Transgenic tobacco plants having leaves expressing antigenic subunit of hepatitis B virus have been produced. This is just a model system, and for actual use, the gene for HBV would be transferred into a food plant. In another example, rabies antigen was expressed in spinach and fed to volunteers. Eight of the fourteen volunteers showed high expression of rabies-specific antibodies. Edible vaccines are undergoing further studies and clinical trials.

16.3.4 Recombinant Protein

The proteins mentioned above including recombinant insulin, recombinant growth hormone, as well as the vaccines are examples of recombinant proteins. The first recombinant protein was somatostatin produced in 1976. From there, a host of other proteins have been cloned and expressed in heterologous systems from bacteria to plants which are widely used in therapy today. Recombinant proteins provide the advantage of producing large number of products at lower cost. It is also safer as it avoids transmission of infection from animal- or cadaver-derived products.

The X-linked disorder hemophilia occurs due to a lack of clotting factors. Hemophilia A is caused by a defect in factor VIII, and hemophilia B is caused by a defect in factor IX. For a long time, plasma-derived clotting factor concentrates were used for the treatment of hemophilia. However, due to lack of proper screening methods, they were discovered to be also transmitting blood-borne viruses including HIV and hepatitis. In 1984, the gene for factor VIII was successfully cloned, paving the way for its production using recombinant technology. By 1992, factor VIII was commercially produced and licensed for therapeutic use. Factor IX was commercially available for people with hemophilia B in 1997. These recombinant proteins provided a safe and effective method for treatment of hemophilia. Table 16.1 lists the recombinant proteins used in clinical treatments.

A range of recombinant proteins including hormones, antibodies, enzymes, and vaccines have been produced which have been used for therapy in certain diseases.

16.4 Biotechnology to Study Animals

Applying novelty of molecular genetics in the field of biotechnology, the study of animals has become one of the important branches of study that includes a vast area of related topics such as animal health, animal in research, transgenic animal, gene pharming, etc. Animal biotechnology is an important branch of biotechnology that includes a wide range of topics such as use of animal in research, clones, transgenic animal, gene pharming, and animal health. In the past history of human beings, animals have been bred to the enormous purposes over 1000 years, such as breeding of (i) working dogs to flock pasture animals, (ii) cows produces more tender and tasty meat, (iii) horses that are easier to tame, and so on. All these things have been

Table 16.1 List of recombinant proteins and their therapeutic applications

Proteins	Therapeutic application
DNase I	Cystic fibrosis
Coagulants factors	Hemophilia A and B
Erythropoietin	Anemia in chronic renal disease
Glucocerebrosidase	Gaucher disease
Growth hormone	Pituitary dwarfism
Insulin	Diabetes
Alpha interferon	Some leukemias, Kaposi's sarcoma, and hepatitis B and C
Gamma-1b interferon	Chronic granulomatous disease
Interleukin-2, interleukin-3, and interleukin-4	Immunotherapy of cancer
Tissue-type plasminogen activator	Acute myocardial infarction and massive pulmonary embolism
Antibodies for cellular immunotherapy	Neoplastic processes
Vaccines	Influenza and hepatitis A and B
Monoclonal antibodies anti-antibodies	Lupus and rheumatoid arthritis

possible by the use of biotechnology. Notably, animal biotechnology is the core result of genetic engineering which includes recombinant technology for transferring exogenous or foreign DNA into germ line. Genetic engineering is a modification of an organism's characteristic by adjusting its genetic material.

Use of Animals in Research

Animal models are an essential part of primary research. The need of medical assessment of various drugs or products produced for disease treatment is supported by the use of animal models. It has been noticed that working on computer models and in vitro cell studies would not reproduce the result as an organism does. Thus, working with computer models and cells will be a supplement to animal research. Regardless of initial failure, recent development in animal biotechnology has changed the fate of agriculture, medicine, and animal breeding, and a prompt effort to conserve the endangered animals to save the flora and fauna is remarkable.

It's very well known that for any new product to be approved for human use, the manufacturer must first demonstrate that it's safe, and therefore, trials on cell culture, in live animals, and on human subjects are facilitated by virtue of this field. The most required animal used for testing is a pure-bred rat, mice, primates, etc., prior to trial on human subjects. Zebra fish, a hardy aquarium fish, is extremely used for valuable research, while dogs are enormously used for study of cancer, heart disease, and lung disorders. Importantly, HIV and AIDS like lethal studies are always conducted on monkey and chimpanzees.

16.4.1 Transgenic Animals

Transgenesis (transfer of significant genes) or transgenic animal is one of the most significant and exciting research tools in biotechnology. Through this advanced technology, inserting new genes in livestock for economical important characteristics such as fertility, resistance, or tolerance to the environmental stress has become a major revolution in animal breeding. Another significant application that transgenic animal brought to us would be producing clotting factors in the milk of domestic livestock.

To elaborate the literal definition of transgenic animal is a deliberate modification of genome of one animal by introducing DNA through recombinant DNA technology and then must be transmitted to the germ line that homogeneously spread to every cell and pass over through the next generations. It means that gene can be altered artificially so that some characters of animals are changed and can pass to all offspring.

In modern history, fundamental to these techniques is an ability to culture early embryos in vitro which allowed a variety of manipulations in the genome to be performed. One of the first genetic manipulations of the embryo is the production of chimeric mice achieved by mixing an early stage of development (eight cells) to develop a single form of embryo carrying chimeric characteristics in adult. Likewise, knocked-out mouse is also another great achievement in the history of biotechnology. First ever knocked was created by Mario R. Capecchi, Martin Evans, and Oliver Smithies in the year 1989 who won the Nobel Prize in 2007 for introducing knockout mice technique. Knockout generally refers to a deletion (knockout) of a gene to inactivate its function.

Reasons that Promote the Production of Transgenic Animal

1. Some transgenic animals are being produced, keeping the goal in mind for specific economic traits which can produce milk containing particular human proteins like protein C (a potential coagulant) and fibrinogen (plasma glycoprotein).
2. The primary goal was to produce advanced cattle breed which is subsequently followed by the second goal of production of disease model. This disease model shows disease symptoms as the result of transgenesis. This model has been manufactured to study several disease studies. For example, OncoMouse® and 3× Tg AD mice are produced to study various human cancer and Alzheimer's disease. These animals successfully show the symptoms of the disease alike humans.
3. One ultimate purpose is to study the interaction between environment and genome. This can be possible with transgenesis that provides a unique opportunity to design unique models for such study.

Methodology for Producing Transgenic Animal

To date, there are several methods in used, but the most efficient methods are as follows:

(a) Recombinant retrovirus.
(b) Embryonic stem cell.
(c) Pronuclear DNA microinjection.

Recombinant Retrovirus: Recombinant retrovirus technique is mostly useful for eukaryotic genome. Retrovirus is a family of virus in which genetic material is RNA and transcribed into DNA by specific enzyme "Reverse Transcriptase (RT)." Of this significant characteristic, RT becomes an essential tool for cloning or transgenesis of eukaryote genome. Eukaryote genome cannot clone in directly bacterial cell because it contains additional introns, which make the genome heavy and lengthy which cannot transfer to the bacterial cells. Thus, mRNA from the desired gene will transfer to this virus which later will be transcribe into single-strand complementary DNA (cDNA) by RT. DNA polymerase will transcribe the second strand, complementary to the first strand to complete the DNA amplification. A direct gene transfer to retrovirus will be followed by injecting virus to the host cell and cultured to maintain the cell line, and cultured cell will develop to a mature embryo. Retrovirus method is mainly used to transfer genetic material into host cell to produce the chimeric characteristics of transgenic animal containing diverse genetic constitution from the donor.

Embryonic Stem Cell

This is one of most common techniques and often being used to produce successful transgenic animal. This method is first initiated with isolation of totipotent cell (a cell that has a capacity to develop into any specialized cell) from the embryo. The gene of interest is being inserted in vitro into these cells (totipotent) and then incorporated into the host embryo resulting in a chimeric animal (Fig. 16.3).

DNA of interest is isolated and injected to the embryonic stem cells by gene delivery method, and cells were cultured and maintained carefully. This embryonic stem cell containing DNA is injected to the blastocysts and implanted to the uterus of foster mother. The first generation will be heterozygous, and mating with wild type will produce the homozygous transgenic strains.

Pronuclear DNA Microinjection

First time, this technique was described by Gordon et al., and mice were the first transgenic animals produced through this method. Male and female pronuclei (nucleus of sperm or egg cell) are microscopically spotted after immediate hours of sperm entry into the cell.

The gene of interest or cluster of recombinant genes in a construct will be subjected to microinject into either of these pronuclei (Fig. 16.4). This manipulated cell is first needed to be cultured in vitro (in a lab, not in an alive organism) to develop to a specific embryonic phase, which is further transferred to the recipient female. The recipient female is always a pseudopregnant that promotes the hormone stimulation in the body to make her uterus receptive to the embryo that has to be transplanted in it. However, transfer of gene by DNA microinjection would not be

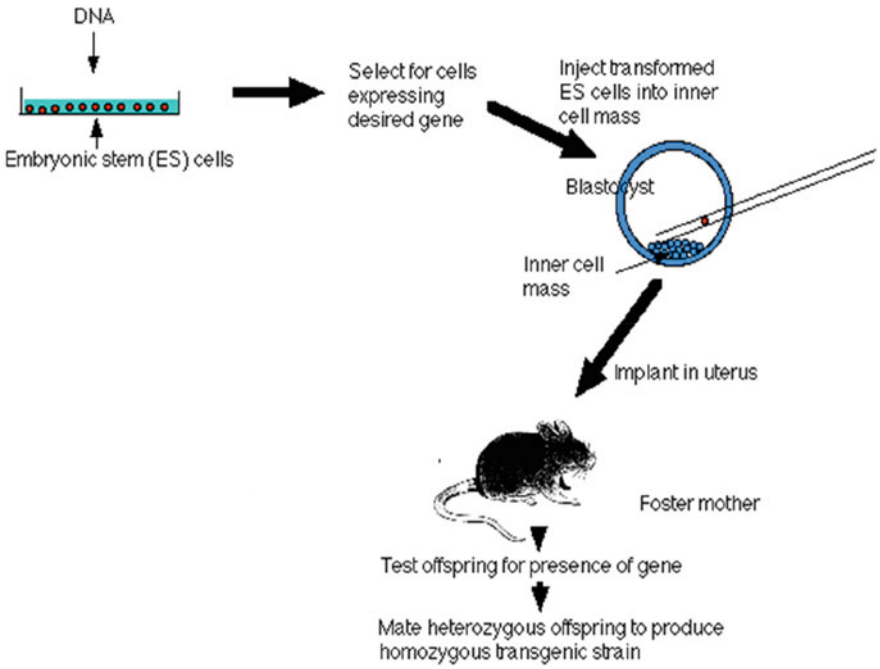

Fig. 16.3 Embryonic stem cell-based transgenic method

homogeneous since it is a random process, so it's not necessary that all pups will be born with expressed desired gene.

