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Viral Vectors for Gene Transfer

A Review of Their Use in the Treatment of Human Diseases

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Abstract

The efficient delivery of therapeutic genes and appropriate gene expression are the crucial issues for clinically relevant gene therapy. Viruses are naturally evolved vehicles which efficiently transfer their genes into host cells. This ability made them desirable for engineering virus vector systems for the delivery of therapeutic genes. The viral vectors recently in laboratory and clinical use are based on RNA and DNA viruses processing very different genomic structures and host ranges. Particular viruses have been selected as gene delivery vehicles because of their capacities to carry foreign genes and their ability to efficiently deliver these genes associated with efficient gene expression. These are the major reasons why viral vectors derived from retroviruses, adenovirus, adeno-associated virus, herpesvirus and poxvirus are employed in more than 70% of clinical gene therapy trials worldwide. Among these vector systems, retrovirus vectors represent the most prominent delivery system, since these vectors have high gene transfer efficiency and mediate high expression of therapeutic genes. Members of the DNA virus family such as adenovirus-, adeno-associated virus or herpesvirus have also become attractive for efficient gene delivery as reflected by the fast growing number of clinical trials using these vectors.

The first clinical trials were designed to test the feasibility and safety of viral vectors. Numerous viral vector systems have been developed for *ex vivo* and *in vivo* applications. More recently, increasing efforts have been made to improve infectivity, viral targeting, cell type specific expression and the duration of expression. These features are essential for higher efficacy and safety of RNA- and

DNA-virus vectors. From the beginning of development and utilisation of viral vectors it was apparent that they harbour risks such as toxicities, immunoresponses towards viral antigens or potential viral recombination, which limit their clinical use. However, many achievements have been made in vector safety, the retargeting of virus vectors and improving the expression properties by refining vector design and virus production.

This review addresses important issues of the current status of viral vector design and discusses their key features as delivery systems in gene therapy of human inherited and acquired diseases at the level of laboratory developments and of clinical applications.

The discovery of the genetic origin of numerous human diseases created the molecular basis for gene therapy. Genes have been characterised that cause disorders by loss of normal function due to genetic alterations such as deletions, mutations, translocation, allele loss, etc. Therefore, the introduction of functional genes for the restoration of normal function or the transfer of therapeutic genes to treat particular diseases such as cancer or viral infections is of growing interest. After the first phase I gene therapy trials for adenosine deaminase (ADA) deficiency and malignant melanoma in the US in 1990,[1,2] more clinical studies rapidly followed aimed at the correction of genetic illnesses, such as cystic fibrosis, low density lipoprotein (LDL)-receptor deficiency, haemophilia A and B, alpha-1-antitrypsin deficiency, and Gaucher's disease (table I). Simultaneously, gene therapy is also employed to treat human malignancies of different origins (melanoma, ovarian carcinoma, brain tumours etc.), employed for vaccination or to fight diseases caused by viral infections, such as AIDS.

In this context, gene transfer systems are needed that are capable of transducing the desired gene to the target cells or tissues at high efficiency and for the period of time required to accomplish therapeutic effects. Viral vectors are still among the most efficient gene transfer vehicles because of their combined ability to infect a high proportion of cells in a population with a great capacity for carrying transgenes in their genetically modified genome. The number of viral vector constructs possessing different features for targeting infection or cell type specific expression, high infectitivity, safety features or the ability to infect resting, differentiated cell populations is rapidly growing. The majority of studies with these vectors are aimed at refining their safety and improving the targeting of gene transfer to efficiently express the transgenes. This is associated with the reduction of potential adverse effects of viral vector application to humans, such as immunogenicity, potential risk of homologous recombination or toxicities. Because gene therapy is being applied to a wide variety of inherited and also acquired diseases, viral vectors have evolved in parallel into a great variety depending on whether long term (if not life-long) or transient expression is required, a particular tropism of viral infection to the target cell population is important, if constitutive or inducible activity of viral promoters is needed, or if virus-mediated target cell killing will be achieved.

Numerous different viral vector systems have been developed for *in vitro*, *ex vivo* and *in vivo* gene transfer which were mainly derived from murine and human DNA- and RNA-viruses depending on the ease of possible manipulation of viral genomes to create recombinant viral vectors. The most commonly used vectors are developed from retroviruses, lentiviruses, adenovirus, herpes simplex virus (HSV) and adeno-associated virus (AAV) because of the comparatively long history of characterisation of particular viruses and their genomes, their valuable characteristics for target cell infectivity, transgene capacity, and accessibility of established helper cell lines for the production of recombinant virus stocks to infect target cells.

Recently, many more different virus vector systems are being developed. These are derived from vaccinia virus, human cytomegalovirus, Epstein-

Table I. Inherited and acquired genetic diseases, and viral infections, which are targets for gene therapy

Disease	Defective gene/therapeutic target	Affected or targeted cell type
Hereditary		
Severe combined immunodeficiency	Adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP)	T lymphocyte, B lymphocyte
Familial hypercholesterolaemia	Low density lipoprotein (LDL) receptor	Hepatocyte
Cystic fibrosis	CF transmembrane conductance regulator	Epithelium of lung and pancreas
Gaucher's disease	Glucocerebrosidase	Macrophage
Fanconi anemia	FAA, FAC genes	CD34+ cell
Hurler syndrome	alpha-L-iduronidase	CD34+ cell
α- and β- thallasaemia	α- and β-globin	Erythrocyte
Chronic granulomatosis	Cytochrome b245	Neutrophil
Haemophilia A and B	Clotting factors VIII and IX	Platelet
Duchenne muscular dystrophy	Dystrophin	Muscle
Emphysema	Alpha-1 antitrypsin	Lung epithelium
Sickle cell anaemia	β-globin	Erythrocyte
Agammaglobulinaemia	Bruton's tyrosine kinase	B lymphocyte
Leukocyte adhesion deficiency (LAD-1)	β chain of CD18	T lymphocyte
Phenylketonuria	Phenylalanine hydroxylase	Hepatocyte
Hyperammonaemia	Ornithine transcarbamylase (OTC)	Hepatocyte
Citrullinaemia	Argininosuccinate synthetase	Hepatocyte
Lesch-Nyhan syndrome	Hypoxanthine phosphoribosyl transferase (HPRT)	Basal ganglia
Acquired		
Solid tumors	Induction of specific/nonspecific immune responses to tumor by cytokine gene transfer or suicide gene transfer for tumor eradication	Tumor cell, dendritic cell, T lymphocyte
AIDS	Inhibition of replication and infection of HIV	T lymphocyte

Barr virus, poxviruses, and foamy virus, some of which have started to enter the clinical phase. These virus vectors, currently under development, are contributing to the broadening of the application spectrum of viral vectors for gene transfer.

1. Retroviral Vectors

Retroviral vectors are efficient transfer systems for the introduction of foreign genes into target cells. These vectors are widely used systems in gene therapy, reflected by the very high percentage (about 40%) of clinical gene therapy protocols which are performed using recombinant retrovirus particles or retrovirus producing cells for gene transfer. The predominance of employment of retroviral vectors is in part due to the longer research history which laid the basis for manipulating the retroviral genome. Retroviral vectors are derived from RNA viruses possessing the main feature of reverse-transcribing their viral RNA genome into a double stranded viral DNA which is then stably inserted into the host DNA (fig. 1). Members of this class of RNA viruses are the murine leukaemia viruses (MuLV) and the lentiviruses, which are extensively used for virus vector engineering.

