GENE THERAPY

Introduction of wild copies of the defective gene causing the disorder into the cells of the affected individuals is termed as gene therapy.

Types of Gene Therapy:

A. Germline gene therapy:

1. Germ cells(sperms and eggs) are modified by the introduction of functional genes which are ordinarily integrated into their genomes.

2. Therefore,the change due to therapy would be heritable and would be passed onto the next generation.

B. Somatic cell gene therapy:

1. The gene is introduced only in somatic cells.

2. Expression of the introduced gene eliminates symptoms of the disorder but this effect is not heritable.

THE MAIN STEPS INVOLVED IN GENE THERAPY:

a. Identification and characterization of gene

b. Cloning of gene

c. Choice of vector

d. Method of delivery

e. Expression of gene

APPROACHES TO GENE THERAPY:

Disease caused by the loss of a functional gene can be treated by introducing extra copies of the normal genes which may produce the amount of normal gene product level after the normal phenotype is restored.

1. Aggressive gene therapy

2. Adenovirus gene therapy

3. Adenoassociated virus gene therapy

4. Retroviral gene therapy

5. Nonviral delivery in gene therapy

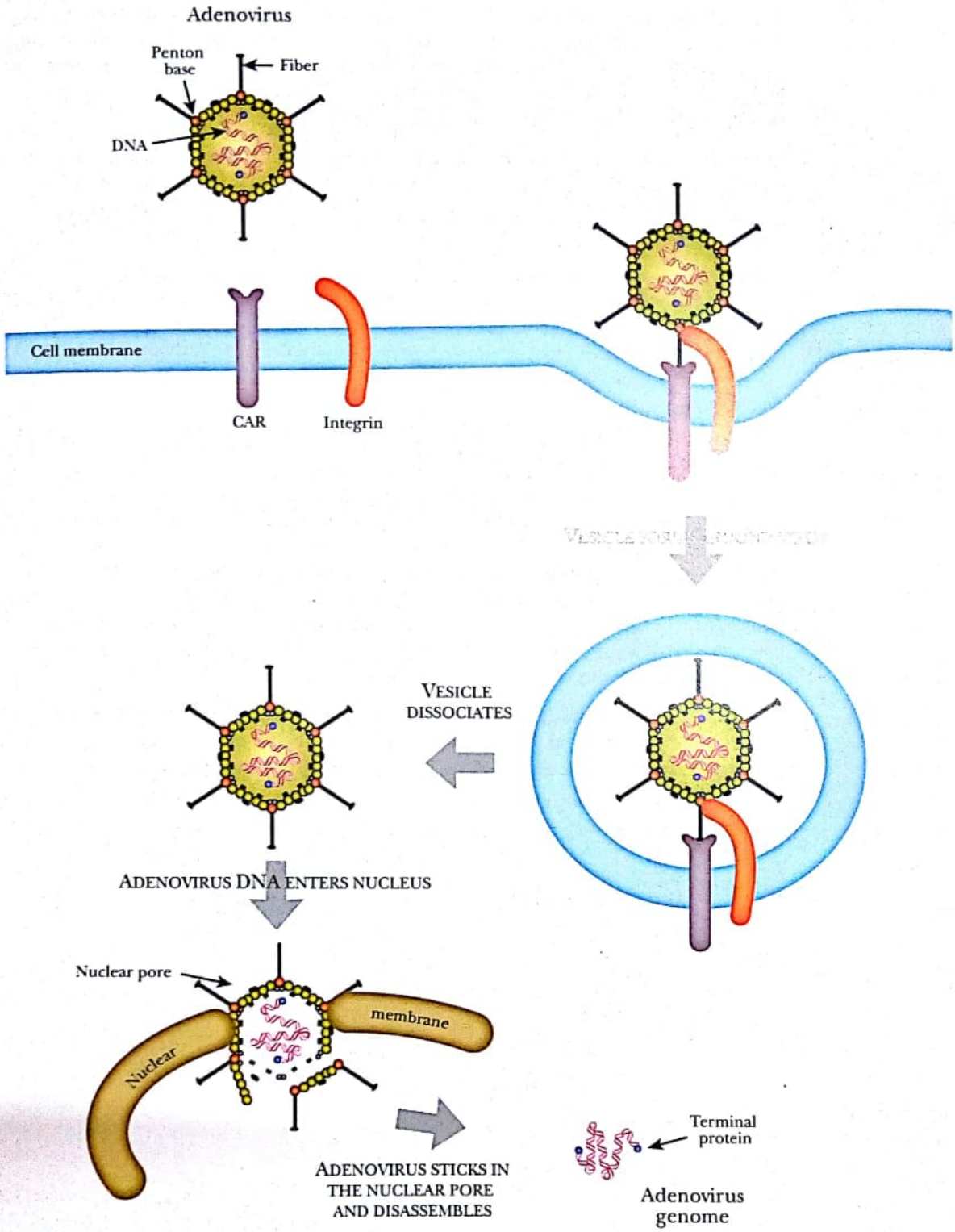

FIGURE 17.3 Adenovirus Entry
Adenoviruses enter human cells by recognizing two receptors, CAR and integrin. The virus is taken into the cell attached to the receptors and surrounded by a membrane vesicle that dissociates in the cytoplasm. The adenovirus injects its DNA into the nucleus through a nuclear pore.

After the fiber tip binds to CAR, the penton base binds to integrins on the host cell surface. (Integrins are transmembrane proteins involved in adhesion.) Next the membrane puckers inward and forms a vesicle that takes the adenovirus inside the cell. The virus is then released into the cytoplasm and travels toward the nucleus. The virion is disassembled outside the nucleus, and only the DNA (with its terminal proteins) enters the nucleus.