Application of Transgenic Animals in Human Welfare

Disease Model: For ages, mice model has been depicted as potential source for studying human disease since mice shares physiological, anatomical, and genomic characteristics with human. To study most effective disease such as cancer, AIDS, and Alzheimer, transgenic animals have been produced to exhibit similar symptoms in order to understand the basic fundamentals of these disease. In short, transgenic animals enable scientists to understand the role of genes in specific diseases.

Growth. Transgenic pigs and sheep are being produced with increased growth and heavy body composition. This attempt had been achieved by transfer of gene that regulates growth hormone. Delivery of proteohormone (peptide hormone) made it possible.

Quality of Animal Products. Improved quality and composition of animal products can be achieved by respective gene transfer. A model for this hypothesis is being proposed by Mercier in the year 1987. He proposed a model that is lacking lactose content in the milk on purpose. Practically, sheep and cattle had been produced carrying lactose gene but combined with udder specific promoter. This construct results lactose degradation into the end product. Thus, milk lacking lactose will be useful for a large population that are suffering with lactose intolerance.

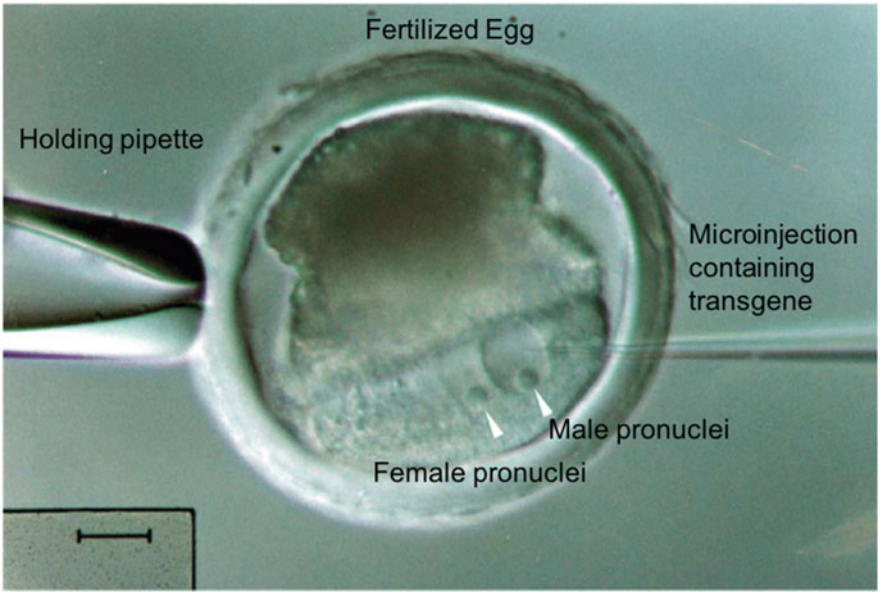

Fig. 16.4 DNA microinjection in pig embryo to inject transgene (image taken on scale bar 20 μm). A DNA construct or recombinant construct of transgene was prepared and injected to the pronuclei (nucleus either egg or sperm cell) with needle or microinjector. This is further followed by cultivating into matured embryo and implantation to the foster mother

Researchers also have produced transgenic animals that promote cysteine synthesis (an essential amino acid) in the animals that enhance the woo growth particularly.

Gene Pharming. Pharming seems a misspelled word for farming, but it isn't at all. This word comes from two different word "farming" and "pharmaceutical." Thus, pharming denoting here is the production or farming of significant genes or proteins by means of secretion in the transgenic animal's blood, milk, saliva, eggs, etc. Altering the gene makeup (modifying its own DNA or splicing) of an animal through transgenesis or transfer of a particular gene for production of valuable proteins for human purpose leads to the idea of gene pharming. In this direction, tissue-specific promoter inducing the protein production in domestic animal is a reliable source for human needs. Therefore, remarkable efforts have been made by scientists, in particular direction to use animals as bioconversion system.

In the year 1987, Gordon et al. and Simons, McClenaghan, and Clark successfully demonstrated that human T-PA (tissue-specific plasminogen activator, to treat the blood clotting) and sheep beta-lactoglobulin were, respectively, expressed in the milk of transgenic mice.

Industrial Applications

Two scientists from Nexia Biotechnologies, Canada (2001), had spliced a spider gene into the cells of lactating goats. Eventually, they observed that goats started to

produce silk in a form of tiny strands from their body along with their milk. The amount of silk was quite enough to commercialize it. This strand was subjected to be extracted and weaved into thread that would be useful for manufacturing objects like military uniform, tennis racket strings, etc.

In 1997, the first cow "Rosie" was produced as a transgenic cow that secreted protein-enriched milk at 2.4 grams per liter. This cow was more nutritional than normal bovine milk. The milk was containing human gene "alpha-lactalbumin."

Ethical Issue

Beside its application in human welfare, transgenic animals had faced many bioethical issues raised by environmentalist and activist and cannot be ignored by scientist, biotechnology industry, policy maker, and public domain. Those ethical issues and doubts we tried to sum up here are as follows:

1. Why transgenesis does not have any universal or standardized protocol?
2. Should only promising research permit to demand such protocol?
3. Why transgenic animals have to be taken into consideration of human welfare only? What about lab animal welfare or other forms of life?
4. Should transgenic method first needed to be examined on in vitro (cultured cells in the laboratory) before using live animals to reduce animal surfing?
5. Do transgenic animals provide an evolutionary benefit in the right direction or instead will result in a drastic consequence for nature and humans?
6. Should patent policies be legitimate to restrict the free exchange of scientific research?

16.4.2 Improved Reproductive Rate

Animal reproduction through animal biotechnologies have endowed many improvements in agriculturally important traits and livestock in which production of development of genetically improved animals for farming is the primary concern for researchers. The very primitive and first biotechnology tool applied to improve the production was artificial insemination as it is well known that reproductive success is of topmost importance for economic efficiency of cattle production in animal pharming. High productive efficiency is required for efficient milk production and meat production and therefore has an influence on herd profitability. Among the most recent of these emerging technologies, reproductive cloning and production of transgenic animals are the best choice to improve the reproductive rate in animals. Nevertheless, the other novel techniques include synchronization of estrus, in vitro fertilization, multiple ovulation (female released more than one egg in a month by intrauterine insemination), embryo transfer, and cloning, and they are all important potential tool for improvement of livestock reproductivity rate. Researchers have found that it is important to avoid the risk and challenges that impede productivity, reproduction rate, and health with adverse environment conditions. This concern has

been prioritized in animal reproduction system that results in encouragement of the practice of techniques mentioned below.

Estrus Synchronization

This is a technique related to regulate the estrus synchronization of female. Estrus synchronization is basically a manipulation of heating time to reduce for a short period (36 to 96 hr). Such synchronization can be achieved by using of one or more hormones. This technique is one of the competent methods that increase the possibility of animals to breed at the beginning of breeding season.

Artificial Insemination (AI)

AI has been practiced worldwide on large scale from more than half of the century. This is one of the most common and efficient method among all. The purpose of this technique is to determine an efficient bull with maximum fertility rate. It has been proven by several studies that seminal plasma contains fertility-associated antigen which defines the differences in fertility rate between males whose seminal composition is the same though. To this reason, even every bull comprised the same amount and composition of semen but may affect the sperm capacitating, fertilization, or related events. Because of technology boom, in recent years, sperm collection and AI have been improved by the advent of sperm sexing or selection which here refers to a possibility to select the sperm carrying X chromosome or Y chromosome with 85–95% of accuracy. This method is common in some domestic animals such as buffalos. At practical ground, this technique is facilitated by using cutting-edge flow cytometric principle, where fluorescent-labeled X chromosome-bearing spermatozoa can separate from the fluorescent-labeled Y chromosome-bearing spermatozoa. In addition to this, the production of more male progeny and the reduction of sex-linked disease are the most beneficial outcomes of this approach. However, this technique has some limitations including reduced number of sexed sperms and variety of damage to the sperm cells, viz., sperm membrane destabilization. Changes in the competence, thereby, life span of spermatozoa would reduce after fertilization in female genital tract.

Embryo Transfer

This technique is one of the tools which provides a faster rate to livestock and an opportunity in which both male and female has contributed equally. This method involves superovulation which is an important step to increase the oocyte number from the superior donor. The first mammalian embryo transfer was reported by Walter Heape in 1890, while the first birth of calf through this method was reported by Betteridge. The first live calves developed from bubaline embryos (using embryo transfer method) were born in 1983 in the USA and later in India.

The essential stages for this method are as follows:

- Donor cow of good pedigree animals and treated with hormones (FSH and LH) to stimulate ovulation and release eggs in large number—multiple ovulation (MO).
- Insemination is performed using semen of a chosen bull.

- Embryos are flushed nonsurgically after 6–7 days of insemination.
- Collected embryos are implanted to the recipient cows whose estrus cycle is at the correct receptive stage due to hormone manipulation.
- Embryo may be frozen and stored for a long time.

In India, embryo transfer and protocols of ET technology are being standardized for cattle, buffalo, sheep, goat, camel, and other species of animals. Embryo transfer is an effective method, and genetic improvement is higher in embryo transfer than AI alone.

In Vitro Fertilization (IVF)

In vitro fertilization is an artificial technique to fertilize the oocytes outside and transfer the developed embryo to the recipient mother. The oocytes can be collected either from slaughterhouse or live animals. This oocyte undergoes the maturation and fertilization in vitro and later will develop into mature and viable embryo. "Pratham" was the first IVF buffalo calf produced in India in 1990. Through this technique, it is now possible to study development process, gene expression, epigenetic modification, and cytogenetic disorder in various species and also provide a model for study of embryogenesis in human. In this method, unfertilized eggs are first fertilized in the laboratory and cultured for a few days until they have successfully developed into early embryos. This embryo is now ready to be implanted in the recipient uterus through a long syringe. It is important to notice that the recipient mother should be in receptive stage of estrus cycle before the implantation. The first live offspring achieved from IVF technology was a rabbit.

Cloning

The literal meaning of clone is an identical copy of any organism or tissue or cell which contains identical copies of genetic information. In cloning, it is possible to reproduce an entire organism from any cell taken from parent organism, and resulted clone is an identical copy of parental organism in every means. The genetic composition of clone is identical to its donor (or parental organism). The main objective of cloning is to increase the number of identical copies of superior livestock to produce high-quality end product although cloning does not change the genetic makeup of the animal.