The advantages of retroviral vectors are determined by their characteristics of stable integration into the host genome, generation of viral titres sufficient for efficient gene transfer, infectivity of the recombinant viral particles for a broad variety of target cell types and ability to carry foreign genes of reasonable sizes [<8kb] (table II). These properties are the essential prerequisite for persistence of the transgene in transduced cells and their progeny cells, and for long term and high level expression of the transgene, which is essential to correct the disease phenotype and to exert therapeutic effects. However, these advantages are accompanied

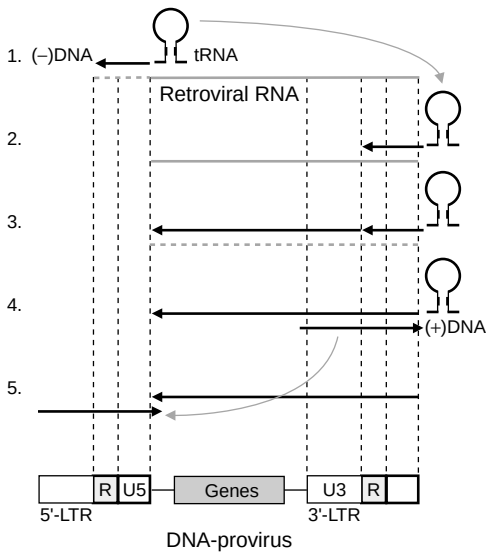

Fig. 1. Schematic representation of retroviral reverse transcription. 1. Reverse transcription of retroviral RNA starts with a tRNA molecule that binds to viral RNA for (-)DNA-strand synthesis. 2. The hybrid RNA, which served as template for the (-)DNA-strand synthesis is then degraded and the (-)DNA-strand moves to the R-region of the 3′-LTR. 3. At this position the (-)DNA-strand synthesis continues, while at the same time viral RNA is degraded. 4. The synthesis of the (+)DNA strand starts at the (-)DNA-strand, synthesizing the 3′-LTR of the DNA-provirus. 5. This (+)DNA-strand moves to the 5'-end of the (-)DNA-strand to complete second strand synthesis for the DNA-provirus consisting of the viral genes (or transgenes) flanked by the 2 LTRs. **LTR** = long terminal repeats.

by disadvantages which can create obstacles, particularly for their clinical application. Among those are the instability of some retroviral vectors, possible insertional mutagenesis by random viral integration into host DNA and the requirement of cell division for integration of MuLV-derived retroviral vectors, which makes certain cell types refractory to viral infection[3] (table II). Furthermore, targeting of retroviral infection and/or therapeutic gene expression still remains unsatisfactory for clinical application. Despite these hurdles for retrovirus application in gene therapy, these vectors will remain the gene delivery system of choice for the majority of gene therapy applications.

1.1. MuLV-Derived Vectors

The retroviral vectors currently used for gene therapy are predominantly derived from the Moloney murine leukaemia virus (MoMuLV)[4,5] possessing the classical properties of a retrovirus. This amphotropic virus is able to infect murine cells and cells of a variety of other species including human cells.[6] Infectitivity of the amphotropic virus is mediated by the envelope protein which binds to the phosphate transporter Ram-1 (Pit-2) on the target cell surface.[7] The MuLV consists of 3 structural genes, *gag*, *pol* and *env*, flanked by the viral long terminal repeats (LTR), which are responsible for regulation and expression of the viral genome such as polyadenylation, replication and integration of the provirus into host DNA. The characteristics of the retrovirus life cycle are important features and advantageous for the development of the retrovirus vector systems. Reverse transcription of viral RNA into proviral DNA makes manipulation of the viral genome easier, since the proviral DNA can be used for construction of viral vectors. Furthermore, the removal of the structural genes in the viral vector and of the packaging signals (*psi*-site) from the packaging vector constructs are the basis for safe and efficient production of recombinant virus particles for infection of the desired target cells[8] (fig. 2). The viral structural genes are supplemented via helper cells, whereas the *psi*-signal, essential for packaging the viral RNA, is present on the viral vector *in cis*. For the construction of MuLV-based vectors the structural genes are removed to create space for insertion of the therapeutic genes, the internal promoters and the resistance genes for possible clonal selection of transduced cells. The removal of the structural genes does not interfere with the capability of the viral RNA to be packaged into infectious retroviral particles.

The variations in arrangement of the therapeutic gene insertion and of the expression-driving promoter and enhancer elements established different classes of MuLV-based retrovirus vector constructs (fig. 3). The simplest retrovirus vector constructs, the single gene vectors, carry only a single gene as replacement for the deleted viral structural genes

Table II. Features of viral vector systems for their application in gene therapy

Viral vector	Advantages	Disadvantages
Retroviral vectors	Insert capacity for transgene <7-8kb, stable integration into host DNA, recombinant virus titers of 10^6-10^7 pfu/ml, broad cell tropism of infectivity, relatively easy manipulation of viral genome for vector engineering	Difficult targeting of viral infection, no infection of non-dividing cells, random integration into host genome, instability of vectors
Lentiviral vectors	Infect dividing and non-dividing cells stable gene expression, insert capacity of 10kb	Potential insertional mutagenesis,presence of regulatory (tat, rev) and of accessory protein sequences in the packaging constructs
Adenovirus vectors	Generation of high virus titers of 10^{10} pfu/ml, high level gene expression, large insert capacity (7-8kb), infects dividing and non-dividing cells	Immune response to viral proteins, no integration into host genome, transient gene expression
AAV vectors	Infect dividing and non-dividing cells, broad cell tropism, potential of targeted integration, low immunogenicity and nonpathogenic	Limited capacity for transgenes (4kb), difficult generation of high virus titers, requirement of adenovirus or herpesvirus for AAV replication
Herpesvirus vectors	Infects a wide variety of cell types, high insertion capacity (up to 50 kb), natural tropism to neuronal cells stable viral particles allow generation of high virus titers (10^{12} pfu/ml)	Possible toxicities, risk of recombination, no viral integration into host DNA,
Poxvirus vectors	High insertion capacity, insertion of large DNA fragments possible high transgene expression level, suited for live recombinant vaccine	Potential cytopathic effects
Epstein-Barr virus vectors	Infects dividing and non-dividing cells with preference for B-cells, high insert capacity (<150kb)	Difficult access to packaging cell lines

AAV = adeno-associated virus; **pfu** = plaque forming units.

in trans, since at this position the inserted genes are not spliced out. This type of vector is utilised for gene correction strategies, introducing the entire gene cassette of a functional gene including its own regulatory units. For efficient gene expression these gene inserts are placed in opposite orientation in relation to the viral LTR. The β-globin gene was inserted in such manner, including introns, promoter and enhancer sequences, allowing the appropriate and tissue specific gene expression.[9,10]

In many retroviral vectors the therapeutic gene expression is controlled solely by the viral LTR, classifying them as LTR-based vectors (fig. 3). In these vectors, the therapeutic gene is expressed from the unspliced viral mRNA or from the spliced envelope message.[11] However, in the majority of viral vector constructs, expression of one gene is under the transcriptional control of the viral LTR, whereas expression of the second gene is driven by an internal promoter. These vectors, which are also classified as internal promoter vectors, often suffer from reduced expression efficiencies due to competitive LTR and promoter interference.[12] The most widely used type of vectors, the double gene vectors, are mostly internal promoter vectors harbouring the gene of interest and a second gene as the selectable marker, such as the neomycin phosphotransferase (*neo*) or hygromycin phosphotransferase (*hgr*) gene, controlled by the LTR and the internal promoter, respectively. The selectable marker genes are employed for the cloning of *ex vivo*/*in vitro* transduced cells. The important feature of these constructs is the potential to exploit internal promoters to achieve cell type-specific or regulatable expression of the desired gene[13] for controlled gene expression that can be restricted to the targeted cell population.

Further variations of MuLV-derived vectors are the double copy vectors, promoter conversion (ProCon) vectors and the self-inactivating (SIN) vectors (fig. 3) which are based on alterations of viral LTR sequences. The double copy vectors harbour cloning sites within their viral 3′-LTR for insertion of the therapeutic gene which is dupli-

