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GENE THERAPY WITH VIRAL VECTORS

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■ **Abstract** A key factor in the success of gene therapy is the development of gene delivery systems that are capable of efficient gene transfer in a broad variety of tissues, without causing any pathogenic effect. Currently, viral vectors based on many different viruses have been developed, and their performance and pathogenicity has been evaluated in animal models. The results of these studies form the basis for the first clinical trials for correcting genetic disorders using retroviral, adenoviral, and adeno-associated viral vectors. Even though the results of these trials are encouraging, vector development is still required to improve and refine future treatment of hereditary disorders.

INTRODUCTION

The major goal of gene therapy is to introduce a functional gene into a target cell and restore protein production that is absent or deficient due to a genetic disorder. Although the basic principle of gene therapy is quite simple, successes rely considerably on the development of the gene transfer vectors.

Over the years, a number of gene transfer vehicles have been developed that can roughly be divided into two categories: synthetic and virus-based gene delivery systems. Synthetic gene delivery systems depend on direct delivery of genetic information into a target cell and include direct injection of naked DNA and encapsulation of DNA with cationic lipids (liposomes). Although these delivery systems exhibit low toxicity, gene transfer in general is inefficient and often transient.

Viral delivery systems are based on replicating viruses that have the ability to deliver genetic information into the host cell. In general, genomes of replicating viruses contain coding regions and *cis*-acting regulatory elements. The coding sequences enclose the genetic information of the viral structural and regulatory proteins and are required for propagation of infectious viruses, whereas *cis*-acting sequences are essential for packaging of viral genomes and integration into the host cell. To generate a replication-defective viral vector, the coding regions of the virus are replaced by the genetic information of a therapeutic gene, leaving the *cis*-acting sequences intact. When the viral vector is introduced

into producer cells providing the structural viral proteins in *trans*, production of nonreplicating virus particles containing the genetic information of a therapeutic gene is established (Figures 1, 3, and 4). The ability to generate replication-defective viral vectors is the backbone of developing virus-based gene delivery vehicles.

Viral vectors currently available for gene therapy are based on different viruses and can roughly be categorized into integrating and nonintegrating vectors. Vectors based on adeno-associated virus and retroviruses (including lentivirus and foamy virus) have the ability to integrate their viral genome into the chromosomal DNA of the host cell, which will possibly achieve lifelong gene expression. Vectors based on adenovirus (Ad) and herpes simplex virus type 1 (HSV-1) represent the nonintegrating vectors. These vectors deliver their genomes into the nucleus of the target cell, where they remain episomal.

In this review, we give an overview of the development of vectors derived from viruses, discussing their specific properties and problems. Furthermore, we give a brief overview of clinical studies using viral vectors.

ADENO-ASSOCIATED VIRAL (AAV) VECTORS

AAV Structure and Replication

AAV is a member of the dependoviruses, a subfamily of the parvoviridae. The virus is nonpathogenic and by itself nonreplicating. As the name suggests, virus replication can be propagated only upon coinfection with a helper virus, which explains why AAV has frequently been found as a contaminant in Ad and herpes virus isolates.

AAV virions are small nonenveloped particles (20–25 nm) that carry a linear single-stranded DNA (ssDNA) genome, which is 4.7 kb in size. Two open reading frames (ORFs), *rep* and *cap*, have been identified in the viral genome and are flanked by T-shaped inverted terminal repeats (ITRs). The *cap* ORF encodes for the structural proteins that form the capsid, whereas the regulatory proteins are produced from the *rep* ORF (Figure 1*A*). [For more details see (1).]

After binding to its receptor, the virus enters the cell through the endocytic pathway and is subsequently transported to the nucleus. Before the viral genome can integrate into the host cell genome, the ssDNA has to be duplicated. This occurs either by annealing with a complementary DNA strand from a second AAV virus or by the host cell machinery. Subsequently, the double-stranded DNA (dsDNA) genome is directed to a specific site in chromosome 19 by *rep* proteins, where it integrates by nonhomologous recombination. After integration, the virus remains silent and persists for the lifetime of the cell.

Subsequent steps in AAV replication depend completely on gene products of unrelated helper viruses, and therefore virus production is propagated only upon coinfection. Ad or herpes virus can serve as a helper virus, and upon infection their viral proteins regulate AAV replication from initiation of transcription to

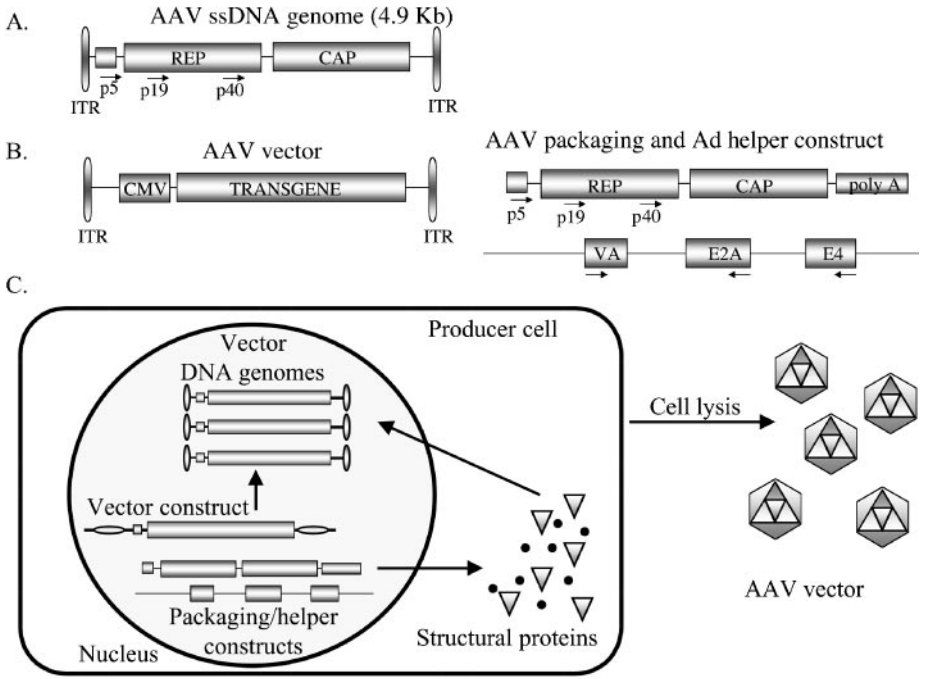

Figure 1 AAV vectors. (*A*) The AAV viral genome containing *rep* and *cap* genes flanked by ITRs. (*B*) In the AAV vector, the *rep* and *cap* genes are replaced by promotor and transgene sequences. The *rep* and *cap* genes are provided from a packaging construct in which expression is regulated by the endogenous promoters p5, p40, and p19. The Ad helper construct provides expression of E2, VA, and E4, which are essential for AAV vector production, whereas Ad E1 genes are provided by the 293 producer cells. (*C*) Nonreplicating AAV vector is produced by simultaneous expression of the viral vector, packaging, and helper constructs in producer cells. Vector ssDNA genomes are packaged by the structural proteins in the nucleus and are released from the cell by lysis.

