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VECTORS DERIVED FROM THE HUMAN IMMUNODEFICIENCY VIRUS, HIV-1

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1. ABSTRACT

The aim of gene therapy is to modify the genetic material of living cells to achieve therapeutic benefit. Gene therapy involves the insertion of a functional gene into a cell, to replace an absent or defective gene, or to fight an infectious agent or a tumor. At present, a variety of somatic tissues are being explored for the introduction of foreign genes with a view towards treatment. A prime requirement for successful gene therapy is the sustained expression of the therapeutic gene without any adverse effect on the recipient. A highly desirable vector should be generated at high titers, stably integrate into target cells (including non-dividing cells), be nonpathogenic, and have little or no associated immune reaction. Lentiviruses have the ability to infect and stably integrate their genes into the genome of dividing and non-dividing cells and, therefore, constitute ideal candidates for development of vectors for gene therapy. This review presents a description of available lentivirus vectors, including vector design, applications to disease treatment and safety considerations. In addition, general aspects of the biology of lentiviruses with relevance to vector development will be discussed.

2. INTRODUCTION

Gene therapy is a promising novel approach to the treatment of a variety of disorders including infectious,

genetic and neurological diseases and cancer (1-5). Gene therapy employs various methods to deliver foreign genes into somatic cells with the ultimate goal of incorporation and stable expression of the gene of interest. Methods used for gene therapy are categorized into non-viral 6 and viral. Non-viral methods of gene delivery include naked DNA on gold particles, cationic and cholesterol-containing liposomes, peptide-lipid vectors, activated dendrimers (branched DNA-binding carbohydrates), bacteria, artificial chromosomes, and artificial viruses (liposomes with viral components). Delivery of genes by non-viral methods is limited by the low efficiency of transfection of primary cells and the inability to propagate in most primary cells receiving the genes. Recombinant viruses carrying the desired gene can infect a greater number of cells and in some instances the virus can stably integrate the desired gene into the chromosome of the target cell without the need for selection methods (e.g., drug selection).

Before mid 1990's, scientists utilizing virus mediated gene delivery methods relied mainly on adeno-associated virus, or modified onco-retroviruses such as Moloney murine leukemia virus (MoMLV), for chromosomal gene transfer, and adenoviral vectors when integration was not needed. The usefulness of onco-retrovirus derived vectors in gene transfer of non-dividing cells was hindered by the requirement for breakdown of the nuclear envelope at mitosis prior to proviral integration (7).

Viruses of the lentivirus subfamily, such as the human immunodeficiency virus type 1 (HIV-1) have the unique ability to stably transfer genetic material into the genomes of non-dividing cells (8-10). Research in lentiviral development has focused on two major areas: (a) development of safe vectors, and (b) increasing the efficiency of transduction.

3. VIRAL VECTORS

Viruses are obligate intracellular parasites, which have evolved to invade cells, often with specificity for a particular cell type. Viruses efficiently transfer their genetic material into the host cell and use the host cellular machinery to produce new viral particles. Requirements for viral vectors include the presence of appropriate promoter elements, a genome size that allows packaging of foreign genetic material, and absence of viral virulence determinants. To decrease pathogenesis while producing a recombinant viral vector, the structural genes of the virus are provided *in trans*, either through integration into the genome of a packaging cell line or on a plasmid cotransfected with the transfer vector into a packaging cell line. Though a large number of viruses have been developed into vectors, interest has focused on four types: retroviruses (including lentiviruses), adenoviruses, adeno-associated viruses and herpes viruses (11, 12). Each specific vector group has its own advantages and limitations for use in human gene therapy (Table 1). For instance, the use of adenoviruses is hampered by their induction of immune responses in the host and their inability to stably integrate into the host genome. The lack of the stable integration has also been a problem when using herpes simplex virus-based vectors. Adeno-associated viruses, although capable of integration into genomic DNA, depend on "helper" viruses (e.g. adenovirus or herpes simplex virus) for productive infection (13). The major drawback of the commonly used onco-retroviral vectors is their inability to transduce non-dividing cells (14).

The family *Retroviridae* comprises a group of viruses that infect primarily vertebrates, although infection of other animals has been described in a few instances (15). A unifying feature of these viruses is that replication involves the process of conversion of the viral RNA genome into double-stranded DNA, hence the designation "retro" (backward) viruses. Members of the Retroviridae family are classified into seven genera: (i) *Avian*-leukemia virus related, (ii) BLV-HTLV, (iii) B-type, (iv) D-type, (v) MLV-related, (vi) spumaviruses or foamy viruses, whose members establish persistent infections in the absence of clinical disease and cause a characteristic cytopathic effect *in vitro*; and (vii) lentivirus, a group of viruses that are associated with slow, progressive diseases affecting the immune and central nervous systems (16).

Lentiviruses include a variety of primate (e.g. human immunodeficiency viruses, HIV-1 and 2, and simian immunodeficiency viruses, SIV) and non-primate viruses (e.g. maedi-visna virus [MVV], feline immunodeficiency virus [FIV], equine infectious anemia virus [EIAV], caprine arthritis encephalitis virus [CAEV] and bovine immunodeficiency [BIV]) viruses). The ability to integrate into the genome of non-dividing cells (10, 17, 18) makes lentiviruses particularly attractive in human gene therapy. Examples of non-dividing cells, which are potential targets for gene therapy, include hepatocytes, neurons, hematopoietic stem cells, myocytes, macrophages and dendritic cells. Lentiviruses that are currently used as the basis for development of gene therapy vectors are HIV-1 and 2, SIV, FIV, EIAV, BIV and CAEV.

4. THE LENTIVIRUS GENOME

The biology of lentiviral infection has been extensively reviewed elsewhere (11, 19, 20). This review will provide an overview of the organization of lentiviral genome and life cycle with emphasis on those aspects that are relevant to gene therapy, using HIV-1 as an example.

Viral particles of all retroviruses contain two homologous or quasi-identical single-stranded, positive sense RNA molecules of approximately 9.5 kb in length. In addition to the structural genes (*gag*, *pol*, and *env*) common to all retroviruses, lentiviruses also contain a plethora of regulatory and accessory genes involved in modulation of viral gene expression, transport of viral nucleic acid into the nucleus, assembly of viral particles and structural and functional alterations of the infected cell (Figure 1 and Table 1). Thus, the genome of lentiviruses has been characterized as “complex”.

Replication, integration, and packaging of lentiviruses are mediated, in part, by *cis*-acting RNA or

Table 1. Description of principal genetic features of HIV-1-based lentiviral vectors

Molecular determinant	Function	Role in lentiviral vector	References
I. *Cis-acting*			
LTRs	Contain sequences required for viral gene expression; reverse transcription; and integration	Essential	56, 76, 79
PBS	Required for the initiation of minus-strand synthesis	Essential	
Ψ	Required for encapsidation of the genomic transfer RNA	Essential	76, 79
RRE	Interacts with Rev. Required for processing and transport of viral RNAs	Beneficial	30-33
PPT	Required for priming of the plus-strand synthesis	Essential	11
cPPT	Required for efficient reverse transcription and nuclear transport of viral DNA	Beneficial	69, 70
att sites	Required for viral DNA integration	Essential	11
II. *Trans-acting*			
gag / pol	Encodes structural proteins and enzymes required for viral replication	Essential	
env	Determines binding and entry into host cell	Essential	47
tat	Necessary for high level expression from the viral LTR	Dispensable	29
rev	Necessary for expression of unspliced and singly-spliced mRNAs in vivo	Beneficial	30-34

DNA sequences,which do not encode proteins (Table 1). Most of such *cis*-acting elements are essential in the design of lentiviral vectors and are usually included in the transfer construct (e.g., the part of a retroviral or lentiviral vector which will integrate in the host cell genome and which encodes the gene of interest).

The *trans*-acting viral elements encode three groups of proteins: structural, regulatory, and accessory. These genes are provided *in trans* by a packaging plasmid. Elimination of the *trans*-acting genes within the transfer vector renders it replication-defective. Lentiviral vectors are defective for replication and are, therefore, unable to produce progeny. Only the early steps (attachment, entry, reverse transcription, nuclear transport, and integration) of the viral life cycle must be kept in a lentiviral vector. Since these early steps do not depend on viral protein synthesis, most *trans*-acting genes can be excluded within the transfer vector so that only the gene(s) of interest is expressed.