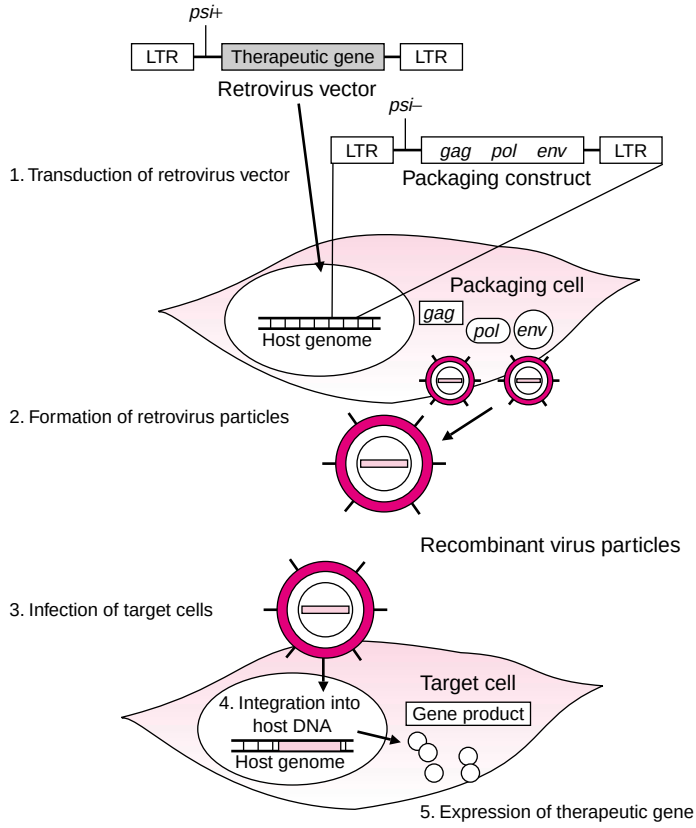

Fig. 2. Principle of retrovirus vector gene transfer. The structural genes *gag*, *pol* and *env* are deleted from the virus vector which instead carries the therapeutic gene and the *psi* packaging signal. Therefore, the vector requires complementation of the deleted structural genes for formation of recombinant infectious virus particles, which is mediated by the helper cell. These cells harbour the 'wild-type' retrovirus lacking the *psi* packaging signal. The retroviral vector is transduced into the helper cell where recombinant virus particles are produced. These particles can be used to infect the target cells via specific receptors to start reverse transcription for random integration of the proviral DNA into the host genome where the vector starts expression of the therapeutic gene.

cated after completion of the reverse transcription, since the 3′-LTR serves as template for the 5′-LTR synthesis[14] (fig. 1). Because of this strategy, the viral LTR promoter activity becomes inactive and therefore the transgene requires an internal promoter for efficient transcription. A similar strategy is used in ProCon vectors, in which the viral U3 region of the 3′ LTR is replaced by a heterologous promoter to improve transcription efficiency and transcriptional targeting of retroviral vectors.[15,16] In SIN vectors, the promoter/enhancer sequences within the U3 region of the viral 3′-LTR is mutated/deleted to prevent insertional activation of genes in the host genome for the safety of retroviral vector use. After viral reverse transcription both the 5′-LTR and 3′-LTR are transcriptionally inactive, which does not interfere with expression of the internally promoted therapeutic genes.[17] Unfortunately this safety feature of SIN vectors is accompanied with low titre of virus stocks of about 10^4 colony forming units (cfu)/ml.

To improve transcriptional efficiency for expression of two or more genes in retroviral vectors, the bicistronic vectors have been established.[18-20]

These vectors carry a putative internal ribosomal entry site (IRES), that was first identified in the 5′ untranslated intercistronic region of picorna viruses. The IRES is placed between the genes of interest leading to co-expression of two or more genes from one single transcript with almost similar gene expression efficiencies for each gene, although genes located downstream of IRES could be expressed at a lower level.[21] It has been shown for interleukin (IL)-12 expression, that IRES vectors can express three separate genes/gene fragments from a single transcript.[22] The advantages of IRES vectors are the possibility for insertion of genes of larger sizes, efficient gene expression and the potential to produce high virus titres.

In all these different vector constructs, retroviral promoters, promoters from other viruses or cellular promoters are employed for transgene expression. However, unfortunately, long term expression, in particular, can be difficult because of silencing effects that interfere with these promoters. Such expression shut-off occurs at variable times and has been observed in haematopoietic and other cells.[23-25] These effects are mediated by specific factors present in the target cells. It has been shown for LTR-driven expression that such factors interact with the viral LTR and the primer binding site.[26] Efforts are being made to modify these sequences to achieve more stable and higher gene expression, or to utilise internal promoters which are not affected by shut-off mechanisms.

As well as the attempts to improve expression of the therapeutic genes by varying the assembly of retrovirus-mediated vector constructs, targeting of viral infection is important for efficient and tolerable retroviral gene therapies. Limitations for efficient viral infection to cell populations of therapeutic interest are that ecotropic retroviruses are only able to infect murine cells mediated by recognition of the cationic amino acid transporter as the receptor for the retroviral envelope protein.[27] Furthermore, for the amphotropic viruses, receptors are limited in number or are poorly competent for infection of desired cell populations. Therefore, much effort has been made to create packaging constructs and

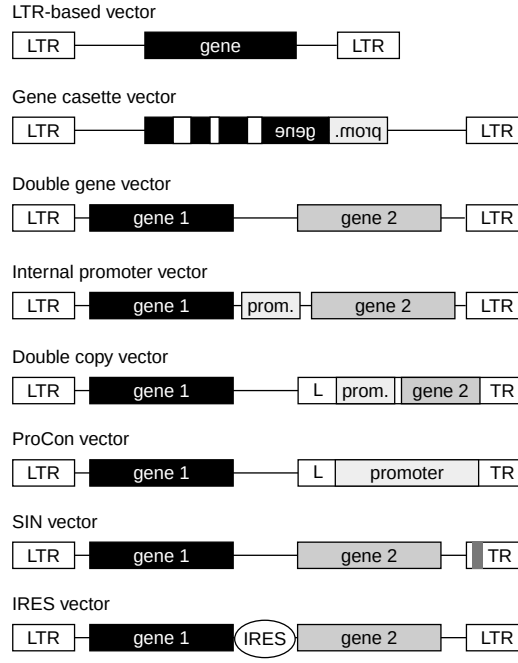

Fig. 3. Representative retroviral vector constructs. The simple viral vector construct (LTR-based vector) consists of viral LTRs and the therapeutic gene, inserted at the *gag, pol, env* position. The gene expression is driven by the LTR. In the gene cassette vector the inserted transgene (in opposite orientation) is the authentic gene sequence carrying intron/exon structures and native regulatory sequences (promoter/enhancer). Double gene vectors and internal promoter vectors carry 2 transgenes, driven either by viral LTR or internal promoters respectively. Double copy vectors carry the transgene and promoter sequences in the U3 region of the viral 3′LTR. The promoter conversion (ProCon) vectors use the U3 region of viral LTR for promoter insertion. In both vector types these sequences are duplicated after reverse transcription. The self-activating (SIN) vectors carry a deleted 3′ U3 region in the LTR inactivating the viral promoter. Insertion of the IRES (internal ribosome entry sites) sequence between the transgenes in the IRES vectors allows efficient transcription from one transcript. **LTR** = long terminal repeats.

packaging cell lines which produce viral particles with altered envelope glycoproteins to redirect retroviral tropism.

To approach the targeting of viral infection, hybrid envelopes were generated by genetic modification of the retroviral env glycoprotein. These genetically

engineered env proteins carry the sequences for polypeptide erythropoietin, CD4 sequences, single-chain antibody genes or sequences coding for short peptides binding to integrins etc. to redirect the cell tropism of ecotropic retrovirus particles[28-33] to cells that express the receptors for these ligands. Although it was demonstrated that such *env*-chimera can permit viral infection, it has been shown in this context that coexpression of the wild-type *env* is essential for efficient infection. However, the coexpression of the wild-type env protein is associated with decrease in virus titre.

The pseudotyping of retroviral core with G protein of vesicular stomatitis virus (VSV-G) was also performed to increase infection efficiency and to stabilise the viral envelope. The pseudotyping by VSV-G improved infection properties of amphotropic MuLV-derived vectors making infection of primary hepatocytes, fibroblasts, peripheral blood leucocytes or human $CD34^+$ and $CD38^+$ cells possible.[34-36] Apart from these pseudotyping strategies another *env* engineering strategy is linking specific single chain antibody-derived (scFv) binding domains to the N terminus of the ecotropic *env* sequences. This alternative has the advantage of preserving the ability of the viral env protein to fold and trimerise properly. In such studies, sequences of scFv were used which have been directed against major histocompatibility complex (MHC) class I molecules, low density lipoprotein (LDL) receptors, epidermal growth factor, the B7-antigen, the receptor binding domain of the urokinase type plasminogen activator antigens of colonic cancer cells, or the glycosphingolipid Lewis A.[37-41] Despite the fact that such chimera permit specific binding of viral particles to the desired target cells, infection efficiencies have been poor and limit their feasibility for clinical use. The additional expression of the wild-type *env* could help to overcome such obstacles, since these were shown to stimulate the incorporation of the *env*-chimera into the virions.[42]

In earlier studies, linkage of viral surface proteins with bispecific antibodies was attempted to permit targeting of viruses to specific cell receptors. Antibodies were used recognising the MuLV gp70 (part of the env protein) or gag protein, and human MHC class I and MHC class II molecules, which were shown to bind the virus to the target cells via these MHC molecules.[43,44] However, infection rates were unsatisfactory in these approaches and it turned out that antibody-mediated binding of the retrovirus particle alone is not sufficient for successful infection, since not all cell surface molecules on target cells are suited for mediating infection.[45] Furthermore, antibody molecules seem to hinder viral penetration and in addition, loss of antibody-mediated vector specificity can occur *in vivo* because of low stability of these complexes.