Adenoviruses were among the first viruses chosen as vectors for use in human gene therapy. Their advantages are as follows:

(a) Adenoviruses are relatively harmless. They cause mild infections of epithelial cells, especially those lining the respiratory or gastrointestinal tracts.
(b) Adenoviruses are nononcogenic (i.e., they do not cause tumors).
(c) Adenoviruses are relatively easy to culture and can be produced in large quantities.
(d) The life cycle and biology of adenovirus are well understood.
(e) The function of most adenovirus genes is known.
(f) The complete DNA sequence is available, in particular for adenovirus serotype 5 of subgroup C.

Although mild, adenoviruses do cause inflammation and can cause serious illness in patients with damaged immune systems. Therefore, when designing an adenovirus vector for gene therapy, the virus needs to be disarmed by crippling its replication system. This is done by deleting the gene for **E1A protein**, a virus protein made immediately on infection. E1A has two functions (Fig. 17.4). First, it promotes transcription of other early virus genes. Second, it binds to host cell Rb protein, which normally prevents the cell from entering S-phase. This prompts the host cell to express genes for DNA synthesis, which the virus utilizes for its own replication. In the lab, crippled adenovirus is grown in genetically modified host cells that have the viral E1A gene integrated into host cell DNA. The virus particles generated by this approach cannot replicate in normal animal cells.

The DNA of adenovirus is packaged by a headful mechanism. If the DNA is more than 5% shorter or longer than wild type, packaging fails (Fig. 17.5). Insertion of a therapeutic gene into an adenovirus will make the DNA longer. If the inserted gene is much longer than the

