Chapter 14

Gene Cloning and DNA Analysis in Medicine

Chapter contents

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Medicine has been and will continue to be a major beneficiary of the recombinant DNA revolution, and an entire book could be written on this topic. Later in this chapter we will see how recombinant DNA techniques are being used to identify genes responsible for inherited diseases and to devise new therapies for these disorders. First, we will continue the theme developed in the last chapter and examine the ways in which cloned genes are being used in the production of recombinant pharmaceuticals.

14.1 Production of recombinant pharmaceuticals

A number of human disorders can be traced to the absence or malfunction of a protein normally synthesized in the body. Most of these disorders can be treated by supplying the patient with the correct version of the protein, but for this to be possible the relevant protein must be available in relatively large amounts. If the defect can be corrected only by administering the human protein, then obtaining sufficient quantities will be a major problem unless donated blood can be used as the source. Animal proteins are therefore used whenever these are effective, but there are not many disorders that can be treated with animal proteins, and there is always the possibility of side effects such as an allergenic response.

We learned in Chapter 13 that gene cloning can be used to obtain large amounts of recombinant human proteins. How are these techniques being applied to the production of proteins for use as pharmaceuticals?

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14.1.1 Recombinant insulin

Insulin, synthesized by the β-cells of the islets of Langerhans in the pancreas, controls the level of glucose in the blood. An insulin deficiency manifests itself as diabetes mellitus, a complex of symptoms which may lead to death if untreated. Fortunately, many forms of diabetes can be alleviated by a continuing program of insulin injections, thereby supplementing the limited amount of hormone synthesized by the patient's pancreas. The insulin used in this treatment was originally obtained from the pancreas of pigs and cows slaughtered for meat production. Although animal insulin is generally satisfactory, problems may arise in its use to treat human diabetes. One problem is that the slight differences between the animal and the human proteins can lead to side effects in some patients. Another is that the purification procedures are difficult, and potentially dangerous contaminants cannot always be completely removed.

Insulin displays two features that facilitate its production by recombinant DNA techniques. The first is that the human protein is not modified after translation by the addition of sugar molecules (p. 236): recombinant insulin synthesized by a bacterium should therefore be active. The second advantage concerns the size of the molecule. Insulin is a relatively small protein, comprising two polypeptides, one of 21 amino acids (the A chain) and one of 30 amino acids (the B chain; Figure 14.1). In humans these chains are

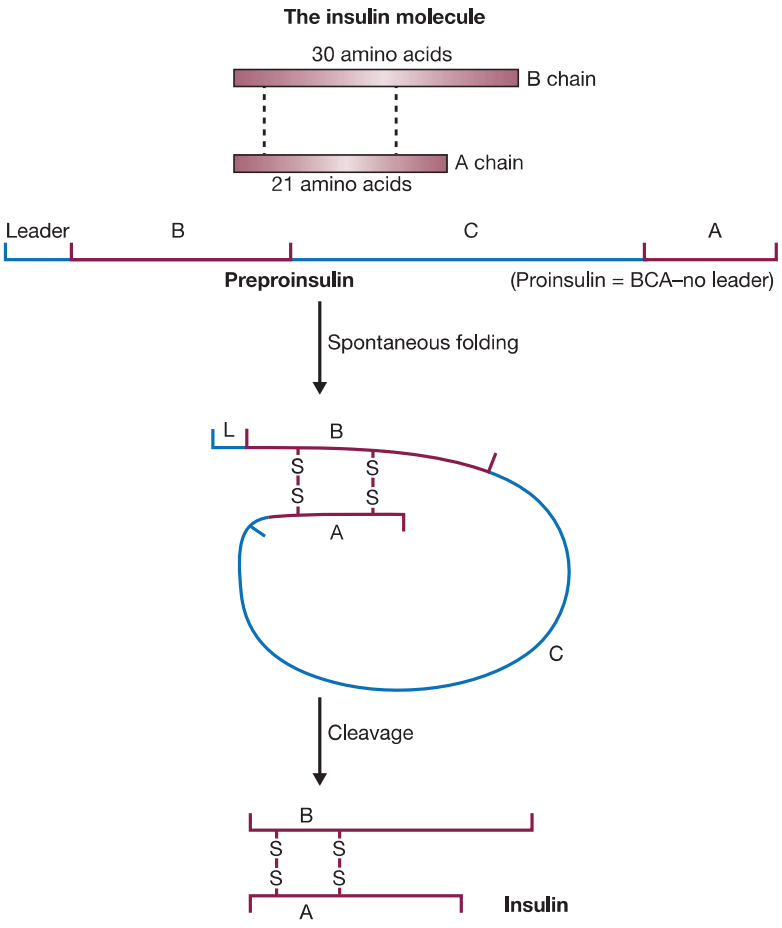


Figure 14.1

The structure of the insulin molecule and a summary of its synthesis by processing from preproinsulin.

synthesized as a precursor called preproinsulin, which contains the A and B segments linked by a third chain (C) and preceded by a leader sequence. The leader sequence is removed after translation and the C chain excised, leaving the A and B polypeptides linked to each other by two disulphide bonds.

Several strategies have been used to obtain recombinant insulin. One of the first projects, involving synthesis of artificial genes for the A and B chains followed by production of fusion proteins in *E. coli*, illustrates a number of the general techniques used in recombinant protein production.

Synthesis and expression of artificial insulin genes

In the late 1970s, the idea of making an artificial gene was extremely innovative. Oligonucleotide synthesis was in its infancy at that time, and the available methods for making artificial DNA molecules were much more cumbersome than the present-day automated techniques. Nevertheless, genes coding for the A and B chains of insulin were synthesized as early as 1978.

The procedure used was to synthesize trinucleotides representing all the possible codons and then join these together in the order dictated by the amino acid sequences of the A and B chains. The artificial genes would not necessarily have the same nucleotide sequences as the real gene segments coding for the A and B chains, but they would still specify the correct polypeptides. Two recombinant plasmids were constructed, one carrying the artificial gene for the A chain, and one the gene for the B chain.

In each case the artificial gene was ligated to a *lacZ′* reading frame present in a pBR322-type vector (Figure 14.2a). The insulin genes were therefore under the control of the strong *lac* promoter (p. 231), and were expressed as fusion proteins, consisting of the first few amino acids of β-galactosidase followed by the A or B polypeptides (Figure 14.2b). Each gene was designed so that its β-galactosidase and insulin segments were separated by a methionine residue, so that the insulin polypeptides could be cleaved from the β-galactosidase segments by treatment with cyanogen bromide (p. 233). The purified A and B chains were then attached to each other by disulphide bond formation in the test tube.