All these efforts in altering the target specificity by manipulating the viral env protein have been of limited success, since such manipulations induce reduced fusogenicity of the env protein, and provoke rapid sequestration and destruction in endosomes, which leads to reduced infectivity.[46] To circumvent these effects, protease sequences or spacer peptides are introduced to separate the env protein from the ligand protein that is introduced for retargeting.[47,48] In this system targeting of the virus can be permitted by the ligand, whereas fusion and infection is mediated by the hidden env protein. The env is unmasked by proteases which cleave the spacer peptides, defined as protease targeting. These proteolytic enzymes are present in the target cell microenvironment. The specific design of such linker sequences creates additional vector targeting, which is mediated by the activity of particular proteases in certain tissue environments, such as in tumour tissues. Such vectors, which are activated by the protease plasmin or matrix (associated with tumour metastasis and invasion), have been tested for the protease targeting, demonstrating the feasibility of this approach.[47,49,50]

Stability of retroviral particles and viral titres are further hurdles for applicable retroviral *in vivo* gene therapy. Low viral titres, viral instability and complement-mediated lysis can be limiting for efficient *in vivo* gene therapy using retrovirus vectors, since it is known that for efficient gene transfer into sufficiently high numbers of cells in the targeted tissue *in vivo* titres of $>10^7$ cfu/ml are re-

quired. Difficulties in obtaining high virus titres during preparation due to shedding of the viral envelope by centrifugation can be overcome by pseudotyping the viral envelope. It has been shown that pseudotyped vectors carrying the aforementioned VSV-G protein can improve the yields of infectious particles during virus concentration by ultracentrifugation of the retroviral supernatants to up to 10^9 cfu/ml.[51,52] The establishment of transient expression systems is also employed to increase viral titres compared with the titres obtained by conventional packaging cell systems.[53-55] Loss in viral infection efficiency can occur as a result of complement-mediated lysis of the viral particles. It has been found that this is dependent upon viral *env* determinants and upon murine packaging cell lines used, producing virions which are susceptible to human complement.[56] This problem might be overcome by the establishment of human producer cell lines that generate complement-resistant virions.[57]

Numerous technical problems still remain to be solved for the efficient clinical application of retroviral vectors for gene therapy. The approaches for improved expression efficacy of the therapeutic genes, the more accurate targeting of retroviral infection and the restriction of transgene expression to the desired cell type by specifically active or regulatable promoters are promising for efficient retroviral delivery of genes in humans.

1.2 Lentiviral Vectors

The human immunodeficiency virus (HIV) is a lentivirus and is known to cause the acquired immune deficiency syndrome (AIDS). This type of retrovirus has certain properties that are of interest for the establishment of lentiviral vector systems (table II). One of these features is the ability of HIV to infect and integrate into nondividing cells, a property which is lacking in MLV-based vectors.[58,59] This ability of lentiviruses is due to their targeting the nucleus without the requirement of mitosis. Specific virus-encoded proteins are responsible for HIV integration in the absence of cellular mitosis, such as the proteins vpr and p17.[60] HIV uses different sets of viral proteins for integration into different target cell types. Based on this feature, the use of HIV-based vectors could be of great value for gene delivery to tissues of nondividing, terminally differentiated cell populations such as neuronal tissue, haematopoietic cells, myofibres etc. To use HIV in the same manner as retroviral vector systems, extensive genetic redesign of the viral genome is necessary to achieve safe gene transfer and an expanded host range beyond their 'natural' target cells, such as $CD4^+$ cells.

During the last few years HIV-based vectors have been established and tested for efficacy of gene transfer and their biological safety to exclude possible reconstitution of the pathogenic replication-competent HIV-1 in the patient. To accomplish this, transient expression systems have been established for the production of recombinant lentivirus particles, where viral packaging genes are located on two separate plasmids, whereas the lentiviral vector carries the transgenes (fig. 4). Furthermore, sequences coding for viral accessory proteins (*vif, vpr, vpu, nef*) had been deleted, to circumvent any interference of these lentiviral proteins with cell cycle control in the recipient cells.[61] The β-*galactosidase* or *luciferase* reporter gene transfer using HIV-1–derived vectors demonstrated that differentiated macrophages and also neurons can be effectively infected *in vitro* and *in vivo*.[62-64] However, in the *in vivo* studies, no direct evidence was presented that the HIV-based vector was stably integrated into the host genome. The complexity of the HIV-genome and regulation mechanisms of viral replication support the recent concerns of potential cell growth dysregulation, mutagenesis and recombination which could have a dramatic impact on the potential clinical use of these vectors. Therefore, extensive studies are needed for biosafety of lentiviral vectors before their application in human gene therapy can be envisaged.

1.3 Clinical Application

Retrovirus vectors have been used for different clinical studies since the early 90s and the number of protocols utilising retroviral vectors has grown

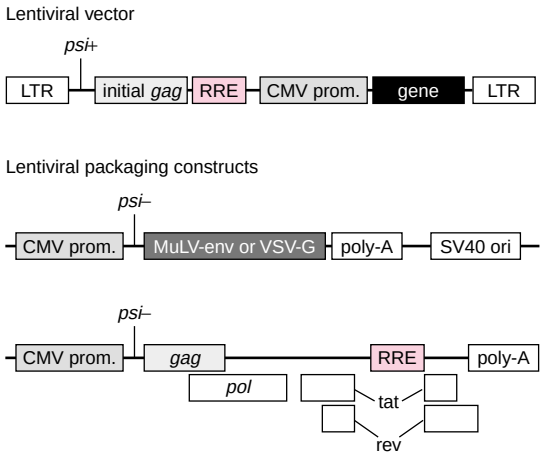

Fig. 4. HIV-based lentiviral vector system. For safety, the vector system packaging components (*gag, pol, env*) are located on separate packaging plasmids and deletions are introduced in sequences coding for the accessory proteins such as *vif, vpr, vpu, nef*. Deletions in the *env* gene are performed for replacement with the vesicular stomatitis virus G (VSV-G) or amphotropic murine leukaemia virus (MLV) envelope (env). The transfer vector harbours the initial *gag* fragment, the *psi* packaging signal and an internal promoter to drive transgene expression.

since then. Meanwhile, more than 150 clinical studies are under way worldwide using retroviral vectors for gene transfer and more than 20 studies employing virus producing cells for gene therapy. The high number of over 1500 patients enrolled in these clinical protocols reflects the high rank of retroviral vectors for human gene therapy to treat cancer or for gene correction therapies.

The first clinical investigations mainly addressed safety and technical issues of retroviral gene transfer in gene-marking studies using neomycin (*neo*) resistance gene expressing vectors. Rosenberg and co-workers[2] initiated the first gene-marking study in 1989 using *ex vivo* transduced tumour infiltrating lymphocytes (TILs) which were then reinfused into the patients.[1] These trafficking studies of transduced TILs provided important information about the fate of transduced cells in the human body and the safety of retroviral gene transfer. Similar protocols were performed by Brenner and Deisseroth, who infected bone marrow cells of patients with myelogenous leukaemia, chronic myelogneous leukaemia (CML), acute lymphocytic leukaemia (ALL) or neuroblastoma. They reintroduced these retrovirally transduced cells to study the mechanisms of relapse.[65-68]

Shortly after the initiation of gene-marking studies, protocols were started which applied cytokine genes [tumour necrosis factor *(TNF)*-α, *IL-2*] into TILs or directly into tumour cells using retroviral vectors for the *ex vivo* gene transfer of these cells. Such approaches are aiming at the improvement of antitumoural immune defence mechanisms via activation of TILs or tumour vaccination strategies using heterologous or isolated autologous tumour cells in tumours of different origin such as melanoma, renal cell cancer or neuroblastoma.[69-71] Meanwhile, many more cytokine genes are used in retroviral gene therapy cancer studies including *interferons*, *IL-12*, *IL-7* and granulocyte-macrophage colony-stimulating factor (*GM-CSF*).