HIV-1 encodes three structural genes: *gag*, *pol* and *env*. The product of *gag* is translated from unspliced mRNA as a precursor protein and cleaved into the following protein subunits: matrix (MA), essential for virion assembly and infection of non-dividing cells (21, 22); capsid (CA), which forms the hydrophobic core of the virion and is essential for virion assembly and maturation; nucleocapsid (NC), which coats viral RNA stoichiometrically and remains tightly associated with viral RNA. MA also associates with the proviral DNA during transport to the nucleus (23). P6 a polyproline-rich protein fragment of the C-terminal portion of *gag* is involved in augmenting viral particle release from the cell (24, 25) and incorporation of the viral accessory protein Vpr into the viral particle (26). Other viral proteins of unknown functions such as p2 and p1 are also produced.

The *pol* gene encodes three enzymes required for viral replication: protease (PR), reverse transcriptase (RT) and integrase (IN). PR cleaves Gag and Gag-Pol polyproteins, which are essential for the maturation of the virion. RT contains three enzymatic activities: RNA-dependent DNA polymerase, RNAse H and DNA-dependent DNA polymerase. IN is encoded at the 3' end of *pol* and is essential for integration of proviral DNA into the chromosome of the host cell. IN contains a Zn2+ finger motif, which may be required for DNA binding (27).

The *env* gene is essential for viral binding and entry into the host cells. It encodes the precursor glycoprotein, gp160, which is cleaved by cellular proteases into a surface moiety, gp120 (SU), and a transmembrane moiety, gp41 (TM). The surface glycoprotein is required for binding to cellular receptors (e.g., CD4 and a chemokine receptor), whereas the transmembrane glycoprotein is responsible for the fusion with the cellular membrane.

The regulatory genes, *tat* and *rev*, encode transactivator proteins essential for viral replication. The *tat* gene is present in all lentiviruses. The *rev* gene is present in all lentiviruses. Doubly-spliced viral mRNAs encoding Tat, Rev, and Nef proteins are the first ones to be synthesized *de novo* following viral integration. Once synthesized, Tat and Rev augment production of viral mRNAs.

The viral protein Tat upregulates viral transcription at the level of elongation *via* interaction with the Tat activation region (TAR) located at the 5' end of all viral mRNAs. In addition to interacting with TAR, the binding to the cyclin-dependent kinase (Cdk)-Cyclin T complex has also been shown to be required for the activity of Tat (28, 29).

Rev contains a nuclear export signal (NES) and allows nuclear export of unspliced and singly-spliced mRNAs that encode viral structural proteins. In the absence of Rev the only mRNAs detected in the infected cells are doubly-spliced ones. Two interactions of Rev are

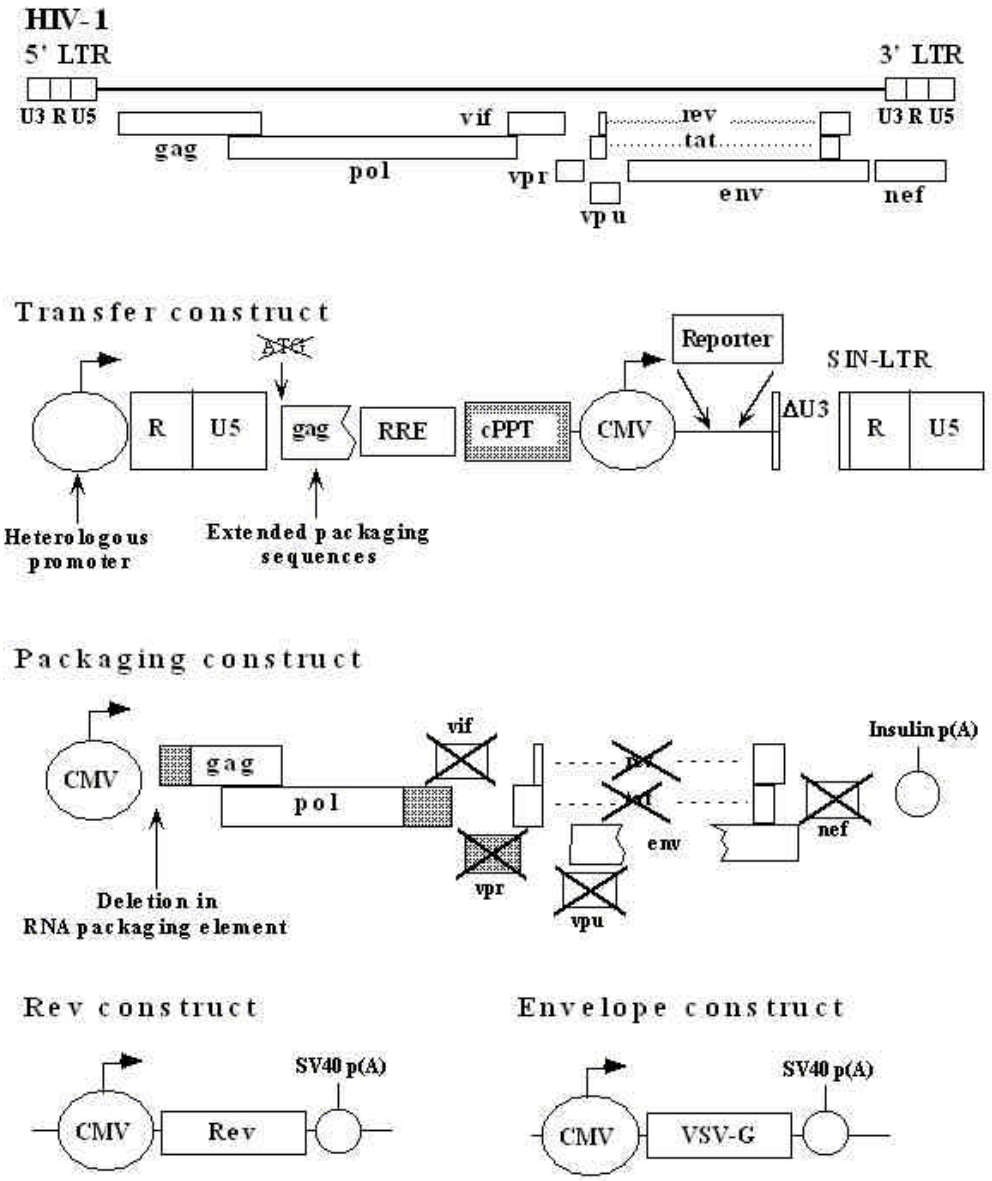

Figure 1. Schematic representation of the HIV-1 genome, and the genetic structure of a lentiviral vector. A latest generation lentiviral vector consists of four genetic elements, as shown. The transfer vector construct, is made up of SIN-LTRs in which the promoter/enhancer region from the 3' LTR (U3) have been deleted. The start site for *gag* is eliminated by frameshift mutation, and the coding sequence is truncated to only include the extended packaging sequences. The reporter gene is expressed from an internal promoter. The structural elements of the virion are provided as two sub-elements: a structural protein construct and a Rev construct. In the structural protein construct, RNA packaging sequence and the accessory genes (*vif*, *vpu*, *vpr* and *nef*) are deleted. The regulatory genes, *tat* and *rev* are also deleted. Shaded areas in the structural protein and transfer constructs denote determinants of infection of non-dividing cells. The envelope construct consists of a CMV driven expression cassette expressing VSV-G protein.

required for attaining this function. First, Rev interacts with the Rev-responsive element (RRE) overlapping the *env* coding sequence (30-34). Second, Rev interacts RAN-GTPase nuclear transport mechanisms to facilitate export of RRE-containing viral mRNA (35) .

The HIV-1 accessory genes are *vif*, *vpr*, *vpu* and *nef*. These genes were named accessory because they are nonessential for virus replication in cell culture (36). Each one of the accessory genes was shown to play multiple functions that usually involve the virus-infected cell relationship. The biology of the HIV-1 accessory genes has been extensively reviewed elsewhere 20, 37, 38. Although all accessory proteins appear to be highly conserved and play important or essential roles in pathogenesis *in vivo*, they are dispensable for the production of lentiviral vectors.

In addition, the effect of deletion of certain accessory genes showed moderate to unnoticeable effects in most cell types (See section 7.3 for details).

5. LIFE CYCLE OF LENTIVIRUSES

The lentiviral life cycle is similar for all the members of the *Retroviridae* family and consists of the steps outlined below.

5.1. Attachment and entry

The interaction of the lentivirus with the target cell occurs via binding of the viral envelope glycoprotein to a specific receptor on the cell membrane, which defines the cellular target for the virus (viral tropism). Primate lentiviruses infect helper T-lymphocytes, macrophages, microglial cell and Langerhans cells by interacting with CD4 and one of several chemokine receptors, most frequently CCR5 or CXCR4 (39-42).