The final step, involving disulphide bond formation, is actually rather inefficient. A subsequent improvement was to synthesize not the individual A and B genes, but the entire proinsulin reading frame, specifying B chain–C chain–A chain (see Figure 14.1). Although this is a more daunting proposition in terms of DNA synthesis, the prohormone has the big advantage of folding spontaneously into the correct disulphide-bonded structure. The C chain segment can then be excised relatively easily by proteolytic cleavage.

14.1.2 Synthesis of human growth hormones in E. coli

At about the same time that recombinant insulin was first being made in *E. coli*, other researchers were working on similar projects with the human growth hormones somatostatin and somatotrophin. These two proteins act in conjunction to control growth processes in the human body, their malfunction leading to painful and disabling disorders such as acromegaly (uncontrolled bone growth) and dwarfism.

Somatostatin was the first human protein to be synthesized in *E. coli*. Being a very short protein, only 14 amino acids in length, it was ideally suited for artificial gene synthesis. The strategy used was the same as described for recombinant insulin, involving insertion of the artificial gene into a *lacZ′* vector (Figure 14.3), synthesis of a fusion protein, and cleavage with cyanogen bromide.

Figure 14.2

The synthesis of recombinant insulin from artificial A and B chain genes.

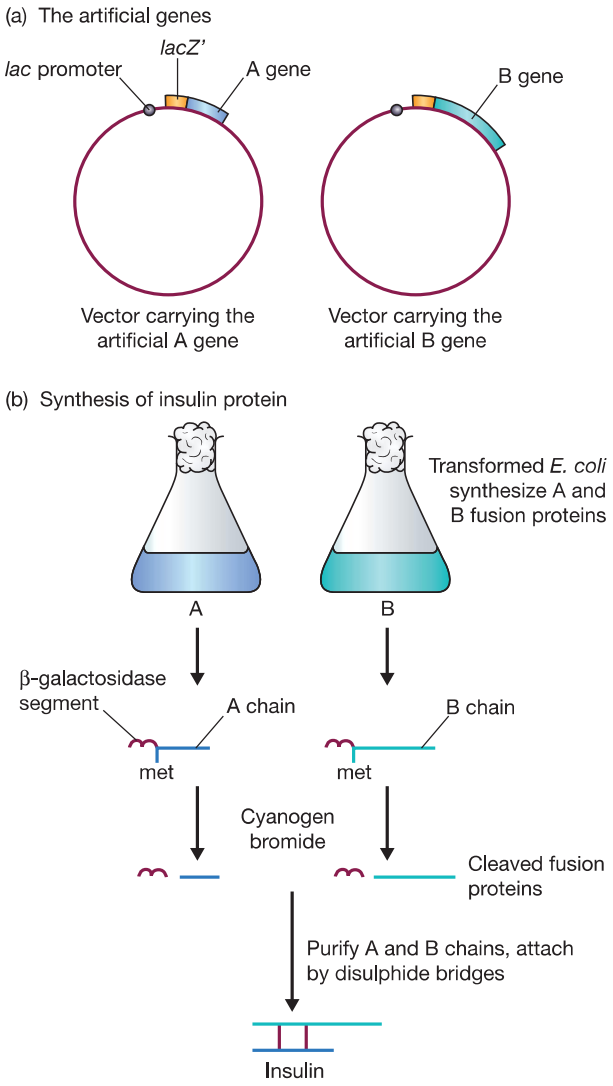
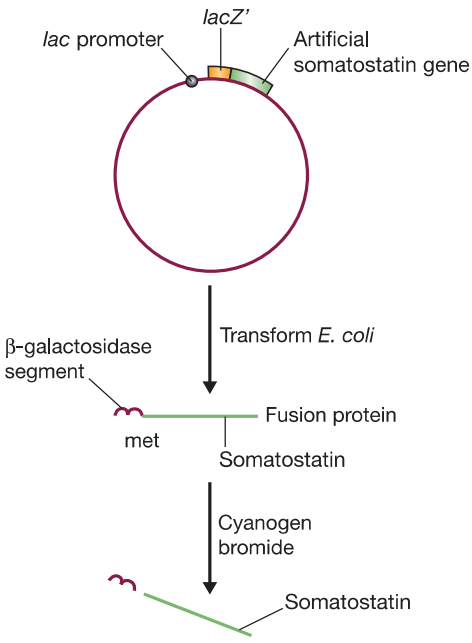


Figure 14.3

Production of recombinant somatostatin.



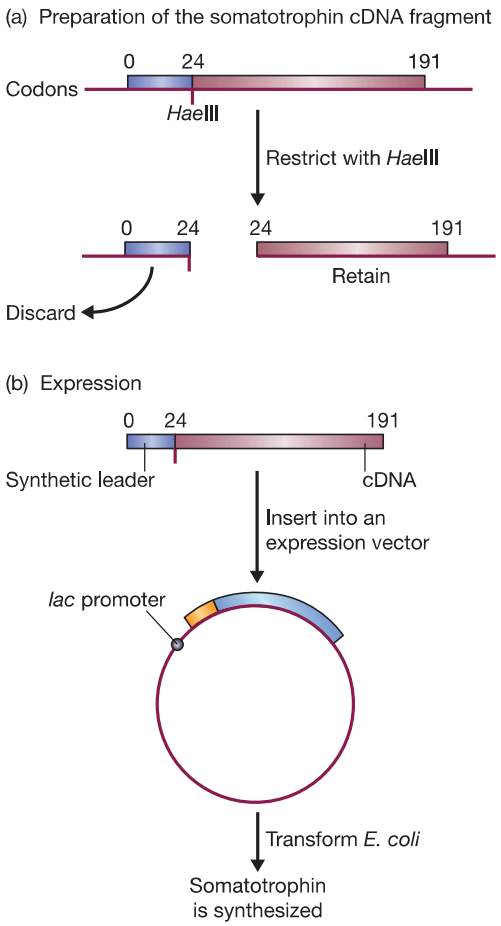


Figure 14.4
Production of recombinant somatotrophin.

Somatotrophin presented a more difficult problem. This protein is 191 amino acids in length, equivalent to almost 600 bp, an impossible prospect for the DNA synthesis capabilities of the late 1970s. In fact, a combination of artificial gene synthesis and complementary DNA (cDNA) cloning was used to obtain a somatotrophin-producing *E. coli* strain. Messenger RNA was obtained from the pituitary, the gland that produces somatotrophin in the human body, and a cDNA library prepared. The somatotrophin cDNA contained a single site for the restriction endonuclease *Hae*III, which therefore cuts the cDNA into two segments (Figure 14.4a). The longer segment, consisting of codons 24–191, was retained for use in construction of the recombinant plasmid. The smaller segment was replaced by an artificial DNA molecule that reproduced the start of the somatotrophin gene and provided the correct signals for translation in *E. coli* (Figure 14.4b). The modified gene was then ligated into an expression vector carrying the *lac* promoter.