Retroviral gene transfer has been further employed for the introduction of suicide genes for more efficient tumour eradication. The suicide gene concept utilises genes coding for specific enzymes which are normally not expressed in the target cell and which are capable of converting nontoxic prodrugs into toxic metabolites. Using this approach, virus producer cells have been used to generate recombinant HSV thymidine kinase gene (*HSV-tk*) carrying virus particles within the tumour to ensure efficient on-site infection of tumour cells. For this, cells were infected *ex vivo*, irradiated, and implanted for suicide gene therapy.[72] These amphotropic vector producer cells released the recombinant virus, which infected the tumour cells. These became sensitive to the prodrug ganciclovir, leading to tumour cell killing. In contrast to the promising results obtained in animal models, the therapeutic effect in clinical studies has been limited.[73] This result may be due to low infection efficacy in the tumour, and this stimulated efforts in establishing vector systems with higher virus titres, improved viral targeting or the use of replication competent retrovirus vectors. Meanwhile, apart from the suicide strategy,

retroviral gene therapy protocols are underway aimed at the substitution of defective tumour suppressor genes to influence tumour growth in the patient. Studies have been initiated to transduce the *BRCA-1* gene into breast, ovarian or brain tumours or the functional *p53* gene into nonsmall cell lung cancer.

Although retroviral gene therapy protocols are mainly performed for cancer treatment, numerous inherited diseases are being targeted with retroviral vectors, since stable integration of retrovirus vectors provides the potential for long lasting gene expression. In this context, retroviral vectors have been utilised for gene therapy of adenosine deaminase (ADA) deficiency. This disease affects the viability of T cell function causing severe combined immunodeficiency (SCID). The function of T cells was partially restored by retroviral *ex vivo* gene transfer of the *ADA* gene and reinfusion of these cells into the patient.[74] This protocol has shown therapeutic effects so far as the T cells of these patients gained normal immune function, although additional application of ADA was still necessary. More recently, cord blood cells from infant patients have been used for retroviral *ADA* gene transfer. It has been shown that ADA-positive T cells in these patients are detectable 2 years after transplantation.[75] However, at present significant gene transfer-mediated improvement in the immune function of these patients has not be seen. The still existing low efficiency of the retroviral gene transfer of stem cells is limiting the success of this therapy.

Retroviral gene transfer was employed for the correction of Gaucher's disease, a lysosomal storage disorder affecting the glucocerebrosidase enzyme, by using enriched $CD34^+$ cells of patients for gene transfer. Genetically engineered $CD34^+$ cells were reinfused into the patients for repopulation with these cells.[76] It has been shown that retroviral gene transfer of the *glucocerebrosidase* gene resulted in an up to 6-fold increase in enzyme activity compared with progenitor cells transduced only with the *neo* gene expressing virus vector.[77,78] A similar approach was used for the introduction of functional the *alpha-L-idorunidase* gene, which is defective in patients with Hurler-syndrome.[79] The retroviral gene transfer was performed in $CD34^+$ enriched cells to evaluate the therapeutic potential for clinical application. Retroviral gene transfer has also been utilised for the treatment of Fanconi anaemia (FA), an autosomal recessive genetic disorder. Autologous haematopoietic progenitor cells were transduced *ex vivo* with the FA group A (*FAA*) and group C (*FAC*) cDNA bearing retrovirus vectors to restore normal function.[80] Engraftment of patients with these CD34-enriched progenitor cells led to the transient presence of the *FAC* gene expression in bone marrow and peripheral blood of these patients, and was associated with improvement in cellularity in the bone marrow and an increase in numbers of haematopoietic progenitor cells.[81] However, stem cell correction could not be achieved in this study, something that will require a more persistent gene expression.

As an alternative to the gene transfer of certain target cell populations such as $CD34^+$ cells, other approaches use *ex vivo* infection of murine or human fibroblasts, which then serve as a source for continuous production of the desired gene product after implantation into the patient. Such cells are admixed with collagen matrix and secrete high levels of the protein as shown for β-glucoronidase and glucocerebrosidase.[82] In a phase I clinical trial in patients with haemophilia B, this strategy was also followed to secrete the human factor IX by implanting retrovirally transduced autologous skin fibroblasts embedded in collagen.[83] This gene therapy lead to a 6 month duration of factor IX expression in treated patients.

The treatment of cardiovascular diseases is also a focus of gene therapy, aimed at the correction of defective, nonfunctional genes. A clinical gene therapy protocol has been developed for familial hyperlipidaemia (FH), which causes atherosclerosis. In this study a retroviral vector was used for the *ex vivo* gene transfer of DNA coding for the normal human LDL receptor (LDL-R) to treat patients who are homozygous for FH.[84] The study showed that engraftment with autologous *LDL-R* gene transduced hepatocytes was well tolerated and could lower

LDL cholesterol levels in patients with FH. However, the level of this decrease in LDL cholesterol was not sufficient to diminish the risk for cardiovascular disease in these patients. Gene expression was observed for up to 4 months in liver biopsies.

The growing number of clinical protocols using retroviral vectors in human gene therapy points to the potential of this transfer system for stable and relatively long lasting expression of therapeutic genes as well as for short term expression studies. However, numerous problems still need to be solved to increase viral titres for efficient and reproducible *in vivo* infection associated with improved targeting of viral infection and transgene expression exclusively in those cell populations the gene therapy is aimed at. Therefore, future studies will focus on the vector design for specific viral targeting combined with regulated and stable high level expression of the desired genes by circumventing transcriptional shut-off or inappropriate gene expression.

2. DNA Virus Vectors

Unlike retroviruses, DNA viruses contain single- or double-stranded DNA as the viral genome. The most prominent DNA viruses, in terms of their use as gene transfer vehicles, are the adenovirus, AAV and HSV. These viruses are extensively used for construction of vector systems because of their great packaging capacities, broad range of target cell infection, and efficient viral infection and gene transfer (table II). Meanwhile, other DNA viruses have become of interest, such as Epstein-Barr virus, vaccinia virus or parvoviruses, and the establishment of useful viral vectors and producer cell lines for the generation of recombinant viruses are under intense investigation.

2.1 Adenoviral Vectors

Adenoviruses are non-enveloped DNA viruses carrying linear double-stranded DNA of about 35kb and were discovered in 1953.[85] 49 serotypes are distinguishable which can be further classified into A to F subgroups based on their haemagglutination properties. The most intensively characterised adenovirus types are type 2 (Ad2) and type 5 (Ad5) which are members of the C subgroup. These adenovirus serotypes were used for the engineering of the first adenoviral vectors, since it has been found that these types are not associated with severe disease in humans and are therefore suitable for *in vivo* applications.[86,87] Modifications of the adenoviral genome are based on deletion of the early gene 1 (*E1A*) to create replication incompetent vectors providing sufficient space for gene insertions, since the *E1A* gene is essential for virus replication. In these first generation adenoviral vectors, in addition to deletion of the *E1A* gene, partial deletions of *E1B* and *E3* genes are made to create more space for gene insertion. The DNA of transgenes is introduced into the *E1*, *E3* regions of the adenoviral genome. The packaging capacity of these adenoviral vectors is 7 to 8kb, which is probably still not the maximum insertion capacity (table II). Helper cells, which provide the essential *E1A* protein in *trans* for efficient viral encapsidation, are needed for the generation of infectious viral particles (fig. 5). The widely used human kidney cell line 293 performs this feature for the production of recombinant virus stocks as the *E1A* gene has been stably inserted into this cell line.[88] The 293 cells are particularly suited as a packaging cell line as they are easy to transfect and produce high virus titres. Viral titres of 10^{10} cfu/ml can be achieved by using these systems, which is a great advantage over retroviral vectors, since such titres ensure efficient *in vivo* gene transfer. Furthermore, adenovirus vectors have a broad cell tropism and can infect nondividing cells. The disadvantage of adenoviral vectors, however, is their episomal status in the host cell allowing only transient expression of the therapeutic gene. Furthermore, expression of the E2 viral protein provokes inflammatory reactions and toxicities that limit the repeated application of adenoviral vectors and thus hinder the application of adenoviral vectors for gene therapies to correct hereditary diseases.[89] It has been shown that the remaining low level of viral replication of first generation vectors induces $CD4^+$ and $CD8^+$ dependent immune responses which leads to a reduced duration of gene expression *in vivo*.[90,91] To prevent

