CHAPTER

11

Gene Therapy

INTRODUCTION

Molecular genetic studies during the last decades have led to an enormous increase in our understanding of the molecular biology of the replication of viruses. The complete nucleotide sequences of many virus genomes have been determined. Information on the origins required for the replication of these genomes, the promoters used to express the information within them, and the packaging signals required for packaging progeny genomes into virions have been established for many. The mechanisms by which viral mRNAs are preferentially translated have been explored. Together with methods for cloning and manipulating viral genomes, this information has made possible the use of viruses as vectors to express foreign genes. In principle, any virus can be used as a vector, and systems that use a very wide spectrum of virus vectors have been described. DNA viruses were first developed as vectors, since it is possible to manipulate the entire genome in the case of smaller viruses, or to use homologous recombination to insert a gene of interest in the case of larger viruses. Recent developments now make it possible to manipulate the entire genomes of even very large DNA viruses as artificial chromosomes, which potentially makes the use of these large genomes for expression of foreign proteins even more appealing. When complete cDNA clones of RNA viruses were obtained, it became straightforward to rescue plus-strand viruses from clones because the viral RNA itself is infectious, and many such viruses have been used to express proteins. The use of minus-strand RNA viruses as vectors was delayed because the virion RNA itself is not infectious, but recent developments has made it possible to rescue virus from cloned DNA by using coexpression of the appropriate viral proteins in a transfected cell. As a consequence, minus-strand RNA viruses have also joined the club of expression systems receiving intense study. Retroviruses have also been widely used because of their capacity to integrate into a host chromosome and potentially express foreign proteins indefinitely.

A sampling of expression systems and their uses is given here to illustrate the approaches that are being followed. Every virus system has advantages and disadvantages as a vector, depending on its intended use. One of the more exciting uses has been the development of viruses as vectors for gene therapy, that is, to correct genetic defects in humans. In the most general sense, gene therapy involves transfer of genetic information into a cell, tissue, organ, or organism with the goal of improving the clinical outcome, either by curing a disease, or alleviating an underlying condition in a patient. Although results have been disappointingly slow in coming, such systems offer great promise. This use represents an example of taking these infectious agents that have been the source of much human misery and developing them for the betterment of mankind. Such expression systems have a wide variety of other potential uses, however. Efforts to engineer viruses to kill cancer cells are also receiving attention. Viruses that express foreign proteins have potential uses in the engineering of new vaccines against other pathogens. Finally, viral expression systems have proved very useful in the expression of proteins in cell culture that can be used for various studies.

VIRUS VECTOR SYSTEMS

A representative sampling of viruses that are being developed as vectors is described next in order to illustrate some of the strengths and weaknesses of the different systems. The viruses used in most clinical trials to date have been the poxviruses, the adenoviruses, and the retroviruses, and these are described here. Several other virus systems that may be used in the future for treatment of humans, or that are useful for other purposes, are also described.

Vaccinia Virus

Vaccinia virus is a poxvirus with a large dsDNA genome of 200 kb (Chapter 7). Until recently, this genome was too big to handle in one piece in a convenient fashion, and homologous recombination has been used to insert foreign genes into it. The large size of the viral genome, however, does mean that very large pieces of foreign DNA can be inserted, while leaving the virus competent for independent replication and assembly. Another advantage of the virus is that it has been used to vaccinate hundreds of millions of humans against smallpox. Thus, there is much experience with the effects of the virus in humans. Although the vaccine virus did cause serious side effects in a small fraction of vaccinees, highly attenuated strains of vaccinia have been developed for use in gene therapy by deleting specific genes associated with virulence. A new approach to the use of poxvirus vectors has been the development of nonhuman poxviruses, such as canarypox virus, as vectors. Canarypox virus infection of mammals is abortive and essentially asymptomatic, but foreign genes incorporated into the canarypox virus genome are expressed in amounts that are sufficient to obtain an immunologic response.

A variety of approaches have been used to obtain recombinant vaccinia viruses that express a gene of interest, but only the first such method to be used, and one that remains in wide use, is described here. This method is illustrated in Fig. 11.1. The thymidine kinase (TK) gene of vaccinia virus is nonessential for growth of the virus in tissue culture. Furthermore, deletion of the TK gene results in attenuation of the virus in humans, which is a desirable trait. Finally, the TK gene can be either positively or negatively selected

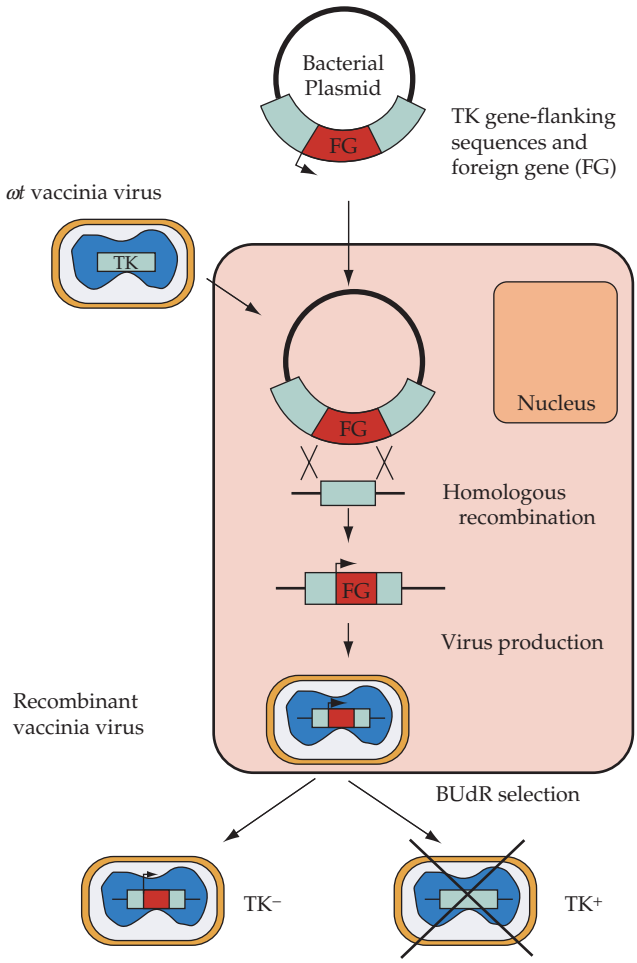

FIGURE 11.1 Construction of recombinant vaccinia virus expression vector. The foreign gene (red) is inserted into a bacterial plasmid adjacent to a vaccinia promoter (black arrow), flanked with sequences from the vaccinia thymidine kinase gene (turquoise). Plasmid DNA is transfected into cells infected with wild-type (TK^+) vaccinia virus. Recombinant progeny from homologous recombination are all TK^-, due to interruption of the TK gene, and can be selected by growth in bromodeoxyuridine (BUdR), since incorporation of BUdR into TK^+ vaccinia is lethal. These TK^- vaccinia will infect normally and express the foreign gene under control of the vaccinia promoter. Adapted from Strauss and Strauss (1997) Figure 2.25 on p. 115.

by using different media for propagation of the virus. The starting point is a plasmid clone that contains a copy of the TK gene that has a large internal deletion. In the region of the deletion a vaccinia virus promoter is inserted upstream of a polylinker. The gene of interest is inserted into the polylinker using standard cloning technology. Thus, we have the foreign gene downstream of a vaccinia promoter, and the entire insert is flanked by sequences from the vaccinia TK gene. The plasmid containing the cloned TK gene with its foreign gene insert is transfected into cells that have been infected by wild-type vaccinia virus. Homologous recombination between the TK gene in the virus and the TK-flanking sequences in the plasmid occurs with a sufficiently high frequency that a reasonable fraction of the progeny have the gene of interest incorporated. These viruses have an inactive TK gene (they are TK^-), because the TK gene has been replaced by the deleted version containing the inserted foreign gene. The next step, then, is to select for viruses that are TK^- by growing virus in the presence of bromodeoxyuridine (BUdR). An active TK enzyme will phosphorylate BUdR to the monophosphate form, which can be further phosphorylated by cellular enzymes to the triphosphate and incorporated into the viral nucleic acid during replication. Incorporation of BUdR is lethal under the appropriate conditions, and thus viruses that survive this treatment are those in which the TK gene has been inactivated.

It is usually necessary to select among the surviving progeny for those that possess the gene of interest, because inactivation of the TK gene can occur spontaneously through deletion or mutation. Selection can be accomplished by a plaque lift hybridization assay in which virus in plaques is transferred to filter paper. Virus plaques on the filter paper are probed with radiolabeled hybridization probes specific for the inserted gene. Virus in plaques that hybridize to the probe is recovered and further passaged. In this way a pure virus stock that will express the gene of interest can be isolated.

Herpesviruses

The herpesviruses also have a large DNA genome that is capable of accommodating large inserts of foreign DNA. HSV-1, in particular, has been studied as an expression vector. Recombination has been used to insert foreign genes and to delete virus genes involved in lytic growth or toxicity. Because HSV-1 is neurotropic, it has been considered as a possible vector for the control or eradication of neural cancers. The viral DNA does not integrate, and the virus is capable of infecting nonreplicating neurons and being maintained in a latent state, properties that suggest it could be used for this purpose. It might also be used as an expression vector that could produce protein for long periods in neurons, and as such might be useful for the treatment of spinal nerve injury, for example, or for pain therapy.

Baculoviruses

The baculoviruses are insect viruses that have a large DNA genome capable of accommodating large DNA inserts. Foreign DNA is inserted by recombination and selection of appropriate viruses. They have been widely used to express high levels of protein in eukaryotic cells (insect cells in this case) that can be used, for example, in crystallization trials to determine protein structure, or to produce protein for immunization of animals, and other uses. Recent studies have suggested that baculoviruses might be useful for gene therapy in humans. The viruses will infect a number of human cells resulting in expression of proteins of interest, but the viruses are nonpathogenic in humans, suggesting a level of safety in their possible application. Whether problems associated with these viruses, such as low levels of expression and the rejection of them by the immune system, can be overcome remains to be determined.

Adenoviruses

Adenovirus infections of humans are common and normally cause only mild symptoms. Deletion of virulence genes from adenovirus vectors further attenuates these viruses. In addition, adenovirus vaccines have been used by the military for some years and, therefore, some experience has been gained in the experimental infection of humans by adenoviruses, although gene therapy trials use a different mode of delivery of adenovirus vectors. Because of their apparent safety, adenoviruses have been developed for use as vectors in gene therapy trials or for vaccine purposes. Two approaches have been used. In one, infectious adenoviruses have been produced that express a gene of interest. In the second approach, suicide vectors are produced that can infect a cell and express the gene of interest, but which are defective and cannot produce progeny virus. Suicide vectors cannot spread to neighboring cells, and the infection is therefore limited in scope and in duration.

The genome of adenoviruses is dsDNA of 36 kbp (Chapter 7). Thus, the genome is smaller than that of poxviruses or other large DNA viruses such as the herpesviruses and the baculoviruses and can accommodate correspondingly smaller inserts. However, inserts large enough for most applications can be accommodated. The genome is small enough that the virus can be reconstituted from DNA clones. Such an approach is inconvenient, however, and homologous recombination is often used to insert the gene of interest into the virus genome.

The foreign gene is inserted into the region occupied by either the adenovirus E1 or E3 genes, one or both of which are deleted in the vector construct. Virus lacking E1 cannot replicate, and such viruses form suicide vectors. For gene therapy, suicide vectors are normally used so as to prevent the spread of the infection. To prepare the stock

of virus lacking E1, the virus must be grown in a cell line that expresses E1. An overview of this process is shown in Fig. 11.2. The complementing cell line, which produces E1 constitutively, supplies the E1 needed for replication of the defective adenovirus. The cells are transfected with the defective adenovirus DNA and a full yield of progeny virions results. The progeny virus is defective and cannot replicate in normal cells, but it can be amplified by infection of the complementing cell line. On introduction of the virus into a human, the virus will infect cells and express the foreign gene, but the infection is abortive and no progeny virus is formed. The stock of defective virus must be tested to ensure that no replication-competent virus is present, since such virus can arise by recombination between the vector and the E1 gene in the complementing cell line.

Adenoviruses with only E3 deleted are often used to express proteins for vaccine purposes. These E3-deleted viruses possess intact E1 and will replicate in cultured cells and in humans, but are attenuated. Because the virus replicates, expression of the immunizing antigen persists for a long time and a good immune response usually results.