Once bound to the cellular receptor, the viral membrane undergoes fusion with the cellular membrane (43). After virion-bound matrix and capsid proteins disassemble and the nucleoprotein complex is delivered into the cells, reverse transcription begins in the cytoplasm.

Because of the limited range of cells HIV-1 is able to infect, it is a disadvantage to use the HIV-1 envelope glycoprotein to produce lentivirus vectors. One property common to most retroviruses is the ability to form pseudotypes, retroviral particles which incorporate a heterologous envelope glycoprotein (44-46). The envelope glycoprotein G of the vesicular stomatitis virus (VSV-G), a rhabdovirus, is commonly used to create such pseudotypes as it provides two major advantages to the development of the gene delivery vectors (47-49). First, entry of VSV into the target cell occurs via binding to ubiquitous phospholipid components of the cell membrane: phosphatidylinositol, phosphatidylserine and GM3 ganglioside (50). This mode of entry provides VSV (and most retroviruses pseudotyped with VSV-G) a broad host range, which includes non-mammalian cells derived from fish, amphibians and insects (47). Second, unlike the HIV-1 envelope glycoprotein, VSV-G envelope forms highly stable viral particles. Owing to this increased stability, concentration of the vectors can be achieved by ultracentrifugation (47, 49). Concentration of vectors leads to increased titers by at least two orders of magnitude. Recent investigations suggest that VSV-G is inactivated by complement and thus inactivation of complement components from serum or avoidance of serum when reconstituting lentivirus vectors may be necessary following concentration (51).

Infection by HIV-1 (VSV) pseudotypes was markedly diminished by ammonium chloride and concanamycin A, a selective inhibitor of vacuolar H+/ATPases, demonstrating that VSV-G-mediated entry required endosomal acidification to achieve productive infection (48). HIV-1 is thus capable of performing all of the viral functions necessary for infection when entry is targeted to an endocytic route *via* a foreign envelope glycoprotein. Pseudotyping HIV-1 with VSV-G markedly suppressed the requirement for Nef for optimal infectivity (48).

5.2. Reverse transcription

Reverse transcription leads to the synthesis of double-stranded, linear DNA from a single-stranded RNA template using cellular $tRNA^{Lys3}$ as a primer. The enzyme that catalyzes this step in viral replication, reverse transcriptase, has low fidelity due to the lack of proofreading ability and, therefore, is partly responsible for the high variability of the viral genome. Whether this lack of proofreading by reverse transcriptase may have undesired mutagenic effects on the transfer gene remains to be determined.

5.3. Integration

Once synthesis of the linear viral DNA is complete, viral integrase performs specific cleavages at the 5' and 3' termini viral DNA and catalyzes integration into the host genome. Integration is essential for retroviral gene expression (52, 53) and allows the provirus to become a permanent genetic element in the host.

The accessibility of different regions of the genome for avian leukosis virus (ALV) was directly studied by Withers-Ward and collaborators (54). This study concluded that there were no preferences for integration within the genome of avian cells, although local differences in DNA structure (as evidenced by DNAse I susceptibility) may affect the integration efficiency. Integration for other retroviruses is expected to follow a similar pattern, although this remains to be directly tested (54). Carteau *et al*. have examined the relationship between chromosome structure and HIV-1 integration and found no strong biases either for or against integration near repetitive sequences such as Alu or LINE-1 elements (55). They also found that centromeric alphoid repeats are selectively absent at viral integration sites. Attempts to manipulate the site of retroviral integration have a moderate but promising degree of success (See Section 7.11 for details).

5.4. Transcription and viral protein synthesis

Early transcription from the provirus results in the production of doubly spliced mRNAs encoding Rev, Tat and Nef. Translation of mRNA leads to accumulation of Tat and Rev. Together, Tat and Rev induce a shift to "late" transcription mode whereby unspliced and singly spliced RNA species are primarily produced. Unspliced and singly spliced mRNAs encode structural genes necessary for the production of viral progeny.

5.5. Virion assembly and release

The viral RNA and structural proteins are packaged into viral particles and released by budding at the plasma membrane. After the polyproteins, Gag and Gag/Pol, are cleaved by the viral protease, mature particles become fully infectious. In currently available lentiviral vectors, *gag/pol* are not present in the transfer construct, but are provided in *trans* by a packaging construct (56, 57). Thus, once the transfer vector is integrated in the host cell,

its inability to direct production of *gag/pol* ensures that there will be no subsequent viral progeny.

6. MOLECULAR DETERMINANTS OF INFECTION OF NON-DIVIDING CELLS

Among retroviruses, the ability to integrate into the genome of non-dividing cells is unique to lentiviruses (58-64). Four molecular elements determine infection of non-dividing cells by HIV-1: MA, IN, Vpr and the central polypurine tract (cPPT) (10, 21, 22, 65, 66). The proteins, MA, IN and Vpr utilize the cellular nuclear import machinery to target the integration complex (PIC) to the nucleus, whereas the cPPT is a *cis*-acting determinant for proper import of the DNA into the nucleus.

The cellular nuclear import system consists of the cytosolic proteins, importin-α and nuclear pore proteins called nucleoporins. Importin-α carries a binding site for nuclear localization sequences (NLS). When NLS-containing proteins bind to importin-α, a conformational change occurs that permits interaction with importin-β. Importin-β then targets the complex to the nuclear pore by binding it to the nucleoporins (67).

Both MA and IN carry conventional NLS and utilize the importin-α/β pathway for nuclear transport. Vpr, on the other hand, does not contain a canonical NLS but binds importin-α at a site that is distinct from the conventional NLS-binding site (68). Moreover, the interaction of Vpr with importin-α increases the affinity of importin-α for the NLS of MA and, therefore, enhances nuclear import of the PIC into non-dividing cells.

The cPPT acts as a second origin of plus-strand DNA synthesis during reverse transcription. Priming at the cPPT leads to the formation of DNA flap by strand displacement. This DNA flap is about 99 nucleotides long and appears to participate in the formation of a triple-stranded structure (69). Inclusion of the DNA flap was shown to increase the efficiency of transduction of non-dividing cells by lentiviral vectors by one order of magnitude (66, 70, 71). It has been proposed that the presence of the DNA flap may have topological consequences on the subcellular localization of the pre-integration complex that may enable tracking along the cytoskeleton or translocation across the nuclear pores, or may act as a docking site for the cellular machinery involved in intracellular trafficking and nuclear transport (66, 70).

7. LENTIVIRAL VECTORS

7.1. Early lentiviral vectors derived from HIV-1

Early HIV-1-based vectors (72-75) were constructed for studies of HIV-1 infectivity and tropism, rather than gene therapy. These vectors were produced by co-transfection of two expression plasmids: an *env*-defective HIV-1 genome and a plasmid that expressed the envelope glycoprotein. In addition to expression plasmids encoding HIV-1 *env*, Page *et al.* and Landau *et al.* used heterologous envelope glycoproteins, including amphotropic and ecotropic MoMLV, and human T-cell leukemia virus Type I (HTLV-I) glycoproteins. The resulting virions possessed an expanded cell tropism and host range, as they were able to infect a variety of cells including CD4+ and CD4- human cells and murine cells.

Buchschacher and Panganiban (76) separated the viral genome into two constructs: a packaging construct and a transfer construct. The packaging construct expressed all the proteins, except Env. Env would be required in *trans* for production of infectious viral particles. The transfer construct contained a reporter gene and the *cis*-acting sequences required for packaging, reverse transcription, integration and expression of viral genes. Inclusion of about 700 bp located at the 5' end of *gag* into the transfer construct increased the transduction efficiency of the viral particles. These vectors had low titers of about 10^2 IU/ml. The use of low-titer vectors for gene therapy can be problematic because of the low transduction efficiency in certain cell types such as hematopoietic cells (77, 78). By using the VSV-G, vector stocks can be concentrated to titers of about 10^7 to 10^9 IU/ml (47, 49).

7.2. First generation of HIV-1-based vectors for gene therapy

Knowledge about the roles of *cis* and *trans*-acting elements of HIV-1 led to the first generation of HIV-1-based vectors intended as a gene delivery vehicles (79). To obtain a lentiviral gene therapy vector, a reporter gene or therapeutic gene is cloned into a vector sequence that is flanked by LTRs and the packaging sequence ("Ψ") of HIV-1. The LTRs are necessary for vector integration into the genome of the target cell. The "Ψ" sequence is necessary for packaging of the transfer RNA with the reporter gene. Viral proteins which make up the virus core and nucleocapsid are provided in the packaging plasmid or cell line, but are not adjacent to LTRs and "Ψ" sequences. Virus particles produced in this manner are replication-deficient and are unable to continue to produce infectious progeny after they deliver their therapeutic content.