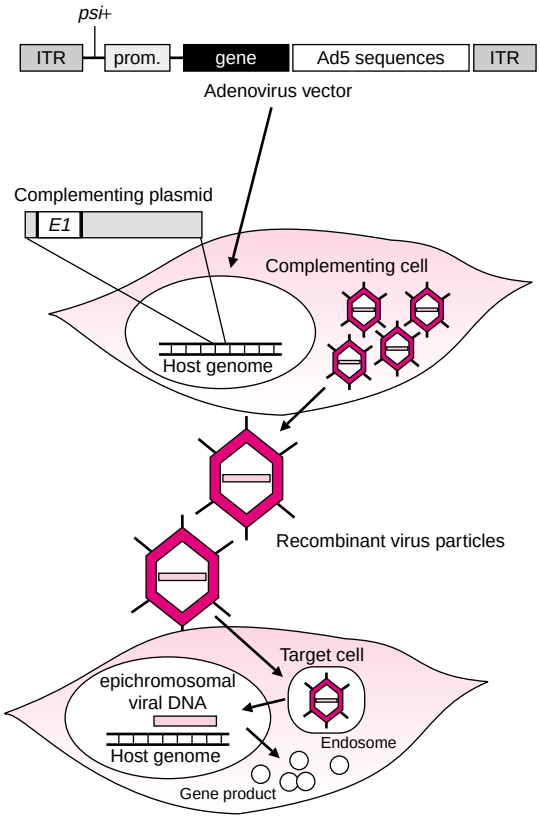

Fig. 5. Structure of adenoviral vectors and principle of adenovirus production. Adenovirus vectors are based on serotypes 2 and 5. Therapeutic genes are placed into the deleted *E1* region of the viral genome, driven by internal promoters. The function of *E1* for production of viral particles is provided by the complementing cell line expressing *E1*. This cell line produces viral stocks at high titres for infection of desired cells and tissues. After infection of target cells, viral particles enter the cytoplasmic endosome and deliver the viral DNA harbouring the therapeutic gene. Gene expression is performed from the epichromosomal viral DNA.

immunogenic reactions after adenoviral gene transfer, newer second generation vectors were established which lack *E2A* gene functions and that are modified by mutation or deletion of the viral *E4* gene.[92,93] These vectors require helper cell lines which can provide the *E4* function[94] required for transcription regulation, transition from early to late viral gene expression, mRNA transport, viral DNA replication, shut-off of host gene expression and assembly of the virion.

As an alternative to engineering the viral genome for reduced immunogenicity, effects of immune suppression on adenoviral application and infectivity have been tested.[95] Nondepleting anti-CD4 antibodies or immunosuppressive compounds etc. have been administered *in vivo* in order to achieve immunological suppression of the recipients receiving adenovirus vectors with promising results so far.[96,97]

More recently defective adenovirus vectors were created in which all viral coding regions were removed leaving only the inverted terminal repeats (ITR), the transgene and *psi* packaging sequences in these 'gutted' or 'gutless' vectors. Such adenoviral vectors were successfully employed for the expression of the full-length *dystrophin* gene and the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene.[98,99] The advantage of such vectors is that they are less immunogenic and produce high virus titres in the 293 cell line. However, separation of 'gutted' vectors from helper virus is difficult.

As well as the aforementioned replication-incompetent adenoviral vector systems, replication-competent adenoviruses are also used for gene therapy approaches, particularly for cancer gene therapy. These vectors are based on a *E1B* deleted wild-type adenovirus. They exploit the cytopathic effect of viral replication in targeted cells in which E1A protein directly mediates tumour cell lysis. Usually, the adenoviral E1B protein blocks p53 mediated apoptosis preventing cells from E1A-mediated and p53-dependent lysis after adenoviral infection. Mutation in the *E1B* gene would therefore create a virus which is unable to productively replicate in normal cells with functional p53. In contrast, such a virus will replicate in cells lacking functional p53. A high percentage of tumour cells possess defective p53, so that these cells are specifically killed by the adenovirus.[100] Thus, in tumour tissues the *E1B*-defective virus can grow and can spread to neighbouring tumour cells, whereas normal cells are not affected. In other studies, such replication-competent adenoviruses have been combined with HSV thymidine kinase (*HSV-tk*) suicide

gene expression for more efficient tumour eradication.

Many strategies are currently being investigated to combine the advantages of adenoviral vectors with reduced immunogenicity and long lasting, high level expression of therapeutic genes. Among these efforts are the design of chimeric vectors such as adenovirus-AAV or adenovirus-retrovirus chimera to achieve viral integration into host DNA in association with efficient infectivity and high level gene expression.[101] The development of adenovirus-retrovirus chimeric vector systems combines the ability of adenoviral vectors to efficiently infect target cells *in vitro* and *in vivo*, and the capability of retroviruses to stably integrate into the host genome which is associated with long term expression.[102] In the chimeric vector system, the adenoviral constructs carry retroviral packaging functions (*gag*, *pol*, *env* and *psi*) and retroviral vector (LTRs) and transgene sequences inserted into the *E1* region of the adenovirus. Target cells can be efficiently infected with these chimera which then produce *in situ* progeny retroviral vectors to infect neighbouring cells. This process leads to the integration of the transgene for long term expression. However, a limitation of this system is that only dividing neighbouring cells can be efficiently infected by the progeny retrovirus. Current developments in such chimeric vectors are aimed at the elimination of problems that arise from possible interference of adenoviral and retroviral gene regulation mechanisms, or the potential immunogenicity of the adenoviral vector. Apart from this, chimeric vectors are promising delivery systems for gene therapy. As an alternative to the generation of such chimera, chimeric Ad5-derived adenoviruses carrying the fibre genes of Ad7 viruses have been shown to possess altered tropism and binding affinity of the viral particles.[103]

2.1.1 Clinical Application

Since adenoviral vectors are very efficient gene transfer vehicles mediating transient but high level expression of the therapeutic gene, these vectors are frequently used in clinical gene therapy studies. There are more than 60 protocols underway for gene therapy of hereditary diseases and cancer. The first clinical protocol using adenovirus vectors was aimed at the life-threatening cystic fibrosis disorder in which the *CFTR* gene is affected leading to a defect in the chloride transport of epithelial tissues. Adenoviruses are known to possess strong tropism to epithelial tissues and therefore such vectors are the transfer system of choice for gene therapy of this disease. For the treatment of cystic fibrosis the *CFTR* gene was transferred into nasal epithelium using Ad2 or Ad5 vectors which led to the transient reconstitution of the chloride transport at the site of vector application in the patients.[104] These clinical trials indicate that topical application of adenoviral vector to the nasal epithelium resulted only in gene transfer of low efficiency and few correction effects at a molecular and a functional level.[105] In another clinical study, nasal and bronchial epithelium was targeted by adenoviral vector aerosol leading to a transient (maximum of 21 days in nasal epithelium) expression of the *CFTR* gene in these tissues.[106] Meanwhile, more than 10 clinical studies have followed in the US and Europe using adenoviral gene transfer for *CFTR* gene therapy. However, it remains to be evaluated whether adenoviral vectors are suited for the long term correction of this disease. All these studies revealed that only transient gene expression and little transfer efficiency can be achieved using adenovirus vectors for *CFTR* gene transfer, whereas adenovirus application was well tolerated in the patients. The modest transfer efficiency and gene expression may be due to the mucosal barrier of targeted epithelia, cellular turnover of airway epithelium, immunoresponse against infected cells and possible promoter shut-off.

In 1996, another gene correction clinical trial commenced in individuals with ornithine tanscarbamylase (OTC) deficiency which affects ureagenesis and results in hyperammonaemia associated with neurological symptoms. Batshaw and colleagues[107] used adenoviral vectors for transduction of the *OTC* gene into the liver of diseased patients to restore normal enzyme activity in the liver tissue, since animal studies had shown that adenoviral gene transfer could restore enzyme function to a near

normal level.[108] It remains to be elucidated whether this protocol will be of clinical benefit.