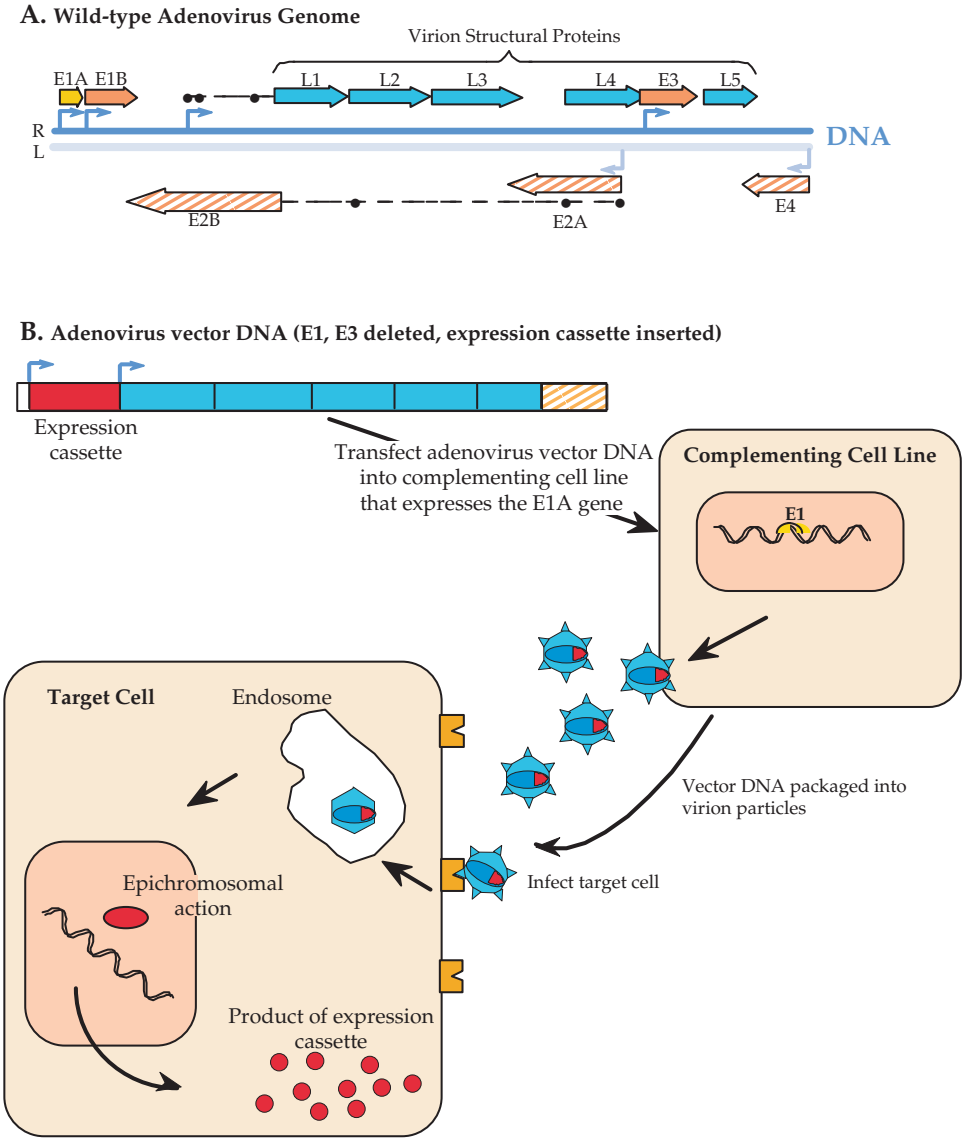

FIGURE 11.2 Generation of a nonreplicating adenovirus expression vector. From the wild-type adenovirus genome, the E1 and E3 genes are removed. The E1 genes are replaced with an expression cassette. This adenovirus DNA is transfected into a complementing cell line that produces E1 protein. The transfection produces particles that are able to infect cells, but which are E1$^-$ and nonreplicating. The DNA genome is delivered to the nucleus where it functions as an epichromosome and directs expression of the inserted foreign gene. Adapted from Crystal (1995).

The procedure for insertion of the gene of interest by homologous recombination resembles that used for the poxviruses. The gene is inserted into a plasmid containing flanking sequences from the E1 or E3 region, and transfected into cells infected with adenovirus. Recombinant viruses containing the gene of interest are selected and stocks prepared. It is also possible to transfect cells with the E1 or E3 expression cassette together with DNA clones encoding the rest of the adenovirus genome, in which case homologous recombination results in the production of virus. In the case of insertions into E1, cells that express E1 must be used to produce the recombinant virus.

Adeno-Associated Viruses

Adeno-associated viruses (AAVs) have a single-stranded DNA genome of 4.7 kb (Chapter 7). They normally require coinfection of a cell by a helper virus, usually an adenovirus or a herpesvirus. They are being developed as expression vectors because they are not pathogenic in humans and because they normally integrate into the host-cell genome in a specific region, thus minimizing the problems of insertional mutagenesis. The genome size is small enough to be readily manipulated as a DNA clone, but the small size also limits the amount of DNA that can be inserted and therefore the applicability of the virus for gene transfer experiments. A related problem is that for expression studies, the genome is normally deleted for the AAV genes with only the ends that function as promoter sites retained. However, site-specific integration requires the activity of the Rep protein. Nonetheless, the system is sufficiently attractive that efforts to develop AAV as a gene therapy vector continue and the virus has been used in a number of clinical trials, as described later.

Retroviruses

Retrovirus-based expression systems offer great promise because the retroviral genome integrates into the host-cell chromosome during infection and, in the case of the simple retroviruses at least, remains there as a Mendelian gene that is passed on to progeny cells on cell division. Thus, there is the potential for permanent expression of the inserted gene of interest. The essential components of a retrovirus vector are the long terminal repeats (LTRs), the packaging sequences known as ψ, the primer-binding site, and the sequences required for jumping by the reverse transcriptase during reverse transcription to form the dsDNA copy of the genome (Chapter 6).

The process of creating and packaging a retrovirus-based expression cassette is illustrated in Fig. 11.3. A packaging cell line is created that expresses the retroviral *gag*, *pol*, and *env* genes, but whose mRNAs do not contain the packaging signal and so cannot be packaged. The vector DNA/RNA is created by modifying a DNA clone of a retrovirus to contain the gene of interest in place of the *gag–pol–env* genes. In the process, all of the essential *cis*-acting signals required for packaging, reverse transcription, and integration are retained. The foreign gene can be under the control of the LTRs, or it can be under the control of another promoter positioned in the insert upstream of it. The resulting DNA clone is transfected into the packaging cell line, and a producer cell line isolated that expresses the vector DNA as well as the helper DNA. Vector RNA transcribed from the vector DNA is packaged into retroviral particles, using the proteins expressed from the helper DNA. These particles are infectious and can be used to infect other cells or to transfer genes into a human. On infection of cells by the packaged vector, the vector RNA is reverse transcribed into DNA that integrates into the host-cell chromosome, where it can be expressed under the control of the promoters that it contains. The limitation on the size of the insert is about 10 kb, the upper limit of RNA size that can be packaged.

Although murine leukemia viruses are not known to cause disease in man, it has been found that these viruses will cause tumors in immunosuppressed subhuman primates. Thus, it is thought to be essential that there be no replication-competent virus in stocks used to treat humans. Replication-competent virus can arise during packaging of the vector by recombination between the vector and the retroviral sequences used to produce Gag–Pol–Env. At the current time, preparations of packaged vectors are screened to ensure that replication-competent viruses are not present. Efforts are being made to reduce the incidence of recombination during packaging in order to simplify the procedure. One approach is to develop vectors that have very little sequence in common with the helper sequences, in order to reduce the incidence of homologous recombination. A second approach is to separate the Gag–Pol sequences from the Env sequences in the helper cell. In this case, recombination between three separate DNA fragments in the producer cell (that encoding Gag–Pol, that encoding Env, and sequences in the vector) are required in order to give rise to replication-competent retrovirus.

In gene therapy trials that use retroviruses, it has been found that the expression of the foreign gene in humans is often downregulated after a period of months. Attempts are being made to identify promoters that will not be downregulated. Different promoters might be required for different uses, and promoters that target transcription to particular cell types would be useful.

A major problem with retroviral vectors is that simple retroviruses will only infect dividing cells. Although they enter cells and are reverse transcribed into DNA, the DNA copy of the genome can enter the nucleus only during cell division. In many gene therapy treatments, it is desirable to infect stem cells in order to maintain expression of the therapeutic gene indefinitely. Because stem cells divide relatively infrequently, it is difficult to infect a high proportion of them by vectors used to date. Attempts are being made to identify methods to stimulate stem cells to divide during *ex vivo* treatment,

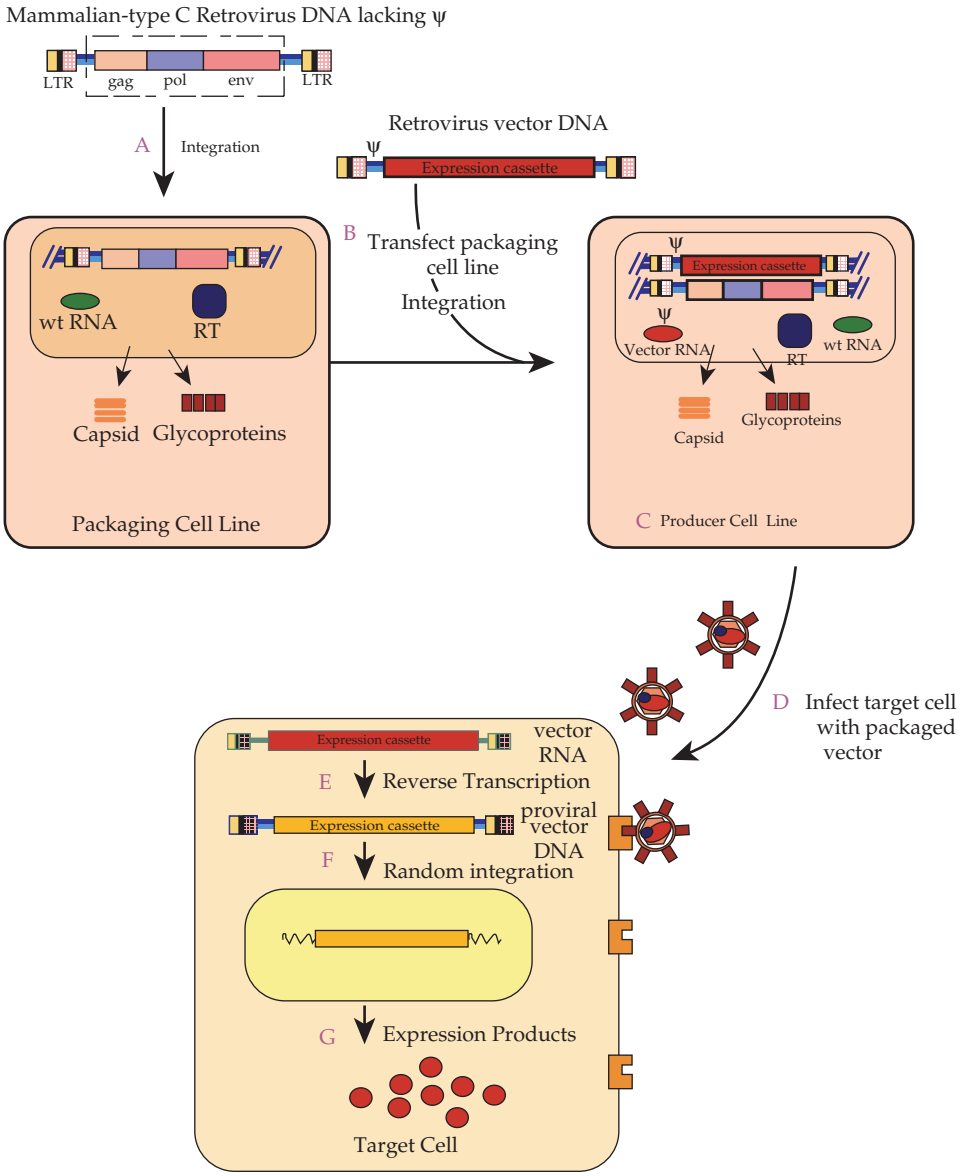

FIGURE 11.3 Scheme for producing a packaged replication-defective retrovirus expression vector. (A) A "packaging" cell line is generated by introduction of DNA encoding *gag*, *pol*, and *env* genes into the chromatin of a fibroblast cell line. This DNA lacks the packaging signal Ψ and RNA transcribed from it is not packaged. (B) The packaging cell line is transfected with a second retroviral DNA, in which the foreign gene (expression cassette) replaces *gag*, *pol*, and *env*, but which has an intact Ψ packaging signal and intact LTRs, to form a "producer" cell line. This producer cell line (C) releases vector particles containing the expression cassette genome packaged with the proteins from the helper genome. (D) These particles enter target cells via specific cell surface receptors, (E) are reverse transcribed, (F) randomly integrate, and (G) produce expression products. Adapted from Dunbar (1996).

so that a larger fraction of them can be infected. A second approach is to develop lentivirus vectors. Lentiviruses, which include HIV, can infect nonreplicating cells and could potentially infect nondividing stem cells during *ex vivo* treatment. Lentivirus vectors could also be useful for therapy involving other nondividing cells, such as neurons.

It would be of considerable utility to be able to target retroviruses to specific cells. One possible approach to this is to replace all or part of the external domains of the retroviral surface glycoprotein with a monoclonal antibody that is directed against an antigen expressed only on the target cells. In principle, this approach is feasible, but whether it can be developed into something practical is as yet an open question. If specific cells could be infected, it would allow protocols in which the therapeutic gene would be expressed only in cells where it would be most useful. It would also allow the specific killing of cells such as tumor cells or HIV-infected cells. For example, the retrovirus could express a gene that rendered the cell sensitive to toxic drugs such as BUdR. A retrovirus vector that expressed such a gene could

also be useful for conventional gene therapy, because it would allow the infected cells to be killed if the infection process threatened to get out of hand.