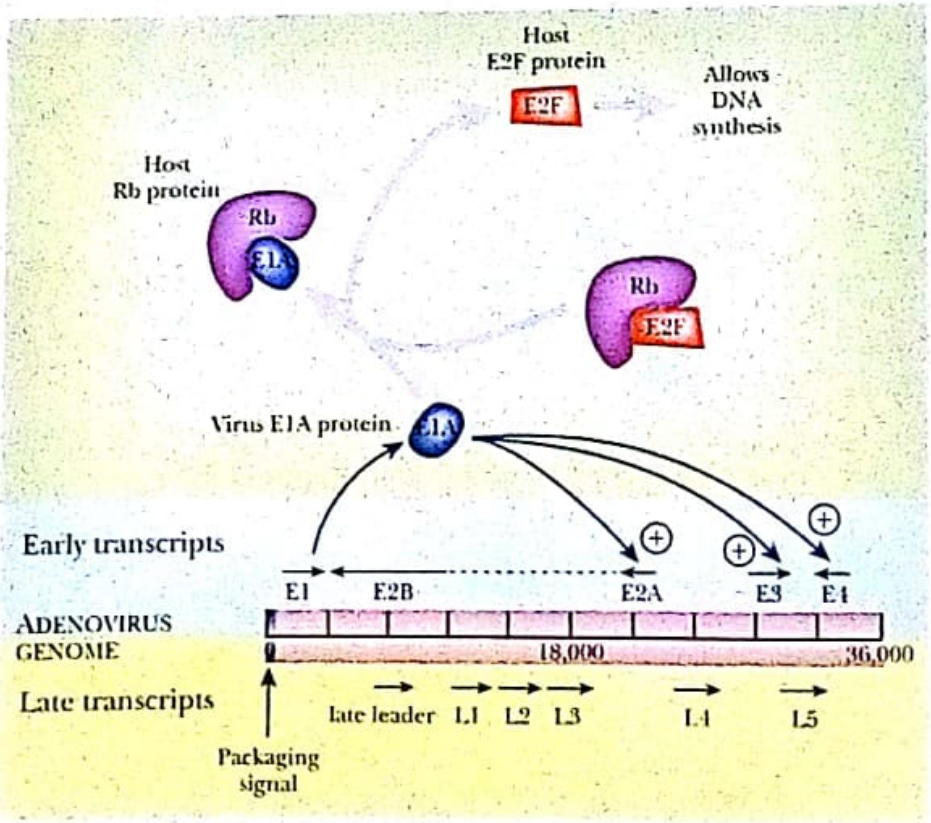

FIGURE 17.4 Role of Adenovirus E1A Protein
Eliminating E1A prevents adenovirus replication. E1A is a transcription factor that activates other adenovirus genes, such as E2A, E3, and E4. E1A also binds host cell Rb protein so releasing host E2F protein to activate DNA synthesis.

deletion used to cripple virus replication, the virus will fail to assemble correctly. Deleting other nonessential virus DNA solves this problem.

Although genes carried on engineered adenovirus have been expressed successfully in both animal and human tissues, there are problems. The major difficulty is that adenovirus infections are short-lived. Thus the therapeutic gene is expressed for only a few weeks before the immune system eliminates the virus. Furthermore, the patient develops immunity to the virus so that a second treatment with the same engineered virus will fail. Thus adenovirus vectors cannot be used for long-term gene therapy for hereditary diseases.

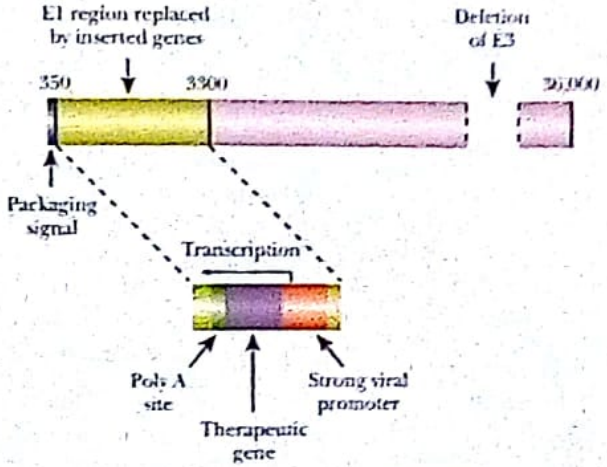

FIGURE 17.5 Engineered Adenovirus
The length of DNA in a virus particle must be very close to the natural adenovirus length of 36,000 base pairs for proper packaging. The therapeutic gene replaces the E1 region. If the gene is much longer than E1A, then a region containing gene E3 is deleted to keep the overall length of the DNA constant.