In nature, cloning is quite usual and frequent. For instance, naturally occurring asexual reproduction in some lower eukaryotes and prokaryotes is similar to those twins reproduced from one fertilized egg.

The major breakthrough in cloning world had appeared in 1996 when Ian Wilmut and his colleagues have successfully produced a clone of sheep named "Dolly" (Fig. 16.5). Dolly was produced from fertilization between cultured adult somatic cell as a donor and enucleated oocyte recipient cell (lacking chromosomal DNA).

The cloning process includes the following steps: (i) Chromosomal DNA will be removed from a mature oocyte. (ii) This egg nucleus will be replaced by the somatic cell nucleus from the donor which is supposed to be cloned. The donor cell now will be fused with enucleated oocyte, and reprogramming of somatic cell genome will be

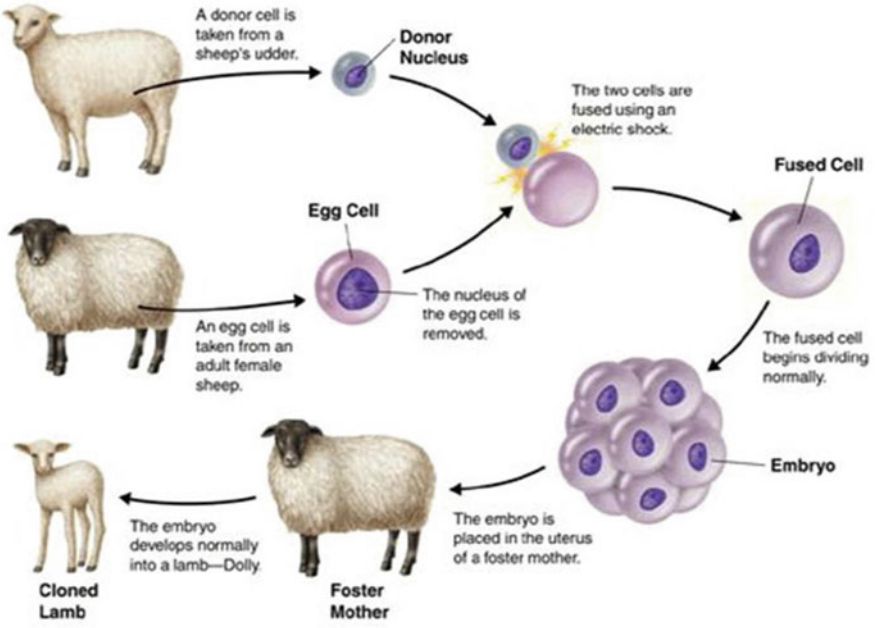

Fig. 16.5 Cloning method to produce "Dolly" sheep. Enucleated oocyte is fused with somatic cell by electric shock. Fused cell developed into fertilized egg and matured embryo, followed by implantation into foster mother. This embryo has a genetic information identical to the donor cell and appeared into Dolly sheep which is a clone

activated as of embryonic genome. Activation of reprogramming can be induced by chemical or electrical pulse. Reconstructed cloned embryo is further cultured, and this viable embryo is transferred to synchronized recipient which carries the live cloned offspring until parturition.

In addition to the genetic cloning of valuable animals, it is also possible to generate the genocopies of exceptional animal from those who are incapable of reproduction. For example, in the case of steers or animals that are dead, cells can be stored.

16.4.3 Improved Health

At the global level, increasing figures of population simultaneously are adding to the requirements of overproduction of high-quality protein, meat, milk, and eggs as essential needs for life, but sustaining a good health and protection from adverse environmental factors which can affect the livestock's longevity are quite questionable and the primary concerns to be taken. Apparently, biotechnology plays a significant role in the diagnosis of livestock diseases and genetically transmitted conditions which reduce animal's health and productivity drastically. Nowadays,

advanced biological technique produces cheaper and more efficient drugs because natural drugs from natural source materials are excessively expensive. In this scenario, drug production utilizing applications of genetic engineering in either microbial or tissue culture system has become a wise decision eventually in favor of human and animal's health. Largely produced human insulin, human growth hormone, and plasminogen activator (used in treating heart disease) are the biggest success in animal biotechnology.

16.4.3.1 Vaccines

Immune system of animals is induced by arrays of vaccine to produce antibodies targeting disease or infection. Emerging recombinant DNA technology has introduced the possibilities to develop a recombinant antibody and vaccines commercially available at low cost. Empirical knowledge in vaccine development and relationship with immune system makes scientist and industries to produce massive range of vaccine that can perform better to boost the body's immune system than the conventional vaccines. Data from the trial history suggested that these engineered vaccines are way safer than the traditional vaccines which may develop the "revert effects" (inactive non-virulent can revert into virulent and cause disease). Therefore, such genetically engineered vaccines have been developed to eliminate this threat to animal health.

Biotechnology industry is booming day by day to produce entirely new engineered vaccines and their new ways of uses. Vaccines have been developed for many purposes, i.e., modulator for growth hormone to increase the growth rate, additives as a feed conversion, stimulator in milk production, enhancer to improve animal carcass and meat quality, and modulator in reproduction system to enhance or to suppress the reproduction rate.

Recombinant or engineered vaccines are useful for those diseases for which vaccine has been not developed. These vaccines do not contain the dangerous infectious agent unlike the traditional vaccines, and this property makes these vaccines efficiently safer. Production of vaccines are considered as less expensive with mass production, and maintenance cost is negligible since it can be stored even at room temperature.

16.4.3.2 Diagnosis

Examination of poor health in cattle, pets, and other domestic animals is an additional responsibility for farmers and biotechnologist. Improvement in diagnosis methods or in tools makes the situation under control for many poultry firm running around. Nearly a decade, scientists from Japan and Taiwan became in spotlight when they invented the DNA to detect the hereditary weakness in poultry pigs during transportation or in slaughterhouse. This test has identified the gene expression associated with "porcine stress syndrome" in pigs. They observed that pig with this gene in active state produce pale and poor quality of meat. Now, it has become an easy job for poultry people to use DNA testing to identify the pig with this active gene and can eliminate during the breeding program to reduce the risk in the offspring.

16.4.4 Feed Additives

The main objective to use feed additives is to enhance the quality of feed for the animal to improve animal's performance and health. Feed additives can be available in many forms, relatively concentrated form, such as vitamins produced by animal or vegetable origin, amino acids, enzymes, minerals, antibiotics and probiotics, and single-cell proteins. For example, yeast products high in protein have been used as a feed additive for many animals: cattle, pigs, and poultry. Rich in nutrition and highly edible, these products also help in creating a healthy balance of bacteria in the digestive tract and prevent bacterial diarrhea. A beneficial bacterial product "phytase," commercially named as "TRANSPHOS," had been used as an inexpensive feed additive. The wide use is to substitute the costly mineral phosphate used as an additive in the feed of monogastric animals. Similar to this, bacteriocin is another feed additive that had been produced and used to fight against livestock pathogen like Listeria monocytogenes, Staphylococcus aureus, etc. Lysine is the most essential supplement for animal growth, and in routine life, animals hardly get this supplement in enough amount. This L-lysine monohydrate is safe, stable, and edible, being produced in many countries from bacteria through fermentation and added to the feed material to increase the quality of nutrition in feed.

Feed additives are categorized as follows:

16.4.4.1 Antibiotics

Antibiotics contain antimicrobial and antifungal properties, usually of plant or fungal origin produced for pharmaceutical purposes, and can be synthesized in laboratories. Antibiotics are meant to be used for the treatment of infections, but there are a few antibiotics available in the market that can improve the growth of animals and increase the feed conversion efficiency. The most common antibiotic used as feed additive is "ionophore." The function of ionophore includes metabolic role in improvising the production efficiency. These ionophores have general metabolic role within the animal to improve the production efficiency.

16.4.4.2 Enzymes

Applying advance biotechnology, many enzymes are produced at large scale and relatively inexpensive (McDonald et al. 2010). These enzymes are widely being used as a feed additive in a nonruminant and ruminant diet. The primary goal was to improve the nutrition value when poor quality and inexpensive ingredients are incorporated during feeding the animal. Many enzymes are available commercially including phytase (phosphorus digestion), hemicellulose (plant cell wall digestion), and cellulase/xylanases as a feed additive. Also, digestibility of amino acid can be improved with phytase supplementation.

16.4.4.3 Probiotics

While antibiotics are designed to be involved as a feed additive to treat any bacterial infection, on the other hand, probiotics are being used to improve the strength of certain strains of bacteria in the gut. Probiotics basically are a microbial population,

which enhance the activity of the digestive system. Apart from all, these probiotics (microbial population) also have been observed to produce vitamin B complex and many digestive enzymes, for protection against toxins, to increase intestinal mucosa immunity, etc.

16.4.4.4 Beta-agonists

Beta-agonist is a natural or synthetic organic compound that shares a common chemical structure with phenethanolamines. Therapeutically, this compound is involved in massive use to maintain smooth muscle mass. Beta-agonist is a type of metabolic modifier which means such compounds modify the metabolism in specific and directed way. These compounds show overall effect on productive efficiency (weight gain or milk production), improving carcass composition (lean vs. fat ratio), increasing milk yield in lactating animals, and decreasing animal waste per production unit. Two main compounds that are popular and commercially available are somatotropins and beta-adrenergic. Such compounds are widely used as feed additive to improve the nutrient amounts in feeds and the productivity of livestock.

16.5 Genetically Modified Organisms (GMOs)

Genetically modified organisms (GMOs) are completely transformed animals whose genetic material has been commodified in a way that it could not occur naturally. It is now possible by cutting-edge application of genetic engineering, and molecular biology together has been involved in modification of genetic makeup of any organism.

In the history of GMs, Europe is the leading place that was producing cloned and GM animals throughout the 90s. The most famous example of genetically modified sheep named "Dolly" had been developed through the cell nucleus transfer from the differentiated cell. This first genetically modified animal was produced at Roslin Institute in Scotland. Afterward, a genetically modified bull named "Herman" was produced by a Dutch biotechnology company "Gene Pharming Europe." The purpose of producing genetic modification was aimed to generate a subsequent trait of female offspring that would be able to produce milk proteins like lactoferrin in their milk and would be an excellent source for food, nutraceutical, and pharmaceutical purposes. Other examples including some experimental animals like genetically modified mouse, pig, fish, and chicken have been developed within European institutions. These animals have been produced with specific advantages and benefits to food production and other areas of application.

Genetic modification of organisms can be understood by the following categories:

- Green genetic engineering (or agrogenetic engineering): Established to develop genetically modified plants in agricultural firms and food sector.
- Red/yellow genetic engineering: Aim to develop medical diagnostic tool, gene therapy, and essential drugs like insulin and vaccines.