7.3 Elements of a prototypical lentiviral vector

Lentiviral vectors are usually produced in a transient transfection system in which a cell line is transfected with three separate plasmid expression plasmids. These include the transfer vector plasmid (portions of the HIV provirus), the packaging plasmid, and a plasmid with a heterologous envelope gene (8, 49, 56, 57).

The transfer plasmid contains retroviral *cis*-acting elements and the gene(s) of interest. The packaging plasmid directs expression of viral structural proteins, except for the envelope. Proteins expressed by the packaging plasmid (Gag/Pol, predominantly) form the capsid and polymerase components, and recognize specific *cis*-acting sequences in the retroviral RNA genome and its reverse-transcribed DNA products (80). These recognition events lead to reverse transcription and integration. The envelope plasmid typically contains a heterologous envelope (e.g. VSV-G). The three expression constructs are maintained in the form of bacterial plasmids and can be

transfected into mammalian cells to produce replication-defective virus stocks.

An example of such a prototypical lentiviral vector (Figure 1) was described by Naldini and collaborators (56). In this vector system, the transfer construct contained a reporter gene under control of an internal human cytomegalovirus immediate-early promoter (HCMV-IE) and the *cis*-acting sequences of HIV-1 including LTRs, splicing signals, the packaging sequence, about 350 base pairs of the *gag* gene, and the RRE. The packaging construct contained all the viral proteins (except Env and Vpu) whose expression was driven by the HCMV promoter. All the *cis*-acting sequences required for packaging ("Ψ"), reverse transcription (PBS, PPT) and integration (*att*) were deleted from the packaging construct. In addition, the 5'LTR was substituted by the HCMV-IE promoter, and the 3' LTR was substituted by a polyadenylation signal from the insulin gene.

8. MODIFICATIONS TOWARDS IMPROVEMENT OF SAFETY AND EFFICIENCY OF LENTIVIRAL VECTORS

The most urgent issue regarding the safety of lentiviral vectors is the potential for recombination leading to the generation of replication-competent retrovirus (RCR), also referred to as "helper virus ". Generation of helper virus in preparations of replication-defective vectors has been documented in numerous instances involving oncoviruses (81-85). In later generations of vectors in which viral protein-coding regions were split in the packaging cells, the frequency of recombination leading to helper was decreased, but not eliminated (86). Helper virus has the potential for inducing pathogenesis as demonstrated by studies in which monkeys were infused with transduced bone marrow cells after ablation of endogenous marrow with gamma irradiation (81-83). In these studies, helper virus gave rise to lymphoma in monkeys. Contemporary lentiviral vectors were made virtually helper-free by segregating vector components into three plasmids. Subsequent modifications to each of these three components have been made to further reduce the possibility of RCR.

8.1. Modifications of the transfer construct

An important development in terms of safety is the creation of "self-inactivating" (SIN) transfer vectors (87-92). A 133-bp deletion in the 3' LTR U3 region results in elimination of the TATA box and the binding sites for Sp1 and NFkappaB. These are unique *cis*-acting sequences within the viral promoter region and are essential for virus replication. The deletion inactivates the LTR promoter function and eliminates the production of vector RNA subsequent to the integration in the transduced cell. The gene to be transferred by the vector is expressed from an exogenous viral or cellular promoter that is inserted into the transfer vector. Conditional SIN vectors have also been developed in which the U3 transcriptional regulatory elements have been replaced with the Tet-responsive element. This Tet responsive element allows vector production in packaging cells expressing a Tet-regulated trasactivator (93). Inactivation of the promoter activity of the LTR reduces the possibility of insertional mutagenesis as the lentiviral products are integrated into the host genome. Also, as expression of the vector RNA is eliminated, the potential for RCR production in the target cell is further minimized.

Certain therapeutic genes may need to be expressed in a highly regulated fashion. Systems to manipulate expression of foreign genes in mammalian cells will be essential in the near future. An inducible lentiviral vector that contains the tetracycline-regulated system has been developed by H. Bujard and colleagues (94-96). The novel vector expresses the GFP reporter gene and the tetracycline transactivator under the control of the tetracycline-inducible promoter and the human CMV promoter, respectively. *In vitro* transduction of human 293 cells resulted in a very low basal expression of GFP in the presence of the effector substance, doxycycline. Withdrawal of doxycyline induced a more than 500-fold increase in transgene expression. Switching transgene expression "off and on" numerous times did not change either the kinetics or the magnitude of induction. Maximal suppression of GFP mRNA transcription was achieved within 24 hours of addition of the drug. In addition, *in vivo* regulation of this promoter system was demonstrated by simply adding or withdrawing doxycycline from the drinking water of transduced rats (97).

Although the above regulatory modifications in the transfer vector will regulate gene expression, epigenetic phenomena, such as positional effects or DNA methylation, may partly account for silencing of the vector. Many gene transfer experiments, performed both *in vitro* and *in vivo*, have demonstrated that the presence of an intron can facilitate gene expression (98). Zufferey *et al.* have shown that apart from regulation at the transcriptional level, post-transcriptional regulation is also possible. Insertion of the posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE) in the 3' untranslated region of coding sequences carried by either oncoretroviral or lentiviral vectors substantially increased their levels of expression in a transgene-, promoter- and vector-independent manner. The WPRE contained at least three distinct *cis*-acting subelements required for maximal function. These elements were involved in increasing the levels of nuclear transcripts, the efficiency of 3' processing and the export of mRNA (99).

The CMV IE promoter within the transfer vector can be replaced with the promoter for the ubiquitously expressed elongation factor 1-α. This pomoter substitution resulted in a significantly more homogenous expression of marker trangene in hematopietic stem cells (100). Similarly, the phosphoglycerate kinase (a cellular, house-keeping gene) promoter could also lead to high level expression of transgene in hematopoietic cells (101).

Transgene expression is strongly affected by the flanking chromosomal DNA sequences. To ensure efficient gene expression, insertion of the locus control region (LCR) derived from the β-globin gene locus or the

CD2 gene into onco-retroviral vectors (102) and HIV-based transfer vectors have been shown to direct efficient and position-independent transgene expression (103).

8.2. Modifications of the envelope construct

Most envelope constructs for lentiviral vector systems use the VSV-G glycoprotein. VSV-G pseudotyped viral particles transduce a wide variety of cells including non-mammalian and invertebrate ones (47).

DePolo *et al.* have shown that VSV-G pseudotyped HIV and FIV vectors produced in human cells are inactivated by human serum complement, suggesting that alternative envelopes may be required for therapeutic efficacy for many clinical applications of lentiviral vectors (51).

Moloney murine leukemia virus (MLV) 4070A amphotropic Env and the rabies virus G glycoprotein not only effectively package retroviral particles but also provide structural stability during concentration by ultra-centrifugation (104).

A potential method for cell-type targeting of retroviral vectors has been the incorporation of single-chain antibody fragments into the viral glycoprotein scaffold. The antibody is expressed as a chimeric protein with the envelope construct (105, 106). In the previous studies, incorporation of the antibody fragment resulted in destabilization of the envelope glycoprotein and affected its function. Wu *et al.* have incorporated a 15-mer peptide, which binds specifically to the $\alpha(v)\ \beta(3)$ integrin, into various locations within the MuLV SU protein (107). Only 8 out of 26 insertion sites tested were tolerated, and were able to bind to $\alpha(v)\ \beta(3)$ integrin in a solid-phase binding assay. However, expanded tropism for $\alpha(v)\ \beta(3)$ integrin-expressing human cells was not demonstrated. These studies highlight the difficulty of engineering the retroviral envelope glycoproteins.

A more successful manipulation of the MuLV Env was achieved by Nguyen *et al.* (108). In this study, the human hepatocyte growth factor (HGF), which binds the c-Met receptor on canine kidney MDCK cells, was fused to the murine amphotropic transmembrane envelope glycoprotein (TM). The resulting chimeric Env was co-expressed with the wild-type Env, and viral particles produced in this fashion infected MDCK cells more efficiently than did unmodified amphotropic retrovirus. Addition of anti-HGF antibodies to the viral vector partially neutralized infectivity of this vector, confirming that increased infectivity was mediated by the HGF moiety.

A vector based on a Filovirus envelope protein (GP) -pseudotyped lentivirus vector efficiently transduces intact airway epithelium from the apical surface, as demonstrated in *in vivo* model systems (109). Although most filoviruses are believed to target endothelial cells and hepatocytes preferentially, the GP-pseudotyped VSV showed greater affinity for epithelial cells than for either of these cell types, indicating that Ebola virus GP does not necessarily have strong tropism toward endothelial cells and hepatocytes (110). Thus ebola virus envelope pseudotype lentivirus vectors can be used in place of VSV-G pseudotyped vectors.