Even with the limitations just described, adenovirus vectors may help deliver a deadly gene to cancer cells. Here only a short period of expression should be needed. The CAR receptor is normally only expressed highly by epithelial cells, which limits adenovirus entry to these cell types. However, many cancers also express the CAR receptor at high levels. Consequently, the majority of gene therapy trials using adenovirus are now aimed at cancer cells (see later discussion).

Adenovirus has been widely used as a gene therapy vector. Eventually the immune system eliminates the virus, restricting its use in long-term therapy for inherited conditions.

CYSTIC FIBROSIS GENE THERAPY BY ADENOVIRUS

Because the lungs are relatively accessible to viral infection, cystic fibrosis (see Chapter 16) has been a prime candidate for gene therapy. The healthy version of the cystic fibrosis gene has been cloned and inserted into a crippled adenovirus. Aerosols containing the engineered adenovirus with the cystic fibrosis gene have been sprayed into the noses and lungs, first of rats, and then of humans. In some instances the healthy cystic fibrosis gene was expressed and normal chloride ion movements were also restored. Unfortunately, expression falls off over a 30-day period and repeated doses of virus have little effect, largely because of recognition and destruction of the virus by the immune system.

It is hoped that in the near future, improved vectors will allow cystic fibrosis to be cured by nasal sprays containing genetically engineered viruses. Note, however, that this sort of gene therapy cures only the symptoms in the lungs; it does not correct the genetic defect in the germline cells. The defect will still be passed on to the next generation.

Cystic fibrosis has been targeted for gene therapy because the lungs are readily accessible. Early attempts using adenovirus vectors were partially successful, but only for a brief period.

ADENO-ASSOCIATED VIRUS

Because of the problems with using adenovirus discussed above, other DNA viruses have been considered as vectors. Although none are yet widely used, the **adeno-associated virus (AAV)** shows considerable promise. AAV is a defective or "satellite" virus that depends on adenovirus (or some herpes viruses) to supply some necessary functions. Consequently, it is usually found in cells that are infected with adenovirus. Unlike adenovirus, AAV seems to be entirely harmless.

The benefits of using AAV are as follows:

(a) It does not stimulate inflammation in the host.
(b) It does not provoke antibody formation and can therefore be used for multiple treatments.
(c) It infects a wide range of animals, as long as an appropriate helper virus is also present. It can therefore be cultured in many types of animal cells, including those from mice or monkeys.
(d) It can enter nondividing cells of many different tissues, unlike adenovirus.
(e) The unusually small size of the virus particle allows it to penetrate many tissues of the body effectively.
(f) AAV integrates its DNA into a single site in the genome of animal cells (the *AAVS1* site on chromosome 19 in humans). This allows the therapeutic gene to be permanently integrated.

One drawback is that the AAV genome is small (4681 nucleotides of single-stranded DNA) and the virus can carry only a relatively short segment of DNA. (AAV is unusual in packaging both plus and minus strands into virus particles. Although each virus particle contains only one ssDNA molecule, a virus preparation contains a mixture of particles, half with plus and half with minus strands.) On entering a host cell, the DNA is converted to the double-stranded replicative form, or RF, which is used for both replication and transcription. In the absence of helper virus, AAV integrates into the host chromosome and becomes latent.