- Gray/white genetic engineering: Involved in production of enzyme or chemicals by utilizing genetically modified microorganism.
- Genetically modified animals: Produced genetically modified organism to the purpose of improvement in the quality of food, milk, meat, and dairy product.

The list of few commercially available genetically modified organisms (GMOs) is as follows:

16.5.1 Glowing Fish

Glowing fish, commercially known as GloFish, is a genetically modified fish and initially had been developed for pollution detection instead for consumption. Naturally bioluminescent fishes are found in the sea and inhabitant of darkest part of sea. Scientists from Singapore adapted this idea and developed a fluorescence transgene that is sensitive to sea pollutant. They had transferred the naturally derived fluorescence gene-incorporated pollutant-sensitive biosensor that will detect the environmental pollutant. Other than its scientific role, GloFish also had captured the public interest for decoration purpose, and so it has become commercial for public to keep in home aquarium. Since 2003, it has become commercial under the license of Yorktown Technologies.

Currently, there are 12 lines of GloFish in the market, including tetras, zebra fish, and barbs, in such colors as electric green, moonrise pink, and cosmic blue.

16.5.2 GM Salmon

Besides insects, fishes had been genetically modified to provide good source of dietary consumption to the consumers. The first genetically modified fish in the market is the AquAdvantage salmon. After three decades of its production, in August 2017, it has become available in Canada. This GM salmon is produced by AquaBounty Technologies which is twice in size and grown in same period of non-GE salmon. GE salmon is produced by recombinant gene containing growth hormone gene from Chinook which is activated by adjacent gene from ocean pout (a fish). AquAdvantage had been approved by US FDA and declared safe to eat. This fish also contains same nutrition as other non-GE Atlantic salmon. There had been no biological side effects observed according to the FDA reports.

16.5.3 GM Mosquito

Mosquitoes are worldwide known to be vector for detrimental diseases such as malaria, dengue, chikungunya, and Zika. In earlier past, to combat these epidemic diseases, scientists were on the mission to reduce the female population.

Scientists at Imperial College London had successfully reduced female population by means of genetic engineering. An enzyme known as I-PpoI is an endonuclease that cut the ribosomal gene sequence (rDNA) in the mosquito. This enzyme specifically destroys the rDNA cluster located on X chromosome. Thus, scientist at Imperial College London had developed the mosquito strain in which sperm cell possesses this enzyme to cut the rDNA cluster located on X chromosome and produced only male offspring. Applying this technology, they were successfully able to produce 95% male offspring which will further inherit this enzyme to the generations.

Another example of GE mosquito is Friendly™ Aedes that had been manufactured at an Oxford-based biotechnology company, Oxitech. This mosquito had been produced by inserting the gene that kills the insect at larval stage. So when male Aedes, which does not consume human blood, mate with wild-type Aedes female, then progeny will be unviable and killed at larval stage before the adulthood.

These mosquitoes had been tested in fields located in different places. First time, Friendly™ Aedes were launched in Brazil (Piracicaba) in April 2015, and a 91% reduction in dengue fever were observed in Dorado district of Brazil after immediate launching in 2016. In total, 12 cases were observed in 2015/2016 that was a huge reduction from 133 in previous years.

16.5.4 Eco-friendly Pig

GM pig had been produced at University of Guelph in Ontario, Canada, in 1999. They develop GM pig that contains phytase enzyme in their saliva. Phytase enzyme digests the plant phytate into phosphorus which is useful for algal growth when the phosphorus is released from the manure of GM pig into the water stream. The origin breed was Yorkshire pigs that genitivally modified for phytase enzyme.

16.5.5 Bird Flu-Resistant Chicken

To fight against most devastating disease for chicken such as bird flu, scientists in the UK had developed transgenic flu-resistant chicken. Thereafter, in another attempt, scientists from University of Cambridge had developed GM chicken with short hairpin RNA. This structure somehow blocks the spread of the influenza virus (mechanism is unknown). Thus, this technology had improved the poultry chicken and environment as well as human health which is prevented by flu infection.

16.5.6 Human Gene Therapy

Gene therapy is referred here as an incorporation of gene to replace a defective gene that might have been involved in many genetic disorders. This is a technique to correct the genetic error which occurs in some genetic disorder like cystic fibrosis,

cancer, ADA, immunodeficiency, etc. First-time gene therapy in human history was performed successfully by William French Anderson, Michael Biase, and Ken Culver in 1990. These guys showed that a severe immunodeficiency, adenosine deaminase (ADA) deficiency, also known as "boy in a bubble disease," can be treated with gene therapy. To spot a bit light over here, ADA is a recessive disease carrying two copies of recessive allele of ADA gene. Normally, two copies of ADA gene promote the production of adenosine deaminase in cells throughout, but error in even one gene will inhibit the conversion of deoxyadenosine (a waste product) into inosine and thus will lead to heavy buildup of deoxyadenosine in the body. This accumulated buildups later undergo phosphorylation, convert into toxic triphosphate responsible for killing T cells, and eventually result in failure of immune system and early death.

Gene therapy can be done in two possible ways:

1. Somatic gene therapy is taking into account that gene transfers into the body cell by means of somatic cell rather than to germ cell (egg or sperm cell). The aim to somatic gene therapy is not to let the gene pass to the offspring in the future but just stay in patient's body till its effective state. Study and trials on somatic therapy has prevailed its success as clinically effective. Gene therapy for ADA that has been discussed above was the first somatic gene therapy in 1990 and 1991 with two patients of ages 4 and 11 years old. Both kids are growing well with the continuity of the treatment. Later in 1992, a 29-year-old woman experiencing familial *hypercholesterolemia*, a genetic condition (defect in the chromosome 19) that is associated with increased cholesterol in the blood due to defective LDL receptor on the liver, was treated with somatic gene therapy. This woman was treated with homozygous FH ex vivo delivery to the liver. This treatment was carried out for 18 months, and liver biopsies demonstrated no discernible abnormalities.

   Consequently, five more patients had been successfully treated with gene therapy since then. After the success of above stories, scientists are focusing on the clinical trial for many other diseases especially chronic genetic disorder and cystic fibrosis.
2. In addition to somatic gene therapy, gene transfer would have been done with germ line cells (eggs and sperm) as well. Gene delivery to the germ cells would modify the genetic makeup of germ line and would definitely pass on the future generations. Germ line gene therapy would be capable to vanish the risk for inherited genetic disorder from the family forever. This type of assurance could be achieved by another method like diagnosis during the IVF if there is any known risk before the implantation. Germ line therapy is a distant prospect and have negative opinion; such therapy is illegal in most of the Europe. However, germ line gene therapy and somatic gene therapy raise different issues. Only somatic gene therapy brings the effective prospect of treatment and have provided a promising cure for few genetic disorders although treatment is complex and success rate is uncertain.

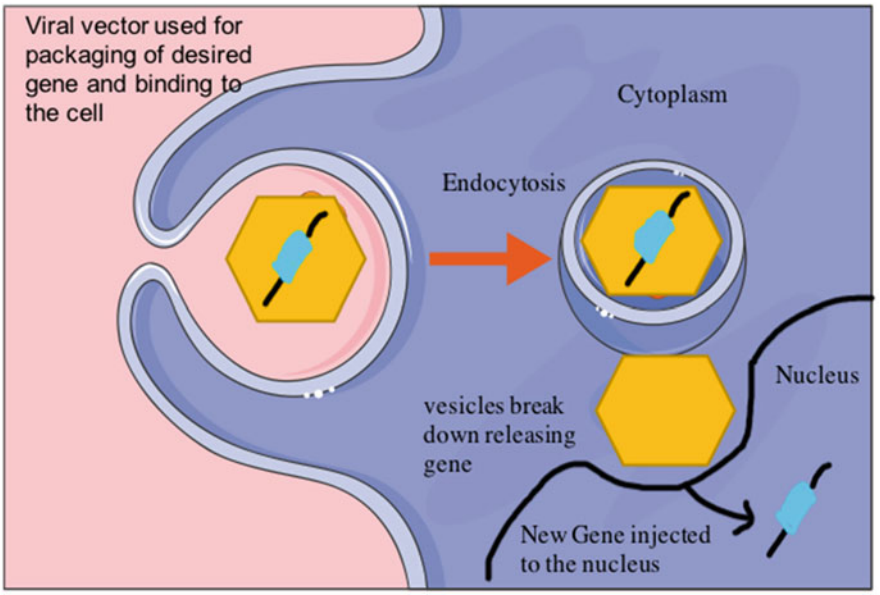

Fig. 16.6 Gene delivery system through a viral vector used in cancer therapy

Despite that techniques are not advanced in gene therapy, researchers are still attempting to develop the methods for gene transfer into the cells in the culture, animals and humans. Within the effect, viral genome was first ever reported as an efficient method for gene delivery into the mammalian cells in the culture. In the beginning of 1980 with the development of retroviral vectors, gene delivery into cultured mammalian cells became widely accepted (Fig. 16.6).

Genome editing has been established as a powerful and efficient tool as a part of gene therapy. Compared to the earlier, current techniques are much more efficient and advanced for modification of DNA. These days, researchers are able to investigate the gene editing in plants, insects, zebra fish, mice, and human cell line in vitro. In theory, gene editing is capable to introduce point mutation to investigate transcription regulation and epigenetic modification. Hence, this technique has a promising contribution in medicine. In recent years, genome engineering is advanced by introducing a powerful and efficient tool: cluster regulatory interspaced short palindromic repeat (CRISPR) nuclease Cas 9. *CRISPR* chops the DNA sequences identified by guide RNA. CRISPR technique is undergoing a widespread use in the research and has already been used for genome engineering of more than dozen species.

Interestingly, a recent study from Chinese research group have demonstrated human embryo genome editing by using *CRISPR/Cas9* system. Unfortunately, this experiment arises significant questions from the scientific and technical point of view upon the risk of these technology over the future. The Chinese research team claimed that the embryo was "*mosaic*" in nature, meaning that only few cells had

desired gene editing but there was enough number of off-target effects or mutation in nontargeted genes that can be harmful if embryo had been viable. Thus, in terms of human welfare, their work further points out a significant concern on social and ethical policies of genome editing especially in human embryos.

Gene delivery system through viral vector in human gene therapy. A viral vector had been used for packaging of gene of interest being absorbed by the cell membrane or through the endocytosis method followed by delivery of gene to the nucleus which is the place of target gene.

This mechanism has been widely used for treatment of cancer, lung cancer, immune deficiency, cystic fibrosis, etc.