14.1.3 Recombinant factor VIII

Although a number of important pharmaceutical compounds have been obtained from genes cloned in *E. coli*, the general problems associated with using bacteria to synthesize foreign proteins (p. 234) have led in many cases to these organisms being replaced by eukaryotes. An example of a recombinant pharmaceutical produced in eukaryotic cells is human factor VIII, a protein that plays a central role in blood clotting. The commonest form of hemophilia in humans results from an inability to synthesize factor VIII,

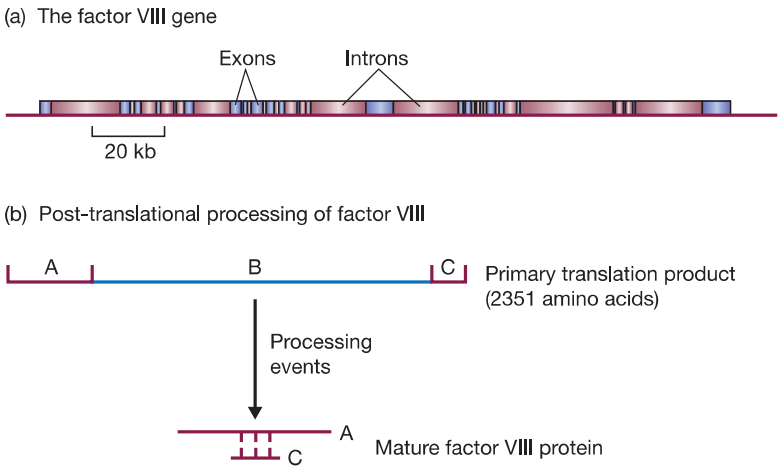


Figure 14.5

The factor VIII gene and its translation product.

leading to a breakdown in the blood clotting pathway and the well-known symptoms associated with the disease.

Until recently the only way to treat hemophilia was by injection of purified factor VIII protein, obtained from human blood provided by donors. Purification of factor VIII is a complex procedure and the treatment is expensive. More critically, the purification is beset with difficulties, in particular in removing virus particles that may be present in the blood. Hepatitis and acquired immune deficiency syndrome (AIDS) can be and have been passed on to hemophiliacs via factor VIII injections. Recombinant factor VIII, free from contamination problems, would be a significant achievement for biotechnology.

The factor VIII gene is very large, over 186 kb in length, and is split into 26 exons and 25 introns (Figure 14.5a). The mRNA codes for a large polypeptide (2351 amino acids), which undergoes a complex series of post-translational processing events, eventually resulting in a dimeric protein consisting of a large subunit, derived from the upstream region of the initial polypeptide, and a small subunit from the downstream segment (Figure 14.5b). The two subunits contain a total of 17 disulphide bonds and a number of glycosylated sites. As might be anticipated for such a large and complex protein, it has not been possible to synthesize an active version in *E. coli*.

Initial attempts to obtain recombinant factor VIII therefore involved mammalian cells. In the first experiments to be carried out the entire cDNA was cloned in hamster cells, but yields of protein were disappointingly low. This was probably because the post-translational events, although carried out correctly in hamster cells, did not convert all the initial product into an active form, limiting the overall yield. As an alternative, two separate fragments from the cDNA were used, one fragment coding for the large subunit polypeptide, the second for the small subunit. Each cDNA fragment was ligated into an expression vector, downstream of the Ag promoter (a hybrid between the chicken β-actin and rabbit β-globin sequences) and upstream of a polyadenylation signal from SV40 virus (Figure 14.6). The plasmid was introduced into a hamster cell line and recombinant protein obtained. The yields were over ten times greater than those from cells containing the complete cDNA, and the resulting factor VIII protein was indistinguishable in terms of function from the native form.

Pharming (p. 241) has also been used for production of recombinant factor VIII. The complete human cDNA has been attached to the promoter for the whey acidic protein

Figure 14.6

The expression signals used in production of recombinant factor VIII. The promoter is an artificial hybrid of the chicken β-actin and rabbit β-globin sequences, and the polyadenylation signal (needed for correct processing of the mRNA before translation into protein) is obtained from SV40 virus.

Table 14.1

Some of the human proteins that have been synthesized from genes cloned in bacteria and/or eukaryotic cells or by pharming.

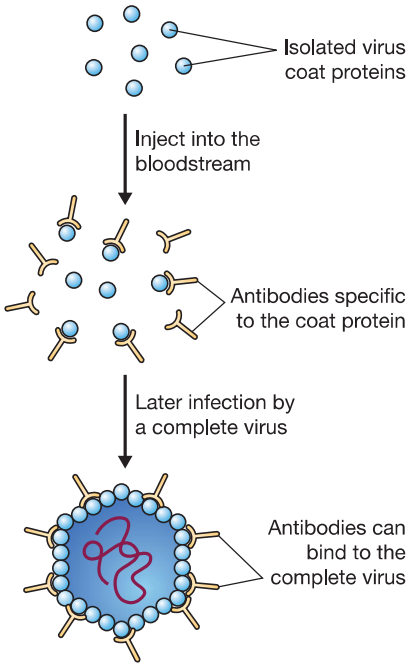
PROTEIN	USED IN THE TREATMENT OF
α_1-Antitrypsin	Emphysema
Deoxyribonuclease	Cystic fibrosis
Epidermal growth factor	Ulcers
Erythropoietin	Anemia
Factor VIII	Hemophilia
Factor IX	Christmas disease
Fibroblast growth factor	Ulcers
Follicle stimulating hormone	Infertility treatment
Granulocyte colony stimulating factor	Cancers
Insulin	Diabetes
Insulin-like growth factor 1	Growth disorders
Interferon-α	Leukemia and other cancers
Interferon-β	Cancers, AIDS
Interferon-γ	Cancers, rheumatoid arthritis
Interleukins	Cancers, immune disorders
Lung surfactant protein	Respiratory distress
Relaxin	Used to aid childbirth
Serum albumin	Used as a plasma supplement
Somatostatin	Growth disorders
Somatotrophin	Growth disorders
Superoxide dismutase	Free radical damage in kidney transplants
Tissue plasminogen activator	Heart attack
Tumor necrosis factor	Cancers

gene of pig, leading to synthesis of human factor VIII in pig mammary tissue and subsequent secretion of the protein in the milk. The factor VIII produced in this way appears to be exactly the same as the native protein and is fully functional in blood clotting assays.