DNA replication. New virus particles assemble in the nucleus, packaging either plus or minus ssDNA viral genomes, and are released from the cell together with the helper virus. [For more details see (1).]

AAV Vector Development and Production

The AAV viral vector system was initially developed by replacing the viral genes *cap* and *rep* with the transgene sequences. During vector production, *cap* and *rep* sequences are provided from a helper plasmid, and an infectious vector is easily rescued by coinfection with Ad (2). However, the viral vector still contains over 400 bp of the viral genome, allowing recombination between homologous sequences in the helper construct and the viral vector, resulting in the emergence of wild-type AAV.

The next generation of AAV vectors contain only the ITRs and 45 adjacent bp that display *cis*-acting functions essential for virus production and integration (3). In essence, these vectors consist of only a promotor and a transgene flanked by ITRs, preventing the formation of replication competent AAV during vector production (Figure 1). *Rep* and *cap* proteins are produced in *trans* in the packaging cells, whereas coinfection with Ad provides the necessary Ad proteins for initiation of vector replication. Although high-titer AAV vectors can be produced using this system, the coproduced wild-type Ad contaminates these vector preparations.

Recently, new vector production systems have been developed that are free of replicating Ad (4). In this system, the Ad proteins E2A, VA, and E4 are expressed from a second helper construct in 293 cells, which provides E1A and E1B gene products (Figure 1) (5). Furthermore, the reduction of *rep* production from the helper construct prevents the cytotoxicity in the packaging cells, which subsequently improves vector production (6).

AAV Vector Tropism and Transduction

AAV serotype 2 (AAV-2) is commonly used for gene therapy studies and shares the natural tropism of the wild-type virus. The primary receptor for AAV-2 is heparan sulfate proteoglycans, whereas fibroblast growth factor receptor 1 and integrin $\alpha_v\beta_5$ serve as coreceptors and facilitate internalization by endocytosis (7–9). Although AAV-2 displays a broad host range, it has been reported that certain cell types are resistant to AAV-2 infection, probably due to the lack of appropriate receptors. Including AAV-2, five serotypes of AAV have been identified. Sequence analysis of the *cap* proteins reveals considerable diversity, indicating that different receptors and coreceptors can be used among the serotypes. AAV-1, AAV-2, and AAV-3 share homology across the *cap* proteins and are all able to bind to heparan sulfate (7), whereas AAV-4 and -5 show more diversity in the *cap* proteins (10, 11). Indeed, differences in cellular tropism have been observed (12–15). Of all serotypes, AAV-1 shows the highest transduction efficiency in muscle and liver, whereas AAV-5 displays high tropism for retina (12). Furthermore, AAV-5 is able to transduce airway epithelia cells (13), and AAV-3 shows tropism for hematopoietic stem cells (14), which were resistant to transduction by AAV-2. These studies suggest that the use of different AAV serotypes may allow targeting of the vector for tissue-specific transduction. Altering the tropism of AAV vectors has also been explored by chemical cross-linking of bispecific antibodies to the viral capsid (16) and by the insertion of receptor specific epitopes in the *cap* proteins (17, 18).

To establish efficient transgene expression, the ssDNA genome of the vectors has to be converted into a dsDNA, and this appears to be the rate-limiting step in AAV transduction (19). Unless the ssDNA genome is converted into a stable dsDNA genome, it is lost rapidly after transduction. Because transduction occurs in the absence of helper virus and *rep* proteins, AAV relies solely on cellular conditions supporting this event. When the ssDNA is converted into dsDNA by the host cell machinery, transgene expression is increased concomitantly (20, 21).

AAV transduction can occur in the absence of cell cycle; however, transduction efficiency is markedly improved in cells in S-phase (22). Furthermore, activation of the cellular DNA repair machinery also supports second strand synthesis, thus improving AAV transduction (23, 24). The latter suggests that transduction of terminally differentiated postmitotic cells may be hampered to some extent due to insufficient second strand synthesis.

AAV vector dsDNA genomes can persist in transduced cells for long periods of time and are able to form concatamers by head to tail recombination of the ITRs. Integration of single and concatameric genomes occurs randomly in the host cell genome at low frequency because vectors are deprived of all *rep* sequences (22, 25). This indicates that the transgene is predominantly expressed from episomal forms, and expression may decline over time due to loss of the episomal genome by degradation.

The major limitation in the use of AAV as a gene delivery vehicle is the small packaging capacity. In order to deliver a large gene, the unique ability of AAV to form concatamers by head to tail recombination of the ITRs has been explored, increasing delivery size up to 10 kb. In this approach, promotor and transgene sequences are split over two AAV vectors (26, 27). When these two vectors were used to transduce cells, expression of a functional gene was obtained after head to tail recombination of the two viral genomes. Although successful transgene expression was demonstrated using this strategy, transduction efficiency was indeed reduced compared with single vector transduction.

Another hurdle in the use of AAV vectors for gene therapy is the presence of circulating neutralizing antibodies against AAV in the majority of the population as a result of natural infection (28). Furthermore, one single injection of AAV vector elicits a strong humoral immune response against the viral capsid, which interferes with re-administration of the vector (29). The use of AAV vectors containing *cap* proteins from different serotypes may overcome the problems of neutralizing antibodies (11).

RETROVIRAL VECTORS

General Features of Retroviruses (Structure and Replication)

Retroviruses are a large family of enveloped RNA viruses found in all vertebrates and can be classified into oncoretroviruses, lentiviruses, and spumaviruses. The enveloped virus particle contains two copies of the viral RNA genome, which are surrounded by a cone-shaped core (30). The viral RNA contains three essential genes, *gag*, *pol*, and *env*, and is flanked by long terminal repeats (LTR). The *gag* gene encodes for the core proteins capsid, matrix, and nucleocapsid, which are generated by proteolytic cleavage of the *gag* precursor protein. The *pol* gene encodes for the viral enzymes protease, reverse transcriptase, and integrase, and is usually derived from the *gag-pol* precursor. The *env* gene encodes for the envelope glycoproteins, which mediate virus entry.