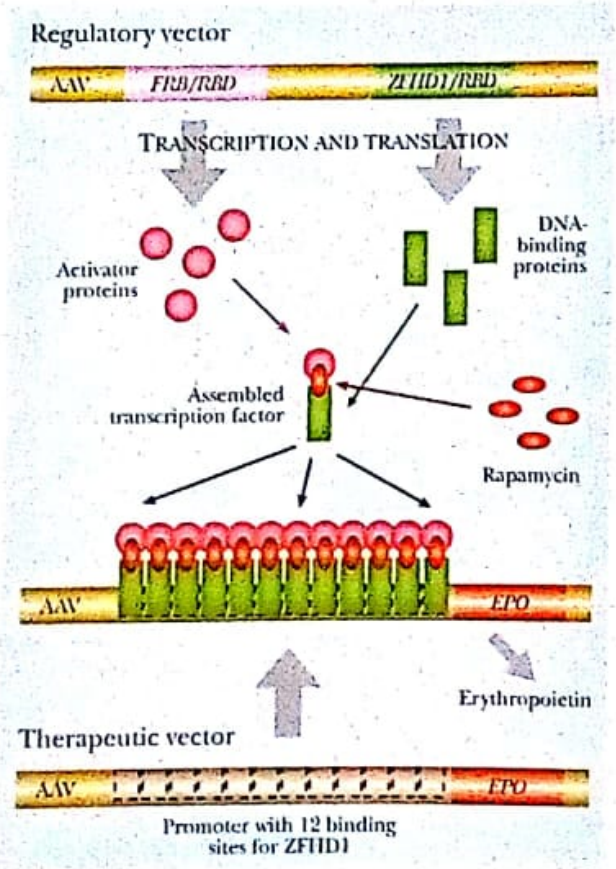

FIGURE 17.6 Double AAV System
Two AAV vectors are used to provide erythropoietin to laboratory animals. The regulatory vector (*top*) has two genes, *FRB/RBD* and *ZFHD1/RBD*, which encode two halves of a transcription factor each linked to a rapamycin binding domain (RBD). The therapeutic vector (*bottom*) has the erythropoietin gene (*EPO*) downstream of the transcription factor binding sites. When rapamycin is added, the transcription factor assembles and activates the *EPO* gene.

Genes that are permanently integrated need to be carefully regulated. This may be tackled by using two AAV vectors. Mice and monkeys have been experimentally treated with a double AAV system that provides **erythropoietin**, a protein required for development of red blood cells. One AAV vector carries the gene for erythropoietin with a promoter that must be activated by a transcription factor. The second AAV vector carries an artificial regulatory system (Fig. 17.6). This consists of two genes encoding hybrid proteins, each with one domain of the transcription factor. The other domain binds rapamycin (used as an immunosuppressant). In the presence of rapamycin, the two hybrid proteins associate via their rapamycin binding domains to form a functional transcription factor. This activates erythropoietin expression.

After delivery of the two vectors to mice, there was no production of erythropoietin. But when the animals were injected with rapamycin, the transcription factor was assembled and the erythropoietin gene was activated. The levels of erythropoietin increased up to 100-fold and the number of red blood cells rose. Even after several months, injection of rapamycin triggered a sharp rise in erythropoietin levels. Preliminary studies are now being performed in cystic fibrosis patients with AAV vectors carrying the *CFTR* gene.

Adeno-associated virus (AAV) is being developed as a gene therapy vector. A major advantage over adenovirus is that AAV does not provoke antibody formation and can be used for multiple treatments.

RETROVIRUS GENE THERAPY

Retroviruses infect many types of cells in mammals. They need dividing cells for successful infection, and will not infect many tissues where host cell growth and division have come to a standstill. Moreover, the genetic material of retroviruses passes through both DNA and RNA stages. This means that introns must be removed from any therapeutic genes before they are cloned into a retrovirus. Despite these extra technical difficulties, a retrovirus has the distinction of carrying the first gene in successful human gene therapy (see later discussion).

The retrovirus particle has an inner nucleocapsid consisting of an RNA genome inside a protein shell and an outer envelope, derived from the cytoplasmic membrane of the previous host cell. The basic retrovirus genome consists of three genes (*gag*, *pol*, and *env*) enclosed between two **long terminal repeats (LTRs)**, although more complex retroviruses such as HIV have extra genes involved in regulation. The LTR sequences are needed for integration of the DNA version of the virus genome into the host cell DNA. Between the upstream or 5′ LTR and the *gag* gene is the **packaging signal** (Fig. 17.7), which is essential for packaging the RNA into the virus particle.