14.1.4 Synthesis of other recombinant human proteins

The list of human proteins synthesized by recombinant technology continues to grow (Table 14.1). As well as proteins used to treat disorders by replacement or supplementation of the dysfunctional versions, the list includes a number of growth factors (e.g., interferons and interleukins) with potential uses in cancer therapy. These proteins are synthesized in very limited amounts in the body, so recombinant technology is the only viable means of obtaining them in the quantities needed for clinical purposes. Other proteins, such as serum albumin, are more easily obtained, but are needed in such large quantities that production in microorganisms is still a more attractive option.

Figure 14.7
The principle behind the use of a preparation of isolated virus coat proteins as a vaccine.



14.1.5 Recombinant vaccines

The final category of recombinant protein is slightly different from the examples given in Table 14.1. A vaccine is an antigenic preparation that, after injection into the bloodstream, stimulates the immune system to synthesize antibodies that protect the body against infection. The antigenic material present in a vaccine is normally an inactivated form of the infectious agent. For example, antiviral vaccines often consist of virus particles that have been attenuated by heating or a similar treatment. In the past, two problems have hindered the preparation of attenuated viral vaccines:

- The inactivation process must be 100% efficient, as the presence in a vaccine of just one live virus particle could result in infection. This has been a problem with vaccines for the cattle disease foot-and-mouth.
- The large amounts of virus particles needed for vaccine production are usually obtained from tissue cultures. Unfortunately some viruses, notably hepatitis B virus, do not grow in tissue culture.

Producing vaccines as recombinant proteins

The use of gene cloning in this field centers on the discovery that virus-specific antibodies are sometimes synthesized in response not only to the whole virus particle, but also to isolated components of the virus. This is particularly true of purified preparations of the proteins present in the virus coat (Figure 14.7). If the genes coding for the antigenic proteins of a particular virus could be identified and inserted into an expression vector, the methods described above for the synthesis of animal proteins could be employed in the production of recombinant proteins that might be used as vaccines. These vaccines would have the advantages that they would be free of intact virus particles and they could be obtained in large quantities.

The greatest success with this approach has been with hepatitis B virus. Hepatitis B is endemic in many tropical parts of the world and leads to liver disease and possibly, after chronic infection, to cancer of the liver. A person who recovers from hepatitis B is immune to future infection because their blood contains antibodies to the hepatitis B

surface antigen (HBsAg), which is one of the virus coat proteins. This protein has been synthesized in both *Saccharomyces cerevisiae*, using a vector based on the 2 μm plasmid (p. 105), and in Chinese hamster ovary (CHO) cells. In both cases, the protein was obtained in reasonably high quantities, and when injected into test animals provided protection against hepatitis B.

The key to the success of recombinant HbsAg as a vaccine is provided by an unusual feature of the natural infection process for the virus. The bloodstream of infected individuals contains not only intact hepatitis B virus particles, which are 42 nm in diameter, but also smaller, 22 nm spheres made up entirely of HBsAg protein molecules. Assembly of these 22 nm spheres occurs during HbsAg synthesis in both yeast and hamster cells and it is almost certainly these spheres, rather than single HbsAg molecules, that are the effective component of the recombinant vaccine. The recombinant vaccine therefore mimics part of the natural infection process and stimulates antibody production, but as the spheres are not viable viruses the vaccine does not itself cause the disease. Both the yeast and hamster cell vaccines have been approved for use in humans, and the World Health Organization is promoting their use in national vaccination programmes.

Recombinant vaccines in transgenic plants

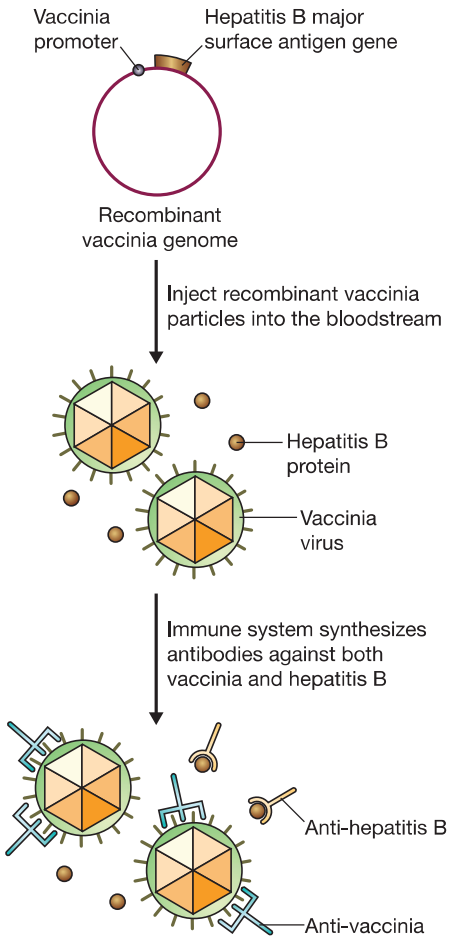
The advent of pharming (p. 241) has led to the possibility of using transgenic plants as the hosts for synthesis of recombinant vaccines. The ease with which plants can be grown and harvested means that this technology might be applicable for developing parts of the world where the more expensive approaches to recombinant protein production might be difficult to sustain. If the recombinant vaccine is effective after oral administration, then immunity could be acquired simply by eating part or all of the transgenic plant. A simpler and cheaper means of carrying out a mass vaccination program would be hard to imagine.

The feasibility of this approach has been demonstrated by trials with vaccines such as HbsAg and the coat proteins of measles virus and respiratory syncytial virus. In each case, immunity was conferred by feeding the transgenic plant to test animals. Attempts are also being made to engineer plants that express a variety of vaccines, so that immunity against a range of diseases can be acquired from a single source. The main problem currently faced by the companies developing this technology is that the amount of recombinant protein synthesized by the plant is often insufficient to stimulate complete immunity against the target disease. To be completely effective the yield of the vaccine needs to make up 8–10% of the soluble protein content of the part of the plant which is eaten, but in practice yields are much less than this, usually not more than 0.5%. Variability in the yields between different plants in a single crop is also a concern. A partial solution is provided by placing the cloned gene in the chloroplast genome rather than the plant nucleus (p. 119), as this generally results in much higher yields of recombinant protein. However, proteins made in the chloroplast are not glycosylated and so those vaccines that require post-translational modification will be inactive if produced in this way. These include most of the relevant viral coat proteins, but not bacterial surface proteins such as the *Vibrio cholerae* B subunit, which can be used to confer immunity against diseases such as cholera. This protein has been synthesized in transgenic tobacco, tomato, and rice plants and shown to elicit an anti-cholera immune response when leaves, fruits, or seeds are fed to mice.