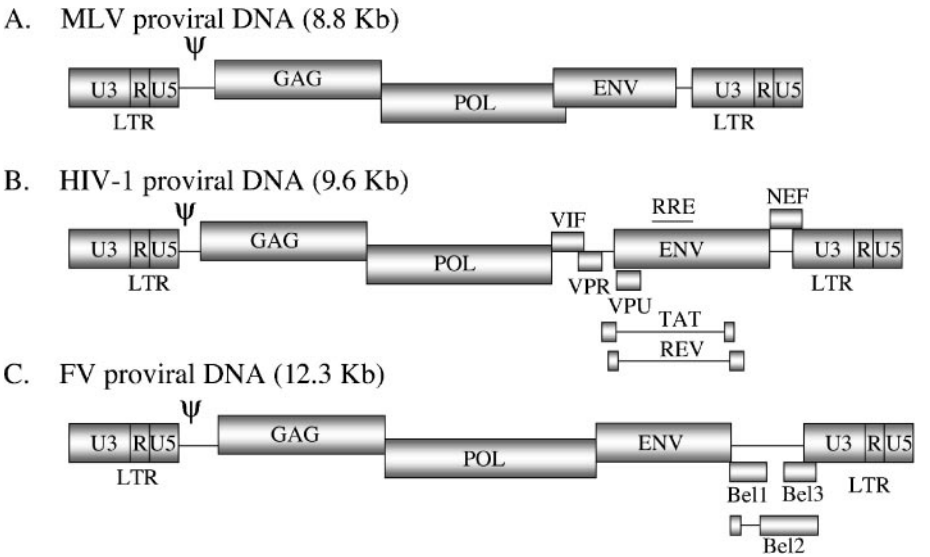

Figure 2 Schematic presentation of the proviral DNA organization of retroviruses. (*A*) The MLV provirus contains *gag*, *pol*, and *env* coding regions flanked by LTRs. The LTR is comprised of three regions, U3, R, and U5, which are essential for reverse transcription, proviral integration, and transcriptional activation. Ψ indicates the packaging signal. (*B*) The HIV-1 proviral DNA encodes for six additional proteins *vif*, *vpr*, *vpu*, *tat*, *rev*, and *nef*, and contains the *cis*-acting element RRE. (*C*) The FV provirus contains three additional ORFs: *bel*1, *bel*2, and *bel*3.

Oncoretroviruses are simple viruses encoding only the structural genes *gag*, *pol*, and *env*, whereas lentiviruses and spumaviruses have a more complex organization and encode for additional viral proteins (Figure 2). Lentiviruses encode three to six additional viral proteins that are essential for virus replication and persistence of infection. Two of the accessory proteins, tat and rev, are present in all lentiviruses and mediate transactivation of viral transcription (31, 32) and nuclear export of unspliced viral RNA, respectively (33). Spumaviruses, also called foamy viruses (FV), contain, in addition to the structural proteins, three ORFs (*tas*/*bel*1, *bel*-2, and *bel*-3), of which *tas*/*bel*1 has been identified as a coactivator of viral transcription (34).

After binding to its receptor, the viral capsid containing the RNA genome enters the cell through membrane fusion. The viral RNA genome is subsequently converted into a double-stranded proviral DNA by the viral enzyme reverse transcriptase. The proviral DNA is heavily associated with viral proteins like nucleocapsid, reverse transcriptase, and integrase (preintegration complex), and translocates to the nucleus where the viral enzyme integrase mediates integration of the provirus into the host cell genome.

Host cell transcription factors initiate transcription from the LTR, and new viral particles are formed at the plasma membrane. *Gag-pol* and *gag* precursors assemble together with two copies of viral RNA, and during the budding process *env* glycoproteins are incorporated into the viral membrane. In the newly formed virion, *gag* and *gag-pol* precursors are subjected to processing by the viral enzyme protease, which results in maturation of the virion. [For more details see (30).]

Retroviral Vector Development and Production

ONCORETROVIRAL VECTORS Viral vectors have been derived from different oncoretroviruses like murine leukemia virus (MLV), spleen necrosis virus, Rous sarcoma virus, and avian leukosis virus, but MLV-based vectors are most frequently used.

Replication-defective MLV vectors are generated by replacing all viral protein encoding sequences with the exogenous promotor-driven transgene of interest (35). Besides the packaging signal, the viral LTRs and adjacent sequences, which are essential for reverse transcription and integration, remain in the vector (36). In this system, vector RNA production is driven by the U3 region of the LTR and results only in low titers of the vector due to the low transcriptional activity of the LTR. Therefore, the U3 of the 5′ LTR is replaced by a CMV promotor resulting in a CMV/LTR hybrid with high transcriptional activity (37). The 3′ U3 region of the LTR remained intact and is copied over to the 5′ LTR during reverse transcription, allowing efficient integration and LTR-driven transgene expression in the transduced cell.

Major concerns in the use of retroviral vectors are the possibility of vector mobilization and recombination with defective (endogenous) retroviruses in the target cell. This led to the development of self-inactivating vectors (SIN) (38). In these vectors, the viral promotor and enhancer regions in the 3′ U3 are deleted, thus preventing LTR-driven transcription in the transduced cells. Furthermore, transgene expression in these vectors is exclusively driven by an internal promotor, which improves the use of regulatory and tissue-specific promoters.

For the packaging of retroviral vectors, the structural proteins are provided in *trans* in packaging cells. The first packaging cell lines expressed *gag*, *pol*, and *env* from a complete proviral DNA lacking only the packaging signal (35). However, sequence homology between the vector and packaging constructs facilitated recombination, resulting in the generation of replication-competent virus. To prevent homologous recombination, packaging cells have been developed expressing *gag*/*pol* and *env* from separate constructs. Furthermore, expression from the packaging constructs is no longer driven by the viral LTR, but by constitutive promoters, thus allowing a high level of virus production (35, 39).

LENTIVIRAL VECTORS Although lentiviral vectors based on primate as well as nonprimate lentiviruses have been developed, lentiviral vector development has mainly focused on human immunodeficiency virus type 1 (HIV-1) because this virus has been studied extensively. Besides the structural proteins, HIV-1 encodes for six

additional accessory proteins (*tat*, *rev*, *vif*, *vpr*, *nef*, and *vpu*) that play an important role in the virus replication and persistence of infection (Figure 2*B*). For example, *tat* transactivates viral transcription (31, 32) and *rev* facilitates nuclear export of unspliced viral RNA (33).