Vectors for gene therapy have been derived from the simpler retroviruses, especially **murine leukemia virus (MuLV)**. The vectors have all the retrovirus genes removed, and as a result they are completely defective in replication. They retain only the packaging signal and the two LTRs (Fig. 17.7) and can carry approximately 6 to 8 kb of inserted DNA. A virus promoter in the 5′ LTR drives expression of the cloned gene. Because the vector lacks *gag*, *pol*, and *env* it cannot make virus particles. Hence these functions must be provided by a **packaging construct**, a defective provirus that is integrated into the DNA of the producer cell (see Fig. 17.7). The packaging construct lacks the packaging signal so although it is responsible for manufacture of virus particles, it is not packaged itself. The virus particles generated contain only the retrovirus vector carrying the cloned gene.

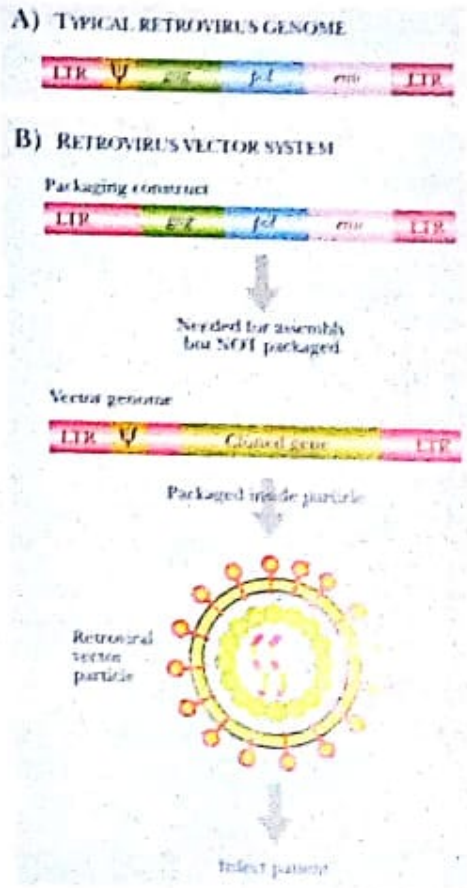

FIGURE 17.7
Retrovirus Genome and Vector System
(A) The retrovirus genome has a packaging signal (Ψ) and the genes *gag*, *pol*, and *env* flanked by two direct repeats known as LTRs. (B) Retrovirus gene therapy uses two virus constructs. The therapeutic vector carries the cloned gene and packaging signal flanked by two LTRs. The packaging vector has the three genes necessary for virus assembly and packaging, *gag*, *pol*, and *env*. Because the packaging vector does not carry the packaging signal, it is never packaged and does not infect the patient. When both constructs are present, the therapeutic vector plus cloned gene is packaged into the capsid.

After infection of the patient, the RNA inside the retroviral vector is reverse transcribed to give a DNA copy. (Although the retroviral vector does not carry a copy of the reverse transcriptase gene, a few molecules of reverse transcriptase enzyme are packaged in retrovirus particles.) Ideally, the cloned gene, enclosed between the two LTR sequences, is then integrated into host cell DNA.

Because the retroviral vectors are completely devoid of genes for retrovirus proteins, they do not cause an immune response or significant inflammation. Furthermore, their ability to integrate into host cell DNA means that the therapeutic gene will become a permanent part of the host cell genome. In principle the retrovirus could integrate into a harmful location, thus disrupting the function or regulation of a host cell gene. In practice, because most DNA in animal cells is noncoding, the chances are low, and only occasional cells would be damaged.

More serious problems are that retroviral vectors can carry only small amounts of DNA (about 8 kb) and cannot infect nondividing cells. However, the lentivirus family of retroviruses (to which HIV belongs) is unusual in being able to infect some nondividing cells. Naturally, using HIV itself is risky, but a future possibility is to transfer this ability into other, safer retroviruses. Alternatively, lentiviruses that infect other mammals, such as FIV (feline immunodeficiency virus), might be used to derive vectors.

Engineered retroviruses are the most frequently used viral vectors in gene therapy. Defective retrovirus vectors are grown in cells with an integrated helper virus to allow formation of virus particles.