Live recombinant virus vaccines

The use of live vaccinia virus as a vaccine for smallpox dates back to 1796, when Edward Jenner first realized that this virus, harmless to humans, could stimulate immunity

Figure 14.8
The rationale behind the potential use of a recombinant vaccinia virus.



against the much more dangerous smallpox virus. The term "vaccine" comes from vaccinia; its use resulted in the worldwide eradication of smallpox in 1980.

A more recent idea is that recombinant vaccinia viruses could be used as live vaccines against other diseases. If a gene coding for a virus coat protein, for example HBsAg, is ligated into the vaccinia genome under control of a vaccinia promoter, then the gene will be expressed (Figure 14.8). After injection into the bloodstream, replication of the recombinant virus results not only in new vaccinia particles, but also in significant quantities of the major surface antigen. Immunity against both smallpox and hepatitis B would result.

This remarkable technique has considerable potential. Recombinant vaccinia viruses expressing a number of foreign genes have been constructed and shown to confer immunity against the relevant diseases in experimental animals (Table 14.2). The possibility of broad-spectrum vaccines is raised by the demonstration that a single recombinant vaccinia, expressing the genes for influenza virus hemagglutinin, HBsAg, and herpes simplex virus glycoprotein, confers immunity against each disease in monkeys. Studies of vaccinia viruses expressing the rabies glycoprotein have shown that deletion of the vaccinia gene for the enzyme thymidine kinase prevents the virus from replicating. This avoids the possibility that animals treated with the live vaccine will develop any form of cowpox, the disease caused by normal vaccinia. This particular live virus vaccine is now being used in rabies control in Europe and North America.

Table 14.2

Some of the foreign genes that have been expressed in recombinant vaccinia viruses.

GENE
Plasmodium falciparum (malaria parasite) surface antigen
Influenza virus coat proteins
Rabies virus G protein
Hepatitis B surface antigen
Herpes simplex glycoproteins
Human immunodeficiency virus (HIV) envelope proteins
Vesicular somatitis coat proteins
Sindbis virus proteins

14.2 Identification of genes responsible for human diseases

A second major area of medical research in which gene cloning is having an impact is in the identification and isolation of genes responsible for human diseases. A genetic or inherited disease is one that is caused by a defect in a specific gene (Table 14.3), individuals carrying the defective gene being predisposed toward developing the disease at some stage of their lives. With some inherited diseases, such as hemophilia, the gene is present on the X chromosome, so all males carrying the gene express the disease state; females with one defective gene and one correct gene are healthy but can pass the disease on to their male offspring. Genes for other diseases are present on autosomes and in most cases are recessive, so both chromosomes of the pair must carry a defective version for the disease to occur. A few diseases, including Huntington's chorea, are autosomal dominant, so a single copy of the defective gene is enough to lead to the disease state.

With some genetic diseases, the symptoms manifest themselves early in life, but with others the disease may not be expressed until the individual is middle-aged or elderly.

Table 14.3

Some of the commonest genetic diseases in the UK.

DISEASE	SYMPTOMS	FREQUENCY (BIRTHS PER YEAR)
Inherited breast cancer	Cancer	1 in 300 females
Cystic fibrosis	Lung disease	1 in 2000
Huntington's chorea	Neurodegeneration	1 in 2000
Duchenne muscular dystrophy	Progressive muscle weakness	1 in 3000 males
Hemophilia A	Blood disorder	1 in 4000 males
Sickle cell anemia	Blood disorder	1 in 10,000
Phenylketonuria	Mental retardation	1 in 12,000
β-Thalassaemia	Blood disorder	1 in 20,000
Retinoblastoma	Cancer of the eye	1 in 20,000
Hemophilia B	Blood disorder	1 in 25,000 males
Tay–Sachs disease	Blindness, loss of motor control	1 in 200,000

Cystic fibrosis is an example of the former, and neurodegenerative diseases such as Alzheimer's and Huntington's are examples of the latter. With a number of diseases that appear to have a genetic component, cancers in particular, the overall syndrome is complex with the disease remaining dormant until triggered by some metabolic or environmental stimulus. If predisposition to these diseases can be diagnosed, the risk factor can be reduced by careful management of the patient's lifestyle to minimize the chances of the disease being triggered.

Genetic diseases have always been present in the human population but their importance has increased in recent decades. This is because vaccination programs, antibiotics, and improved sanitation have reduced the prevalence of infectious diseases such as smallpox, tuberculosis, and cholera, which were major killers up to the mid-20th century. The result is that a greater proportion of the population now dies from a disease that has a genetic component, especially the late-onset diseases that are now more common because of increased life expectancies. Medical research has been successful in controlling many infectious diseases: can it be equally successful with genetic disease?

There are a number of reasons why identifying the gene responsible for a genetic disease is important:

- Gene identification may provide an indication of the biochemical basis to the disease, enabling therapies to be designed.
- Identification of the mutation present in a defective gene can be used to devise a screening programme so that the mutant gene can be identified in individuals who are carriers or who have not yet developed the disease. Carriers can receive counseling regarding the chances of their children inheriting the disease. Early identification in individuals who have not yet developed the disease allows appropriate precautions to be taken to reduce the risk of the disease becoming expressed.
- Identification of the gene is a prerequisite for gene therapy (p. 259).

14.2.1 How to identify a gene for a genetic disease

There is no single strategy for identification of genes that cause diseases, the best approach depending on the information that is available about the disease. To gain an understanding of the principles of this type of work, we will consider the most common and most difficult scenario. This is when all that is known about the disease is that certain people have it. Even with such an unpromising starting point, DNA techniques can locate the relevant gene.

Locating the approximate position of the gene in the human genome

If there is no information about the desired gene, how can it be located in the human genome? The answer is to use basic genetics to determine the approximate position of the gene on the human genetic map. Genetic mapping is usually carried out by **linkage analysis**, in which the inheritance pattern for the target gene is compared with the inheritance patterns for genetic loci whose map positions are already known. If two loci are inherited together, they must be very close on the same chromosome. If they are not close together, then recombination events and the random segregation of chromosomes during meiosis will result in the loci displaying different inheritance patterns (Figure 14.9). Demonstration of linkage with one or more mapped genetic loci is therefore the key to understanding the chromosomal position of an unmapped gene.