The development of lentiviral vectors closely resembles the retroviral vector design. Similar to retroviral vectors, the HIV-1-based lentiviral vector is deprived of all viral sequences apart from the *cis*-acting sequences like LTRs and the packaging signal. In addition, the *rev* responsive element (RRE) is included in the vector. This region in the viral RNA binds the *rev* protein, which is provided in *trans*, ensuring efficient nuclear export of the full-length viral RNA genomes (40).

Vector RNA expression in this system is driven by the endogenous LTR and relies on transactivation by the *tat* protein for its transcriptional activity. Subsequent construction of a CMV/LTR hybrid markedly increased vector production and, more importantly, made vector production independent of *tat* expression (41).

Furthermore, the biosafety of the vectors is markedly improved by the development of SIN vectors. These vectors contain large deletions in the transcriptional activation unit in the 3′ U3 region of the LTR (42, 43), which results in inactivation of LTR and reduces the risk of recombination with wild-type virus. Recently, another *cis*-acting sequence has been added to the vector. This sequence, the central polypurine tract (cPPT) from the *pol* ORF, has been demonstrated to improve nuclear import of the proviral DNA and subsequently accelerates transduction (Figure 3) (44, 45).

During lentiviral vector production, all of the essential viral gene products are provided in *trans* from a packaging construct. The first versions of the packaging construct consist of the full-length proviral DNA lacking a packaging signal and contain a frame shift in the *env* and *vpu* ORF leaving the RRE intact, thus promoting nuclear export of the mRNAs in the presence of *rev*. Furthermore, the LTRs are removed from the construct and transcription is driven by a CMV promotor, which allows a high level of viral protein expression and reduces the sequence homology between the vector and packaging construct (40).

To improve the biosafety of the lentiviral vectors, all nonessential viral sequences are removed from the packaging constructs. The HIV-1 accessory genes *vif*, *vpr*, *nef*, and *vpu* are dispensable for lentiviral vector production and transduction and were deleted from the packaging construct (41). Subsequent modifications in the transfer vector constructs made vector production independent of *tat* expression, resulting in the third generation packaging system containing only the coding region for *gag*/*pol* and the *cis*-acting RRE, whereas *rev* is expressed from a separate construct (Figure 3) (46).

Given the restricted host range of the HIV-1 *env* glycoprotein, lentiviral vectors are pseudotyped with the vesicular stomatitis virus glycoproteins (VSV-G). The VSV-G *env* displays a broad host range and is expressed from a distinct construct during vector production (Figure 3) (40, 41, 46).

Recently, stable packaging cell lines have been developed. These stable producer cells express the structural proteins from minimal packaging constructs and

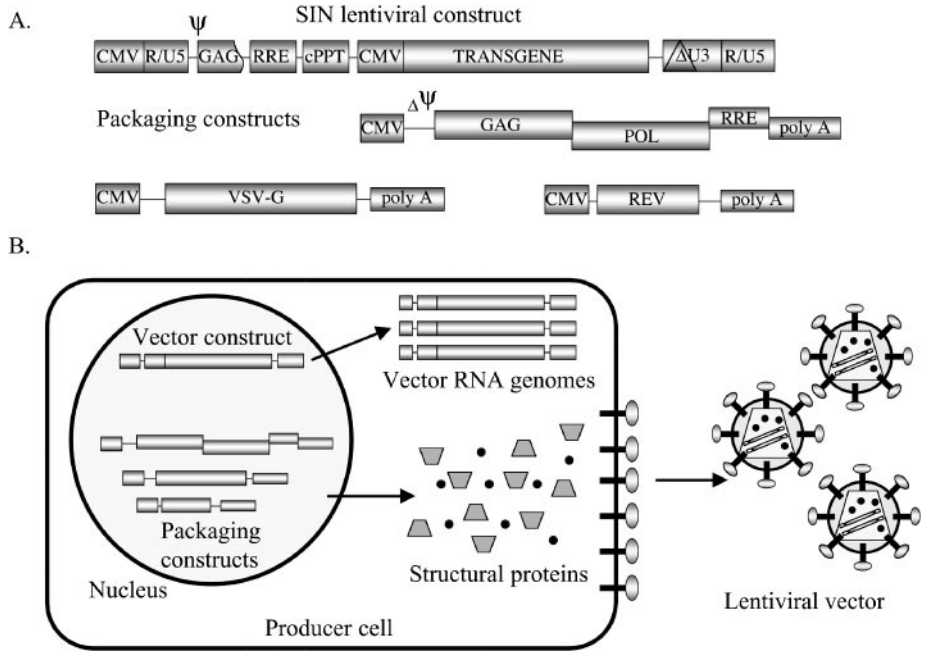

Figure 3 Lentiviral vectors. (*A*) In the HIV-1-based lentiviral vector, the viral genes *gag*, *pol*, and *env* have been replaced by promotor and transgene sequences and are flanked by the viral LTRs. Packaging of the viral RNA genome is ensured by the presence of the packaging signal (Ψ) comprised of the 5′ untranslated region and the 5′ sequence of the *gag* ORF. In addition, the vector contains two additional *cis*-acting sequences, the RRE, which is essential for nuclear export of unspliced viral RNA in the presence of *rev*, and the cPPT, which supports nuclear import of the proviral DNA in the transduced cell. The 3′ LTR contains a large deletion in the U3 region (depicted as ΔU3) to prevent transcription from the LTR. The lentiviral packaging system consists of three constructs encoding for *gag*/*pol*, VSV-G, and *rev*. The *gag*/*pol* construct contains the *cis*-acting RRE and requires expression of *rev* for efficient nuclear export similar to the vector construct. (*B*) Lentiviral vectors are produced by transient transfection of the vector construct together with the packaging constructs in producer cells. Vector RNA genomes are packaged by *gag* and *gag*/*pol* precursor proteins at the cellular membrane. Subsequently, the vector particles bud through the cellular membrane, obtaining the viral envelope that contains the VSV-G glycoproteins.

expression is driven by an inducible promotor to minimize the toxicity of the VSV-G envelope protein (47–49).

FOAMY VIRAL VECTORS FV vectors have only recently been developed and are quite similar to retroviral and lentiviral vectors. In addition to the packaging signal that consists of the 5′ untranslated region and the 5′ portion of the *gag* ORF present

in all retroviral vectors, FV vectors contain the 3′ region of the *pol* ORF, which is critical for efficient packaging of these vectors (50, 51). Similar to other retroviral vectors, the 5′ U3 region of the LTR in the vectors plasmid has been replaced by a CMV promotor, which increased vector expression and made vector production independent of *tas*/*bel1*.