RETROVIRUS GENE THERAPY FOR SCID

Severe combined immunodeficiency (SCID) occurs when both the B cells and T cells of the immune system are defective and results in an almost totally defective immune response. Children with SCID have to be shielded from all contact with other people and are kept inside special sterile plastic bubble chambers. Without immune protection any disease, even a cold, could prove fatal. Several genetic defects are known that cause SCID. About 25% are due to mutations in the ***Ada* gene** that encodes the enzyme **adenosine deaminase**. This is needed for the metabolism of purine bases, and its absence prevents development of lymphocytes (white blood cells including both the B cells and T cells).

The first successful instance of human gene therapy used a retroviral vector to provide a functional copy of the *Ada* gene to a child with SCID. The cells affected by SCID are the lymphocytes that circulate in the blood, where they carry out immune surveillance. They are produced by division of bone marrow cells (Fig. 17.8). Gene therapy involves removing bone marrow cells from the patient and maintaining them in cell culture outside the body. Because bone marrow cells constantly replenish the blood supply, they divide often and are suitable for retroviral infection. While in culture, the bone marrow cells are infected with genetically engineered retrovirus carrying the *Ada* gene and are then returned to the body.

Since 1991, several children have been treated by this approach. However, because T cells live for only 6 to 12 months, the procedure must be repeated at intervals. This problem has been tackled by using blood **stem cells**, which divide to provide the precursors to all types of blood cells and also give rise to more stem cells. These are found in bone marrow but only in very small numbers. However, umbilical cord blood has a much higher proportion of stem cells. So in 1993, blood stem cells were obtained from the umbilical blood of several newborn babies who were known to carry homozygous defects in *Ada*. The *Ada* gene was introduced into the stem cells on a retroviral vector, which has resulted in a long-term supply of healthy T cells.

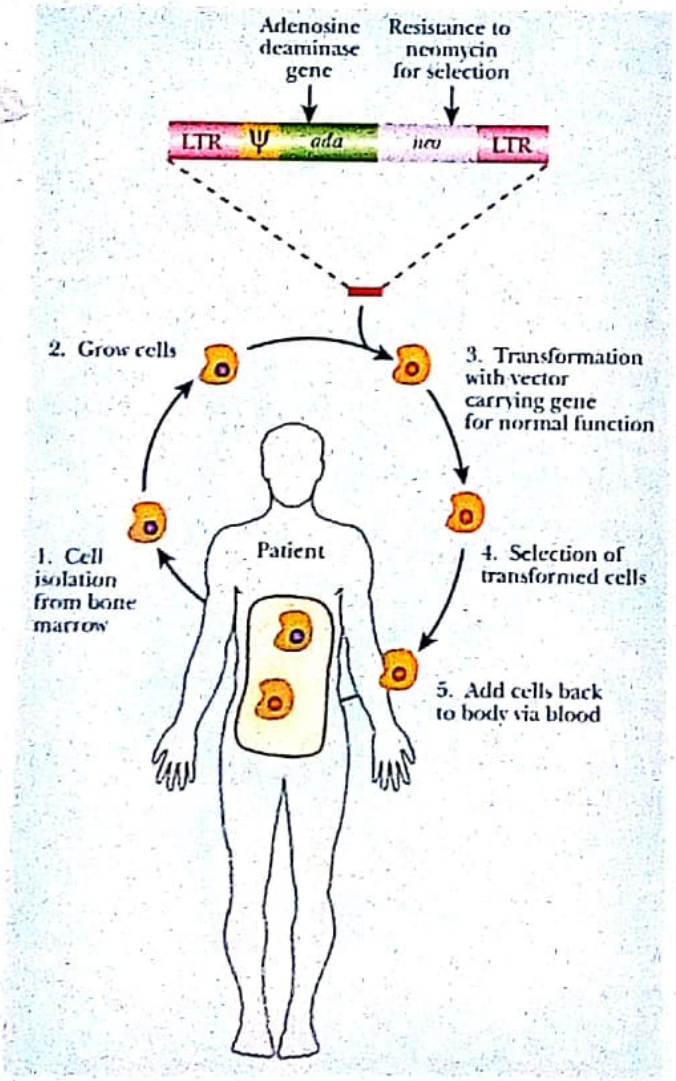

FIGURE 17.8
***Ex Vivo* Retroviral Gene Therapy for *Ada* Deficiency**
Gene therapy for SCID requires the removal of bone marrow cells from the patient. The cells are cultured and the mutant *Ada* gene is replaced with a functional copy. The bone marrow culture is treated with neomycin to kill nontransformed cells. The bone marrow cells are then replaced in the patient and repopulate the blood supply with normal blood cells.