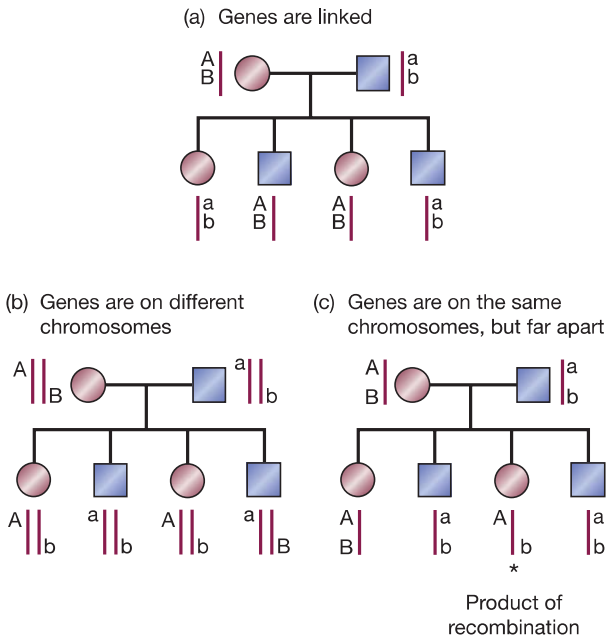


Figure 14.9

Inheritance patterns for linked and unlinked genes. Three families are shown, circles representing females and squares representing males. (a) Two closely linked genes are almost always inherited together. (b) Two genes on different chromosomes display random segregation. (c) Two genes that are far apart on a single chromosome are often inherited together, but recombination may unlink them.

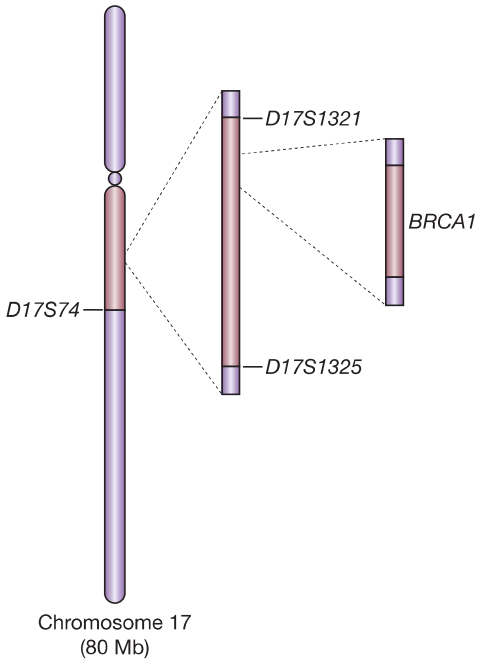
With humans it is not possible to carry out directed breeding programs aimed at determining the map position of a desired gene. Instead, mapping of disease genes must make use of data available from **pedigree analysis**, in which inheritance of the gene is examined in families with a high incidence of the disease being studied. It is important to be able to obtain DNA samples from at least three generations of each family, and the more family members there are the better, but unless the disease is very uncommon it is usually possible to find suitable pedigrees. Linkage between the presence/absence of the disease and the inheritance of other genes could be studied, but as DNA samples are being analyzed, linkage to DNA markers is more usually tested (p. 180).

To illustrate how linkage analysis is used we will look briefly at the way in which one of the genes conferring susceptibility to human breast cancer was mapped. The first breakthrough in this project occurred in 1990 as a result of **restriction fragment length polymorphism (RFLP) linkage analyses** carried out by a group at the University of California at Berkeley. This study showed that in families with a high incidence of breast cancer, a significant number of the women who suffered from the disease all possessed the same version of an RFLP called *D17S74*. This RFLP had previously been mapped to the long arm of chromosome 17 (Figure 14.10): the gene being sought—*BRCA1*—must therefore also be located on the long arm of chromosome 17.

This initial linkage result was extremely important, as it indicated in which region of the human genome this breast cancer susceptibility gene was to be found, but it was far from the end of the story. In fact, over 1000 genes are thought to lie in this particular 20 Mb stretch of chromosome 17. The next objective was therefore to carry out more linkage studies to try to pinpoint *BRCA1* more accurately. This was achieved by first examining the region containing *BRCA1* for short tandem repeats (STRs) (p. 181), which are useful for fine scale mapping because many of them exist in three or more allelic forms, rather than just the two alleles that are possible for an RFLP. Several alleles of an STR might therefore be present within a single pedigree, enabling more detailed mapping to be carried out. STR linkage mapping reduced the size of the *BRCA1* region from 20 Mb down to just 600 kb (Figure 14.10). This approach to locating a gene is called **positional cloning**.

Figure 14.10

Mapping the breast cancer gene. Initially the gene was mapped to a 20 Mb segment of chromosome 17 (highlighted region in the left drawing). Additional mapping experiments narrowed this down to a 600 kb region flanked by two previously mapped loci, *D17S1321* and *D17S1325* (middle drawing). After examination of expressed sequences, a strong candidate for *BRCA1* was eventually identified (right drawing).



Identification of candidates for the disease gene

One might imagine that once the map location of the disease gene has been determined the next step is simply to refer to the genome sequence in order to identify the gene. Unfortunately a great deal of work still has to be done. Genetic mapping, even in its most precise form, only gives an approximate indication of the location of a gene. In the breast cancer project the researchers were fortunate in being able to narrow the search area down to just 600 kb—often 10 Mb or more of DNA sequence has to be examined. Such large stretches of DNA could contain many genes: the 600 kb breast cancer region contained over 60 genes, any one of which could have been *BRCA1*.

A variety of approaches can be used to identify which of the genes in a mapped region is the disease gene:

- The expression profiles of the **candidate genes** can be examined by hybridization analysis or reverse transcription–polymerase chain reaction (RT–PCR) (p. 161) of RNA from different tissues. For example, *BRCA1* would be expected to hybridize to RNA prepared from breast tissue, and also to ovary tissue RNA, ovarian cancer frequently being associated with inherited breast cancer.
- Southern hybridization analysis (p. 142) can be carried out with DNA from different species (these are called **zoo blots**). The rationale is that an important human gene will almost certainly have homologs in other mammals, and that this homolog, although having a slightly different sequence from the human version, will be detectable by hybridization with a suitable probe.
- The gene sequences could be examined in individuals with and without the disease to see if the genes from affected individuals contain mutations that might explain why they have the disease.
- To confirm the identity of a candidate gene, it might be possible to prepare a knockout mouse (p. 214) that has an inactive version of the equivalent mouse gene. If the knockout mouse displays symptoms compatible with the human disease, then the candidate gene is almost certainly the correct one.