8.3. Structural modifications of the packaging construct

8.3.1. Accessory gene deletion in packaging constructs

Pathogenesis of lentiviruses is dependent on the presence of accessory genes (20). Thus, several groups tested the effect of eliminating accessory genes from lentiviral vectors (111-115). Infectious titers and the ability to transduce non-dividing cells were then measured. These studies concluded that viral accessory genes are dispensable for efficient gene delivery into non-dividing cells, including primary cells.

8.3.2. Split genomes and RCR

Split-genome designs of the packaging construct reduce the probability of RCR, as the functional genes are spread over a number of plasmids and therefore would require multiple recombination events to form a functional unit. Dull *et al.* have shown that regulatory protein components of the plasmid construct such as Tat and Rev can either be eliminated or provided in *trans* (116). Co-transfection of the *tat*-free transfer construct, VSV-G construct, a plasmid expressing *gag-pol* and a plasmid expressing *rev*, yielded high-titer viral particles, demonstrating the efficiency of such a split-genome packaging system.

Due to the presence of homologous regions in the *gag-pol* expression cassette and the lentiviral transfer vector, the risk of recombination still remains. Several groups have constructed a synthetic HIV-1 *gag-pol* gene (SYNGP) where the nucleotide sequence has been altered in the majority of codons to retain the primary amino acid sequence but to exploit the favored codon usage of human cells. The altered sequence contains no substantial regions of homology with any naturally occurring HIV-1 *gag-pol* sequence and has reduced potential for recombination with *gag* sequences in the transfer vector genome. In addition, the expression of SYNGP is Rev-independent. Therefore, the RRE can be removed from the *gag-pol* expression cassette, thus eliminating another region with recombination potential (117, 118).

The possibility of generating RCR through genetic recombination of packaging and transfer vectors raises concerns for safety when dealing with lentivirus vectors derived from HIV-1. A novel packaging system, termed “trans-lentiviral vector”, was recently developed that splits gag/gag-pol into two parts: one that expresses gap/gag-PRO and another that expresses RT/IN as fusion partners with VPR. Fusion with VPR allows RT/IN to be encapsidated into the virions, because VPR naturally binds to the p6 domain of GAG. Since an intact gag-pol structure is required for retroviral DNA mobilization and generation of RCR, the trans-lentiviral vector significantly reduces the risk of RCR development (119).

8.4. Regulatory modifications of the packaging construct

Gasmi and co-workers (120) chose a similar approach to that of *Dull et al.* (116) to create a safe gene

therapy vector: they deleted *rev* from the packaging construct thus reducing it to *gag*, *pol* and *tat*. The requirement for Rev was bypassed by replacing RRE in the packaging construct with the constitutive transport element (CTE) of Mason-Pfizer monkey virus (MPMV). Although the CTE-containing vector allowed Rev/RRE-independent expression of Gag, its titer on 293T cells was 10-fold lower than that of an RRE-containing vector. This finding indicated that CTE could only partially substitute for the function of Rev/RRE, and that vectors could be generated most efficiently if Rev and RRE were present in the packaging construct.

Previous work has shown that spleen necrosis virus (SNV) long terminal repeats (LTRs) are associated with Rex/Rex-responsive element-independent expression of bovine leukemia virus RNA and supports the hypothesis that SNV RNA contains a *cis*-acting element that interacts with cellular Rex-like proteins (121). Butsch *et al.* have shown that the R/U5 region at the 5' RNA terminus of SNV contains a unique *cis*-acting posttranscriptional control element that interacts with hypothetical cellular Rev-like proteins and contains stimulatory sequences that function in a 5'-proximal position to enhance initiation of translation of a non-retroviral reporter gene RNA. This facilitates Rev/RRE independent transport and efficient translation of HIV-1 RNA (122, 123).

Other regulatory controls include regulation of expression of the structural proteins. This is usually accomplished by expressing the structural proteins under the control of an inducible promoter, in a packaging cell line (See Section 7.6). Packaging and envelope constructs have also been developed which employ the ecdysone inducible promoter in order to decrease the risk of cytotoxicity associated with constitutive expression of HIV protease and VSV-G envelope proteins (124).

8.5. Heterologous RNA packaging

Retroviral particles which package a heterologous genomic RNA are referred to as RNA pseudotypes (57, 125, 126). RNA pseudotype vectors have the potential advantage for decreased risk of homologous recombination between the packaging and the transfer vectors, due to sequence dissimilarity between the transfer and packaging constructs.

Since the different constructs of the conventional HIV-1-based vector were originated from the same virus, the possibility of homologous recombination exists. Corbeau and coworkers (127) created an RNA pseudotype by using an HIV-1 packaging cell line and an HIV-2 transfer vector. This HIV-1/HIV-2-based vector exhibited titers of 10^4 IU/ml and was capable of transducing non-dividing cells such as primary human macrophages. Kaye *et al.* (128) have also shown that HIV-1 based packaging systems can efficiently package HIV-2 transfer constructs. However, the HIV-2 helper virus was unable to package the HIV-1 vector RNA, indicating a non-reciprocal RNA packaging relationship between these two lentiviruses. Chimeric proviruses based on HIV-2 were constructed to identify the regions of the HIV-1 Gag protein conferring RNA-packaging specificity for the HIV-1 packaging signal. Replacement of the HIV-2 nucleocapsid by that of HIV-1 generated a virus with normal protein processing which could package the HIV-1-based vector. The chimeric viruses retained the ability to package HIV-2 genomic RNA, providing further evidence for a lack of reciprocity in RNA-packaging ability between the HIV-1 and HIV-2 nucleocapsid proteins. Inclusion of the p2 domain of HIV-1 Gag in the chimera significantly enhanced packaging (128).

In the system described by White *et al.* (57), an HIV-1-derived transfer vector is encapsidated by SlVmac virus core particles. These core particles are pseudotyped with vesicular stomatitis virus glycoprotein G-protein. Because the nucleotide homology between HIV-1 and SlVmac is low, the likelihood of recombination between vector elements should be reduced. Encapsidation of a heterologous transfer vector by SlVmac-derived viral proteins is specific because it occurs when providing a transfer vector from a closely related virus, HIV-1, but not from a murine retrovirus (57). This vector system was shown to retain key features of a lentiviral vector (infection of non-dividing cells and integration) and may constitute safe alternative to HIV-1 derived systems.

Due to the significant phylogenic distance from primate lentiviruses, FIV is becoming the lentivirus of choice for human gene transfer systems (see section 8.4). Like SIV and HIV, FIV can cross package its RNA genomes into both primates particles but not nonlentivirus particles such as Mason-Pfizer monkey virus (129).

8.6. Packaging cell lines

The lentiviral vectors described above were produced by transient transfection of multiple plasmids. Although relatively high titers of RCR-free virus can be produced by this method, transient transfection systems have certain drawbacks. First, the infectious titer of the transiently produced vectors is subject to high batch-to-batch variability. Second, the volume of vector particles produced by this method is limited. Thus, stable packaging cell lines have been constructed for large-scale production of replication-defective vectors. These cell lines provide in *trans* all viral proteins required for assembly of viral particles.

In the HIV-1 packaging cell line designed by Corbeau *et al.* (130), the major packaging site, including 37 nucleotides between the major splice donor site and the beginning of *gag* was deleted from HIV-1 genome. Although this packaging line was able to synthesize HIV-1 proteins, virions produced by these cells were noninfectious due to the inability of the genomic RNA to be packaged.

A potential problem with this type of packaging cell is that a single recombination event between the HIV-1 sequences in the packaging cells and the transfer vector could lead to the production of the RCR. To overcome this problem, a split genome approach was developed by Srinivasakumar *et al.* (131). To create the packaging cell line, CMT3-COS were stably transduced with five separate

plasmids. One of the plasmids expressed *gag-pol* and *vif* and the remaining plasmids expressed one of the other genes, including *env*, *nef*, *tat* or *rev*. The 5' sequences required for packaging were deleted from each plasmid. When transfected with a transfer vector, the packaging cells produced helper-free viral particles able to infect HeLa-CD4 cells with titers of up to 10^4 IU/ml.

Packaging lines have been developed in which viral proteins are regulated by inducible promoters. The constitutive expression of HIV-1 proteins (e.g. Vpr and protease) is highly cytotoxic. To overcome this problem, inducible packaging cell lines were developed (132-134). These packaging cell lines produce viral proteins only after the removal of tetracycline from the culture medium and yield low-titer (0.6-2 x10^3 IU/ml), RCR- free viral particles upon transfection with an HIV-1 transfer vector.