Alphaviruses

The genomes of plus-strand RNA viruses are self-replicating molecules that replicate in the cytoplasm, and they can express very high levels of protein. These properties make them potentially valuable as expression vectors.

The alphaviruses possess a genome of single-strand RNA of about 12 kb (Chapter 3). Their genomes can be easily manipulated as cDNA clones, and infectious RNA can be transcribed from these clones by RNA polymerases, either *in vivo* or *in vitro*. RNA transcribed *in vitro* can be transfected into cells and give rise to a full yield of virus, whereas RNA transcribed *in vivo* will begin to replicate and produce virus. The structural proteins are made from a subgenomic mRNA, making it easy to insert a foreign gene under the control of the subgenomic promoter. Two approaches that have been used are illustrated in Fig. 11.4. In one approach, a second subgenomic promoter is inserted into the genome downstream of the structural proteins, or between the structural proteins and the nonstructural proteins (Fig. 11.4C). Two subgenomic mRNAs are transcribed, one for the structural proteins and the second for the gene of interest. The size of the insert must be relatively small, on the order of 2000 nucleotides or less, because longer RNAs are not packaged efficiently. However, this system has the advantage that the resulting double subgenomic virus is an infectious virus that can be propagated and maintained without helpers.

A second approach is to delete the viral structural proteins and replace them with the gene of interest. In this case, there is room for an insert of about 5 kb that will still allow the resulting replicon to be packaged. The replicon is capable of independent replication, and transcription of a subgenomic messenger results in expression of the gene of interest. The replicon constitutes a suicide vector. It cannot be packaged unless the cells are coinfected with a helper to supply the structural proteins, or unless a packaging cell line that expresses the viral structural proteins is used.

Alphavirus replicons can be extremely efficient in expressing a foreign gene. In some cases as much as 25% of the protein of a cell can be converted to the foreign protein expressed by the replicon over a period of about 72 hours. Wild-type replicons are cytolytic in vertebrate cells, inducing apoptosis, and the infection dies out. However, replicons have been produced with mutations in the replicase proteins that are not cytolytic and will produce the protein of interest

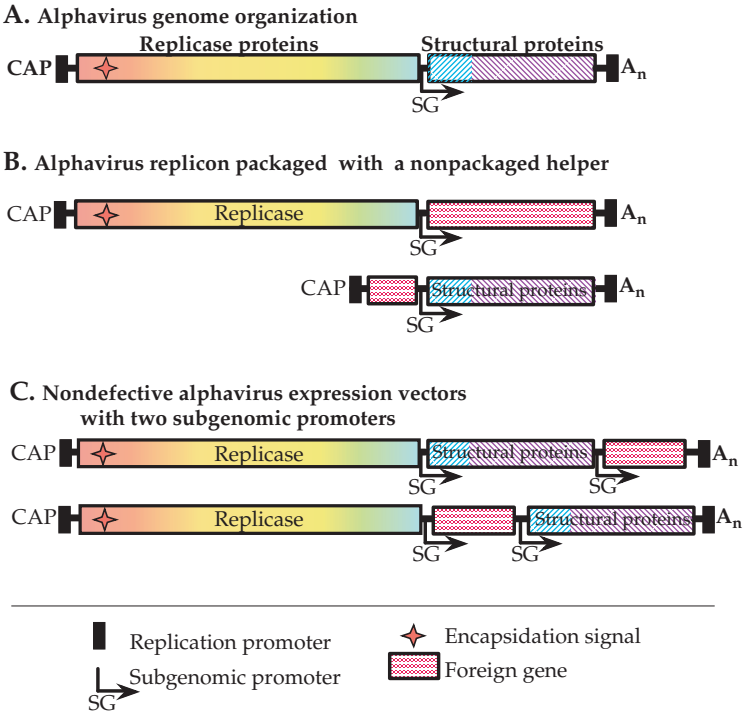

FIGURE 11.4 Alphavirus expression vectors. (A) The genome organization of a typical alphavirus with the location of the promoters for replication and production of subgenomic RNA as well as the RNA-packaging signal indicated. (B) A simple alphavirus replicon. The structural proteins of the virus have been replaced with the foreign gene to be expressed. If packaging of the replicon is required, the structural proteins of the virus are supplied on a DI RNA lacking a packaging signal. (C) Packaged expression vectors with two subgenomic promoters. These constructs are unstable if the foreign gene is much larger than 2 kb. Adapted from Strauss and Strauss (1994) Figure 23.

indefinitely. Thus, a wide sprectrum of choices is available, and the system chosen can be adapted to the needs of a particular experiment or treatment.

Viral expression systems would be more useful if they could be directed to specific cell types. An approach that uses monoclonal antibodies to direct Sindbis virus to specific cells has been described. Protein A, produced by *Staphylococcus aureas*, binds with high affinity to IgG. It is an important component of the virulence of the bacterium because it interferes with the host immune system. The IgG-binding domain of protein A has been inserted into one of the viral glycoproteins. Virions containing this domain are unable to infect cells using the normal receptor. However, the virus will bind IgG monoclonal antibodies. If an antibody directed against a cell surface component is bound, the virus will infect cells expressing this protein at the cell surface. Thus, this system has the potential to direct the virus to a specific cell type. One of the advantages of this approach is that the virus, once made, can be used with many different antibodies and thus directed against a variety of cell types. This approach is potentially applicable to any enveloped virus, and perhaps to nonenveloped viruses as well.

A modification of the alphavirus system is to use a DNA construct containing the replicon downstream of a promoter for a cellular RNA polymerase, rather than using packaged RNA replicons. On transfection of a cell with the DNA, the replicon RNA is launched when it is transcribed from the DNA by cellular enzymes. Once produced, the RNA replicates independently and produces the subgenomic mRNA that is translated into the gene of interest. As described in Chapter 10, naked DNA can be used to transfect muscle cells and perhaps other cells.

Polioviruses

Plus-strand viruses that do not produce subgenomic mRNAs, such as the picornaviruses and flaviviruses, present different problems for development as vectors. The translated product from the gene of interest must either be incorporated into the polyprotein produced by the virus and provisions made for its excision, or tricks must be used to express the gene of interest independently. Two approaches with poliovirus will be described as examples of how such viruses might be used as vectors.

Poliovirus replicons have been constructed by deleting the region encoding the structural proteins and replacing this sequence with that for a foreign gene. The foreign gene must be in phase with the remainder of the poliovirus polyprotein, and the cleavage site recognized by the viral 2A protease is used to excise the foreign protein from the polyprotein. Because the poliovirus replicon lacks a full complement of the structural genes (it is a suicide vector), packaging to produce particles requires infection of a cell that expresses the polioviral structural proteins by some mechanism. A construct that uses this approach to express the cytokine tumor necrosis factor alpha (TNF-α) is illustrated in Fig. 11.5. A poliovirus "infectious

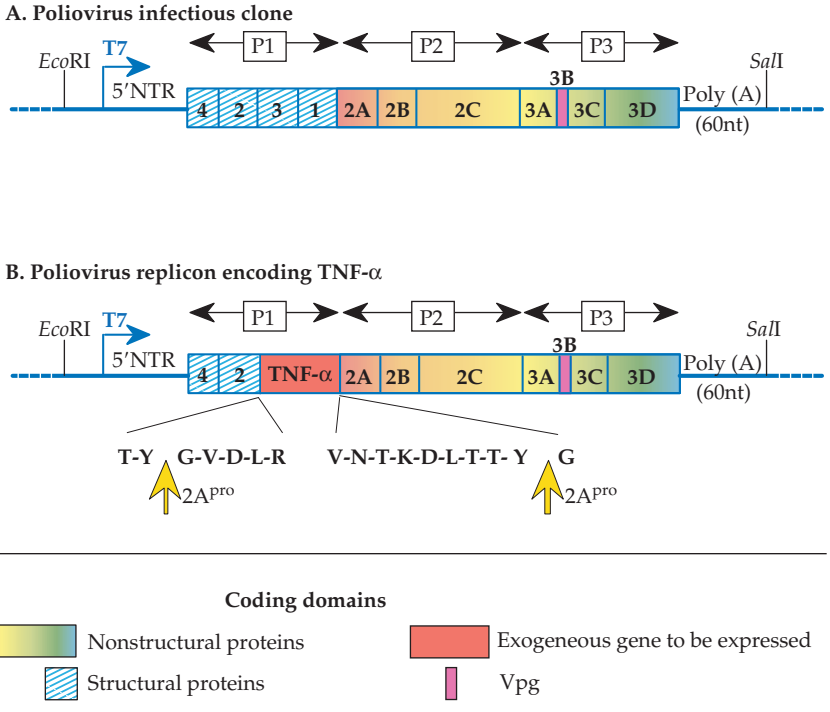

FIGURE 11.5 Generation of poliovirus replicons for expression of foreign genes in motor neurons. Based on an earlier construct to express interleukin-2 via a poliovirus replicon, the gene for wild-type murine tumor necrosis factor alpha (TNF-α) was positioned between the VP0 and 2A proteins of poliovirus, replacing VP3 and VP1. It was flanked on either side by sites for cleavage by the poliovirus 2A protease. These constructs were injected into transgenic mice expressing the poliovirus receptor, and expression of murine TNF-α was monitored. Adapted from Bledsoe *et al.* (2000).

clone" in which a DNA copy of the viral genome is positioned downstream of a promoter for T7 RNA polymerase is modified by replacing the genes for VP3 and VP1 with the gene for TNF-α. Recognition sites for the poliovirus 2A protease are positioned on both sides of the TNF-α gene. The TNF-α protein is produced as part of the poliovirus polyprotein, and cleaved from the polyprotein by the 2A protease. Packaged replicons were used to infect transgenic mice that expressed the polio receptor (Chapter 1). One of the interests of this system is that poliovirus exhibits an extraordinary tropism for motor neurons in the central nervous system (CNS) (Chapter 3). The packaged replicons, on introduction into the CNS, infected only motor neurons, and therefore the foreign gene was expressed only in motor neurons. Such replicons may be useful to treat CNS diseases in which motor neurons are affected.

A second approach to the use of poliovirus replicons is to use a second internal ribosome entry site (IRES) (Chapter 1) to initiate the synthesis of the nonstructural proteins. If the foreign gene replaces the structural genes, it will be translated from the 5′ end of the genome. If the poliovirus nonstructural genes are placed downstream of a second IRES, internal initiation at this IRES results in production of a polyprotein for the nonstructural proteins. This approach is similar to the approach shown in Fig. 3.3, where the structural proteins are replaced by a gene of interest.

Coronaviruses as Expression Vectors and Vaccine Candidates

The coronaviruses have long been considered as potential candidates for experimental expression vectors or as candidate vaccines. The viruses have the largest nonsegmented RNA viral genomes, up to 31 kb (see also Chapter 3), which is both an advantage in that they could potentially accommodate a large amount of heterologous nucleic acid, and a disadvantage due to the difficulties of manipulating large RNA molecules. The essential genes are arranged 5′-replicase-S-E-M-N-3′, and are interspersed with a number of nonessential genes which are group specific. Recent studies have revealed a number of characteristics which make them even more attractive as vector candidates. The first was that the deletion of the nonessential genes is sufficiently attenuating that no further mutations in the essential genes are required to produce an avirulent virus. Second, as the precise domains on the S protein which interact with the species-dependent cellular receptors were determined, it was found that both species and tissue specificity could be altered by relatively minor changes in the sequence of S. Third, it is possible to rearrange the linear order of the genes, and while this altered the relative amounts of the products, it reduces the possibility that the vector (vaccine) could undergo recombination with field strains. Fourth, it is possible to insert heterologous genes anywhere in the genome by simply incorporating a cassette comprised of the gene of interest preceded by the specific intergenic sequence of the parental virus.

The generation of a recombinant MHV (mouse hepatitis virus) encoding *Renilla* luciferase is shown in Figure 11.6 to illustrate the strategies employed. These include maintenance of a replication defective genome as a bacterial plasmid under the control of a T7 promoter, transcription of RNA *in vitro*, electroporation of RNA into feline cells previously infected with a murine coronavirus engineered to infect feline cells, *in vivo* recombination, and selection for recombinants on murine cells. Due to the high frequency of recombination in coronaviruses, many recombinants are unstable and not suitable for use as vaccines. However, the location of the foreign gene within the genome, the particular coronavirus used, and the identity of the particular heterologous gene all have significant effects on stability, and coronaviruses may yet prove to be useful for targeted gene delivery.