Adenovirus vectors have also been used for the gene therapy of vascular diseases based on the concept of therapeutic angiogenesis to stimulate the development of arteries in coronary and peripheral ischaemia.[109] The targeted cells or tissues are ischaemic lower limb myoblasts or the ischaemic myocardium. For the stimulation of angiogenesis adenoviral vectors were constructed carrying the genes for vascular endothelial growth factor (*VEGF*) or for the fibroblast growth factor (*FGF-5*). Adenoviral gene transfer is performed *in vivo* by intracoronary application for gene therapy of ischaemic myocardium and into the lower limb for the treatment of ischaemic myoblasts. Since these studies only started in 1998, conclusions regarding therapeutic benefit can not be drawn at this stage.

As mentioned in section 2.1, adenovirus vectors have their limitations in long term gene expression because of the epichromosomal status of the vectors which causes the loss of vector DNA during cell divisions of transduced cells. However, adenoviruses are of great value for cancer gene therapy since these vectors achieve temporal high level transgene expression combined with high gene transfer efficacy of the recombinant virus particles. Numerous clinical protocols are in progress using adenoviral vectors for transfer of the *HSV-tk* suicide gene for the treatment of head and neck cancer, non-small cell lung cancer, ovarian cancer, brain tumours and prostate cancer. Furthermore, adenoviral vectors are also employed for the gene transfer of immunostimulatory cytokine genes such *IL-2* or *GM-CSF* into tumours. Alternatively, adenovirus vectors are used for the transfer of the *p53* tumour suppressor gene to induce growth arrest in tumours that are defective in normal *p53* function. Similar to the suicide gene therapy approach, transfer of *p53* has the potential for the bystander effect. This effect extends the activity of the transduced gene product to non-transduced cells as well, as it has been first described for *HSV-tk* transduced tumour cells and their neighbouring nontransduced cancer cells.[110] Meanwhile, more than 10 clinical trials have been started for different tumours including malignant glioma, head and neck and nonsmall cell lung cancers.[111]

Apart from using adenovirus vectors as carriers for transgenes, the virus itself provides antitumour activity targeting tumour cells with the defective p53. In this context, replication competent vectors were generated lacking the *E1B* 55-kDa gene which binds and inactivates the normal p53 for efficient viral replication.[100] Therefore, the mutant adenovirus (ONYX-015) only replicates in p53-deficient tumour cells, leading to cytopathic effects and virus spread within the tumour.[112] In contrast, cells with functional p53 suppress replication of this adenovirus vector. Kirn and co-workers[113] have started a phase II clinical trial to test the applicability of this approach for gene therapy of squamous head and neck cancers. They have shown that treatment with ONYX-15 leads to tumour necrosis and observed clinical benefit for some patients. Adenoviral replication has been shown in tumour biopsies from patients. The treatment was associated with low toxicity and encouraged further clinical phase I and II studies in pancreatic cancer and in colon cancer metastases in the liver.

2.2 Adeno-Associated Virus Vectors

The AAV is a single stranded DNA dependovirus and belongs to the family of Parvoviruses. A broad range of cell types are susceptible to AAV infection. Although the majority of the human population is seropositive for AAV, no pathology has been observed with viral infection.[114] The AAV contains 2 genes, *rep* and *cap*, encoding for polypeptides essential for the replication and encapsidation of AAV (fig. 6). The 2 genes are flanked by viral ITRs. The AAV requires an adenovirus or a herpes virus for viral replication and needs stimulation by, for example, adenoviral E1 and E4 proteins[115] (table II). Furthermore, adenoviral E1B and E4 proteins are required for a productive AAV infection. The adenoviral E4 protein also plays a crucial role in the synthesis of the second AAV-DNA strand. This process is important for the conversion of the single-stranded AAV genome to the

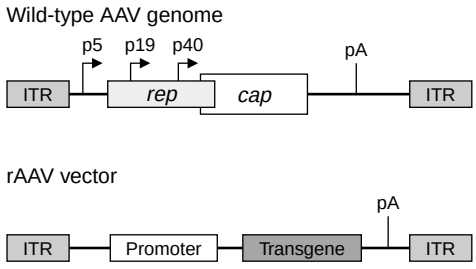

Fig. 6. Structure of adeno-associated virus (AAV) vectors. The wild-type AAV consists of the viral genes *rep* and *cap* coding for the different rep (Rep78, Rep68, Rep52, Rep42) and cap (VP1, VP2, VP3) proteins, the AAV promoters (p5, p19, p40), the polyadenylation site (pA) and the inverted terminal repeats (ITR). In rAAV vectors, the viral *rep* and *cap* genes are replaced by a transgene cassette carrying the promoter, the transgene and the pA-site.

transcription-competent double-stranded DNA molecule.[115] The difficulty of complete removal of helper viruses is still hindering application of recombinant AAV (rAAV) vectors for human gene therapy. The generation of rAAV particles requires superinfection with an adenovirus causing difficulties in obtaining high-quality viral stocks. Recently, high titre packaging cell lines have been established improving access to rAAV stocks of better quality and eliminating the appearance of replication-competent AAV.[116,117] Newer rAAV production systems circumvent the problems associated with adenovirus-dependent generation of rAAV by replacing adenovirus with adenovirus helper plasmids. The helper plasmid carries the adenoviral *E2A, E4* and *VA* genes, which are expressed by their authentic promoters. Cotransfection of rAAV vectors with the adenovirus helper plasmid in 293 cells generates rAAV particles at high titres.[118,119]

The wild-type AAV integrates into a specific region of the human chromosome 19 (between q13.3 and qter) after infection,[120] whereas the recombinant virus has lost the site-specificity of integration, possibly by deletion of the *rep* gene in rAAV vectors. In contrast to adenoviruses, AAV has low immunogenicity, which is important for the application of rAAV vectors in human gene therapy. For the construction of rAAV-based vectors, the *rep* and *cap* genes are replaced by the therapeutic genes and internal promoters regulating transgene expression (fig. 6). However, the rAAV has only limited capacity for insertion of foreign genes ranging only from 4.1 to 4.9kb.[121] It has been shown that rAAV vectors are suitable for *in vitro* and *in vivo* gene transfer into muscle, brain, haematopoietic progenitor cells, neurons, photoreceptor cells and liver cells.[122-125] This indicates that use of rAAV vectors may be advantageous for gene therapy of a variety of inherited monogenic defects as it has been shown that rAAV-mediated transfer of the β-*galactosidase* and the human *clotting factor IX* gene resulted in long lasting expression for up to two years in myofibres or mouse liver, respectively.[126,127] Preclinical studies are in progress for rAAV-based therapy of beta-thalassaemia, sickle cell anaemia, Fanconi anaemia, chronic granulomatous disease, Gaucher's disease, metachromatic leukodystrophy, Parkinson's disease and cystic fibrosis.[128] Numerous *in vivo* studies have demonstrated the efficacy of rAAV vectors in different animal models of human diseases. The rAAV–mediated simultaneous transfer of *tyrosine hydroxylase* and *L-amino acid decarboxylase* genes into striatal neurons improved behavioural deficits in a rat model of Parkinson's disease.[129] In an another gene therapy concept, the rAAV vectors proved useful for transduction of muscle and liver tissue to establish an *in vivo* reservoir of vital factors which are limited or absent in particular diseases. Using this approach, transfer of *factor IX*, β-*glucuronidase*, α*1-antitrypsin* or *erythropoetin* genes by rAAV vectors led to sustained production of these factors and their release into the serum associated with correction in the targeted diseases in the animal models.[130-134]

A phase I/II clinical trial using rAAV vector to transduce the *CFTR* gene has been started in the US for treatment of cystic fibrosis.[135] A phase I clinical trial for rAAV-mediated *CFTR* gene transfer into epithelial cells of the maxillary sinus of 10 patients indicated persistence of the transgene for up to 10 weeks.[136] In these patients, only minor or no immune responses were observed associated

with functional effects for the targeted epithelia. These data indicate that rAAV vectors are of great value for gene correction therapies requiring long term and high level gene expression in targeted tissues. In this context, great efforts are being made for the generation of efficient packaging systems to obtain high quality, high viral titres for clinical application.