Stable packaging cell lines that are devoid of HIV-1 *tat*, *vif*, *vpr*, *vpu* and *nef* have been developed. In these cell lines HIV protease and VSV-G are under control of the ecdysone inducible system. Using these packaging cell lines, titers as high as 10^8 IU/ml could be generated (124).

Packaging cell lines have one common drawback: they produce low vector titers when compared to transiently transfected producer cells. To address this problem, Kafri and co-workers (134) developed a tetracycline-inducible HIV-1 packaging cell line that produced VSV-G pseudotyped vectors. In this system, the VSV-G cDNA and an HIV-1 packaging cassette, which included all HIV-1 genes except *env*, were cloned into tetracycline-inducible vectors and stably transfected into the SODk0 cell line. The resulting HIV-1 vectors displayed titers of greater than 10^6 IU/ml and could be concentrated by ultracentrifugation to 10^9 IU/ml. Moreover, these vectors were capable of transducing growth-arrested HeLa cells, human embryonic fibroblasts, and adult rat neurons *in vivo.*

8.7. Lentivirus vectors as a tool to combat HIV-1 replication

Conditionally replicating vectors (crHIV) capable of inhibiting HIV-1 infection were constructed by Dropulic et al. (135). These vectors contained deletions in all of the viral genes and encoded a U5-specific hammerhead ribozymes that selectively cleaved HIV-1 RNA. The U5 region of crHIV RNA was rendered ribozyme-resistant by substituting critical oligonucleotides at the cleavage site. Such vectors were only able to replicate in the presence of live HIV-1 and, when co-transfected into Jurkat cells, inhibited viral replication.

Conditionally replicating VSV-G pseudotyped HIV based lentivirus vectors expressing envelope antisense mRNA have been made which enable permissive CD4+ cells to resist infection by HIV. The effect was observed in that not only did envelope antisense mRNA inhibit production of envelope but the vector competed with the wild type infecting virus for encapsidation (136).

Several groups have recently demonstrated that HIV-1-derived vectors can inhibit HIV-1 replication in the absence of anti-HIV genes (137-140). Lentiviral vector-mediated inhibition of HIV-1 is essentially different from previously described mechanisms of retroviral interference. This type of interference does not involve CD4 downmodulation, does not involve expression of *env*, *nef* or *vpu*, and appears to be independent of viral entry (141)

Importantly,several groups demonstrated that the lentiviral vectors were mobilized by wild-type HIV to the initially untransduced cells (138-140).

The mechanism(s) of HIV-1 inhibition by lentiviral vectors are unclear. Corbeau and Wong-Staal (137) attributed it to TAR and RRE decoy effects of the vectors. An *et al.* (138) and Bukovsky *et al.* (139) demonstrated that competition for substrates necessary for reverse transcription and RNA encapsidation, are, at least in part, responsible for vector-induced HIV-1 inhibition.

Another possible reason for the inability of HIV-1 to propagate in vector-transduced cells involves formation of defective HIV/vector recombinants caused by the dimerization of HIV and vector RNAs at the dimer linkage structure (DLS) (142). The defective heterodimers could be packaged into virions and inhibit HIV replication by either competing for the packaging or, later, interfering with the strand transfers. Ding and co-workers (143) demonstrated such co-packaging and subsequent inhibition of HIV replication by Moloney MLV-based vectors that expressed sense or antisense RNA containing HIV-1 TAR and the extended HIV-1 packaging signal including the DLS and RRE.

The efficiency of the inhibition and the ability of these vectors to be trafficked to untransduced cells may prove to be extremely useful for the purpose of HIV gene therapy where one may be able to protect a large number of target cells by initially transducing a small number of cells to allow subsequent trafficking.

8.8. Multigene vectors

The ability to induce expression of multiple genes from a lentiviral vector may present important benefits. An example of a gene therapy protocol that would benefit from the availability of multigene lentiviral vectors is immune therapy against cancer. In order to provide an effective immune response against tumor antigens, transduction of various stimulatory cytokines and co-stimulatory molecules may be required. To accomplish this, Stripecke and collaborators (144) constructed lentiviral vectors in which expression of two open reading frames on the same mRNA was facilitated by inclusion of a translational *cis*-acting element, the encephalomyocarditis virus internal ribosome entry site (IRES) (145). This strategy had previously been used in the construction of onco-retroviral vectors (89, 146). The ability to insert two genes in a lentiviral vector also allows sensitive detection of transduced cells for pilot studies (147). A schematic diagram of an IRES-containing lentiviral vector is shown in Figure 2, panel A.

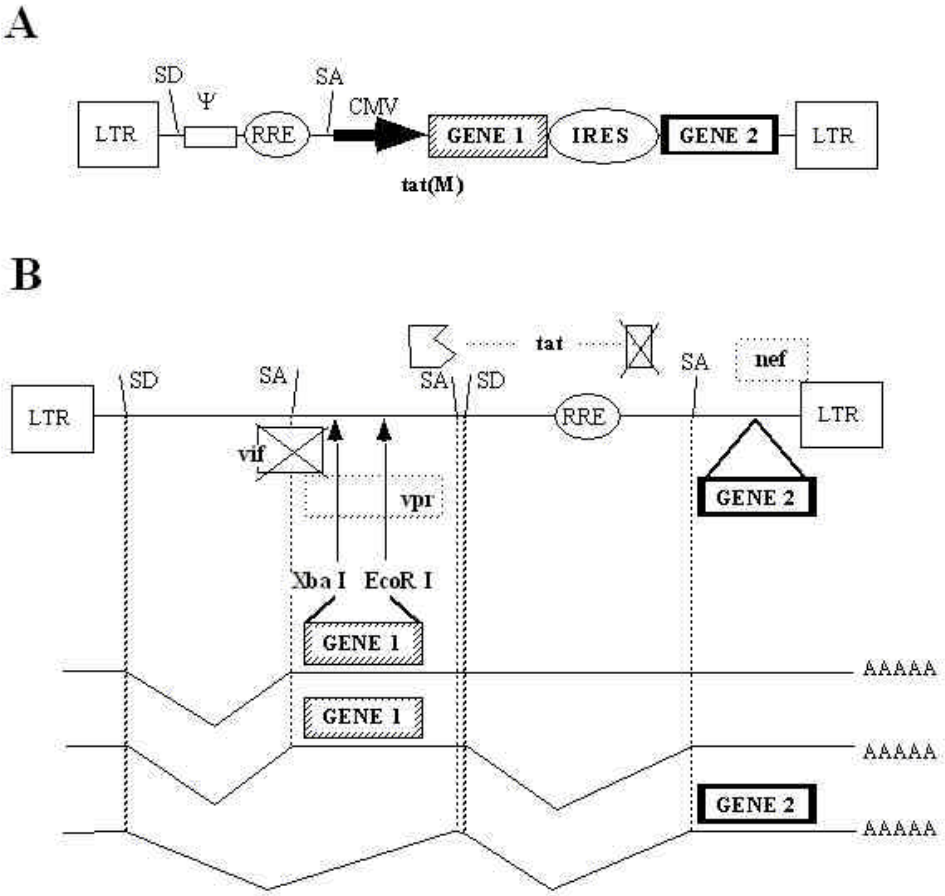

Figure 2. Design of multigene lentiviral vectors. A. Multigene vector based on the use of an internal ribosome entry site (IRES). B. Multigene vector based on natural HIV-1 splicing patterns. Discontinuous lines depict splicing events. SD, splice donor; SA, splice acceptor. Only splice donors and acceptors controlling expression of the reporter genes are shown. Mutations in the *tat* and *rev* open reading frames. The *rev* gene was inactivated by deletion of its second coding exon. The mutant, tat(M), was constructed by introducing a frameshift mutation after codon 24 of *tat*.

The genomes of HIV-1 and other lentiviruses encode nine known genes. Transcription is initiated from the "R" region of the 5' LTR. Lentiviruses utilize a combination of strategies to extract the maximal coding potential from their genomes. These strategies include overlapping reading frames, translational frameshifting, weak translational start and stop codons, and differential splicing. Vector development can, potentially, take advantage of the above strategies in order to direct multigene expression from lentiviral vectors. The ability of lentiviruses to direct differential splicing has recently been exploited in the construciton of bicistronic lentiviral vectors. Nakajima *et al.* developed a novel lentiviral vector system based on a non-pathogenic simian immunodeficiency virus from African green monkeys (SIVagm), that carries a unique dual gene expression system. Using these vectors, two reporter genes could be expressed simultaneously at equal levels, and modifying the length of the RRE could alter relative expression levels of both genes (148). When pseudotyped with VSV-G, the SIVagm-based vectors could transduce both growth-arrested human cells and terminally differentiated neuronal cell lines.