FV vectors are produced by transient transfection of the vector construct and the packaging constructs encoding for the structural proteins *gag*, *pol*, and *env* in 293T cells. Because the FV envelope has a broad cellular host range, it is used by the vector and therefore the *env* sequences are included in the packaging construct or expressed from a separate construct (50, 51). The FV *env* contains, in contrast to other retroviruses, an ER sorting signal, which allows FV particles to bud from intracellular membranes, and, therefore, the majority of the infectious virions are cell associated (52). Consequently, the infectious particles have to be released from the packaging cells by freeze-thawing.

Retroviral Cellular Tropism and Transduction

The limited cellular tropism of the natural envelope of wild-type viruses is one of the barriers for retroviral transduction. However, retroviruses have the ability to incorporate *env* glycoproteins from related as well as unrelated viruses, thus allowing pseudotyping with alternative glycoproteins. Retroviral vectors are usually pseudotyped with glycoproteins from the VSV-G, a member of the rhabdovirus family, or the amphotropic MLV envelope in order to broaden their host range (53–59). Moreover, pseudotyping allows transfer of specific tropisms to the vector. Neurotropism and retrograde axonal transport were accomplished by the vector by pseudotyping with the G protein of Mokola lyssaviruses (60, 61), and the filovirus (Ebola Zaire) envelope supported transduction of airway epithelia, whereas only minimal transduction was observed with VSV-G (62). Additionally, glycoproteins obtained from other retroviruses (MLV 10A1, gibbon ape leukemia virus, RD114/endogenous feline virus) have been used for pseudotyping, which proved to be useful for transduction of hematopoietic progenitor cells (63, 64).

Interestingly, the entry pathway of the retroviral vector has evidently no effect on the transduction efficiency. Retroviral glycoproteins mediate virus entry by membrane fusion, whereas VSV-G pseudotyped vectors enter the cell through the endocytic pathway. Apparently, the virus is able to escape from the endosome by membrane fusion, induced by VSV-G at low pH, without being degraded.

Reverse transcription and nuclear translocation of the preintegration complex are thought to be limiting steps in retroviral transduction, especially in terminally differentiated postmitotic cells. Proviral DNA synthesis of all retroviruses depends strongly on cellular conditions, and low nucleoside pools or absence of cellular cofactors may explain the incomplete reverse transcription in quiescent or stationary cells (56, 65–70).

In contrast to other retroviral vectors, FV vector particles can contain fully reverse transcribed viral DNA-activation through reverse transcription before

virus assembly (71, 72). This suggests that FV vector gene transfer may be more efficient in certain postmitotic cells in which reverse transcription is limited.

A major limitation in the use of oncoretroviral vectors is their inability to transduce nondividing cells. Oncoretroviruses are unable to transport their preintegration complex containing the proviral DNA across the nuclear membrane in the absence of cell division. During mitosis, the nuclear membrane breaks down and only then is the large preintegration complex able to enter the nucleus (73, 74).

Lentiviral vectors on the other hand are able to transduce nondividing cells. The HIV-1 preintegration complex has karyophilic properties due to the presence of nuclear localization signals (NLS) in the viral proteins matrix and integrase. These unique features allow the preintegration complex to cross the nuclear membrane using the cellular nuclear import machinery in the absence of mitosis (75–78).

FV, however, is unable to replicate in nondividing cells despite efficient nuclear localization of the preintegration complex due to a NLS in *gag* (79, 80). Nevertheless, efficient transduction of postmitotic cells has been demonstrated using FV vectors in which transgene expression is driven by an internal CMV promotor (81), indicating that virus replication in nondividing cells is probably blocked at the transcriptional level.

Because HIV-1 is a human pathogen, there is some concern about the use of HIV-1-based lentiviral vectors. The current HIV-1 lentiviral vector system is deprived of all accessory proteins (except *rev*) and viral sequences in the vectors have been minimized; therefore, replication of these vectors is highly disabled and the possibility of homologous recombination is minimized. In addition, codon-optimization of the packaging construct further decreases the risk of homologous recombination. Furthermore, changes in the codons makes the production of the structural proteins independent of *rev*, and therefore additional viral sequences (RRE) can be eliminated from the packaging construct (82).

The use of vectors based on other primate lentiviruses (83, 84) may also eliminate some of the concerns; however, HIV-2 and simian immunodeficiency virus (SIV) are closely related to HIV-1. Therefore, vectors based on nonprimate lentiviruses like feline immunodeficiency virus (FIV), equine infectious anemia virus, and visna may be more acceptable (85–87). These viruses do not cause infection in humans due to restrictions in the envelope tropism. However, the risk associated with the introduction of nonhuman lentiviral vectors in human tissues is unknown, and the actual safety of these lentiviral vectors remains to be evaluated.

Because many steps in lentiviral infection (reverse transcription, nuclear transport, and integration) depend on cellular cofactors (65, 66, 68–70), there may be serious limitations in the use of nonhuman lentiviral vectors in primary human tissues. Replication of lentiviruses is highly adapted to their natural host, indicating that cross species variability of cellular factors essential for virus replication, and thus vector transduction, may impair the transduction efficiency of nonhuman lentiviral vectors in human cells. These restrictions may be overcome by the use of chimeric lentiviral vectors. Indeed, cross-packaging of FIV RNA by HIV-1 and SIV packaging systems has been demonstrated, and viral proteins were able to

support transduction, indicating that recognition of *cis*-acting sequences is highly promiscuous among lentiviruses (88). Furthermore, cross-packaging of nonhuman viral RNA by HIV-1-based virions may further eliminate any sequence homology between the vector and packaging constructs. However, for biosafety reasons, the introduction of chimeric lentiviral vectors for gene therapy is less desirable.

ADENOVIRAL VECTORS

Adenovirus Structure and Replication

Ad are icosahedral particles existing of a viral capsid that surrounds the viral core containing the large DNA genome of 36 kb. The viral linear dsDNA genome coding region is flanked by ITRs, and contains five early transcription regions (E1A, E1B, E2, E3, E4) and one late transcription region from which five families of late mRNAs (L1-5) are generated (Figure 4*A*). The Ad genome is intimately associated with viral proteins (core) and is packaged in the viral capsid, which consists primarily of three proteins: hexon, penton base, and knobbed fibers (89).