2.3 Herpes Virus Vectors

The HSV-1 is a DNA virus possessing a double stranded linear genome of 150kb encoding 70 to 80 genes, which determines the large packaging capacity of this virus for insertion of foreign genes of 30 to 50kb (table II). The virus has a broad range of target cell types, and can infect dividing and nondividing cells. The infection with HSV-1 can be lytical or establish a latent infection persisting for years. The cytotoxic effects of HSV infection are mediated by viral proteins such as the infected cell proteins (ICP) ICP4, ICP22 or ICP27.[137] Currently, HSV-1 vectors are engineered which are defective in these particular *ICP* genes leading to reduced toxicity. The virus does not integrate into the host genome, which is the reason for only transient expression in infected cell populations. HSV is of interest mainly because of its large insertion capacity for foreign DNA and its natural tropism toward neuronal cells which can be exploited for either corrective gene therapies or cancer gene therapies for neurogenic tumours.[138] Furthermore, the HSV-1 particles are relatively stable and can be concentrated to high virus titres of up to 10^{12} cfu/ml which is important for effective *in vivo* gene therapy. Two major technologies are followed for the generation of HSV-based vectors, which either use insertion of the therapeutic gene directly into the virus establishing the recombinant HSV-vector or insertion of the foreign DNA into an amplicon plasmid vector. This vector harbours an HSV-origin of replication and packaging signal, and requires a superinfection with HSV helper virus for propagation. In both HSV-vector systems, disabling the recombinant or the helper HSV is of crucial importance in order to prevent cell damaging effects caused by lytic replication of the virus.

The potential suitability of HSV-1–based vectors for gene therapy was tested *in vitro* and *in vivo* by transferring the β-*galactosidase* reporter gene,[139] the β-*glucoronidase* gene as an approach for gene correction therapy of mucopolysaccharidosis VII,[140] or delivery of canine *factor IX* and of the *hepatitis B virus surface antigen* into murine hepatocytes.[141] An amplicon plasmid-based HSV vector has been employed to transfer the *tyrosine hydroxylase* gene into rat striatal neurons to treat Parkinson's disease in this animal model.[142] The abnormal behaviour of infected animals could be restored by the gene transfer, however it was associated with considerable adverse effects of vector toxicity. The studies generally demonstrated the applicability of HSV-vectors for long term expression of the transgenes particularly in neuronal cells.

Apart from the great efforts to create efficient replication incompetent HSV-1 vectors and complementing packaging systems, the majority of investigations of gene therapy of brain tumours utilise replication competent HSV-1 vectors as virus therapy, since replication of the virus in proliferating tumours is oncolytic and causes destruction of the tumour.[143] To improve these oncolytic HSV-1 vectors for gene therapy of brain tumours and to reduce neurotoxicity, attenuated vectors have been developed carrying mutations in the viral genes that are essential for replication in nondividing cells but not for replication in dividing cells and/or genes which are directly associated with neurotoxicity such as the *HSV-tk* and *ribonucleotide reductase* genes.[144] *In vivo* experiments have shown that intratumoral application of such HSV-1 vectors can prolong the survival of animals with malignant glioma.[145-147] Although clinical trials have started recently using HSV-1 vectors for gene therapy of human glioblastoma, there are still numerous safety and technical issues to be addressed such as reduction of toxicity after viral infection, possible inactivation of circulating virus vectors by host antibodies, prevention of formation of rep-

lication competent viruses by recombination and prevention of silencing of exogenous promoters.

2.4 Poxvirus Vectors

Viruses which are members of the Poxviridae family, such as vaccinia virus (VV), avipoxviruses (canarypox virus) and numerous others, are widely used for high level cytoplasmatic expression of transgenes. The advantage of these viruses is their high insertion capacity for multiple foreign genes. More than 25 kbp of foreign DNA can be stably integrated into the viral genome. The insertion of the transgene sequences is somewhat different from the techniques used in other vector systems and utilises homologous recombination or *in vitro* ligation for construction of recombinant vaccinia virus vectors (rVV).[148,149] Viral promoters are required for the expression of foreign genes, since poxviruses replicate in the cytoplasm using their own transcriptional machinery. To circumvent cytopathic effects after immediate or late expression events, such as viral DNA replication, early viral promoters or more recently modified or efficient synthetic tandem early/late promoters are employed.[150] Recombinant poxviruses are of interest for vaccination strategies to express proteins important for the generation of immune responses against infectious diseases or cancer as they are known to induce cell mediated and humoral immune responses. In this context, live recombinant vaccines have been tested in clinical trials using rVV or canarypox virus for expression of the HIV-1 envelope, and the major Epstein-Barr virus membrane glycoprotein or the rabies virus glycoprotein[151-153] for the induction of immune responses. This property of poxvirus vectors is extensively used for their application as live cancer vaccines, since poxviruses induce T cell responses. Numerous clinical trials are in progress testing the anticancer efficiency of poxviruses expressing tumour specific antigens such as prostate specific antigen, carcinoembryonic antigen or costimulatory molecules such as CD80 (B7-1) and cytokines (IL-2, IL-12) to treat prostate cancer, colorectal cancer, breast and lung cancer.[154] The rVV vectors were also used for expression of *E6* and *E7* genes of human papilloma virus types 16 and 18 in cervical cancer patients to induce tumour regression.[155] A study demonstrated that immunisation with rVV induces papilloma virus-specific memory cytotoxic T lymphocytes in patients.[156] The difficulties with poxvirus vectors are based on their complex structure and biology, and further studies are required to improve their safety and to reduce the risk for cytopathic effects.

3. Other Virus Vectors

Apart from virus vector systems which have approached testing in clinical trials, numerous other vectors are under development to exploit their specific features for gene therapy. The Epstein-Bar virus (EBV) [table II], as a herpesvirus with a double stranded DNA genome,[157] is engineered to express large DNA fragments or entire authentic genes including the native regulatory sequences of these genes for efficient gene expression in target cells.[158] Since EBV establishes itself in the host nucleus in a latent state as extrachromosomal circular plasmid, this virus is suitable for long term retention in the target cell.[159] Because of the properties of the EBV, EBV-derived vectors have been tested for corrective gene therapies[160] and also for immune therapy of cancer such as B cell lymphoma employing the natural B cell tropism of the virus.[161] Alphaviruses, which are single-stranded RNA viruses, have attractive features for utilisation as gene transfer systems. They have a broad host range and the production of high virus titres is possible. Viral vectors have been constructed for Semliki forest virus (SFV), Sindbis virus (SIN) and Venezuelan equine encephalitis virus (VEE) virus.[162-164] *In vivo* studies using alphaviruses point to their applicability as vaccine vectors to induce antigenic responses. Recombinant VEE vectors have been used for expression of the influenza *haemagglutinin* gene[165] or the HIV-1 *gag* gene,[166] and resulted in humoral and cellular immune response in the treated animals.

Among others, human cytomegalovirus, herpes virus saimiri, influenza virus, and foamy virus[167-170] have been used to engineer vectors with feasible insertion capacities, expression efficacies and cell

tropism of viral infection. Many attempts have been made to improve these systems for the future application to gene therapy of infections, cancer and inherited human diseases.

4. Discussion

The great interest in gene therapy has forced the efforts in development for gene transfer systems which are feasible for *in vivo* studies or clinical application. Because of their many advantages, viral vectors are still in focus of worldwide research programmes, which aim at the improvement and the safety testing of viral vectors for clinical application in humans. The hurdles to overcome in efficient gene therapy are successful transfer of the therapeutic genes, appropriate expression levels associated with sufficient duration of gene expression, and the specificity of gene transfer to achieve therapeutic effects in the patient. In this context, numerous improvements have been made for targeted gene transfer and regulated gene expression reflected by the demonstration of successful *ex vivo* and *in vivo* viral gene transfer in humans. The experiences in basic research and clinical phase I and II trials indicate that specific diseases and applications require their specific viral vector system, depending on what is to be accomplished: long term correction of inherited genetic diseases or timely restricted and high level expression of a specific therapeutic gene product. Apart from all the advantages and disadvantages, those viral vectors which have recently been used in clinical trials in the majority appear to be safe and well tolerated by patients. However, the majority of clinical trials using viral vectors for gene therapy in humans still lack a significant clinical success, defining the still existing barriers to achieving clinical benefits with gene therapy.

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