Lentiviral vectors that use differential splicing for multigene expression depend on efficient transcription from the viral LTR. This, in turn, requires the presence of the viral transactivator protein, Tat. However, expression of this viral gene product is associated with cell cytotoxicity (149, 150). In an effort to overcome this limitation, Zhu and collaborators (151) constructed a *tat* truncation mutant, denominated Tat(M), that is devoid of the sequences involved in induction of apoptosis, but maintains residual transactivation ability. Tat(M) was able to transactivate the HIV-1LTR with approximately 20 % of the efficiency of wild-type Tat (151). The Tat(M) mutant was constructed in the context of a lentiviral vector regulated by splicing, in which two reporter genes were cloned into the *vpr* and the *nef* loci, respectively. The decreased activity or Tat(M) did not appear to affect vector production when using a packaging construct, pCMVΔR8.2Δvpr (138), that expressed wild-type Tat *in trans*. At the level of gene expression in the transduced cells, only a slight reduction in average intensity levels of fluorescence for both reporter genes was detected. Therefore, the diminished ability of Tat(M) to transactivate the LTR did not have deleterious effect on vector production or gene expression.

8.9. Targeted integration

An ideal integrating viral vector would be locus-targeted. However, the factors that determine the site of retroviral DNA integration are not completely understood (See Section 4.3 for details).

A certain degree of success has been achieved in targeting retroviral DNA and HIV-1 DNA to specific DNA sequences. Goulaouic et al. have shown that HIV-1 integrase fused to the E. coli LexA protein at the C-terminus, preferentially integrated viral DNA into the fragment containing a DNA sequence specifically bound by LexA protein (152, 153). Bushman et al. have shown that by fusing a sequence-specific DNA-binding domain of the zinc finger protein, zif26, the retroviral cDNA could be targeted to a specific site on the DNA *in vitro* (154, 155). Although initial results showed that the IN-zif286 fusion protein did not support the production of infectious virus, virions produced in the presence of mixtures of wild-type IN and the fusion protein were infectious. Pre-integration complexes from these viral particles showed specific integration in vitro (154, 155).

Shibagaki *et al*. have shown that purified IN from HIV-1 and FIV displayed different patterns of integration preference (156). The central core domain of IN is responsible for such patterns. Furthermore, manipulation of the amino acid sequences of the central core domain of IN could be used to change the site of integration in the target DNA (156).

9. VECTORS BASED ON LENTIVIRUS OTHER THAN HIV-1

The use of HIV-1-based vectors in human gene therapy faces a number of obstacles due mainly to the fact that HIV-1 is a deadly pathogen. Thus, the possibility of the appearance of RCR in preparations of HIV-1-derived vectors though remote, has prompted efforts to develop vectors based on other lentiviruses.

9.1. HIV-2-based vectors

HIV-2-derived vectors have potential advantages over HIV-1-derived ones such as decreased pathogenicity and segregation of the genes responsible for infection of non-dividing cells (*vpx*) and growth arrest (*vpr*) (157). *Cis*-acting regions of HIV-2 responsible for genomic encapsidation were defined by Poeschla *et al.* (158) who found that deletion of 61 nucleotides from the region between the major 5' splice donor and the *gag* initiation codon prevented HIV-2 packaging and replication, but not expression of viral genes. The packaging plasmid was derived by deleting the 61-nucleotide fragment and replacing 3' LTR with the bovine growth hormone polyadenylation signal. Transient co-expression of the packaging construct with VSV-G and a transfer vector bearing *lacZ* yielded titers of 10^6 IU/ml before concentration and 10^8 IU/ml after concentration. These viral particles were able to efficiently transduce human T and monocytoid cell lines, growth-arrested HeLa cells, human macrophages, NTN2 neurons and human hematopoietic progenitor cells. Poeschla *et al*. used their packaging plasmid to develop an HIV-2 packaging cell line, which was capable of stable expression of HIV-2 structural proteins, but did not produce replication-competent virus (159).

9.2. SIV-based vectors

The usefulness of an HIV-1 based vector for delivery of an anti-HIV strategies would be limited by the inhibitory effect of the vectors upon itself. Vectors based on SIV would overcome such a limitation because it is unlikely that certain anti-HIV reagents would inhibit the SIV vector. Non-pathogenic SIV strains would be ideal candidates for gene therapy vectors. A number of SIV strains which are apathogenic in experimentation animals have been used for design of gene therapy vectors, such as SIVagm (148) and SIVmac1A11 (57). Since these strains are not pathogenic in animals they are perceived to be safe for use in humans. SIVmac251-based vectors have also been developed which do not generate RCR but still able to transduce cells with similar efficiency as HIV-1 based lentivirus vectors. Similar to HIV-1 based vectors, SIV-based vectors can also be self inactivating to increase safety (117) and removal of accessory gene still allows for transduction of non-replicating cells without the effects of *vif*, *vpr*, *vpx*, and *nef* (115, 117).

9.3. FIV-based vectors

The feline immunodeficiency virus, FIV, does not productively infect human or other non-feline cells. This property, together with the greater phylogenetic difference from primate lentiviruses, make FIV vectors very attractive in terms of safety. To explore the possibility of creating an FIV-based vector with extended tropism which could be used for human gene therapy, Poeschla and coworkers (160) used a three-plasmid expression system. The packaging plasmid was constructed by replacing the 5' LTR with the HCMV promoter and the 3' LTR by the bovine growth hormone polyadenylation signal. In addition, 875 nucleotides from the *env* gene were deleted. In the transfer construct, the U3 element within 5' LTR was replaced by fusing the HCMV promoter to the R repeat. A frameshift mutation was introduced into the *gag* gene and either *lacZ* or GFP reporter genes driven by the HCMV promoter were inserted. When co-transfected with VSV-G expression plasmid, viral particles were produced that exhibited titers of 10^5 IU/ml and were capable of transducing primary human macrophages and postmitotic NTN2 neurons. Most importantly, this work revealed that the 5' U3 region of FIV limits the productive phase of FIV replication in human cells and that the substitution of this region with the heterologous promoter (HCMV) allows the expression of FIV proteins in human cells.

Johnston *et al.* studied the minimal requirement for an FIV based vector. They constructed a series of packaging and transfer plasmids from which viral gene expression was minimized and unnecessary *cis*-acting sequences were deleted. Pseudotyped vector particles could transduce various cell lines including contact-inhibited human skin fibroblasts and growth-arrested HT1080 cells. Replacement of the FIV U3 promoter with the CMV IE promoter resulted in 50-fold-higher titers. Deletion of *vif*

and *orf2* accessory gene of FIV did not affect vector efficiency. However, vector production was Rev/RRE dependent (161).

Second and third generation three-plasmid vector systems, termed FELIX, were constructed from FIV. The FIV LTR was replaced with the CMV promoter, CTE was used to replace FIV Rev/RRE, and Gag-Pol was the only viral protein present. These vectors could be produced in high titers and could efficiently transduce dividing, non-dividing and primary human cells such as DCs, hepatocytes and macrophages (162).

An FIV vector was formulated with a calcium chelator by mixing the vector at a 1:1 (vol/vol) ratio with 12 mM EGTA/HEPES/saline solution. This solution was thought to disrupt the epithelial junction and enhance transduction from the apical surface of airway epithelial cells. The vectors transduced fully differentiated non-dividing human airway epithelia when applied to the apical surface. An FIV-based vector encoding the cystic fibrosis transmembrane conductance regulator cDNA corrected the transport defect in differentiated CF airway epithelia for the life of the culture (>3 months), and FIV vector expressing β-galactosidase transduced 1-14% of adult rabbit airway epithelia. Transduced cells were present in the conducting airways, bronchioles, and alveoli. Importantly, gene expression persisted, when cells with progenitor capacity were targeted (163). Thus FIV-based lentivirus vectors may be useful for the treatment of genetic lung disease.

9.4. Other potential lentivirus vectors

Other non-primate lentiviruses are under investigation for potential use as gene delivery vehicles. For example, replication-defective vectors based on caprine arthritis encephalitis virus (CAEV) were constructed by Mselli-Lakhal *et al.* (164). The CAEV-derived vectors were only tested on goat cells lines and had a low transduction efficiency (3-4 logs lower than that of parental virus, CAEV). Similar vectors based on Visna virus have now been produced (165).