16.5.7 Complications and Issues in Gene Therapy

Besides its great success in various disease treatments, gene therapy has rose many ethical issues in the society. One of the great accomplishment of gene therapy is gene editing is now no more an obstacle for scientists. World's advanced technique CRISPR has made this opportunity possible for biologist to edit any gene required. Thus, it had become a grave concern that the day is not far for parents to achieve when they will be desiring a customized baby, and they can decide a list of new features such as redhead, blue eyes, and extrovert to be added in their child's genome. While everyone is in the race to make their kids smarter, so why wouldn't you?

It's ethically and economically a dilemma, questioning upon fate and fairness, about vanity and values. Considering all the issues of grave concern, a vigilance committee including not only scientist but also lawyers, doctors, religious, and ethicist have decided to permit somatic cell gene therapy to cure genetic disease but should not extend to the germ line gene transfer which will cause the gene editing for preferred child appearances, which would further pass on to offspring. This action may lead to breach the policies of availing gene therapy and will reason for harmful outcome for human society.

There is also sensitive concern about safety highlighted in 1999 after an incident when a patient was participating as volunteer for gene therapy trial through viral vector, but shockingly, this patient had a fatal immune reaction after injecting viral vector for treatment of his metabolic disorder.

16.6 Molecular Markers

A molecular marker is referred to as a tool to detect variation at DNA or gene level among the individuals. Variation occurs due to base pair changes, rearrangements/translocation/inversion of sequence, insertion or deletion of sequence, and variation in the number of tandem repeats. Molecular markers can be categorized into protein markers such as allozymes, DNA marker such as mitochondrial DNA (mtDNA), and nuclear DNA marker such as microsatellites, tandem repeats, RAPD, RFLP, AFLP,

etc. Molecular markers have been widely used to study genetic variation because of its dynamic properties such as ubiquitous, stably inherited, contain multiple alleles for each marker, devoid of pleiotropic effects, detectable in all tissues, and long shelf life of DNA sample.

16.6.1 Types of Nuclear Molecular Marker

16.6.1.1 Restriction Fragment Length Polymorphism (RFLP)

RFLP represents a molecular marker that utilizes the information about restriction enzyme recognition sites in different individual. However, this technique is not so often being used nowadays, but it was the only technique being used for DNA analysis in forensic science widely. The principle of this technique is that any small mutation like base substitution, insertion, deletion, duplication, and inversion within the whole genome can remove or incorporate new restriction site. Thus, any two individuals will show differences by a great as many as RFLPs.

RFLP analysis distinguishes that where the disease gene lies on the chromosome. In RFLP assay, a cloned DNA fragment is used as a probe and must bind to one site alone which reveals a different-sized restriction fragments as an RFLP locus on Southern blot.

In model organism, a set of strain or individual that provides standard RFLP loci has been utilized for RFLP mapping of that particular species. Human RFLP mapping has been established on a set of individuals in 61 families worldwide and an average of eight children per family. Mapping of human RFLP loci was taken into consideration that disease allele may be linked to the restriction site, and this linkage information of each individual may provide a sustainable approach to assess the probability of a person getting disease in next generation. RFLP mapping is a useful tool to predict any genetic disease linkage in pedigree analysis. Despite the fact that RFLP is one of the primitive and efficient method, it is still a very time-consuming and tedious method compared to the new advanced DNA analysis techniques. Moreover, it requires comparatively large sample size to determine RFLP mapping. Remember that RFLP is always considered as a "single locus marker" since we investigate the single restriction enzyme recognition site on a DNA fragment which reflects similar to the single locus on an allele.

16.6.1.2 Random Amplification of Polymorphic DNA (RAPD)

RAPD is a PCR-based molecular technique to develop DNA markers. This technique has been established by Welsh and Maclelland in 1991. RAPD was designed with the concept of the DNA sequence-based polymorphism at a very large number of loci among the individual. RAPD is a pre-DNA sequencing free technique that makes it more conceivable. This technique has been beneficial for certain purposes in animal breeding such as to identify and classify the accessions of the breed, for identification of the breed, and to study genetic diversity.

RAPD principle works on the virtue of a set of primers, a short oligonucleotide sequence that binds to many different loci. This binding results in the amplification

of *random sequence* in the complexed DNA sample. Amplification is done in the PCR and amplified PCR product influenced by the size of both primer and template DNA. Let's consider that two different genomic DNA from two different individuals create a different pattern of the amplified PCR product. A specific fragment found in individual 1 not present in individual 2 will represent a *DNA polymorphism*, and this difference can be used as a genetic marker. An advantage of RAPDs is that it is technically simplified irrespective of demanding any prior DNA sequence information. It has been observed that this method is more advanced and constructive compared to *fingerprinting* and RFLP though this technique has a significant disadvantage of the fact that polymorphism can only detect the presence or absence of a particular amplified DNA sequence in the sample that appears a band of a specific molecular weight. This information is regardless of homozygosity or heterozygosity of that particular DNA fragment. Also, reproducibility of the data is a technical demerit in RAPD.

16.6.1.3 Amplified Fragment Length Polymorphism (AFLP)

AFLP is another PCR-based molecular marker and very specific to the species and subspecies. The common use is determining the close relationship between species or subpopulation in mostly plants, humans, animals, fungi, and bacteria. This marker prevails the application in investigation of genetic variation, population structure, and differentiation. AFLP is capable to generate hundreds of replicable markers from small size DNA sample. In this way, AFLP provides high-resolution genotyping quality. Pertaining its advantageous features, AFLP is known as a substantial genetic marker, used in many broad applications, for example, molecular systematic studies, phylogenic studies, DNA fingerprinting, quantitative trait loci (QTL) mapping, and population genetics. AFLP analysis is more convenient because it generates large numbers of marker fragment for any organism without having prior knowledge of genomic sequence. Additionally, AFLP requires only small fraction of starting template, and it shows much higher reproducibility compared to RAPD. On the basis of factors like time consumption, cost-effectiveness, resolution, and reproducibility, AFLP is equal to the other markers, i.e., RAPD, RFLP, and microsatellite.

Template fragments are generated by (1) digestion with combination of two restriction enzymes and (2) ligation of restriction enzyme-specific adaptors (red and blue) to each end (3). After adaptor ligation, a preamplification step is completed by using combination of primers that matches any of these adapter sequences, and each primer carries selective nucleotide that is represented here with N. (4) Selective PCR amplification step completed by adding additional nucleotides to the primers (specific to two restriction sites) (5). Gel electrophoresis image shows amplified AFLP product.

However, the major drawback of this technique is that AFLPs cannot distinguish dominant homozygous individuals from dominant heterozygous individuals as it is a dominant biallelic marker (i.e., SNP, two possible nucleotide variation for a single position). AFLP is widely used in population genetics and genome typing and subsequently more suitable for detecting genetic polymorphism and tracing animal genetic resources.

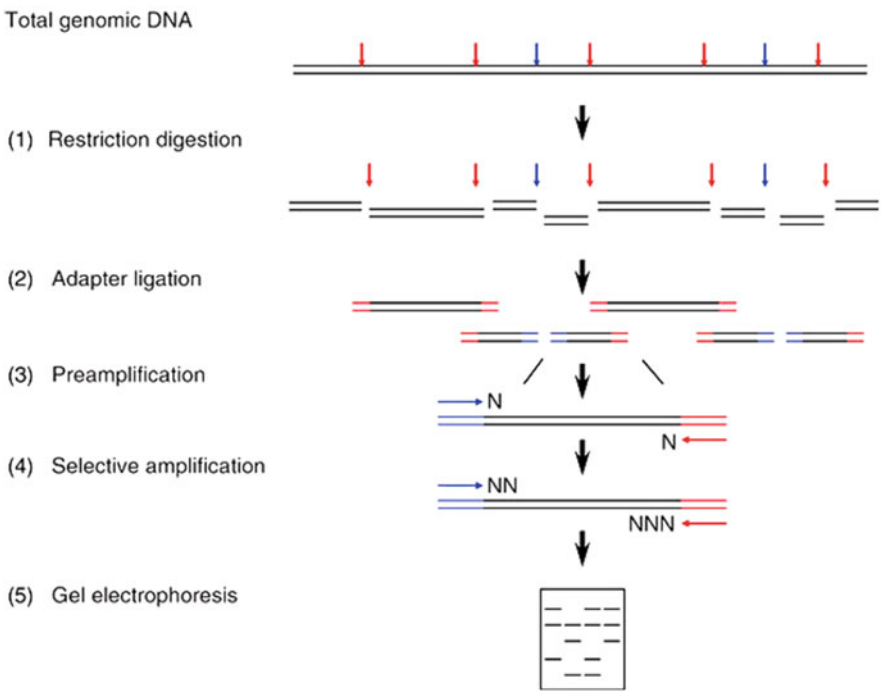

Fig. 16.7 Schematic representation of AFLP method

For a high-impact AFLP, researcher should choose an appropriate primer combination that will generate the sufficient polymorphic marker to study. Literature suggested that at least minimum combination of three primers from the dozens of combinations (that have been used for examining polymorphism in a few individuals, and the results enable them (primers) to identify optimal pairs) will be used for a sufficient gain. AFLP success from the practical point of view requires four major factors including (i) standardized reaction condition, (ii) optimized reagents, (iii) a robust and reliable electrophoresis platform, and (iv) accurate analysis software (Fig. 16.7).

16.6.1.4 Sequence-Tagged Microsatellite

Microsatellites or *simple sequence repeated* (SSR) loci are also known as variable number of *tandem repeats* (VNTRs) and simple sequence length polymorphisms (SSLPs). These repeats are very common in eukaryotes genome and, to some extent, in prokaryotes genome. *Microsatellites* vary from one to six nucleotides in length. Depending upon the numbers of nucleotide in the repeats, they are categorized as mono (A), di (AA), tri (AAA), tetra (AAAA), penta (AAAAA), and hexa (AAAAAA). Di-, tri-, and tetranucleotide repeats are so often used for genetic studies. These repeats are present in repeated manner at least 5–20 times with the minimum length of 12 nucleotide.

Microsatellite provides ease to perform high score, high polymorphism, and a strong automation for thousands of markers at a time and makes this technique suitable for a number of genetic studies including (i) DNA fingerprinting, (ii) genetic mapping, and (iii) paternity analysis and genetic diversity. Microsatellite sequence at specific loci can be easily detected by PCR. Primer for PCR should be specific to conserve flanking region of repeats. A sequence tag is used as a primer to amplify the corresponding SSR and resulted to amplified sequences known as sequence-tagged microsatellite (STMs). These sequence tags must have been taken from the group of enormous amplified restriction fragments or from the genomic DNA. Primer for STM amplification is designed by combination of universal primers that have been used for microsatellite repeats and anchored at 5′ end of the targeted microsatellite repeat. Thus, primer designing for STM is cost-effective and cut down the cost of developing SSR marker (constructing a microsatellite marker) direct to the synthesis of a single primer specific to a conserved flanking region (conserved DNA sequence) of SSR.