Rhabdoviruses and Other Negative Strand Viruses

In minus-strand RNA viruses, the genomic RNA is not itself infectious. Ribonucleoprotein containing the N, P, and L genes is required for replication of the viral RNA, and thus for infectivity, and only recently have methods been devised to recover virus from cDNA clones. A schematic diagram of how virus can be recovered from DNA clones of the rhabdovirus vesicular stomatitis virus (VSV) (Chapter 4) is shown in Fig. 11.7. A cell is transfected with a set of cDNA clones that together express N, P, and L as well as the genomic or antigenomic RNA. The antigenomic RNA usually works better, probably because it does not hybridize to the mRNAs being produced from the plasmids. Encapsidation of the antigenomic RNA by N, P, and L to form nucleocapsids allows it to replicate and produce genomic RNA that is also encapsidated. Synthesis of mRNAs from the genomic RNA, together with continued replication, results in a complete virus replication cycle and production of infectious progeny virus that have as their genome the RNA supplied as a cDNA clone. The yield of infectious virus is small, but sufficient to isolate individual plaques and thus obtain viruses from the cDNA clones.

The ability to rescue virus from a cDNA clone makes it possible to manipulate the viral genome. Since the rhabdovirus genome is transcribed into multiple mRNAs, one for each gene, and the transcription signals recognized by the enzyme are well understood, it is relatively simple to add or delete genes. A modified VSV that was produced by using DNA clones is illustrated in Fig. 11.8. In this VSV, the surface glycoprotein present on the VSV particle, called G, has been deleted and replaced with CD4, the cell surface protein that is used as a receptor by HIV. In addition a new gene has been inserted, the gene encoding the HIV coreceptor CXCR4, so that the virus now contains six genes. The virions produced

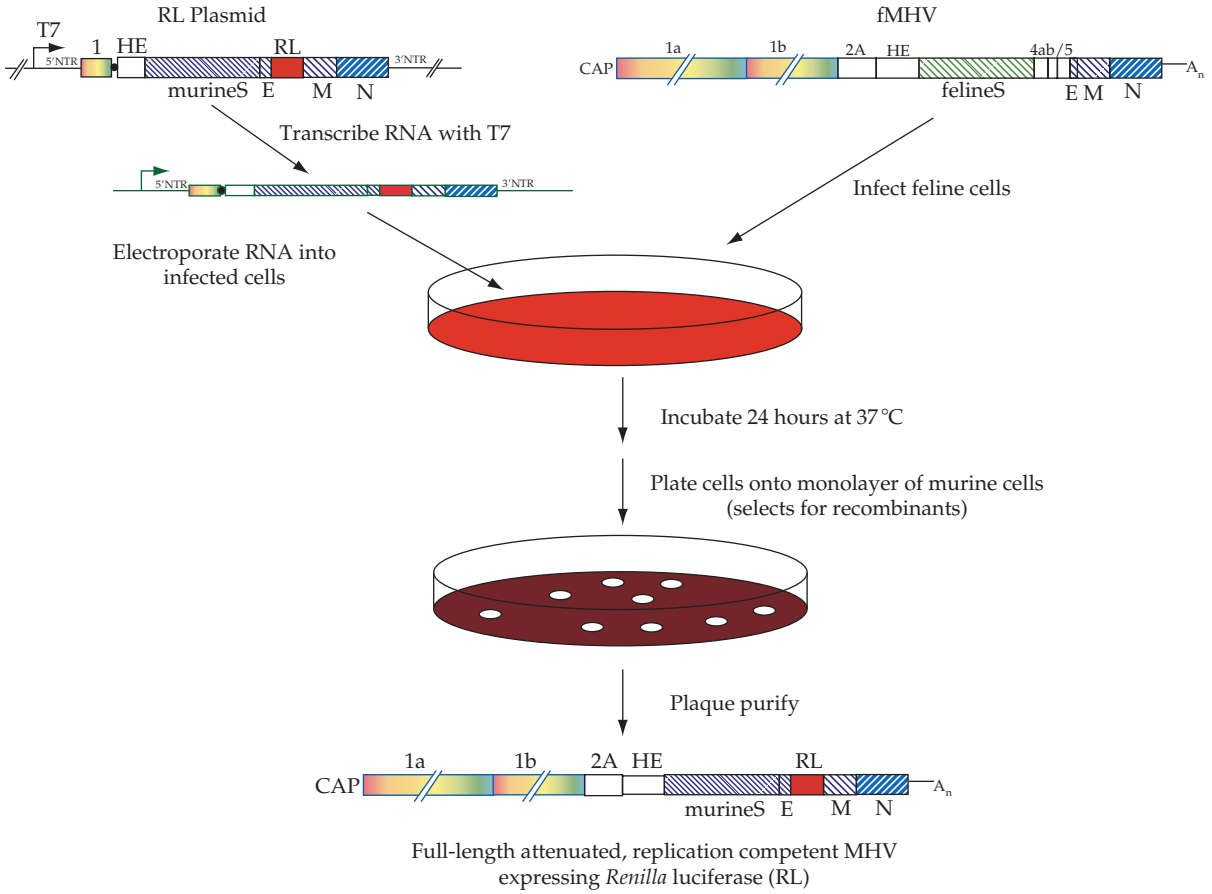

FIGURE 11.6 Construction of a coronavirus expression plasmid. An attenuated and nonreplicating plasmid from MHV is constructed in which the 5′ end of the ORF 1 sequence is fused to the last 28 codons of HE. In addition, all of the accessory protein genes are deleted, and the gene for *Renilla* luciferase (RL) inserted. RNA is transcribed *in vitro* from this plasmid using T7 polymerase and the RNA electroporated onto feline cells which have been infected with MHV virus in which the spike S protein has been mutated to recognize receptors on feline cells (fMHV). After 24 hours these cells are plated out onto a monolayer of murine cells to select for coronaviruses that have undergone recombination between the plasmid and the helper virus. Recombinants are plaque-purified and stocks grown.

are unable to infect the cells normally infectable by VSV because they lack the G protein. However, because they contain the HIV receptor and coreceptor on their surface, they do infect cells that express the HIV glycoprotein on their surface, such as cells infected by HIV. Since VSV is a lytic virus, the HIV-infected cells are killed.

USE OF VIRUSES AS EXPRESSION VECTORS

Viruses have been widely used as vectors to express a variety of genes in cultured cells. This use is of long standing and has led to important results. Of perhaps more interest are efforts to develop viruses as vectors for medical purposes. The manipulation of virus genomes to develop new vaccines is very promising. Although no licensed human vaccines have been introduced using this technology, clinical trials are ongoing and it is to be expected that several such vaccines will be licensed in the near future. There is also expectation that viruses will be useful as vectors for gene therapy, and numerous clinical trials are taking place. The results to date have been disappointing, but the promise remains.

Expression of Proteins in Cultured Cells

The use of viruses to express foreign genes in cultured cells is well established and only a few examples are cited to illustrate the range and purpose of such use.

Hepatitis C virus (HCV) does not grow in cultured cells to titers sufficient to allow studies on the expression of viral proteins. The only experimental model for the virus is the chimpanzee, which severely restricts the number and nature of experiments that can be done. Thus, most of what we know about the expression of the HCV genome has been obtained through expression of parts of

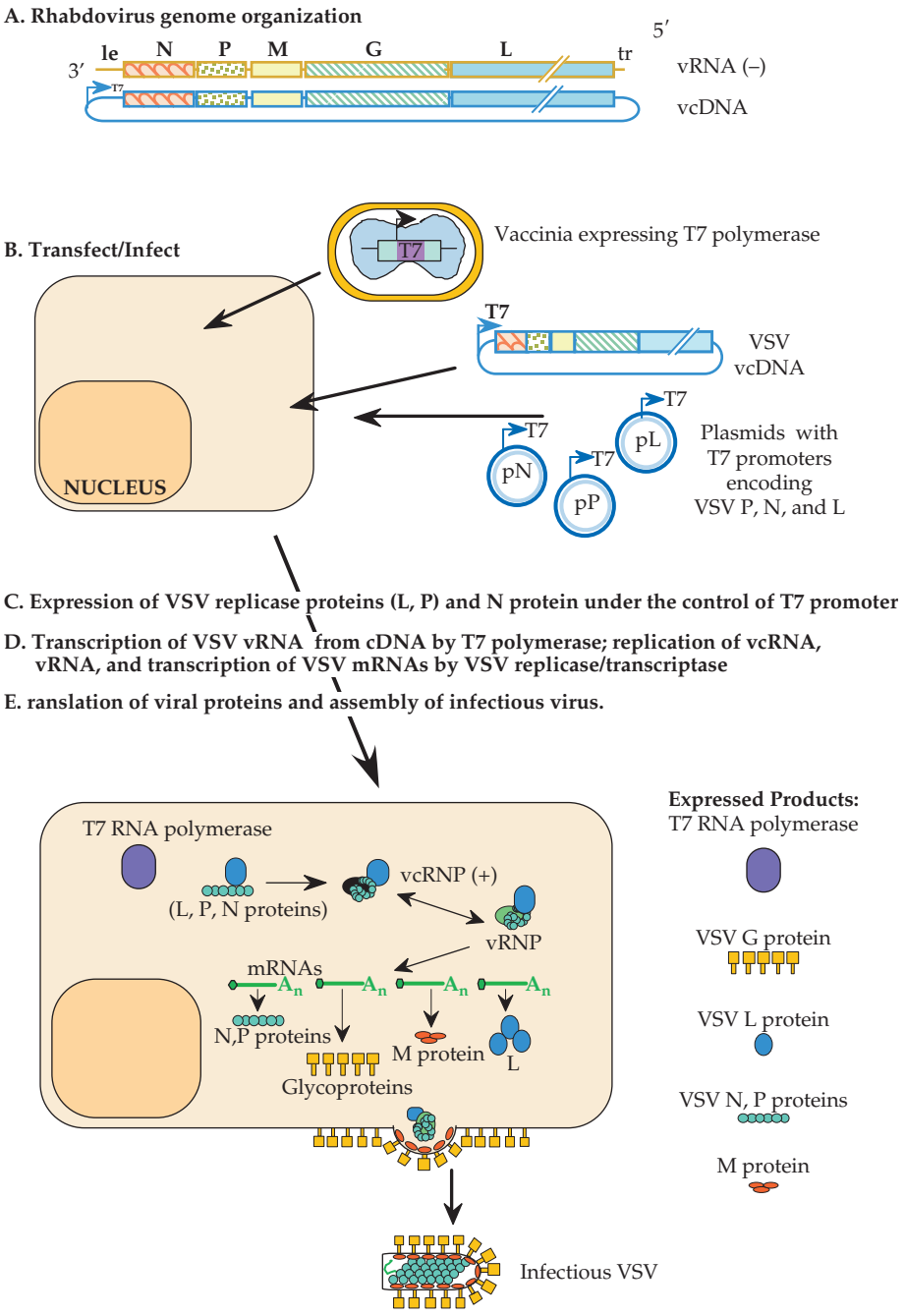

FIGURE 11.7 Rescuing infectious VSV virions from cDNA clones. (A) The rhabdovirus genome organization, and a schematic of a cDNA clone containing the genome sequence (cDNA copy of vRNA). (B) A susceptible cell is infected with vaccinia virus expressing the T7 RNA polymerase, and transfected with four separate plasmids: the genome plasmid from which plus strand anti-genome RNA is transcribed, and 3 individual plasmids expressing VSV N protein, P protein, and L protein, all under the control of T7 promoters. (C), (D), (E) Steps in the synthesis of infectious virus are described within the figure. Infectious virions bud from the cell and can infect a new susceptible cell. Adapted from Conzelmann and Meyers (1996).