When applied to the breast cancer region, these analyses resulted in identification of an approximately 100 kb gene, made up of 22 exons and coding for a 1863 amino acid protein, that was a strong candidate for *BRCA1*. Transcripts of the gene were detectable in breast and ovary tissues, and homologs were present in mice, rats, rabbits, sheep, and pigs, but not chickens. Most importantly, the genes from five susceptible families contained mutations (such as frameshift and nonsense mutations) likely to lead to a non-functioning protein. Although circumstantial, the evidence in support of the candidate was sufficiently overwhelming for this gene to be identified as *BRCA1*. Subsequent research has shown that both this gene and *BRCA2*, a second gene associated with susceptibility to breast cancer, are involved in transcription regulation and DNA repair, and that both act as tumor suppressor genes, inhibiting abnormal cell division.

14.3 Gene therapy

The final application of recombinant DNA technology in medicine that we will consider is gene therapy. This is the name originally given to methods that aim to cure an inherited disease by providing the patient with a correct copy of the defective gene. Gene therapy has now been extended to include attempts to cure any disease by introduction of a cloned gene into the patient. First we will examine the techniques used in gene therapy, and then we will attempt to address the ethical issues.

14.3.1 Gene therapy for inherited diseases

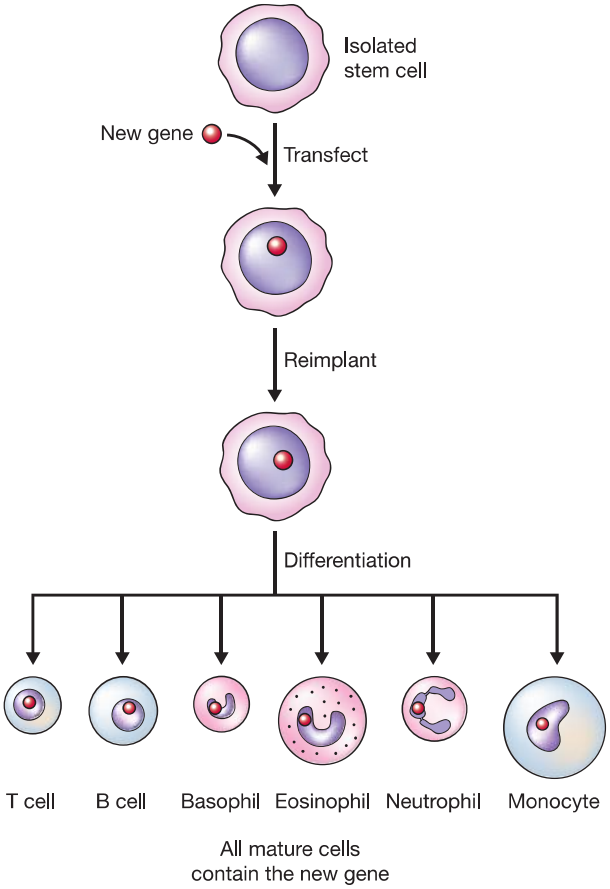
There are two basic approaches to gene therapy: germline therapy and somatic cell therapy. In germline therapy, a fertilized egg is provided with a copy of the correct version of the relevant gene and re-implanted into the mother. If successful, the gene is present and expressed in all cells of the resulting individual. Germline therapy is usually carried out by microinjection of a somatic cell followed by nuclear transfer into an oocyte (p. 241), and theoretically could be used to treat any inherited disease.

Somatic cell therapy involves manipulation of cells, which either can be removed from the organism, transfected, and then placed back in the body, or transfected *in situ* without removal. The technique has most promise for inherited blood diseases (e.g., hemophilia and thalassaemia), with genes being introduced into stem cells from the bone marrow, which give rise to all the specialized cell types in the blood. The strategy is to prepare a bone extract containing several billion cells, transfect these with a retrovirus-based vector, and then re-implant the cells. Subsequent replication and differentiation of transfectants leads to the added gene being present in all the mature blood cells (Figure 14.11). The advantage of a retrovirus is that this type of vector has an extremely high transfection frequency, enabling a large proportion of the stem cells in a bone marrow extract to receive the new gene.

Somatic cell therapy also has potential in the treatment of lung diseases such as cystic fibrosis, as DNA cloned in adenovirus vectors (p. 123) or contained in liposomes (p. 85) is taken up by the epithelial cells in the lungs after introduction into the respiratory tract via an inhaler. However, gene expression occurs for only a few weeks, and as yet this has not been developed into an effective means of treating cystic fibrosis.

With those genetic diseases where the defect arises because the mutated gene does not code for a functional protein, all that is necessary is to provide the cell with the correct version of the gene: removal of the defective genes is unnecessary. The situation

Figure 14.11
Differentiation of a transfected stem cell leads to the new gene being present in all the mature blood cells.



is less easy with dominant genetic diseases (p. 255), as with these it is the defective gene product itself that is responsible for the disease state, and so the therapy must include not only addition of the correct gene but also removal of the defective version. This requires a gene delivery system that promotes recombination between the chromosomal and vector-borne versions of the gene, so that the defective chromosomal copy is replaced by the gene from the vector. The technique is complex and unreliable, and broadly applicable procedures have not yet been developed.

14.3.2 Gene therapy and cancer

The clinical uses of gene therapy are not limited to treatment of inherited diseases. There have also been attempts to use gene cloning to disrupt the infection cycles of human pathogens such as the AIDS virus. However, the most intensive area of current research concerns the potential use of gene therapy as a treatment for cancer.

Most cancers result from activation of an oncogene that leads to tumor formation, or inactivation of a gene that normally suppresses formation of a tumor. In both cases a gene therapy could be envisaged to treat the cancer. Inactivation of a tumor suppressor gene could be reversed by introduction of the correct version of the gene by one of the methods described above for inherited disease. Inactivation of an oncogene would, however, require a more subtle approach, as the objective would be to prevent expression of the oncogene, not to replace it with a non-defective copy. One possible way of doing this would be to introduce into a tumor a gene specifying an **antisense** version of the