When we have to apply STMPs in large complex DNA, complications have been reported in isolation of restriction fragments containing target SSRs from the pool of amplicon which has been used to construct a sequence tag profile. This problem has been solved by enriching the pool of SSR-rich restriction fragments available from the same DNA template which were used to construct the sequence tag profile, reported in Hayden and M. J. et al. In spite of many advantages, STMSs also possess some limitations including extensive use of high cost of cloning, sequencing, and primer synthesis. Still standard protocol uses radioisotope, and primer selection procedure suffers from a number of problems like redundancy of clones and the occurrence of artificial chimeras.

16.7 DNA Fingerprinting

This technique is also known as DNA profiling and DNA typing. DNA fingerprinting is usually collections of fragmented DNAs from individuals to be compared for particular purpose and generate a DNA-specific profile in a term of fingerprinting. DNA fingerprinting is nonetheless but a distinctive pattern of DNA fragments according to the length isolated by gel electrophoresis. In a forensic field, DNA sample is first isolated and purified from the suspects and victim in order to suspect a crime scene. These samples are further digested by restriction enzyme, amplified by PCR, and profiled using electrophoriesis. DNA fingerprinting is first invented by Alec Jeffreys in 1985 in England. He used restriction enzyme to cut the DNA into fragments because PCR had not been developed that time. Initially, fragments were used with radioactivity-labeled DNA, but now this technique is improvised with the discovery of PCR and fluorescent dye. Routine fingerprinting testing is accommodated with repeated sequence or short tandem repeats which allow to distinguish DNA fragments more effectively. Nowadays, DNA fingerprinting is always performed by PCR assay.

To understand the analysis result of fingerprinting, we can consider the DNA fragments on autoradiograph image below. This radiograph contains five essential lanes describing (i) markers, a known size of DNA fragments; (ii) control lane (TS), a sample from positive source and expected to bind with DNA probes favorably; (iii) experimental lane which denoted the blood collection from defendant, defendant's shirt, and jeans; and (iv) blood sample from victim's shirt and victim's blood.

The given example shows that blood on defendant's shirt and jeans are similar to the blood of victim. Defendant was lying here because his own blood doesn't match with victim's blood and blood on his clothes (Fig. 16.8).

DNA analysis has become the routine job in many forensic labs for solving the crime scene and identifying the suspects. In the beginning of DNA technology, scientists have realized that DNA database from the crime scene would provide a criminal justice in no time, and with more efficiency also, some local DNA database have been created on purpose. Before the DNA fingerprinting, protein assay was being used for several years for solving the cases, but this technique was not reliable because of less availability of technical support and protein degradation was the major issue. DNA collection was much easier, and DNA is more resistant to physical damage compared to the protein. DNA analysis can be performed from any tissue, i.e., blood, hair, saliva, semen, skin, and bones, while protein markers are restricted to the cells where these proteins are expressed. Besides, this technique has also been used to analyze the relationships and source of another organism in the population. For example, DNA fingerprinting has revealed that anthrax has been transmitted to the population having same source of origin.

16.8 Fluorescent in Situ Hybridization (FISH)

If a part of genome is possible to clone, then it can be used to make a labeled probe for hybridization to chromosome in situ. The logic of this approach is identical to Western or Southern blot just in case this probe does not bind with any DNA or protein instead binds to largely intact chromosome since the probe is cloned for chromosome specific. This method involves a few steps to be performed such as isolation of chromosome by tearing cells chemically or mechanically and spread on the microscopic glass. The chromosome on slide is supposed to be denatured so that the double-stranded long DNA can convert into single stranded. Thereafter, denatured labeled probe is added to this mixture. In result, the probe will be hybridized to homologous sequence in situ with the chromosomal DNA on the slide and apparently location of hybridization on the chromosome will be detected by bright fluoroscent spot on the chromosome DNA under the fluoroscent microscope (Fig. 16.9). With the advance technology, FISH can also be used to localize and detect various RNA target (mRNA and miRNA) within the cells and tissue.

Now, the probe sequence will be used to map the position of hybridization on the chromosome by observing the banding pattern related to centromere or any other cytological feature. Unfortunately, this technique does not allow to observe recombinational mapping due to low resolving power, as in example of two genes that

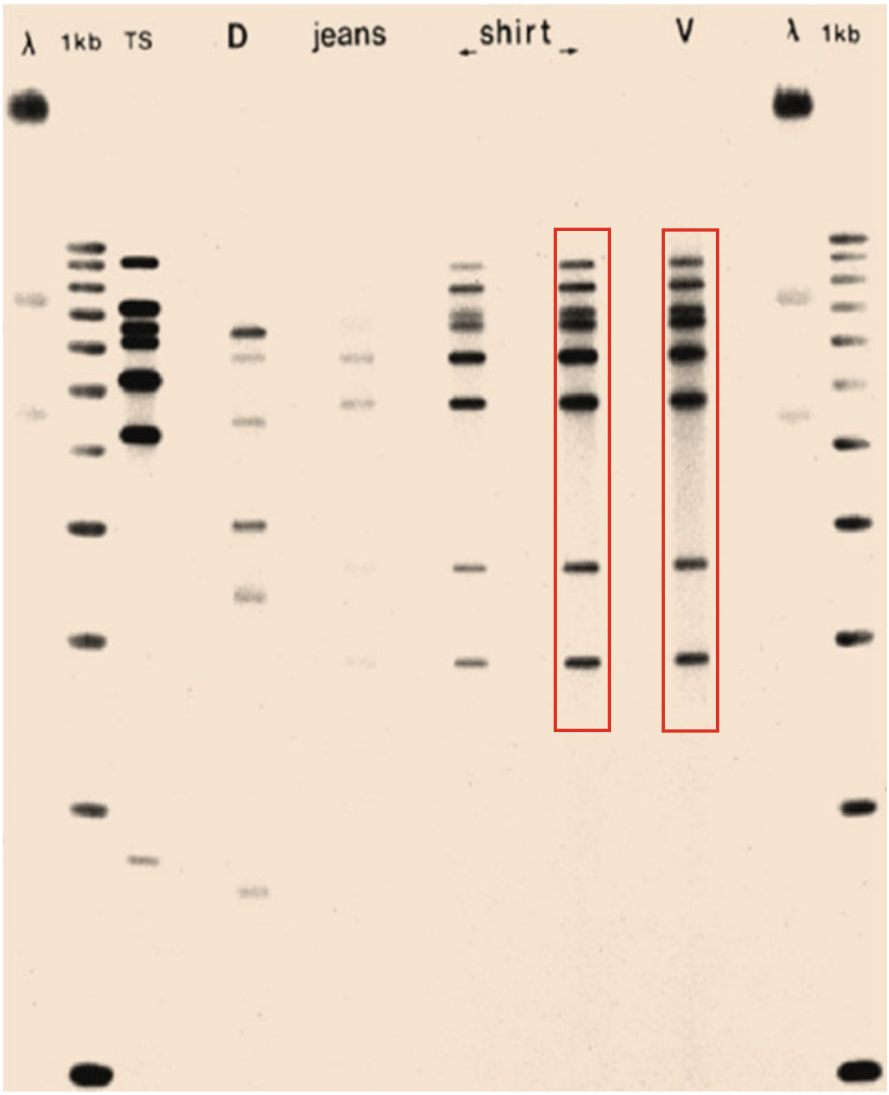

Fig. 16.8 DNA fingerprinting/profiling from a crime scene. The DNA samples collected from the victim (V) were found on defendant's (D) clothing (jeans/shirt). First lane shows DNA ladder (λ), and second lane shows positive control (TS). This profile shows a successful DNA fingerprint assay from a crime scene resulting in prediction of criminal

positioned 5 cM apart to each other on the human chromosome, but in situ hybridization mapping technique would not be able to distinguish this recombinational-like feature.

In advance in situ hybridization technique, probe can be a cDNA, cRNA, or synthetic oligonucleotide. While choosing the probe, researchers should consider the

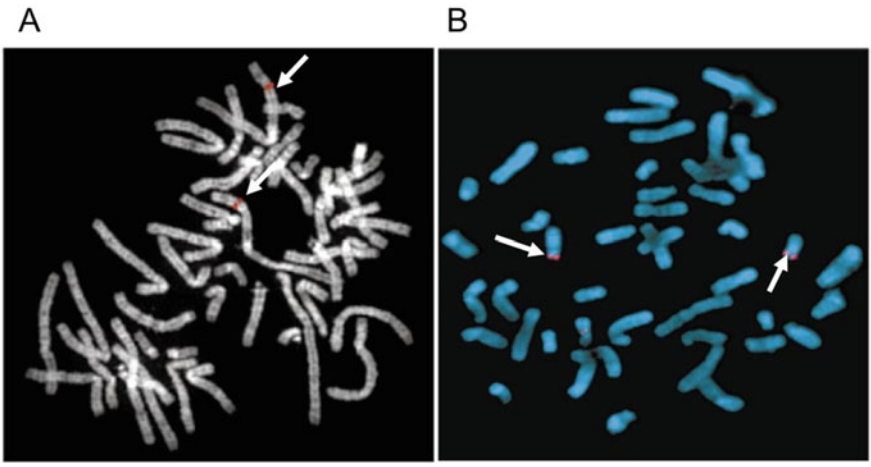

Fig. 16.9 FISH image of chromosome shows locus-specific fluorescent signals. (**a**) Cytogenetic bands (gray) with a hybridized probe with chromosome spotted in red. (**b**) A clone selected from patient suffering with multiple congenital malformations and mental retardation. FISH analysis is used for locating the break point of a translocation on chromosome 11 or 19, and FISH was able to show the red signals split between chromosomes 11 and 19 where the translocation took place

binding sensitivity, specificity, production facility probe penetration strength, and stability of hybrid. These probes are basically conjugated with fluorescein, biotin, or digoxigenin.

FISH probes can be categorized (Fig. 16.9) as (i) locus-specific probe or (ii) chromosome paint probes. Locus-specific probes are particular to detect a distinct gene or chromosomal area, and such probes help detect any deletion or amplification of DNA sequence (if any occur), while *chromosome paint* (Fig. 16.10) probes are completely specific for detecting structural abnormalities within the chromosome structure or rearrangement of chromosome. The optimal size of probe should be of length 50–300 bases.

Box 16.1: Production of Recombinant Human Proinsulin in the Milk of Transgenic Mice (Qian X et al.)