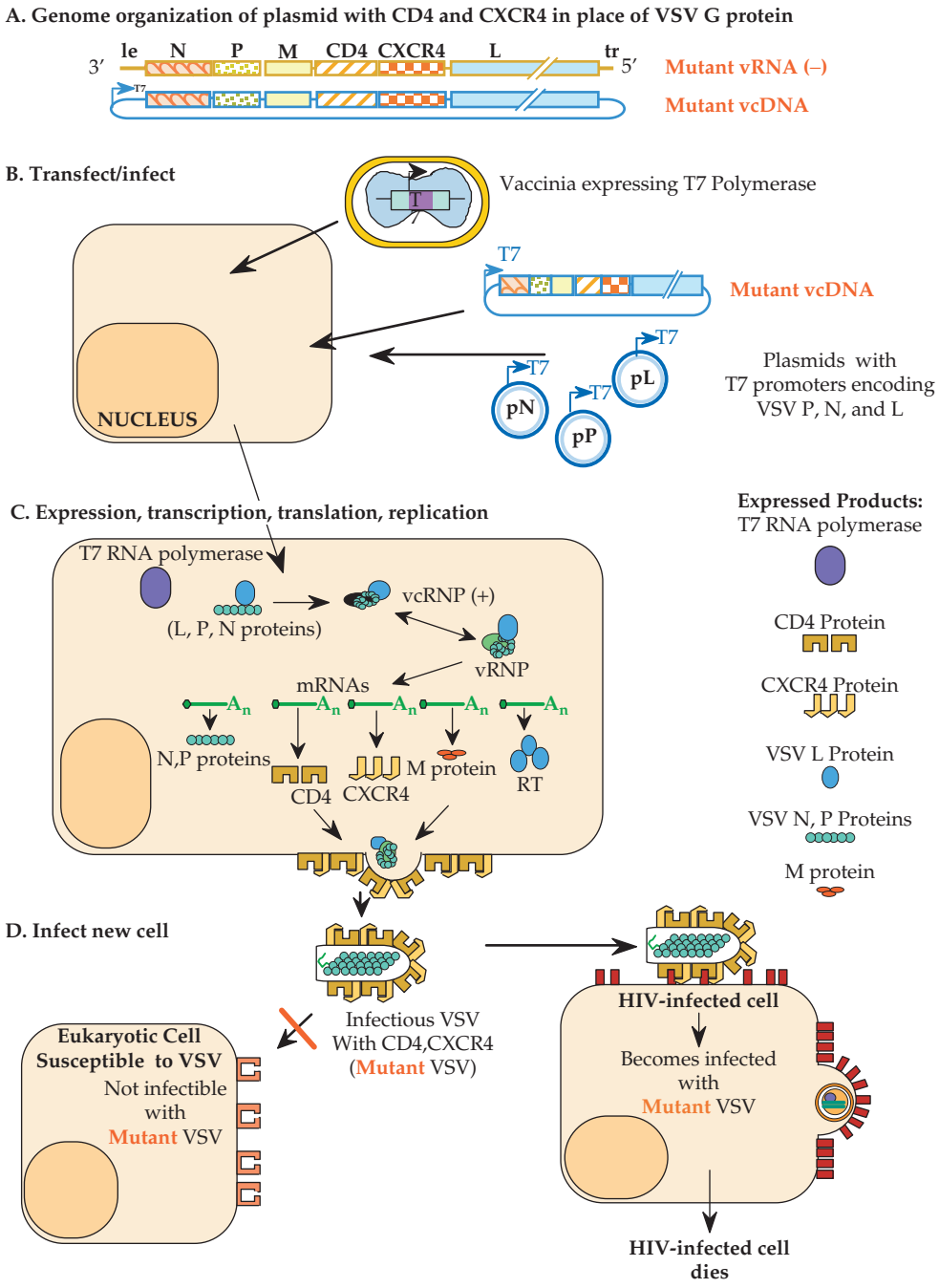

FIGURE 11.8 Producing a mutant VSV targeted to kill HIV-infected cells. (A) Genome of a rhabdovirus in which the glycoprotein G gene has been replaced with sequences encoding CD4 and CXCR4, the HIV primary receptor and coreceptor, and a schematic of a cDNA clone containing the genome sequence (cDNA copy of vRNA). (B) A susceptible cell is infected with vaccinia virus expressing the T7 RNA polymerase, and four separate plasmids: the mutant genome plasmid from which full-length vc (plus strand) RNA is transcribed, and three individual plasmids expressing VSV N protein, P protein, and L protein, all under the control of T7 promoters. (C) Plus-strand mutant vcRNA is transcribed and encapsidated with N, P, and L. The RNP then replicates and both viral proteins and CD4 and CXCR4 are expressed from individual mRNAs transcribed from genome sense RNPs. Virions bud from the cell and (D) cannot infect a new susceptible cell as before in Fig. 11.7, but can infect an HIV-infected cell expressing HIV *env* proteins on its surface. Infection with VSV is cytolytic and the HIV-infected cell dies. Adapted from Conzelmann and Meyers (1996).

the genome by virus vectors, often by recombinant vaccinia virus. These studies have resulted in an understanding of the two viral proteases within the HCV genome, the processing pathway through which the polyprotein translated from the genome is processed, the function of the viral IRES, and the function of the viral replicase, among other results. The use of virus vectors means that such studies on HCV can be conveniently conducted in mammalian cells under conditions that are related to the natural growth cycle of the virus.

Norwalk virus is another virus for which there is no cell culture system. The virus can be grown only in human volunteers, again limiting the range of studies that can be done. Virus particles isolated from the stools of infected volunteers are often degraded and difficult to purify to homogeneity. Thus, structural studies of infectious virus have been limited. Expression of cDNA copies of the structural proteins of the virus in baculovirus vectors has allowed the production of large amounts of viral structural proteins that spontaneously assemble into virus-like particles. These virus-like particles have been studied by cryoelectron microscopy, and detailed information on the structure of the virus has been obtained in this way.

Baculoviruses are also widely used to prepare large amounts of protein for crystallographic studies. Such studies require 20 mg or more of protein, and the baculovirus system can be used to prepare such quantities. An advantage of the system is that the protein is made in a eukaryotic cell, which can be important for obtaining the protein folded into its correct three-dimensional conformation. Also of importance is the use of secretion sequences in the constructs that lead to the secretion of the protein from the infected cell, making it easier to purify the protein.

Even for viruses for which cell culture systems exist, the use of virus vectors that express to higher levels can be advantageous. There are cell culture systems in which rubella virus will grow and plaque, and there is a full-length cDNA clone of rubella virus from which infectious RNA can be recovered. However, the cell culture systems produce only low amounts of virus proteins, especially of the nonstructural proteins, and it has been difficult to study the expression and processing of the nonstructural polyprotein. Expression of the nonstructural region of rubella virus in vaccinia virus vectors or in Sindbis virus vectors has allowed the production of much larger quantities of the polyprotein precursor. This has been used to determine the processing pathways, the identification of the virus nonstructural protease, and the identification of the cleavage sites that are cleaved by this protease.

As a final example, vaccinia virus vectors and Sindbis virus vectors have been used to map T-cell epitopes for a number of viruses (Chapter 10). For this, defined regions of a viral protein are expressed in order to determine whether a particular T-cell epitope lies within that region.

Viruses as Vectors to Elicit an Immune Response

Much effort is being put into the development of viruses as agents to immunize against other infectious agents, including other viruses. Such an approach has a number of advantages. There is a large body of experience in the use of attenuated or avirulent viruses as vaccines. Many of these, such as vaccinia virus or the yellow fever 17D virus, both of which have been used to immunize many millions of people, can be potentially developed as vectors to express other antigens, such as those of HCV or HIV. Use of a live virus as a vector to express antigens of other pathogens has many of the advantages of live virus vaccines. This includes the fact that only low initial doses are required, and therefore the expense of vaccine production may be less; that subsequent virus replication leads to the expression of large amounts of the antigen over an extended period of time, and the antigen folds in a more or less native conformation; and that a full range of immunity, including production of CTLs as well as of humoral immunity, usually develops.

No human vaccines have been licensed that use such recombinant viruses, but there are ongoing clinical trials of several potential vaccines. Several trials of candidate vaccines against HIV have been conducted that use vaccinia virus or retrovirus vectors to express the HIV surface glycoprotein. These trials have been moderately successful in the sense that immune responses to HIV glycoprotein were obtained, but these immune responses were not particularly vigorous and it is not known if the immune response is protective. HIV is able to persist in infected patients despite a vigorous immune response, and sterilizing immunity might be required. Further, the HIV surface glycoprotein is highly glycosylated and neutralizing antibodies are difficult to obtain. Studies in monkeys with related vaccines against simian immunodeficiency virus have given mixed results. In most such trials, immune responses were generated, but these were not fully protective. One recent trial did generate a protective response, however, giving hope that continued efforts in this direction will ultimately work out. Recent studies with anti-HIV drugs given very soon after infection found that limiting the replication of the virus early appears to allow the generation of a protective immune response in at least some patients. Although such studies remain preliminary, they do suggest that a nonsterilizing immune response that restricts virus replication early might prove to be protective.

Other clinical trials have also tested poxviruses as vectors. Vaccinia virus has been used in an attempt to immunize against Epstein–Barr virus, and canarypox virus has been used as a vector for potential immunization against rabies virus.

Although no licensed human vaccines use poxvirus vectors, veterinary vaccines that are based on poxvirus vectors are in use. One such vaccine consists of vaccinia virus that expresses the rabies surface glycoprotein. This vaccine has been used

to immunize wildlife. The recombinant vaccinia viruses are spread in baits that are eaten by wild animals that serve as reservoirs of the virus, such as skunks, raccoons, foxes, and coyotes. This approach has been useful in limiting the spread of rabies in wildlife populations. Other poxvirus-based vaccines include vaccinia virus vectors to protect cattle against vesicular stomatitis virus and rinderpest virus, and to immunize chickens against influenza virus; pigeonpox virus vectors to immunize chickens against Newcastle disease virus; fowlpox virus vectors to immunize chickens against influenza, Newcastle disease, and infectious bursal disease viruses; a capripox virus vector to immunize pigs against pseudorabies virus; and a canarypox virus vector used to immunize dogs against canine distemper virus. Thus, it should be possible to develop human vaccines based on poxvirus vectors.

In a quite different approach, clinical trials of a novel vaccine against Japanese encephalitis (JE) virus have been conducted. JE is a scourge in parts of Asia, causing a large number of deaths and neurological sequelae in people that survive the encephalitis (Chapter 3). Vaccines in widespread use are inactivated virus vaccines, and the difficulties in preparing the large amounts of material required and delivering it to large segments of the population are significant. An attenuated virus vaccine, SA14-14-2, has been prepared in China by passing the virus in cultured cells and in rodent tissues. This vaccine is safe but overattenuated, so that the effectiveness is only 80% after a single dose. In contrast, the yellow fever virus (YF) 17D vaccine has an effectiveness of virtually 100% after a single dose, and immunity is long lasting, probably lifelong. A candidate JE vaccine has been developed that consists of the 17D strain of YF virus in which the prM and E genes have been replaced with those of JE, as illustrated in Fig. 11.9. Four chimeric viruses were tested. The JE structural proteins were taken from either the virulent Nakayama strain or from the attenuated SA-14-14-2 strain. In both cases, chimeras containing all three structural proteins from JE were tested

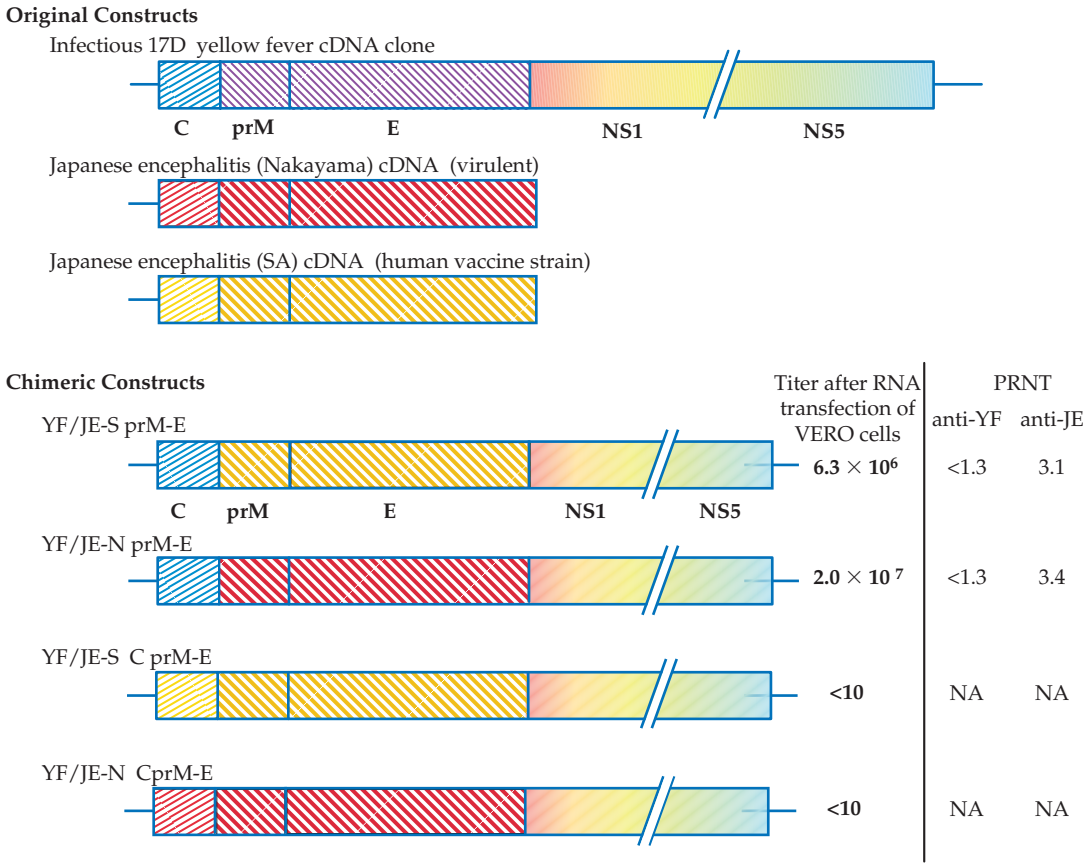

FIGURE 11.9 Construction of yellow fever/Japanese encephalitis chimeric viruses. Starting with the full-length cDNA clone for 17D yellow fever virus, a number of chimeric viruses were constructed in which the M and E proteins were replaced with those of different strains of Japanese encephalitis virus. However, when C, M, and E of JE were put into the yellow fever clone, no viable virus was obtained. Both prME chimeras grew well in tissue culture, and were neutralized by anti-JE antiserum. YF/JE-S prM-E was attenuated, and did not kill adult mice by intracerebral inoculation, but YF/JE-N prM-E was neurovirulent. PRNT is the log reciprocal of the dilution yielding 50% plaque reduction neutralization, based on 100 PFU on LLV-MK cells, using either YF or JE hyperimmune ascitic fluid. Adapted from Chambers *et al.* (1999).

as well as chimeras that contained only prM and E from JE. Chimeras containing C, prM, and E from JE were not viable, whereas chimeras containing only prM and E from JE were viable and grew well in culture (Fig. 11.9).