The Ad replication cycle can be divided in two phases, early and late. During the early phase of the replication cycle, the viral DNA is transported to the nucleus and transcription of early viral genes is initiated. Early gene products interfere with antiviral host cell defense mechanisms and direct the host cell to enter the cell cycle, supporting transcription and DNA replication. As soon as DNA replication is initiated by the E2 gene product, late events in the viral replication start. During this phase, gene expression of mRNA regulated by the late promotor increases, which results in high production of structural proteins that assemble together with viral genomes in the nucleus. The newly synthesized virions are released from the cell by the induction of cell lysis. [For more details see (89).]

Adenoviral Vector Development and Production

The first recombinant Ad vectors have been generated by deleting the E1 and/or E3 gene regions in the viral genome, allowing the introduction of promotor and transgene sequences up to 6.5–8.3 kb (90, 91). However, removal of the E1 gene from the vector, markedly hampers transcription of E2 genes and, consequently, DNA replication and production of structural viral proteins. E1-deleted Ad vectors can be efficiently propagated in 293 cells, complementing E1 gene products (5). Moreover, deletions in the E2 and/or E4 regions were made that increased the packaging capacity of the vector but also required the development of suitable complementing cells additionally expressing E2 and/or E4 (92, 93).

One major concern in the use of Ad recombinant vectors is the emergence of replication competent Ad virus as a result of recombination events between the viral sequences. Ad protein expression by replication-competent Ad virus, but also from the vector itself, results in the in vivo induction of a potent immune response.

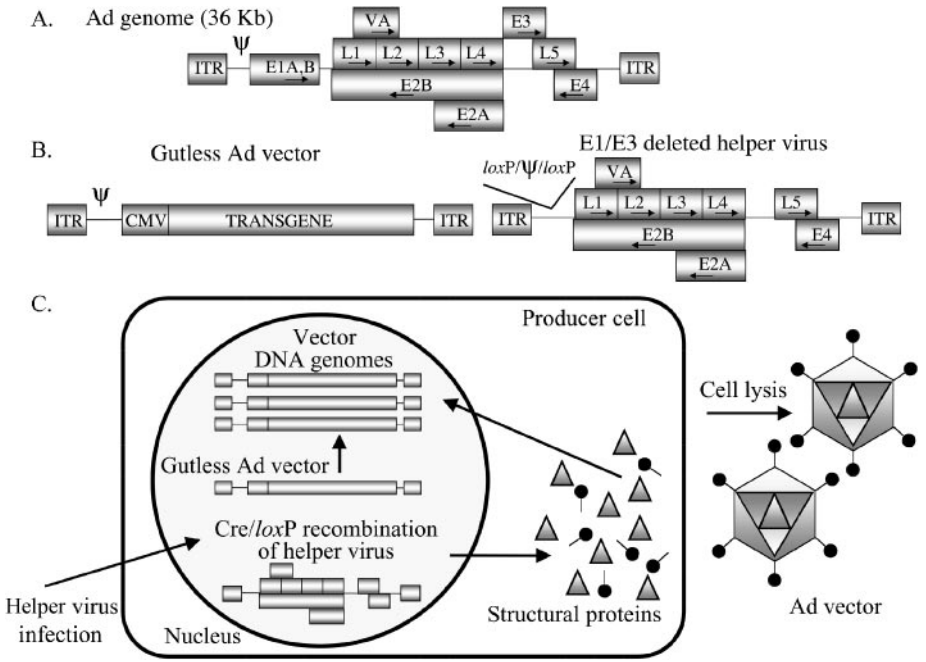

Figure 4 Gutless Ad vectors. (*A*) Schematic presentation of the organization of the Ad viral genome. (*B*) Gutless Ad vectors contain, besides promotor and transgene sequences, the *cis*-acting ITRs and the packaging signal (Ψ). Structural and regulatory Ad genes essential for vector production are provided from an E1/E3 deleted helper virus in which the packaging signal is flanked by *lox*P sites. (*C*) The gutless Ad vector is produced in 293 cells, which complement for the Ad E1 and express Cre recombinase. The vector construct is transiently transfected into the producer cells and vector production is propagated by infection with the E1/E3 deleted helper virus. Packaging of the helper virus is prevented by excision of the Ψ element by Cre/*lox*P recombination.

The immune system eliminates transduced cells that express viral proteins as well as the transgene, and therefore only transient transgene expression is observed.

More recently, Ad vectors have been developed that are deprived of nearly all viral genes. These so-called gutless Ad vectors retain only the viral ITRs and the packaging signal and require a helper virus for their replication (94). The helper virus, in general, is an E1/E3 deleted recombinant Ad vector that provides the necessary viral proteins in *trans*. However, the helper virus is also packaged in this system, resulting in the generation of replication competent Ad. To prevent packaging of the helper virus genome, the packaging signal is removed by Cre/*lox*P recombination in the producer cells expressing Cre recombinase (Figure 4). This helper virus maintains the ability to facilitate efficient virus replication but minimizes the generation of replication competent virus.

Because the majority of the viral coding sequences have been deleted in the gutless Ad vector, the addition of stuffer DNA is essential to maintain the optimum packaging size of the vector (95). Moreover, removal of viral genes from the vector prevents the induction of an immune response, and long-term gene expression can be obtained using the gutless Ad vector.

Adenoviral Vector Tropism and Transduction

Ad serotype 5 is commonly used for gene therapy studies, and consequently the Ad vector host range is similar to that of the wild-type virus. Recently, the viral receptor and coreceptor of this serotype have been identified. The virus binds to the cellular receptor CAR (coxsackievirus and adenovirus receptor) (96), a member of the immunoglobulin superfamily, through the knob of the fiber, and virus entry occurs through clathrin-mediated endocytosis after binding of the penton to integrins ($\alpha V\beta 3$ and $\alpha V\beta 5$ integrin) (97). Although the receptors for Ad are ubiquitously expressed, inefficient transduction due to low level expression of the receptors has been observed in some tissues like airway epithelia (98). To permit virus entry in cells lacking the CAR receptor or to target specific cell types, retargeting of Ad vectors was essential, and this has been explored extensively. Bispecific conjugates or retargeting complexes that cross-link the virus with alternate receptors have been developed. Retargeting complexes have been designed using either Ad neutralizing antibodies (fiber or penton base specific) chemically linked to ligands or antibodies specific for cellular receptors (99–101) or fusion proteins of the ectodomain of the Ad receptor CAR and ligands (102). Using this method, retargeting of Ad vector via epidermal growth factor (99, 102) and E-selectin (101) has been demonstrated. Alternatively, the Ad vector host range can be altered by incorporation of binding motifs in the C terminus of the fiber protein and by modifications in the RGD motif of the penton base (103–106).