Another potential candidate for use in human gene therapy is the equine infectious anemia virus (6, 166). The only EIAV proteins required for packaging activity are Gag/Pol and the only accessory protein required for vector production is Rev. The *pol* gene encodes a dUTPase activity that is found in all non-primate lentiviruses. The vectors can be pseudotyped with a range of envelopes including rabies G and MLV 4070A and can be concentrated to high titers. The ability of EIAV to infect mitotically inactive cells makes this vector an attractive alternative to the immunodeficiency viruses for gene therapy (166). EIAV vectors have been employed for transfer genes toi human Fanconi anemia hematopoietic cells and compared with HIV- and MLV- based systems. EBV-tranformed lymphoblasts from Fanconi anemia group C which administered with EIAV, HIV and MLV-based vecors transduced cells to similar levels. The cells were resitant to the effects of mitomycin- C This study indicates the usefulness of EIAV-based vectors in treating hematopoietic disorders.

Bovine immunodeficiency virus and its related Jembrana virus are also been considered as the starting points for novel gene therapy vectors (167, 168). BIV-based vectors were able to transduce most cell types including nonhuman primate hematopietioc stem cells and irradiated endothelial cells. The virion preparations transduced cells with an efficiency of up to 90% with titer of unconcentrated virus up to 5 $x10^5$ infeccious doses/ml (169).

10. LENTIVIRUS VECTORS AND GENE THERAPY

The applicability of a safe lentiviral vector in human disease is broad because (i) the host range of lentiviruses can be virtually unlimited when using vesicular stomatitis G glycoprotein to produce envelope pseudotypes; (ii) many relevant targets for gene therapy are non-dividing cells (neurons, hepatic cells, hematopoietic stem cells, myocytes); and (iii) the stability of the transgene is potentially indefinite due to chromosomal integration.

Lentiviral vectors offer potential for treatment of a wide variety of syndromes, including genetic/metabolic deficiencies. Inherited genetic defects such as adenosine deaminase deficiency, familial hypercholesterolemia, cystic fibrosis, mucopolysaccharidosis type VII, types I and II diabetes, classical phenylketonuria and Gaucher disease may be overcome by lentiviral vector-mediated gene therapy because they constitute single-gene deficiencies for which the involved genes are known.

Examples of such broad applicability can be found both *in vitro* and in animal experiments. An HIV-1-based vector was constructed that successfully delivered the cystic fibrosis (CF) transmembrane conductance regulator (CFTR) into human undifferentiated bronchial epithelium cells derived from the CF patient implanted into nude mice. Expression of CFTR resulted in functional correction of the CF defect after maturation of the airway epithelium (170).

Lentiviral vectors efficiently transduce human CD34(+) cells that mediate long-term engraftment of non-obese diabetic/severe combined immunodeficient mice. They could efficiently transduce stem cells that maintain normal hematopoiesis without impairing self-renewal and normal lineage specification *in vivo.* Efficient gene delivery into murine stem cells with lentiviral vectors will allow direct tests of genetic therapies in mouse models of hematopoietic diseases such as sickle cell anemia and thalassemia, in which corrected cells may have a selective survival advantage (171).

Other genetic diseases in which lentiviral based vectors have potential for application are retinitis pigmentosa, in which the target cells would be the retinal photoreceptor cells (172), hematopoietic diseases such as sickle cell anemia and **?** -thalasemia.

Individuals with certain types of cancer may benefit from the use of lentiviral vectors. Hypoxia and lack of vascularization lead to the generation of tumor cells

which exhibit limited or no proliferation. Partly because of the lack of growth, these cells are highly resistant to genotoxic agents. A lentiviral vector may prove to be a useful vehicle for delivery of a "lethal" gene (such as herpes virus thymidine kinase) to such quiescent tumor cells. Cell vaccines engineered to express immunomodulators have shown feasibility in eliminating leukemia in murine models. Vectors for efficient gene delivery to primary human leukemia cells are required to translate this approach to clinical trials.

Stripecke *et al.* have shown that lentiviral vectors expressing immunomodulators could be used to elicit anti-leukemic immune responses and, therefore, have potential for application in the clinic (144). Lentiviral vectors driving expression of granulocyte-macrophage-colony-stimulating factor and CD80 were used to transduce primary acute lymphocytic leukemia cells. Transduced cells were then able to stimulate T-cell proliferation in autologous mixed lymphocyte reactions.

Professional antigen-presenting cells, such as dendritic cells (DCs) and macrophages are target cells for gene therapy of infectious disease and cancer. However, transduction of DCs and macrophages has proved difficult by most currently available gene transfer methods. Several recent studies have shown that lentiviral vector systems can efficiently transduce PBMC-derived DCs and macrophages, and other cells of the hematopoietic system including stem cells (173, 174). Lentivirus vector-transduced dendritic cells can induce naïve T-cells to develop into strong antigen-specific cytotoxic T-lymphocytes (175).

The use of potent drugs currently in use for treatment of AIDS only suppresses active viral replication. There is an urgent need to develop anti-HIV therapies that can effectively kill the infectious virus, or provide resistance to infection. Many strategies have been proposed to achieve this effect. For some of these strategies, phase I studies have recently begun in humans. Preliminary studies have dealt with defective murine oncoviruses for delivery of anti-sense RNAs, ribozymes and trans-dominant proteins against HIV replication (176-181).

Lentiviral vectors have been used to introduce glial cell line-derived neurotrophic factor (lenti-GDNF) gene into degenerating nigrostriatal neurons in nonhuman primate models of Parkinson's disease. Lentivirus containing GDNF was injected into the striatum and substantia nigra of nonlesioned aged rhesus monkeys or young adult rhesus monkeys treated 1 week prior with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). Extensive GDNF expression with anterograde and retrograde transport was seen in all animals. In aged monkeys, GDNF augmented dopaminergic function. In MPTP-treated monkeys, GDNF reversed functional deficits and completely prevented nigrostriatal degeneration. Additionally, the GDNF transduced by lentivirus vectors in rhesus monkeys revealed expression for as long as 8 months. In MPTP-treated monkeys, GDNF treatment reversed motor deficits in a hand-reach task. These data indicate that GDNF delivery using a lentiviral vector system can prevent nigrostriatal degeneration and induce regeneration in primate models of Parkinson's disease and might be a viable therapeutic strategy for Parkinson's disease patients (182).

Since the inception of the Human Genome Project in 1990, considerable progress has been made in our understanding of human genetics (183). More disease-related genes are being constantly identified and characterized widening the range of applications for human gene therapy.

The beginning of the new millennium has seen several extraordinary clinical successes in the field of gene therapy. Although these successes have not involved the use of lentiviral vectors, these studies are worthy of mention here because they are likely to provide necessary impetus to the field of gene therapy. One of these studies involved a gene therapy protocol that was able to provide full correction and clinical benefit in severe combined immunodeficiency-X1 patients. This trial used a Moloney murine leukemia retrovirus-derived vector encoding the γ-c cytokine receptor subunit of interleukins 2, 4, 7, 9, and 15 receptors and *ex vivo* infection of CD34+ cells (184). Other promissing studies involved adenovirus-mediated delivery of the wild-type p53 gene as a surgical adjuvant in advanced head and neck cancers (185). Thus, although the field of gene therapy is still in its infancy stages, demonstrations of its potential application in human disease are beginning to appear more frequently.

We anticipate that the inclusion of lentiviral vectors in clinical trials will not be immediate. Major safety concerns, extensively discussed in this review, will have to be addressed. Although sophisticated genetic engineering methods have yielded vector systems deemed to be safe and recombination-free, the ultimate answer to the issue of safety may only be obtained through actual trials in humans.

Development of universal “helper” detection assays for lentiviral vectors will be necessary prior to testing in humans. A standard assay for detection of potential replication-competent virus generated in defective lentiviral systems does not exist at the moment. It will be crucial to the safety of novel gene therapy vectors to develop rapid and sensitive assays, which will detect such recombinants in vector preparations. We envision that this may be accomplished by the use of indicator cells, which show apparent cytopathic effects (syncytium formation) and/or express inducible reporter genes under the control of a lentivirus promoter. Development of a helper assay should be a concerted effort among many groups who specialize in the production and use of lentiviral vectors, so that detection conditions can be standardized.

The use of lentiviral vectors in humans will also require investigators to develop large-scale production methods, which are currently unavailable. This would also have to include methods by which the process can be

standardized so as to prevent batch-to-batch variation. Once these obstacles are overcome, the potential for modification of the human genome using lentiviral vectors is virtually unlimited.

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