The viable chimeras were first tested in mice. The chimera containing the Nakayama strain proteins caused lethal encephalitis in mice, as does the YF 17D virus (even though it is safe for use in humans). However, the chimera containing prM and E from the attenuated JE strain was fully attenuated in mice and did not cause illness. The fully attenuated chimera was chosen for testing in monkeys, and was found to be safe and to protect monkeys against challenge with JE virus.

Clinical trials of this candidate vaccine have taken place in humans. The vaccine appears to be safe and more effective than the JE vaccines now in use. Furthermore, this approach is applicable to other flaviviruses, such as the dengue viruses, for which no licensed vaccines exist, or West Nile virus, which spread recently to the Americas where it caused a number of fatal cases of human encephalitis. Recombinant YF 17D expressing the prM and E proteins of all four serotypes of dengue viruses and recombinant viruses expressing prM and E of West Nile virus are also in clinical trials with encouraging results.

Yet another possible approach to developing new generations of vaccines using the power of biotechnology is to attenuate a virus by making changes in the laboratory that are expected to cripple the virus. Such an approach can be used with virtually any virus. A candidate vaccine strain of dengue virus has been constructed by making deletions in the 3′ nontranslated region of the genome that attenuate the virus, and such viruses are being tested in early trials.

GENE THERAPY

A number of genetic diseases result from the failure to produce a specific protein, usually due to a single defective gene. One of the more exciting possible uses for virus vectors is for the expression of a missing protein as a cure for the genetic defect associated with its absence. Some of these "monogenic diseases" that might be curable through the use of gene therapy are listed in Table 11.1. For successful treatment, expression of the missing protein must be long-term and preferably lifelong, the levels of protein produced must be sufficient to alleviate the symptoms of the disease, the protein must be expressed in or translocated to those cells that require the normal protein for function, and infection with the virus vector must be free of disease symptoms. Because of the requirement for long-term expression, viruses whose DNA integrates into the host chromosome, such as the simple retroviruses as well as the lentiviruses, and adeno-associated viruses, offer the most promising system for many diseases. To date, several hundred patients have been treated with vectors based on Moloney murine leukemia virus in clinical trials. Clinical trials have also been conducted that use adenovirus. adeno-associated virus, poxvirus, and herpesvirus vectors (Fig. 11.10).

Clinical trials in humans, which require extensive prior testing in animals, are divided into three phases. Phase I involves relatively few, usually healthy individuals. The objective of Phase I trials is to test the safety of a vaccine or treatment as well as the dosage that is tolerated, and the individuals are closely monitored during the trial. Phase II trials involve more individuals and test the efficacy of the treatment, and patients are again closely monitored. If a treatment

TABLE 11.1 Genetic Defects That Are Candidates for Gene Therapy

Disease	Defect	Incidence	Target cells
Severe combined immunodeficiency (SCID)	Adenosine deaminase (ADA) in 25% of SCID patients	Rare	Bone-marrow cells or T lymphocytes
Hemophilia A	Factor VII deficiency	1:10,000 males	Liver, muscle, fibroblasts or bone marrow cells
Hemophilia B	Factor IX deficiency	1:30,000 males	
Familial hypercholesterolemia	Deficiency of low-density lipoprotein (LDL) receptor	1:1 million	Liver
Cystic fibrosis	Faulty transport of salt in lung epithelium	1:3000 Caucasians	Airways in the lungs
Hemoglobinopathies thalassemias	(Structural) defects in the α or β globin gene	1:600 in certain ethnic groups	Bone marrow cells that are precursors to red blood cells
Gaucher's disease	Defect in the enzyme glucocerebrosidase	1:450 in Ashkenazi Jews	Bone marrow cells, macrophages
α1 antitrypsin deficiency inherited emphysema	Lack of α1 antitrypsin	1:3500	Lung or liver cells
Duchenne muscular dystrophy	Lack of dystrophin	1:3500 males	Muscle cells
Xeroderma pigmentosa	Impaired DNA repair, leading to severe sensitivity to sunlight	Rare	Fibroblasts

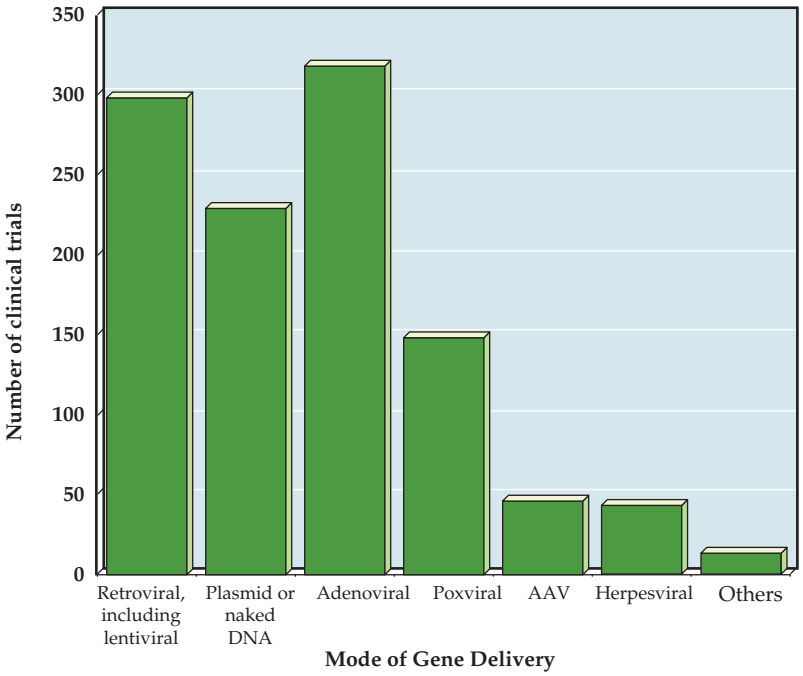

FIGURE 11.10 Vectors used in gene therapy trials as of January 2007. The "others" category includes flavivirus (5), measles virus (3), Newcastle disease virus (1), poliovirus (1), Semliki Forest virus (1), Sendai virus (1) and SV40 (1). Data are from Gene Therapy Clinical Trials Worldwide Web site of the *Journal of Gene Medicine* at: http://www.wiley.co.uk/genetherapy/clinical/.

passes both of these tests, Phase III trials can begin in which thousands of individuals are treated to test the efficacy of treatment. Virtually all of the clinical trials in gene therapy conducted to date are Phase I or Phase II; fewer than 2% of trials have progressed to Phase III (Fig. 11.11), and no gene therapy treatments have been licensed to date.

In addition to the possible treatment of genetic defects, virus vectors may also be useful for the treatment of a number of acquired diseases. These include cancer, HIV infection, Parkinson's disease, injuries to the spinal cord, and vascular diseases such as restenosis and arteriosclerosis. A partial listing of acquired diseases that have been suggested as candidates for gene therapy is given in Table 11.2, and the number of trials for a number of different conditions is shown in Fig. 11.12. Despite the large efforts to use gene therapy in clinical settings, the progress has been disappointingly slow, and many of the trials have been aborted due to unforeseen adverse consequences.

Nevertheless, as infectious clones of viruses continue to be developed, a large body of research is being devoted to construction of vectors, especially now to second and third generation vectors, as the problems associated with the initial systems are becoming clear. A comparison of various virus systems that are being considered for gene therapy is shown in Tables 11.3 and 11.4. Naked DNA has also been used in a recent trial for coronary artery disease, and the properties of this system are included in Table 11.4. Most of the modern vectors have had more and more of the dispensable viral genes deleted. Deletion of these genes reduces pathogenicity, and prevents the production of immunogenic viral antigens. Often only the gene of interest and the viral transcriptional regulatory elements are left, and to prepare the vectors for use in trials, all other functions must be supplied by a helper virus or a packaging cell line. Another advantage of such stripped down vectors is the fact that it is improbable that the vector can recombine with wild-type viruses, either exogenous or endogenous, to cause disease.

A partial listing of clinical trials that attempt to treat several different genetic defects by using virus vectors to deliver specific genes is given in Table 11.5. There have been few successes to date and the table is more of a compendium of the variety of genes and diseases, as well as the variety of delivery schemes, that are being examined. Also included in the table is an impending trial for the vaccination of humans against HPIV-3 using a bovine virus.

Retrovirus Vectors to Genetically Mark Cells

Retroviruses have been used in a number of clinical trials to genetically tag cells. Although this use does not fall within the narrow definition of gene therapy, it does provide background experience in the use of retrovirus vectors in humans. One such use has been in bone marrow transplantation for leukemia. Severe forms of leukemia can sometimes be treated by ablation of the hematopoietic system with chemotherapy and/or X-rays in order to kill all tumor cells, followed by reconstitution of the system by transplantation of bone marrow from a compatible donor. Although often successful, the leukemia sometimes recurs and it is desirable

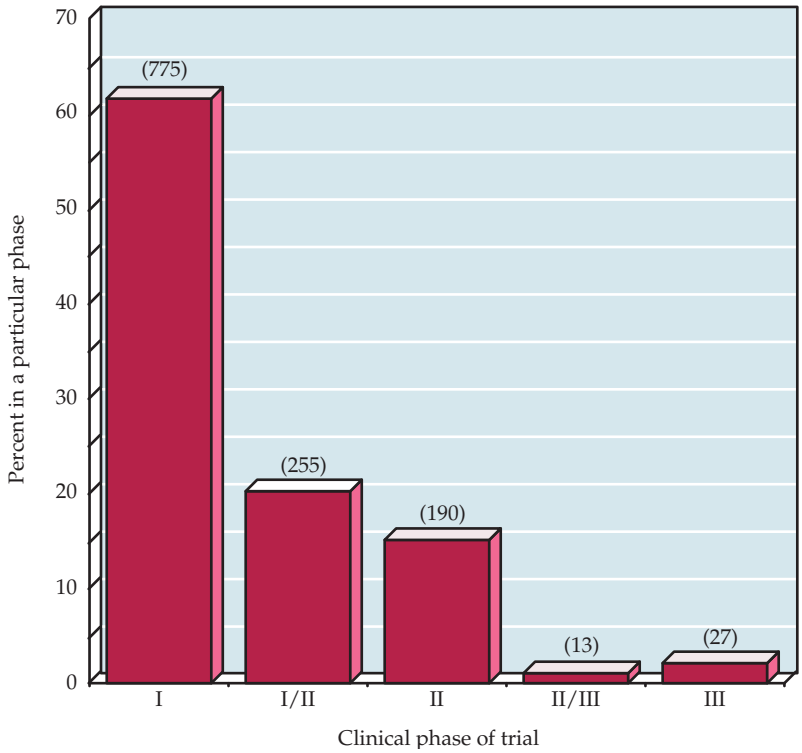

FIGURE 11.11 Percent of gene therapy clinical trials that are in each phase as of January 2007. The numbers above the bars are the actual number of trials. Data from Gene Therapy Clinical Trials Worldwide from the Web site of the *Journal of Gene Medicine* at: http://www.wiley.co.uk/genetherapy/clinical/.

to know whether it recurs because of incomplete destruction of the patient's leukemic cells or whether the donor cells are the source of the leukemia. Experiments in which the donor cells have been tagged using retroviruses that express a marker gene have been used to answer this question, which is important for the design of transplantation protocols.

Gene Therapy for ADA Deficiency

Patients who lack the enzyme adenosine deaminase (ADA) will die early in life unless treated. Lack of ADA results in the failure to clear adenosine from the body and, consequently, the accumulation of adenosine in cells throughout the body. Adenosine is toxic at high concentrations, producing a variety of symptoms. The most serious symptom results from the extreme sensitivity of T cells to elevated adenosine ***c***oncentrations. Loss of T cells results in SCID, ***s***evere ***c***ombined ***i***mmuno***d***eficiency. Both CTL responses (which are T-cell based) and humoral responses (which require T-helper cells) are impaired. People with SCID syndrome are unable to mount an immunologic response to infectious agents, and SCID is invariably fatal early in life unless treated in some way. ADA deficiency accounts for about 25% of SCID syndromes in humans.