Differences in the host-range of Ad serotypes indicate that besides CAR, other cellular receptors are involved, and consequently, the cellular tropism of Ad vectors can also be altered by the use of alternative serotypes. For instance, Ad17 has enhanced tropism for airway epithelia (107), Ad35 is able to infect hematopoietic progenitor cells (108), and Ad subgroup D shows higher infectivity in the central nervous system (109).

Ad vectors efficiently transduce dividing as well as nondividing cells, and high levels of transgene expression have been observed from the episomal dsDNA genome. However, the synthesis of viral proteins from the vector elicits a strong cellular and humoral immune response (110). This results in clearance of the transduced cells by cytotoxic T cells, whereas the humoral immune response precludes re-administration of the vector.

The development of the gutless Ad vector, which is deprived of nearly all viral sequences, prevents elimination of transduced cells by the immune system, thus allowing long term transgene expression. However, the vector DNA genome exists episomally and is nonreplicating. Therefore, transgene expression may be lost over

time due to dilution in replicating cells, whereas the episomal genome is subjected to degradation in nondividing cells. Recently, Ad/AAV and Ad/retrovirus hybrid vectors have been developed (111, 112). These vectors are devoid of all viral genes, and the transgene sequence is flanked by AAV ITRs or retroviral LTRs. These *cis*-acting elements from unrelated viruses allow integration of the transgene by host cell enzymes in the absence of viral proteins (AAV *rep* and retroviral integrase) supporting these events. Successful transduction and integration has been observed using these hybrid vectors, albeit at low efficiency.

One of the major concerns in the use of Ad vectors for gene therapy purposes is the induction of an innate immune response. Administration of an Ad vector causes inflammation of the infected tissue, especially in the liver (110, 113–115). The induction of chemokine production at the infected site attracts neutrophils, leading to necrosis and apoptosis in the liver. The development of gutless Ad vectors markedly reduce inflammatory responses and cellular infiltration (116). However, inflammation induced by the viral capsid proteins itself cannot be prevented (110).

HERPES SIMPLEX VIRUS TYPE 1 (HSV-1) VECTORS

HSV-1 Structure and Replication

The HSV-1 virion is approximately 20 nm in diameter and consists of four components: envelope, tegument, capsid, and viral genome. The envelope is derived from the cellular membrane and contains approximately 12 viral glycoproteins essential for viral entry. The tegument is the protein layer between the capsid and the envelope and contains at least 10 viral proteins, including VP16 (essential for transactivation and virion envelopment), VP22 (membrane translocation domain), and virion host shut off (*vhs*) protein. The capsid consists of 7 viral proteins and contains the linear dsDNA genome, which is 152 kb in size.

The viral genome can be divided in unique long (U_L) and unique short (U_S) regions flanked by terminal repeats (TR), and encodes for at least 80 viral proteins. However, the function of a large number of these viral proteins is still unknown.

The fate of HSV-1 infection can be either lytic or latent. During the lytic replication cycle, which occurs in mucosal or epithelial cells, the host cell protein synthesis is shut off by the tegument *vhs* protein immediately after infection. Subsequently, the viral capsid releases the viral DNA into the nucleus where it will circularize.

Within several hours after infection, protein expression from the circular genome is initiated. Protein expression occurs in a highly regulated fashion and can be divided into three groups of sequentially expressed proteins: α- or immediate early genes, β- or early genes, and γ- or late genes. Once β-gene products are present, DNA replication and γ-gene production is initiated and progeny virus is produced. In the nucleus, capsid proteins assemble together with the viral DNA genomes and the newly formed viral capsids bud through the nuclear membrane. On their way

to the Golgi apparatus, the virion obtains the tegument and the viral envelope and, subsequently, the virus is released from the cell through secretory vesicles.

In neurons, HSV-1 infection can result in a latent infection. Upon entry, the virion is transported to the nucleus by retrograde transport along the axon. It is currently unknown which viral genes are involved in the establishment of a latent infection, but de novo viral protein synthesis is not required. However, latency is related to the expression of latency associated transcripts (LAT), which are expressed from a promotor that is highly active in neurons. LATs prevent the lytic replication cycle by down-regulation of genes associated with lytic infection. Reactivation of the latent virus can be induced by different stimuli like stress and UV irradiation. [For more details see (117).]

HSV-1 Vector Development and Production

The development of vectors based on HSV-1 has produced two different viral vector systems: recombinant HSV-1 vectors and HSV-1 amplicons.

Recombinant HSV-1 vectors contain a number of deletions in the α-genes and the *vhs* and can harbor large transgenes up to 30 kb in size (118, 119). These vectors are nonreplicating and can be propagated in complementing cell lines providing the essential α-genes in *trans*. Nevertheless, recombinant HSV-1 vectors still retain large proportions of the HSV-1 genome and can express viral genes that induce cytotoxicity and immune responses. Moreover, transgene expression by recombinant HSV-1 vectors is usually transient.

The HSV-1 amplicon vector system is based on the ability of HSV-1 to package defective genomes containing the *cis*-acting sequences *ori* (origin of viral DNA replication) and *pac* (packaging and cleavage signal). HSV-1 amplicon vectors contain, besides these *cis*-acting elements, no viral genes (120–122). However, packaging of the amplicon vectors requires a replicating helper virus, resulting in high level contamination with replication competent virus. This problem has been overcome by the development of a helper-free packaging system in which viral genes are provided in *trans* from five cosmids spanning the HSV-1 genome but lacking the *pac* signal (123). This packaging system markedly decreases the generation of replication competent virus and cytotoxicity; however, only low titers of amplicon vectors are generated. Recently, the entire HSV-1 genome lacking the *pac* signal has been cloned as a bacterial artificial chromosome (*bac*) that simplifies amplicon packaging and results in increased vector titers (124, 125).

HSV-1 Vector Tropism and Transduction

HSV-1 virus entry is mediated by multiple glycoproteins present in the envelope and is a rather complex process. Initial adhesion to the cellular membrane is mediated by the interaction of gC and gB with glycosaminoglycan heparan sulfate. Subsequently, gD binds to a specific cellular receptor, either a member of the TNF receptor family [herpes virus entry mediator A (HveA)], immunoglobulin superfamily (HveB, HveC), or 3-O-sulfated heparan sulfate. Then, the virus enters the cell through membrane fusion promoted by the gH/gL complex and gB (117).