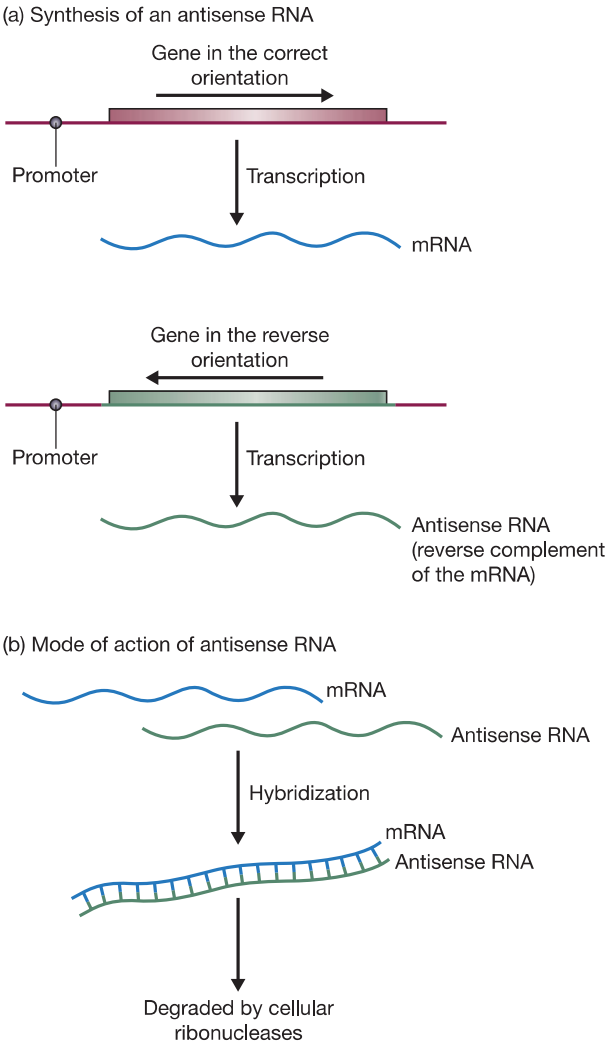


Figure 14.12
Antisense RNA can be used to silence a cellular mRNA.

mRNA transcribed from the oncogene (Figure 14.12a). An antisense RNA is the reverse complement of a normal RNA, and can prevent synthesis of the protein coded by the gene it is directed against, probably by hybridizing to the mRNA producing a double-stranded RNA molecule that is rapidly degraded by cellular ribonucleases (Figure 14.12b). The target is therefore inactivated.

An alternative would be to introduce a gene that selectively kills cancer cells or promotes their destruction by drugs administered in a conventional fashion. This is called **suicide gene therapy** and is looked on as an effective general approach to cancer treatment, because it does not require a detailed understanding of the genetic basis of the particular disease being treated. Many genes that code for toxic proteins are known, and there are also examples of enzymes that convert non-toxic precursors of drugs into the toxic form. Introduction of the gene for one of these toxic proteins or enzymes into a tumor should result in the death of the cancer cells, either immediately or after drug administration. It is obviously important that the introduced gene is targeted accurately at the cancer cells, so that healthy cells are not killed. This requires a very precise delivery system, such as direct inoculation into the tumor, or some other means of ensuring that the gene is expressed only in the cancer cells. One possibility is to place the gene under control of the human telomerase promoter, which is active only in cancerous tissues.

Another approach is to use gene therapy to improve the natural killing of cancer cells by the patient's immune system, perhaps with a gene that causes the tumor cells to synthesize strong antigens that are efficiently recognized by the immune system. All of these approaches, and many not based on gene therapy, are currently being tested in the fight against cancer.

14.3.3 The ethical issues raised by gene therapy

Should gene therapy be used to cure human disease? As with many ethical questions, there is no simple answer. On the one hand, there could surely be no justifiable objection to the routine application via a respiratory inhaler of correct versions of the cystic fibrosis gene as a means of managing this disease. Similarly, if bone marrow transplants are acceptable, then it is difficult to argue against gene therapies aimed at correction of blood disorders via stem cell transfection. And cancer is such a terrible disease that the withholding of effective treatment regimens on moral grounds could itself be criticized as immoral.

Germline therapy is a more difficult issue. The problem is that the techniques used for germline correction of inherited diseases are exactly the same techniques that could be used for germline manipulation of other inherited characteristics. Indeed, the development of this technique with animals has not been prompted by any desire to cure genetic diseases, the aims being to "improve" farm animals, for example by making genetic changes that result in lower fat content. This type of manipulation, where the genetic constitution of an organism is changed in a directed, heritable fashion, is clearly unacceptable with humans. At present, technical problems mean that human germline manipulation would be difficult. Before these problems are solved we should ensure that the desire to do good should not raise the possibility of doing tremendous harm.

Further reading

FURTHER READING

Brocher, B., Kieny, M.P., Costy, F. et al. (1991) Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine. *Nature*, 354, 520–522.

Broder, C.C. & Earl, P.L. (1999) Recombinant vaccinia viruses—design, generation, and isolation. *Molecular Biotechnology*, 13, 223–245.

Goeddel, D.V., Heyneker, H.L., Hozumi, T. et al. (1979) Direct expression in *Escherichia coli* of a DNA sequence coding for human growth hormone. *Nature*, 281, 544–548. [Production of recombinant somatotrophin.]

Goeddel, D.V., Kleid, D.G., Bolivar, F. et al. (1979) Expression in *Escherichia coli* of chemically synthesized genes for human insulin. *Proceedings of the National Academy of Sciences of the USA*, 76, 106–110.

Itakura, K., Hirose, T., Crea, R. et al. (1977) Expression in *Escherichia coli* of a chemically synthesized gene for the hormone somatostatin. *Science*, 198, 1056–1063.

Kaufman, R.J., Wasley, L.C. & Dorner, A.J. (1988) Synthesis, processing, and secretion of recombinant human factor VIII expressed in mammalian cells. *Journal of Biological Chemistry*, 263, 6352–6362.

Liu, M.A. (1998) Vaccine developments. *Nature Medicine*, 4, 515–519. [Describes the development of recombinant vaccines.]

Miki, Y., Swensen, J., Shattuck Eidens, D. et al. (1994) A strong candidate for the breast and ovarian cancer susceptibility gene *BRCA1*. *Science*, 266, 66–71.

Paleyanda, R.K., Velander, W.H., Lee, T.K. et al. (1997) Transgenic pigs produce functional human factor VIII in milk. *Nature Biotechnology*, 15, 971–975.

Schepelmann, S., Ogilvie, L.M., Hedley, D. et al. (2007) Suicide gene therapy of human colon carcinoma xenografts using an armed oncolytic adenovirus expressing carboxypeptidase G2. *Cancer Research*, 67, 4949–4955. [An example of suicide gene therapy using the telomerase promoter and an enzyme that converts a non-toxic pro-drug into the toxic form.]

Smith, K.R. (2003) Gene therapy: theoretical and bioethical concepts. *Archives of Medical Research*, 34, 247–268.

Tiwari, S., Verma, P.C., Sing, P.K. & Tuli, R. (2009) Plants as bioreactors for the production of vaccine antigens. *Biotechnology Advances*, 27, 449–467.

van Deutekom, J.C.T. & van Ommen, G.J.B. (2003) Advances in Duchenne muscular dystrophy gene therapy. *Nature Reviews Genetics*, 4, 774–783. [A example of the use of gene therapy.]