SCID can be treated by bone marrow transplantation if a suitable donor can be found. In the case of SCID due to ADA deficiency, weekly or twice weekly injections of ADA

TABLE 11.2 Some Acquired Diseases That Are Candidates for Gene Therapy

Disease	Defect	Incidence	Target cells
Cancer	Many causes, including genetic and environmental	1 million/year in United States	Variety of cancer cell types, in liver, brain, pancreas, breast, kidney
Neurological diseases	Parkinson's, Alzheimer's spinal-cord injury	1 million Parkinson's and 4 million Alzheimer's patients in the United States	Neurons, glial cells, Schwann cells
Cardiovascular	Restenosis, arteriosclerosis	13 million in United States	Arteries, vascular endothelial walls
Infectious diseases	AIDS, hepatitis B	Increasing numbers	T cells, liver, macrophages
Rheumatoid arthritis	Autoimmune inflammation of joints	Increasing numbers with aging population	Intra-auricular delivery and expression of IL-1 and TNF-α inhibitors

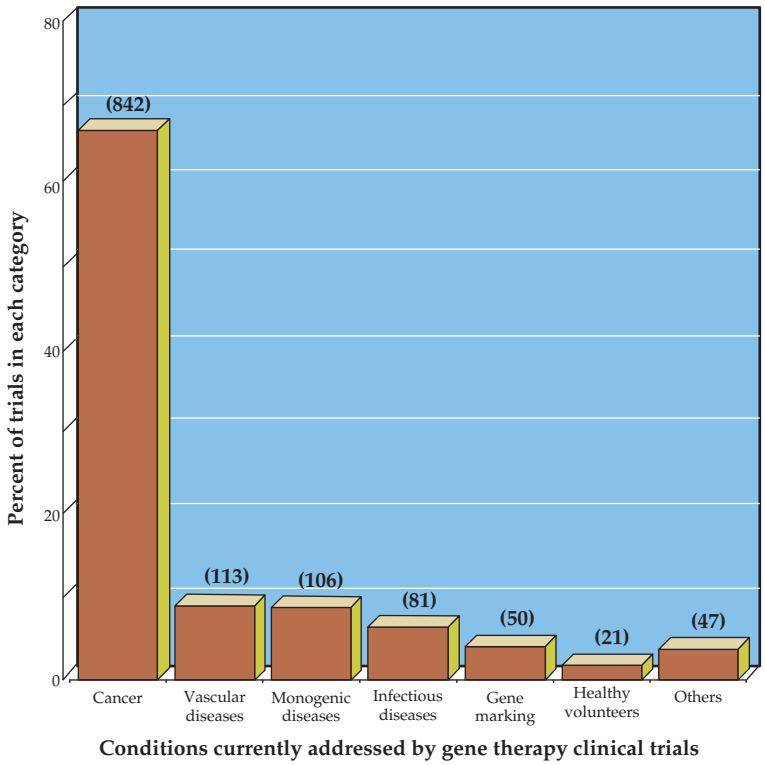

FIGURE 11.12 Percent of gene therapy trials which are directed toward various conditions as of January 2007. The numbers above the bars are the actual number of trials. Data from Gene Therapy Clinical Trials Worldwide from the Web site of the *Journal of Gene Medicine* at: http://www.wiley.co.uk/genetherapy/clinical/.

TABLE 11.3 Comparison of Properties of Various Vector Systems Based on RNA Viruses and Retroviruses

Features	Simple retroviral	Lentiviral	Alphaviral	Coronaviral	Negaviral[a]
Maximum insert size	7–7.5 kb	7–7.5 kb	5 kb	2.7 kb	~4 kb[b]
Concentration in viral particles/ml	$>10^8$	$>10^8$	$>10^9$	$>10^8$	$>10^9$ for VSV, PIVs 10–1000 fold lower
Route of gene delivery	*Ex vivo*	*Ex/in vivo*	*In vivo*	*In vivo*	*In vivo*
Integration	Yes	Yes	No	No	No
Duration of expression *in vivo*	Shorter than theorized	Long	Short	Variable	Not known
Stability	Good	Not tested	Good	Dependent on background	Good
Ease of preparation scale up	Pilot scale up to 20–50 liters	Not known	Not known	Not known	Not known
Preexisting host immunity	Unlikely	Unlikely, except in AIDS patients	No	Unlikely	Unlikely
Safety problems	Insertional mutagenesis?	Insertional mutagenesis?	Few	Recombination with wild strains	—
Other advantages	—	Replicate in nondividing cells	—	—	Recombination virtually unknown; naturally atttenuated viruses exist

[a] Includes consideration of VSV based and PIV based vectors.
[b] Longer inserts are tolerated but vectors are too attenuated *in vivo* to be useful.
Source: Verma and Somia (1997), Jolly (1994), and Bukreyev *et al.* (2006).

TABLE 11.4 Comparison of Properties of Various Vector Systems Based on DNA Viruses

Features	Vectors based on DNA viruses					Naked/lipid DNA
	Adenoviral	AAV	Herpesviral	Vaccinia	Baculovirus	
Maximum insert size	7.5 kb	<4 kb	~30 kb	25–75 kb	>38 kb	Unlimited size
Concentration in viral particles/ml	$>10^{10}$	$>10^{12}$	$>10^{10}$	10^{7}–10^{9}	$>10^{8}$	No limitation
Route of gene delivery	*Ex/in vivo*	*Ex/in vivo*	*Ex vivo*	*Ex/in vivo*	*Ex/in vivo*	*Ex/in vivo*
Integration	No	Yes/no	No	No	No	Very poor
Duration of expression *in vivo*	Short	Long	Short/long in CNS?	Short	Short	Short
Stability	Good	Good	Unknown	Good	Good	Very good
Ease of preparation scale up	Easy to scale up	Difficult to purify, difficult to scale up	Not yet tried	Vaccine production facilities exist	Easy to scale up	Easy to scale up
Immunologic problems	Extensive	Not known	Not known	Extensive	Not known	None
Preexisting host immunity	Yes	Yes	Yes	Diminishing as unvaccinated population grows	Unlikely	No
Safety problems	Inflammatory response, toxicity	Inflammatory response, toxicity	Neurovirulence? Insertional mutagenesis	Disseminated vaccinia in immuno-compromised hosts	None?	None?

Source: Verma and Weitzman (2005), Jolly (1994), Boulaiz *et al.* (2005), Hu (2006).

mixed with polyethylene glycol (PEG) have been used to successfully treat about 60 patients in whom bone marrow transplantation cannot be used because of the lack of compatible donors. Of these, about 10 patients have also been treated with retroviral vectors that express ADA. In these experiments, T cells were taken from the patient (or in the case of three newborns, umbilical cord cells were used), infected *ex vivo* with the retrovirus vector using a number of different cell culture and infection protocols, and the cells reinfused into the patient. Many of the patients continue to produce ADA from the vector several years after treatment. However, all of the patients continue to receive ADA–PEG injections, which is known to be an effective treatment. Although some patients who have received retroviral therapy have been partially weaned from the supplementary ADA–PEG, it appears that some of these, and perhaps all, do not produce enough ADA to be cured. Thus, although no cures were effected in these early trials, the results were encouraging and suggested that future protocols might be more successful. Two areas of retroviral therapy that needed improvement were to increase the efficiency with which stem cells are infected, and the need to prevent the retroviral promoter from being downregulated.

A more recent trial involving two SCID-ADA patients in Italy was more successful (Table 11.5). Two infants for whom no compatible donor existed and for whom no PEG-ADA treatment was available were treated with improved retroviral therapy. Both patients developed functional immune systems and no adverse events have been reported. The improved results appear to arise from improved protocols as well as to selection in the patient for lymphoid progenitor cells that expressed adequate amounts of ADA.

Treatment for SCID Caused by IL2RG Deficiency

SCID disease can also be caused by a failure to produce the receptor for the cytokine interleukin-2 (Chapter 10). Thirteen SCID patients with this deficiency were treated in two different clinical trials with retroviruses that expressed the defective gene (Table 11.5). The results illustrate the highs and the lows of gene therapy trials. All 13 patients developed functional immune systems, and the trials at first appeared to be a complete success. However, three of the patients later developed T-cell leukemia and one has died of the leukemia. The leukemia was at first suspected to arise from insertional mutagenesis, a chronic worry with vectors that insert into the host DNA, and many gene therapy trials that used retroviral vectors were suspended. Recent studies indicate that the disease is not due to insertional mutagenesis, however, but rather due to the oncogenic potential of the IL2RG gene itself, as studies have shown that overexpression of this gene in mice results in leukemia.

TABLE 11.5 Recent Human Gene Therapy Trials

Disease	Therapeutic gene	Total patients	Vector/ promoter	Method of delivery	Outcome
Monogenic diseases					
OTC deficiency	OTC cDNA	18	E1E4 deleted adenovirus/CMV	*In vivo* injection to hepatic artery	No clinical benefit; **1 death**
Factor IX deficiency (hemophilia B)	Modified Factor IX gene	8	rAAV2/ CMV	*In vivo* intramuscular	No sustained clinical benefit
		7	rAAV2/APOE-SERPINA1	*In vivo* injection to hepatic artery	Transient Factor IX expression, no sustained benefit
SCID-X1 (French trial)	IL2RG cDNA	10	Retrovirus/MLV-LTR	*Ex vivo* transduction of $CD34^+$ cells	9 patients developed functional immune system; **3 developed T-cell leukemia, 1 death**
SCID-X1 (British trial)	IL2RG cDNA	4	GALV-pseudotyped retrovirus/MFG-LTR	*Ex vivo* transduction of $CD34^+$ cells	4 patients developed functional immune system; no adverse results reported in first 2 years
SCID-ADA (Italian trial)	ADA cDNA	2	Retrovirus/MLV-LTR	*Ex vivo* transduction of $CD34^+$ cells	2 patients developed functional immune system; no adverse results reported in first 4 years
CGD	GP91PHOX	2	Retrovirus/SFFV-LTR	*Ex vivo* transduction of $CD34^+$ cells	2 patients developed functional neutrophils and **clonal myeloproliferation (cancer)**
Duchenne muscular dystrophy	Dystrophin	9	Plasmid/CMV	Intramuscular injection	Low dystrophin expression in 6 of 9, no adverse effects (Phase I)
	Microdystrophin	?	AAV/CMV	Intramuscular biceps injection	Phase I/II ongoing
	2OMeAOs	9	Oligonucleotide	Injection into extensor digitorum brevis	Successful non-human primate study; human Phase I/II not yet opened for patient recruitment
Vaccine for infectious disease					
HPIV-3 vaccine	F and HN surface glycoproteins	—	BPIV-3	Infection	Successful non-human primate study; human Phase I trial not yet opened for patient recruitment

APOE-SERPINA1, α1-antitrypsin promoter linked to APOE enhancer; BPIV-3, bovine parainfluenzavirus 3; CGD, chronic granulomatous disease; CMV, cytomegalovirus promoter; GALV, gibbon ape leukemia virus; HPIV, human parainfluenzavirus; LTR, long terminal repeat; MFG, derivative of MLV; MLV, Moloney murine leukemia virus; OTC, ornithine transcarbamylase; rAAV, recombinant adeno-associated virus serotype 2; SCID-ADA, severe combined immunodeficiency secondary to adenosine deaminase deficiency; SCID-IX, severe combined immunodeficiency secondary to mutations in the IL2RG gene; SFFV, Friend mink cell spleen focus-forming virus.
Source: Data for this figure came from Porteus *et al*. (2006), Foster *et al*. (2006), Bukreyev *et al*. (2006).

Cystic Fibrosis

Cystic fibrosis results from loss of the cystic fibrosis transmembrane conductance regulator (CFTR), which regulates epithelial transport of ions and water. Although lack of this protein results in damage to the epithelium in many parts of the body, the most serious manifestation is lung disease accompanied by chronic bacterial infection of the airways. Clinical trials using adenoviral vectors, which infect respiratory epithelium, to express CFTR in the lungs have been conducted. The first such studies were encouraging, but a more recent trial that was carefully controlled found no relief of symptoms. Inflammation produced by the high doses of adenovirus used in trials is also a problem. It is difficult to get efficient delivery to the lung, especially through the thick mucus that is a characteristic of cystic fibrosis. In addition, the expression needs to continue for the life of the patient, which means either a very stable (integrated?) gene being expressed, or a system of repeated administration of vector that can be tolerated without immunologic consequences. Lentiviruses have been proposed as an attractive vector, but there have been concerns about probable pathogenesis due to the lentivirus itself. However, for this disease, the most promising mode of gene delivery so far developed has been DNA compacted into nanoparticles with polycations, in particular PEG-polylysine, nicknamed "polyplexes." A clinical trial in humans used CFTR polyplexes, and the Phase I trial showed no adverse effects. For this disease a nonviral approach may well be the best solution.

Duchenne Muscular Dystrophy

Duchenne muscular dystrophy (DMD) is a severe muscle wasting disorder due to the lack of functional dystrophin protein. It occurs in 1/3500 male births. There are a number of difficulties in attempting to cure DMD with gene therapy, including the size of the protein, which is encoded in a cDNA of 11kb, and the need to deliver the vector to a large proportion of the body mass, that is to all of the striated muscles and cardiac muscle. Several approaches have been tried, and the first human clinical Phase I trial has just been completed (see Table 11.5). In this study, a plasmid containing the entire gene under the control of a CMV promoter was injected intramuscularly. Although only low levels of dystrophin expression were observed in 6 out of 9 patients, there was no evidence for adverse reactions and the trial was considered a success.

A second approach has been to attempt to introduce the gene in an AAV vector, especially in one of the many human isolates to which most of the population show no preexisting immunity, or into a nonhuman AAV. However, here the size of the gene is a problem, but it has been shown that the dystrophin protein contains a large number of repeated elements (Fig. 11.13) and that "mini-dystrophin" and "micro-dystrophin" are functionally active in the mouse model for the disease, the *mdx* mouse. Low-pressure intravenous injection of AAV6 expressing micro-dystrophin into a mouse could transfect 90% of muscle, but high titers of the AAV6 vector were required. A Phase I/II clinical trial has been initiated for delivery of micro-dystrophin in AAV under the control of a CMV promoter into human patients, but there are no results as yet.