Diabetes is the third most worldwide health issue among the people and characterized with high blood sugar which can lead to a number of serious complications such as heart disease, stroke, kidney failure, blindness, nerve damage, Alzheimer's, etc. The main causes of diabetes are lacking insulin production (Type I) and failure of its use after production (Type II). After success of medical trial, insulin treatment has become one of the promising treatments for diabetes. At molecular level, human insulin gene in vivo is first transcribed and translated in single-chain precursor known as "proinsulin"

(continued)

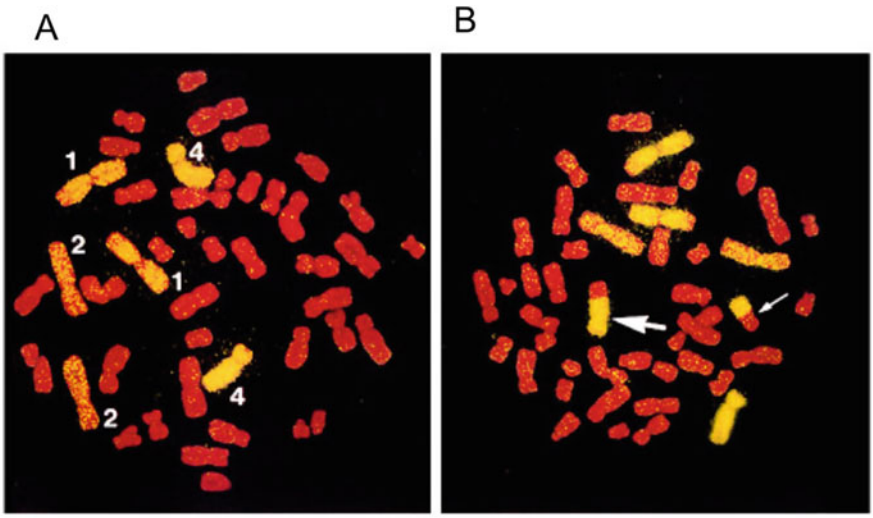

Fig. 16.10 FISH image of human metaphase chromosome painting. (a) Chromosomes 1, 2, and 4 were labeled yellow and rest painted red. (b) Image shows reciprocal translocation between chromosoms appeared in bicolor chromosom indicated with white arrow. FISH technique used to detect translocation between chromosomes is stained yellow, while chromosome is stained red

Box 16.1 (continued)
which is 110-amino acid (aa)-long peptide in the ß cells of the islet of Langerhans in the pancreas. This signal peptide, first 23–24 aa at the N terminus, is removed during the protein folding in the endoplasmic reticulum which results in proinsulin (86 aa, 9.5 kDa). Molecular structure of protein has three domains: an amino terminal B chain of 30 aa, 3.4 kDa, a carboxy terminal A chain (21 aa, 2.4 kDa), and a connecting C chain (34 aa, 3.0 kDa). This structure is generally considered as a premature protein structure, but a cut by neuroendocrine cell-specific prohormone convertase (PC1 and PC2) in order to remove C chain and remaining A and B chain together is bound with a sulfide bond known as mature insulin (5.8 kDa). With an alarming need of insulin production for diabetes treatment, recombinant DNA technology has become a boom for the medical field. Human insulin now biosynthetically can be possible to manufacture at large scale for clinical use worldwide. Primitively, chain A and chain B successfully were produced in two separate bacterial strains and followed by purification and sulfide bond formation by air oxidation. Furthermore, insulin and its analogues were produced in a yeast as inactive proinsulin that further goes for enzymatic cleavage of C chain by using enzymes such as trypsin and carboxypeptidase

(continued)

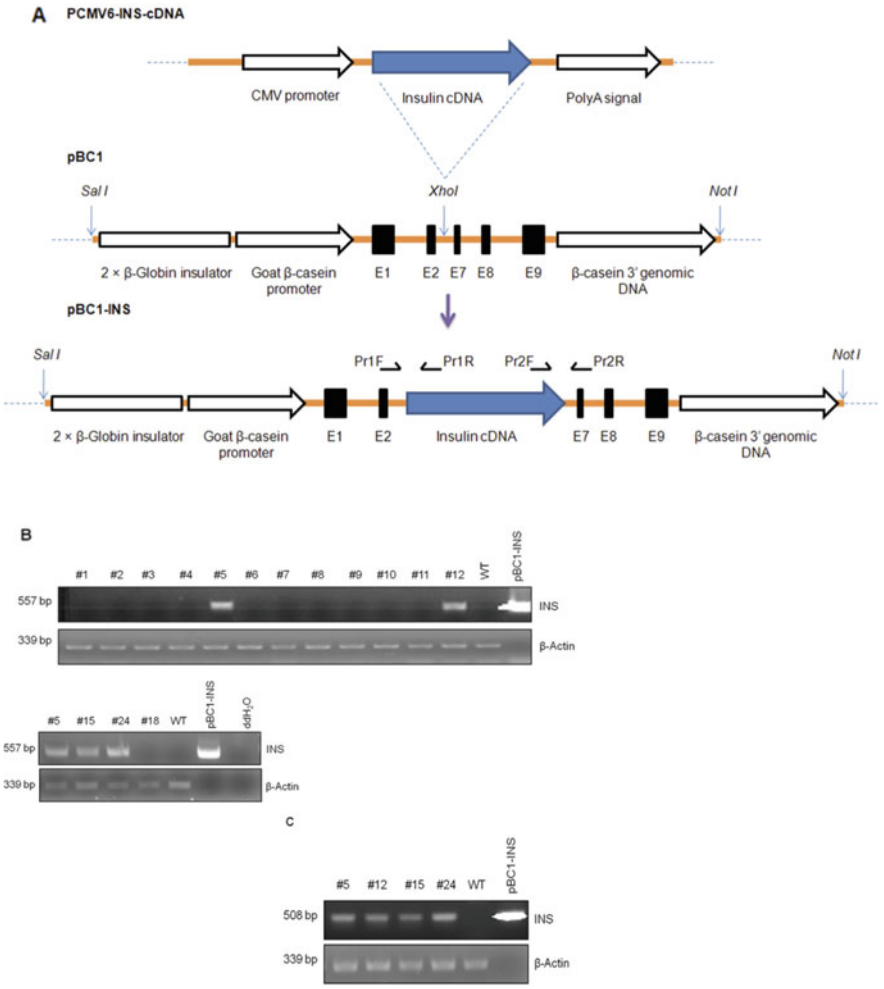

Fig. 16.11 Transgene construction and the identification of transgenic mice. (**a**) Schematic representation of the transgene construction. The full length of insulin cDNA in the pCMV6-XL5-INS-cDNA was amplified by PCR and inserted into the pBC1 vector at the Xho I site, generating the pBC1-INS construct. Before microinjection, the pBC1-INS construct was excised with Sal I and Not I. From left to right, the linearized pBC1-INS comprises the 2 × β globin insulator; the goat β-casein promoter and untranslated exons E1 and E2; human insulin cDNA; untranslated goat b-casein exons E7, E8, and E9; and 39 genomic DNAs. Pr1F, Pr1R, Pr2F, and Pr2R primers were used in PCR for the identification of the transgenic mice. (**b** and **c**) Identification of the transgenic mice by PCR using the Pr1 primer pair (**b**) and Pr2 primer pair (**c**). Non-transgenic wild-type (WT) mouse DNA was used as a negative control, and the DNA used for microinjection served as a positive control. b-actin was amplified to show the same amount of DNA used in each PCR reaction

Table 16.2 List of proinsulin concentration in the collected milk of transgenic mice at the midlactation period

Transgene copy numbers and human proinsulin expression levels in multiple lines of transgenic mice			
Line F0 (gender[a])	Offspring F1 (gender[a])	Transgene copy number	Expression level in milk[b] (μg/μl)
5 (F)		13	1.4
	5_1 (F)	7	1.2
	5_2 (F)	6	2.0
12 (F)		20	7.8
	12_1 (F)	10	7.2
	12_2 (F)	6	8.1
15 (F)		7	4.1
	15_1 (F)	5	2.1
	15_2 (F)	3	1.6
24 (M)		9	NA
	24_1 (F)	5	5.7

[a]F = female, M = male.
[b]Milk samples were collected at mid-lactation, and the concentration of human proinsulin was quantified by ELISA.

Box 16.1 (continued)
B to obtain full potential mature insulin. However, this production method has some limitations on the net production rate, and according to diabetic federation, in 2030, one out of ten people will suffer with insulin deficiency or diabetes which gives rise to the possibility of increased demand of insulin production. Current insulin production methods are insufficient to cover the targeted demands to cure the diabetes. Thus, the biopharmaceutical protein production in the mammary glands of transgenic animals is one of the exclusive and extensive trial to increase the proficiency of insulin production for medical uses. This approach has achieved the heights of current expectations in the medical field as it promises the production of high-quality therapeutic medicine for human at very efficient cost. In the current scenario, production of human proinsulin in the milk of transgenic mice provides a possibility and feasibility for scaling up the potential proinsulin production by using transgenic dairy animals.

To generate the transgenic mice expressing the human proinsulin milk, full length of human insulin cDNA was amplified in the pCMV6 vector andinserted into the mammary gland-specific expression vector pBC1

(continued)

Box 16.1 (continued)
(Fig. 16.11a). pBC1 vector contains goat ß-casein promoter and flanked by 5′ to 3′ untranslated sequence of goat ß-casein gene (Fig. 16.11a) This transgene was first linearized in the vector and injected into the fertilized mouse eggs followed by transfer into the recipients. Recipient female mice were crossed with wild type male, which gives F1 transgenic mice pups. However, qPCR for identification of copy number of transgenes in the founder and offspring reveals a slight difference in the numbers of transgenes in the offspring from its founder (Table 16.2) which can be due to the loss of transgene transfer efficiency. However, loss of transgenes is a common phenomenon that explains that transgenes are usually exogenous fragmented DNA supposed to be introduced to some random sites into the host genome and will not result in 100% transfection capability of vectors (Fig. 16.11)

Secretion of proinsulin is targeted only in the milk product of transgenic mice, but to confirm the negligible secretion of insulin in the blood or plasma of these animals, further ELISA test was performed. ELISA test confirmed that there is no difference between the insulin levels in the blood of transgenic and non-transgenic mice. Also, no glucose amount was observed in the plasma of non-transgenic mice which was similar to the transgenic mice. To further obtain the bioactivity of proinsulin or conversion into mature insulin, this proinsulin which was collected from the milk was further digested with enzymes. Digested product was targeted to the CHO cells which express the enormous insulin receptor on its surface. Treated CHO cells were harvested and analyzed for tyrosine phosphorylation of insulin receptor. Levels of total phosphorylation were compared with positive control, undigested transgenic milk, and digested and undigested non-transgenic milk. Results from these samples do not show any detectable activity compared to the digested transgenic milk which confirms the bioactivity of proinsulin produced from the milk of transgenic mice. This observation further clarifies that mammary gland does not produce the mature insulin since the digestion of proinsulin was done exogenously, and therefore, no insulin bioactivity was observed in undigested proinsulin. This mammary gland may not express PC1 and PC2, enzymes required to release the C chain from the proinsulin, but mammary gland can recognize the insulin signal peptide and able to secret the proinsulin protein in the milk. This proinsulin production is empirically favorable in the human health since it does not affect the blood glucose and blood insulin levels that reduce the systemic side effects on diabetic patients.