Although the HSV-1 envelope has a broad host range, efforts to target and alter the cellular tropism of the vectors have been made. The HSV-1 vector host range can be altered by the incorporation of the VSV-G envelope, which circumvents the receptor-specific binding of gD and supports attachment and entry of gD-deficient HSV-1 vectors, albeit at low levels (126). Furthermore, construction of a chimeric gC-containing ligands for cellular membrane receptors allows targeting of HSV-1 vector deficient for gC and lacking the receptor-binding domain in gB. However, the vector enters the cell by endocytosis instead of membrane fusion, which results in degradation of the vector in the endosome (127).

The major problem of recombinant HSV-1 vectors is their cytopathic effect and the induction of an immune response by viral gene expression. The development of amplicon vectors and a helper virus–free packaging system has overcome this problem to a great extent (123–125). However, additional deletion of nonessential genes from the *bac* packaging system may be necessary to further prevent the cytotoxicity of this vector system.

The large packaging capacity of HSV-1 amplicons (up to 152 kb in theory) may be very useful for gene therapy purposes to deliver complex genes and regulatory sequences or to deliver multiple copies of the transgene. However, long-term gene expression using HSV-1 amplicons has not been demonstrated. Because the amplicon DNA exists extra-chromosomal in a circular form and does not integrate, it is subjected to loss by cell division and degradation. The incorporation of elements from the Epstein-Barr virus (*oriP* and EBNA-1) has been shown to maintain the viral DNA and subsequently prolongs transgene expression (123).

VIRAL VECTORS IN CLINICAL TRIALS

In recent years, many clinical trials have been conducted and the first successes have been reported. Most prominent is the treatment of two young children suffering from a fatal form of severe combined immunodeficiency-X1 (SCID-X1). This disease is an X-linked hereditary disorder characterized by an early block in the development of T and natural killer (NK) cells due to mutations in the γc cytokine receptor subunit. Hematopoietic stem cells from the patients were stimulated and transduced ex vivo with an MLV-based retroviral vector expressing the γc cytokine receptor subunit and were re-infused into the patients (128). During a 10-month follow-up, γc-expressing T and NK cells could be detected, and cell counts and function were comparable to aged-matched controls. The selective advantage of the γc-expressing lymphocyte progenitors, enabling the development of mature T and NK cells, contributed considerably to the success of this study. Two additional patients have since been treated and the outcome is very promising. However, earlier attempts to treat SCID patients suffering from adenosine deaminase (ADA) deficiency using retroviral vectors failed to show a long-term beneficial effect even though long-term reconstitution from transduced progenitor cells was observed at low levels. Patients in these studies received, in addition to the transduced cells, ADA enzyme preparations, which

may have prevented the selective outgrowth of the transduced progenitor cells (129–133). Indeed, discontinuation of ADA replacement therapy showed a selective increase in the number of peripheral blood mononuclear cells containing the transgene. Although T cell counts and function remained within normal limits, a loss of B and NK cells was observed in these patients. Moreover, the accumulation of toxic adenosine metabolites in erythrocytes required reinstatement of the therapy (133, 134) and indicated that ADA production was insufficient due to limited numbers of transduced cells or low ADA expression levels.

Although successful transduction of hematopoietic stem cells has been demonstrated using MLV-based retroviral vectors, the extensive manipulation of the hematopoietic stem cells to achieve efficient transduction is undesirable and may induce differentiation of the early progenitors. The development of lentiviral vectors, which are able to transduce hematopoietic progenitor in the absence of cytokines, may further improve stem cell gene therapy. Although these vectors have not yet been approved for use in clinical trials, some remarkable results have been obtained in animal models. Lentiviral vectors were successfully used to introduce a functional β-globin gene into hematopoietic stem cells and corrected β-thalassaemia and sickle cell disease in mice models (135, 136). Furthermore, lentiviral vectors hold great promise in the treatment of neurological diseases as demonstrated in a rhesus monkey model for Parkinson's disease (137) and a mouse model for metachromatic leukodystrophy, a lysosomal storage disease affecting the central nervous system (138).

In another clinical study, patients suffering from hemophilia B, which is a bleeding disorder caused by a deficiency of coagulation factor IX, were treated with AAV vectors expressing human factor IX (139). These patients participated in a Phase I trial and received intramuscular injections of AAV vectors. Although only very low levels of secreted factor IX could be detected in the plasma of one patient, the treated patients showed some clinical benefits and a reduced intake of factor IX infusions. Moreover, no vector-related toxicity and germ line transmission was observed.

The treatment of genetic diseases using Ad vectors has recently been tempered. As a result of in vivo studies, it became clear that administration of Ad vector induced a potent immune response and inflammation of the transduced tissue (113, 114). The significance of this problem became even more evident after the tragic death of a participant in a Phase I trial receiving Ad vector therapy (115), emphasizing the need to understand and control the host responses.

CLOSING REMARKS

This review focuses on viral vectors commonly used in gene therapy studies. Their specific properties are summarized in Table 1. Although remarkable progress has been made in the development of viral vectors, the ideal vector remains elusive. The wide variety of diseases that may benefit from gene therapy will mandate specific

TABLE 1 Properties of viral vector systems

		Retroviral			Adenoviral		HSV-1	
	AAV	**Onco**	**Lenti**	**Foamy**	**Recomb**	**Gutless**	**Recomb**	**Amplicon**
Viral genome	ssDNA	RNA	RNA	RNA dsDNA	dsDNA	dsDNA	dsDNA	dsDNA
Packaging size[a] (transgene)	4.9	8.8	9.6	12.3	36 (8.3)	36	152 (30)	152
Integration	Y/N[b]	Y	Y	Y	N	N	N	N
Nondividing cells	Y	N	Y	Y	Y	Y	Y	Y
Duration of expression	L	L	L	L	S	S/L	S	S
Induction of CTL response	N	N	N	N	Y	Y	Y	Y
Pre-existing immunity	Y	N	N[c]	N	Y	Y	Y	Y
Safety concerns	Insertional mutagenesis		Insertional mutagenesis		Inflammation cytotoxicity		Inflammation cytotoxicity	

[a]Size of the viral genome packaged by the virion (Kb).

[b]Integration is inefficient in the absence of *rep* protein.

[c]With the exception of HIV-1 patients.

Y: yes; N: no; L: long; S: short.

requirements of viral vectors, such as tissue-specific transduction and regulated gene expression. Therefore, it is unlikely that one single vector system will suffice for all gene therapy purposes. Nevertheless, gene therapy approaches have great promise to influence human health in the future.

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