In all the cases just discussed, the patients have been receiving injections of purified ADA enzyme as well as gene therapy. It is therefore unclear how much of their improvement is due to the gene therapy, even though these patients now have functional T cells in their blood. However, a clear-cut result has been obtained more recently with another variant of SCID. This variant is due to defects in the receptor for several **interleukins**, including IL-7, a protein that promotes development of T cells from stem cells. Because B cells need helper T cells in order to function, both B and T cells are inactive. In this case, the gene for the missing subunit of the IL-7 receptor was inserted into cultured stem cells using a retroviral vector.

Because purified protein is not used in treating this type of SCID, the patients had to depend on gene therapy alone. The patients did develop normal numbers of T cells and were successfully vaccinated against several infectious diseases, indicating a proper immune response. Consequently they were able to leave their bubbles and enjoy normal lives. Although most of the dozen patients

treated so far are still doing well, one developed leukemia, presumably due to retrovirus insertion into other genes. Thus the use of retrovirus for gene therapy needs further safeguards before it can be used more routinely.

Some types of severe combined immunodeficiencies have been successfully treated by gene therapy using a retrovirus vector.

NONVIRAL DELIVERY IN GENE THERAPY

However sophisticated a viral delivery system may be, nonviral vectors are inherently safer. Nonetheless, they have been relatively neglected because viruses were more efficient. However, several unfortunate incidents have occurred with viral vectors, especially retroviruses, including the occasional appearance of leukemia-like disease. This has resulted in renewed interest in nonviral delivery systems.

About 75% of gene therapy trials have used viral vectors. A variety of alternative approaches have also been investigated, though few have been effective or widely used so far. These include:

(a) Use of naked nucleic acid (DNA or less often RNA). Many animal cells can be transformed directly with purified DNA. The therapeutic gene may be inserted into a plasmid and the plasmid DNA used directly. Some 10% to 20% of gene therapy trials have used unprotected nucleic acid.

(b) Particle bombardment. DNA is fired through the cell walls and membranes on metal particles. This method was originally developed to get DNA into plants and is therefore discussed in Chapter 14. However, it has also been used to make transgenic animals and is occasionally used for humans.

(c) Receptor-mediated uptake. DNA is attached to a protein that is recognized by a cell surface receptor. When the protein enters the cell, the DNA is taken in with it.

(d) Polymer-complexed DNA. Binding to a positively charged polymer, such as polyethyleneimine, protects the negatively charged DNA. Such complexes are often taken up by cells in culture and may in principle be used for *ex vivo* gene therapy.

(e) Encapsulated cells. Whole cells engineered to express and secrete a needed protein may be encapsulated in a porous polymeric coat and injected locally. Foreign cells excreting nerve growth factor have been injected into the brains of aging rats. The rats showed some improvement in cognitive ability, suggesting that this approach may be of value in treating conditions such as Alzheimer's disease.

(f) Liposomes are spherical vesicles composed of phospholipid. They have been used in around 10% of gene therapy trials (see later discussion).

A variety of approaches, other than viruses, can be used to get foreign DNA into target cells. These include using naked DNA, DNA bound to artificial polymers or proteins, particle bombardment, and liposomes.

LIPOSOMES AND LIPOFECTION IN GENE THERAPY

About 10% of gene therapy trials have used **liposomes**. These are hollow microscopic spheres of phospholipid, and can be filled with DNA or other molecules during assembly. The liposomes will merge with the membranes surrounding most animal cells and the contents of the liposome end up inside the cell (Fig. 17.9), a process known as **lipofection**. Although lipofection works reasonably well, it is rather nonspecific, because liposomes tend to merge with the membranes of any cell.