16.9 Summary

- Molecular genetics is being used for a long time, which has been engaged in enormous applications in the field of animal biotechnology, transgenic animal, production of genetically modified organisms, human gene therapy, development of molecular markers, and forensic science.
- The most advantageous contribution of molecular genetics is in development of transgenic animal. Transgenic animal is produced by injecting DNA into fertile egg that contains foreign DNA (of desired requirement or trait) that is integrated into a chromosome. Knockout mice is a transgenic mouse in which genes are disabled of particular role.
- In molecular genetics, it is now possible to improve the reproduction system and health of cattle and domestic animals by enrichment of diet, improved diagnosis tool, providing feed additives, adding nutrient supplements, etc.
- In human gene therapy, detrimental diseases are now possible to treat by altering the disease-associated gene in human cells.
- Variation in DNA sequence of individuals or studying polymorphism in a population can be assessed by analyzing molecular markers such as RFLP, RAPD, AFLP, and microsatellite.
- DNA fingerprinting and in situ hybridization are mostly used in forensic science. Both techniques are being used in understanding the crime scene and analyzing the samples through these methods to identify criminals.

Further Reading

Amos B, Schlotterer C, Tautz D (1993) Social structure of pilot whales revealed by analytical DNA profiling. Science 260:670–672

Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C et al (1999) Production of goats by somatic cell nuclear transfer. Nat Biotechnol 17(5):456

Brinster RL (1974) The effect of cells transferred into the mouse blastocyst on subsequent development. J Exp Med 140(4):1049–1056

Brumlop S, Finckh MR (2011) Applications and potentials of marker assisted selection (MAS) in plant breeding. BfN-Skripten (Bundesamt für Naturschutz) 298

Campbell KH, McWhir J, Ritchie WA, Wilmut I (1996) Sheep cloned by nuclear transfer from a cultured cell line. Nature 380(6569):64

Cao D, Oard JH (1997) Pedigree and RAPD-based DNA analysis of commercial US rice cultivars. Crop Sci 37(5):1630–1635

Chakravarthi PV, Sri Balaji N (2010) Use of assisted reproductive technologies for livestock development. Vet World 3(5)

Charters YM, Robertson A, Wilkinson MJ, Ramsay G (1996) PCR analysis of oilseed rape cultivars (Brassica napus L. ssp. oleifera) using 5′-anchored simple sequence repeat (SSR) primers. Theor Appl Genet 92(3–4):442–447

Cheung VG, Nowak N, Jang W, Kirsch IR, Zhao S, Chen XN et al (2001) Integration of cytogenetic landmarks into the draft sequence of the human genome. Nature 409(6822):953

Chial H (2008) Rare genetic disorders: learning about genetic disease through gene mapping, SNPs, and microarray data. Nature. Education 1(1):192

Choudhary KK, Kavya KM, Jerome A, Sharma RK (2016) Advances in reproductive biotechnologies. Vet World 9(4):388
Chuang CK, Chen CH, Huang CL, Su YH, Peng SH, Lin TY et al (2017) Generation of GGTA1 mutant pigs by direct pronuclear microinjection of CRISPR/Cas9 plasmid vectors. Anim Biotechnol 28(3):174–181
Clark AJ, Simons P, Wilmut I, Lathe R (1987) Pharmaceuticals from transgenic livestock. Trends Biotechnol 5(1):20–24
Dubock A (2019) Golden rice: to combat vitamin a deficiency for public health. In: Leila Queiroz Zepka, Veridiana Vera de Rosso and Eduardo Jacob-Lopes. IntechOpen, London
Dzau VJ, Ralph J (2015) Cicerone. Responsible use of human gene-editing technologies. 411–412
Fuller R (1989) Probiotics in man and animals. J Appl Bacteriol 66(5):365–378
Gatehouse AM, Ferry N, Edwards MG, Bell HA (2011) Insect-resistant biotech crops and their impacts on beneficial arthropods. Philos Trans R Soc Lond Ser B Biol Sci 366(1569): 1438–1452
Gordon K, Lee E, Vitale JA, Smith AE, Westphal H, Hennighausen L (1987) Production of human tissue plasminogen activator in transgenic mouse milk. Bio/Technology 5(11):1183
Grossman M, Raper SE, Kozarsky K, Stein EA, Engelhardt JF, Muller D et al (1994) Successful ex vivo gene therapy directed to liver in a patient with familial hypercholesterolaemia. Nat Gen 6(4):335
Gupta PK, Varshney RK (2000) The development and use of microsatellite markers for genetic analysis and plant breeding with emphasis on bread wheat. Euphytica 113(3):163–185
Hammer RE, Pursel VG, Rexroad CE, Wall RJ, Bolt DJ, Ebert KM et al (1985) Production of transgenic rabbits, sheep and pigs by microinjection. Nature 315(6021):680
Hanahan D, Wagner EF, Palmiter RD (2007) The origins of oncomice: a history of the first transgenic mice genetically engineered to develop cancer. Genes Dev 21(18):2258–2270
Hayden MJ, Good G, Sharp PJ (2002) Sequence tagged microsatellite profiling (STMP): improved isolation of DNA sequence flanking target SSRs. Nucleic Acids Res 30(23):e129–e129
Hernandez Gifford JA, Gifford CA (2013) Role of reproductive biotechnologies in enhancing food security and sustainability. Anim Front 3(3):14–19
Howlett DR (2011) APP transgenic mice and their application to drug discovery. Histol Histopathol 26(10):1611
Jensen E (2014) Technical review: in situ hybridization. Anat Rec 297(8):1349–1353
Lathe R, Clark AJ, Archibald AL, Bishop JO, Simons P, Wilmut I (1986) Novel products from livestock. Oxford University Press, Oxford
Liras A (2008) Recombinant proteins in therapeutics: haemophilia treatment as an example. Int Arch Med 1(1):4
Maksimenko OG, Deykin AV, Georgiev PG (2013) Use of transgenic animals in biotechnology: prospects and problems. Acta Nat 5.1(16)
Manmohan S, Niraj K (2010) Transgenic animals: production and application. Int J Pharmac Sci Res 2:12–22
McCarthy C (n.d.) What are GloFish? https://www.petmd.com/fish/what-are-glofish
McNicol AM, Farquharson MA (1997) In situ hybridization and its diagnostic applications in pathology. J Pathol 182(3):250–261
Mercier JC (1986) Genetic engineering applied to milk producing animals: some expectations. Exploiting new technologies in animal breeding: genetic developments/edited by C. Smith, JWB King, and JC McKay
Nandani K, Thakur SK (2014) Randomly amplified polymorphic DNA-a brief review. Am J Anim Vet Sci 9(1):6–13
Nascimento IP, Leite LC (2012) Recombinant vaccines and the development of new vaccine strategies. Braz J Med Biol Res 45(12):1102–1111
Ormandy EH, Dale J, Griffin G (2011) Genetic engineering of animals: ethical issues, including welfare concerns. Can Vet J 52(5):544

Paun O, Schönswetter P (2012) Amplified fragment length polymorphism: an invaluable fingerprinting technique for genomic, transcriptomic, and epigenetic studies. In: Plant DNA fingerprinting and barcoding. Humana Press, New York, pp 75–87

Pierce BA (2008) Genetics: a conceptual approach, vol 1. Freeman, New York, NY

Plaschke J, Ganal MW, Röder MS (1995) Detection of genetic diversity in closely related bread wheat using microsatellite markers. Theor Appl Genet 91(6–7):1001–1007

Qian QIAN, Cheng H, Sun Z, Zhu L (1996) The study on determining true and false hybrid rice II you 63 using RAPD molecular markers. Zhongguo Shuidao Kexue 10(4):241–242

Rezaei M, Zarkesh-Esfahani SH (2012) Optimization of production of recombinant human growth hormone in Escherichia coli. J Res Med Sci 17(7):681–685

Röder MS, Korzun V, Wendehake K, Plaschke J, Tixier MH, Leroy P, Ganal MW (1998) A microsatellite map of wheat. Genetics 149(4):2007–2023

Ross MJ, Olson KC, Geier MD, O'Connor JV, Jones AJS (1986) Recombinant DNA synthesis of human growth hormone. In: Raiti S, Tolman RA (eds) Human growth hormone. Springer, Boston, MA

Selden RC, Springman K, Hondele J, Meyer J, Winnacker EL, Kräußlich H et al (1985) Production of transgenic mice, rabbits and pigs by microinjection into pronuclei. Reprod Domest Anim 20(4):251–252

Singh B, Chauhan MS, Singla SK, Gautam SK, Verma V, Manik RS et al (2009) Reproductive biotechniques in buffaloes (Bubalus bubalis): status, prospects and challenges. Reprod Fertil Dev 21(4):499–510

Smith C, Gibson JP (1987) On the use of transgenics in livestock improvement. Anim Breed Abstr 55:1–10

Tseten T, Murthy K (2014) Advances and biotechnological applications in biofuel production: a review. Open J Renew Sustain Energ:29–34

Virk PS, Ford-Lloyd BV, Jackson MT, Newbury HJ (1995) Use of RAPD for the study of diversity within plant germplasm collections. Heredity 74(2):170

Vize PD, Michalska A, Ashman R, Seamark RF, Wells JR (1987) Improving growth in transgenic farm animals. In EMBO Workshop Germline Manipulation of Animals

Ward KA, Franklin IR, Murray JD, Nancarrow CD, Raphael KA, Rigby NW, et al. (1986a) The direct transfer of DNA by embryo microinjection

Ward KA, Franklin IR, Murray JD, Nancarrow CD, Raphael KA, Rigby NW, Byrne CR, Wilson BW, Hunt CL (1986b) The direct transfer of DNA by embryo microinjection. Proceeding of 3rd World Congress on Genetics Applied to Livestock Production Lincoln, Nebraska

Weber JL, May PE (1989) Abundant class of human DNA polymorphisms which can be typed using the polymerase chain reaction. Am J Hum Genet 44(3):388

Winter P, Pfaff T, Udupa SM, Hüttel B, Sharma PC, Sahi S et al (1999) Characterization and mapping of sequence-tagged microsatellite sites in the chickpea (Cicer arietinum L.) genome. Mol Gen Genet MGG 262(1):90–101

Wolff JA, Lederberg J (1994) An early history of gene transfer and therapy. Hum Gene Ther 5(4): 469–480