A third approach attacks the specific nature of the genetic defect. It has been shown that 75% of DMD is caused by a frameshift mutation in one of the exons, such that no dystrophin is produced. Since severely truncated dystrophin (like micro-dystrophin) can be functional (Fig. 11.13), therapy to get rid of the exon causing the problem is being developed. Modified antisense oligonucleotides (AOs) can be used to alter the splicing pattern of the gene such that the exon in which the frameshift occurs is skipped, thereby restoring the reading frame. Following success of an AO with a 2′*O*-methyl-phosphorothiolate backbone (2OMe AOs) to restore function in the *mdx* mouse, two clinical Phase I trials have been initiated, one in the Netherlands using 2OMe AOs and one in Great Britain using morpholino AOs. Since both are targeting the exclusion of exon 51, it will be possible to directly compare the two chemically modified AOs.

Rheumatoid Arthritis

Rheumatoid arthritis is a chronic, progressive inflammatory disease of the joints. An estimated 5 million people in the United States suffer from it. There is no cure. Drug therapies are used that ameliorate the symptoms, but most of these drugs have side effects and cannot be taken indefinitely. If the disease progresses far enough, joint replacement may be required. The disease is associated with the release of inflammatory cytokines in the affected joints. Clinical trials have started that use retroviruses to deliver the gene for an anti-arthritic cytokine gene to the joints. The gene encodes the interleukin (IL)-1 receptor antagonist, which inhibits the biological actions of both IL-1α and IL-1β. It is hoped that such treatment might damp out the disease or at least keep it from progressing.

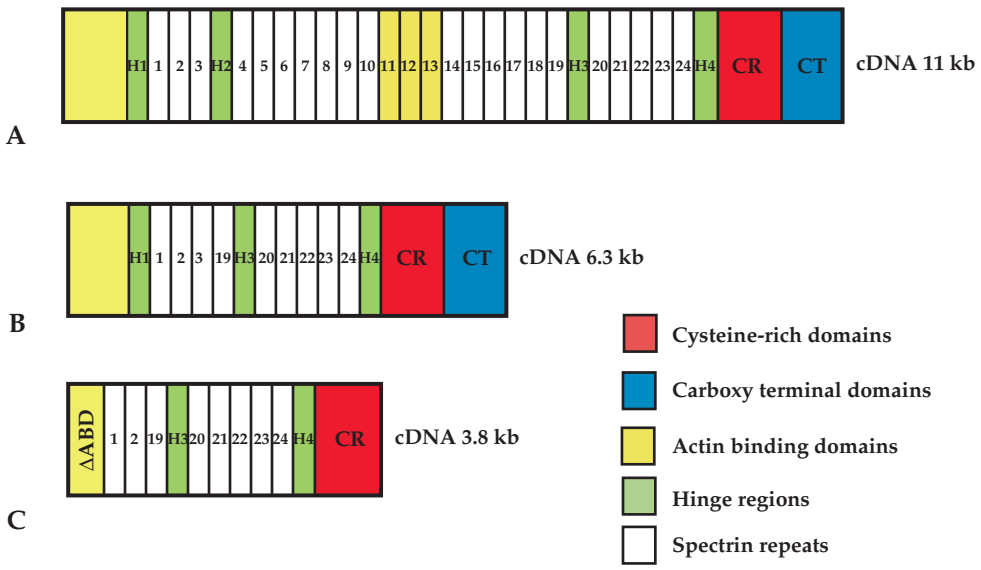

FIGURE 11.13 Forms of the dystrophin protein. (A) Full-length dystrophin containing all functional domains. (B) Mini-dystrophin from a Duchenne muscular dystrophy patient who was only mildly impaired. (C) Micro-dystrophin engineered for delivery in AAV vectors. Adapted from Figure 1 in Foster *et al*. (2006).

A Gene Therapy Failure

Patients who have deficiencies in enzymes that participate in the urea cycle have increased concentrations of ammonia in the blood. High concentrations of ammonia result in various symptoms, which can include behavioral disturbances or coma. Severe deficiencies in these enzymes result in early death, but moderate deficiencies can result in delayed appearance of symptoms and may be partially controlled by diet. One such enzyme is ornithine transcarbamylase (OTC), which is found on the X chromosome. Deficiencies in OTC are therefore more common in males than in females.

Gene therapy trials that use virus vectors recently received a major setback when a relatively fit 18-year-old male with an inherited deficiency for OTC died 4 days after an adenovirus vector was injected into his liver. A high dose of adenovirus (4×10^{10}) that expressed OTC was injected in an effort to achieve adequate levels of enzyme production. The virus unexpectedly spread widely and a systemic inflammatory response developed, inducing a fever of 40.3°C. He went into a coma, his lungs filled with fluid, and he died of asphyxiation. This unfortunate result makes clear the possible drawbacks to experimental treatments and the difficulties in designing protocols that allow an adequate margin of safety while trying to achieve a clinically relevant result.

Treatment of Restenosis

A gene therapy trial in patients with heart disease gave very encouraging results. Although this study did not involve virus vectors, a brief description will be given since it serves as an incentive for continuation of gene therapy trials. Coronary artery disease is common in older people. Angioplasty or bypass surgery is used to open clogged arteries, but in many patients the arteries close up again (a process called restenosis). Thirteen patients with chronic chest pain who had failed angioplasty or bypass surgery or both were injected in the heart muscle with DNA encoding vascular endothelial growth factor. This factor promotes the growth of blood vessels, a process called angiogenesis. Two months after treatment, all patients exhibited an improvement in vascularization of damaged areas of the heart, as shown by imaging and mapping studies. All patients reported a decrease in disease symptoms, and all had an improved performance in treadmill tests. Although the number of patients is small, the uniformly positive results are encouraging.

Viruses as Anticancer Agents

A large number of clinical trials have examined the possibility of using viruses as anticancer agents. Table 11.6 lists a number of trials that were active in the year 2000, to give a flavor of what is being tried and the number of malignacies that are being considered as candidates for treatment using gene therapy approaches. These trials are all Phase I or I/II, but more than 1000 patients were participating in the trials listed in the table. As shown in Fig. 11.12, the development of gene therapy approaches for the control of cancers continues to attract much effort, such that the majority of clinical trails to date have been directed against cancers. Although progress has been painfully slow and disappointing, the prospect remains that effective treatment may yet be obtained for at least some cancers using such approaches.

In most of the trials in Table 11.6, viruses are used to express proteins that control the growth of tumors or that are toxic to tumor cells. A number of different cytokines are being tried as antitumor agents, such as IFN-γ, IL-2, TNF, and GM-CSF. Another approach is to try to repair the defective regulatory gene in the tumor cell, which is often p53. Many other gene products are also being tested. The viruses used to express these products include the retroviruses, the adenoviruses, or the poxviruses. More recent trials have used additional viruses as well, in particular the herpesviruses and the adeno-associated viruses. Herpes simplex type 1 would seem to be particularly appropriate for control of brain tumors, because the virus is neurotropic but sets up a latent infection in neurons. One idea would be to engineer herpes to express a protein that is only toxic in dividing cells but which would be nontoxic in mature, nondividing neurons. The table also lists a number of trials that use lipofection to introduce the gene of interest into target cells.

Further afield, thought is being given to the possibility of using viruses to express proteins that are overexpressed in tumor cells in an attempt to stimulate the immune system to respond by killing tumor cells. This is in essence an attempt to vaccinate a person against a tumor. For this approach to succeed, an antigen overproduced by a tumor cell, such as a melanoma cell, must be identified, inserted into a suitable vector, and the person with the tumor infected with the virus vector in an attempt to stimulate the immune system. In principle, this approach may be feasible, but only time will tell whether it is in fact practical.

Another approach is to try to direct the virus, more or less specifically, to infect the tumor cells, so that upon infection the cells are killed. Cell death might result either because the virus itself is cytolytic or because the virus expresses a protein that renders the cell sensitive to a toxic agent such as BUdR. A number of the trials listed in Table 11.6 use the TK gene for this, since cells that express TK are sensitive to BUdR. One possible approach is to engineer the virus so that its surface glycoprotein expresses a monoclonal antibody directed against an antigen expressed only on the tumor cell, while at the same time causing the virus to be unable to infect cells that do not express the antigen. Experiments have established the possibility of this approach, at least in principle, with viruses such as the alphaviruses. Another approach was illustrated by the experiments with VSV to design a virus that could infect only HIV-infected cells.

TABLE 11.6 Clinical Trials of Gene Transfers for Cancer Therapy in the United States in 2000

Location	Gene[a]	Vector	Number of trials	Number of patients	Phase[b]
Brain cancers					
Neuroblastoma	IFNγ	Retrovirus	1	4	I
	IL-2	Retrovirus	1	12	I
	IL-2	Adenovirus	1	6	I
Central nervous system	TK	Adenovirus	2	22	I
Pediatric tumor	TK	Retroviral producing cells	1	2	I
Adult brain tumor	TK	Retroviral producing cells	1	15	I
Ovarian cancer	HSV-TK	Adenovirus	1	10	I
	TK	Retroviral producing cells	3	42	I
	BRCA-1	Retrovirus	1	40	I/II
	p53	Adenovirus	1	16	I
Small cell lung cancer	IL-2+NeoR	Lipofection	1	8	I
	Anti-sense to k-*ras*	Retrovirus	1	9	I
	p53	Adenovirus	2	59	I/II
Prostate cancer	GM-CSF	Retrovirus	1	8	I/II
	PSA	Poxvirus	1	3	I
	HSV-TK	Adenovirus	1	18	I
Breast cancer	BRCA-1	Retrovirus	1	21	I
	E1A	Lipofection	1	16	I
	MDR-1+NeoR	Retrovirus	4	39	I
	CD80	Lipofection	1	15	I
	CEA	Poxvirus	4	53	I
	CEA	RNA transfer	1	30	I
Melanoma	GM-CSF	Gene gun	1	17	I
	GM-CSF	Retrovirus	2	29	I
	HLA-B7/β2 m	Lipofection	8	165	I/II
	IL-2+NeoR	Retrovirus	5	115	I
	IFNγ	Retrovirus	3	91	I
	TNF+NeoR	Retrovirus	1	12	I/II
	MART-1	Adenovirus	1	33	I
	MART-1	Poxvirus	2	16	I
	gp100	Poxvirus	1	19	I
	gp100	Adenovirus	1	7	I
	CD80	Lipofection	1	17	I
Miscellaneous carcinomas	p53	Adenovirus	1	26	I
	HLA-B7/β2m	Lipofection	4	76	II
	IL-2	Lipofection	1	11	I
	CEA	Poxvirus	1	8	I
Lymphomas and solid tumors	IL-2	Retrovirus	2	29	I
	TK	Retrovirus	1	11	I
	IL-12+NeoR	Retrovirus	1	31	I
Bladder cancer	p53	Adenovirus	1	5	I
Colo/rectal, renal, and liver cancers	GM-CSF	Retrovirus	1	18	I
	HLA-B7/β2m	Lipofection	4	53	I/II
	CD	Adenovirus	1	6	I
	IL-4	Retrovirus	1	18	I
	TNF+NeoR	Retrovirus	1	12	I

(Continued)

[a]Abbreviations: IFNγ, interferon gamma; IL-2, interleukin 2; TK, thymidine kinase (sometimes used with bromodeoxyuridine); HSV-TK, herpes simplex thymidine kinase, often coupled with gancyclovir treatment; BRCA-1, breast cancer 1, early onset; PSA, puromycin-sensitive aminopeptidase; CEA, carcinoembryonic antigen; GM-CSF, granulocyte-macrophage colony stimulating factor; MDR-1, multi-drug resistance protein 1 (used to insert chemotherapy-resistance genes into the hematopoetic lineage); CD80, protein involved in T-cell activation; CD, cytosine deaminase; TNF, tumor necrosis factor.

[b]Definitions of phases in a clinical trial: Phase I usually fewer than 100 healthy volunteers, primarily to gauge adverse reactions, and to determine optimal dose and best route of administration. Phase II are generally pilot efficacy studies usually involving 200–500 volunteers randomly assigned to control and study groups. Phase II will test for immunogenicity in the case of vaccines, and duration of expression and amelioration of symptoms for gene therapy. Note that none of these trials have proceeded beyond Phase II, and most are in Phase I.

Source: Wiley Journal of Gene Medicine/Clinical Trial Database at: http://www.wiley.co.uk/genetherapy/clinical/.

The possible use of herpesviruses to control brain tumors, especially gliomal tumors, has been cited earlier. Simple retroviruses are also being examined for this purpose. These viruses can only replicate in dividing cells. Thus, they should be able to infect only tumor cells in the brain, since most neuronal cells are terminally differentiated and do not divide. If the retroviruses express a protein that renders the cells sensitive to a toxin, it might be possible to kill replicating cells and therefore only the tumor cells.

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