THE JOURNAL OF GENE MEDICINE
J Gene Med 2004; **6**: S125–S138.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.501

REVIEW ARTICLE

Lentiviral vectors: optimization of packaging, transduction and gene expression

Christophe Delenda*

Genethon III, CNRS URA 1923, 1 bis rue de l'Internationale, Evry, France

*Correspondence to:
Christophe Delenda, Genethon III, CNRS URA 1923, 1 bis rue de l'Internationale, Evry, France.
E-mail: delenda@genethon.fr

Summary

Gene transfer vectors based on retroviruses including oncogenic retroviruses and lentiviruses provide effective means for the delivery, integration and expression of exogenous genes in mammalian cells. Lentiviral (LV) vectors provide attractive gene delivery vehicles in the context of non-dividing cells. This review summarizes the different optimized LV genetic systems that have been developed to date. In all cases, the production of LV-derived vectors consists of a genetically split gene expression design. The viral elements that are specifically required are (i) the LV packaging helper proteins consisting of at least the *gag-pol* genes, (ii) the LV transfer vector RNA containing the transgene expression cassette, and (iii) an heterologous glycoprotein. While the genetic requirements and performances of the two former viral elements will be treated herein, the latter element relative to the envelope pseudotyping of LV vectors will not be further described (cf. review by Cosset in this issue).

Keywords lentiviral vectors; packaging; transduction; gene expression; gene therapy

Introduction

Several laboratories are currently using different kinds of lentiviral (LV) vectors originating from individual species (Figure 1). These include the immunodeficiency viruses derived from human (HIV-1 [1] and HIV-2 [2]), simian (SIV [3]), feline (FIV [4]) or bovine (BIV [5]) origins, the caprine arthritis encephalopathy virus (CAEV [6]), the equine infectious anemia virus (EIAV [7,8]) and the Jembrana disease virus (JDV [9]) of bovine origin. The minimal packaging activities for these different LV vectors will be described and the newly optimized LV genetic systems will be discussed in term of production yield, transduction efficiency and biosafety parameters.

Generation of minimal helper packaging elements

To reduce the number of viral sequences and therefore the risk of homologous recombinations, various LV elements in the packaging helper constructs have been omitted or replaced by additional heterologous sequences. These include modifications of *cis*-acting sequences such as the replacement of the long terminal repeat (LTR) elements by heterologous promoting and polyadenylation signals and the deletion of the encapsidation signal ($\Delta\psi$). The envelope (*env*) gene has been deleted in all LV packaging constructs and the pseudotyping by other exogenous viral glycoproteins, such as the vesicular stomatitis virus glycoprotein (VSV-G), has been intensely

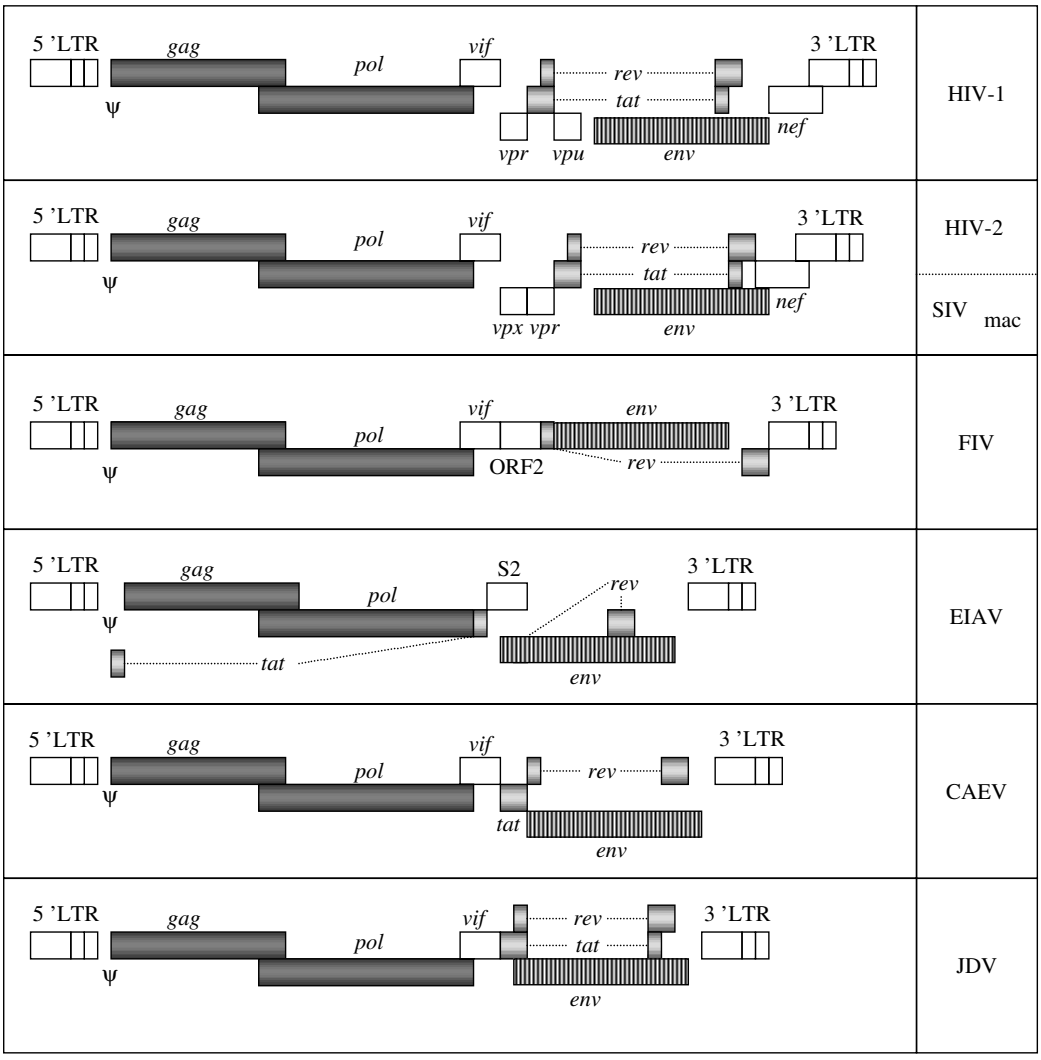

Figure 1. Genomic description of the different lentiviruses that have been used as gene transfer vectors. Viral gene open reading frames are displayed with the three possible genetic codes. The *gag-pol* genome is represented by dark bars whereas the bars figuring the *env* genes are hatched because of their absence in pseudotyped LV vector compositions. The accessory genes (*vif, vpr, vpu, nef, vpx*, ORF2 and S2) and the regulatory genes (*tat* and *rev*) are shown as white and grey bars, respectively

used to circumvent a wide host range tropism. Additionally, removals of the accessory and/or regulatory genes as well as modifications in the *gag-pol* genes have been undertaken in some LV vector designs (Figure 2).

Removal of accessory genes

Several HIV-1 genes (*vif, vpr, vpu, nef*) are not essential for viral replication *in vitro*, but crucially important for viral pathogenesis *in vivo*. Their presence in vectors may raise safety concerns because the protein they encode has cytotoxic or cytostatic activities. For instance, *vpr* induces G2 cell cycle arrest [10,11] and *nef* alters cellular activation pathways [12,13]. Cell surface molecules such as CD4 and the class I major histocompatibility complex are down-regulated by *nef* and *vpu* [14]. Moreover, *nef* [15], *vif* [16] and *vpr* [17] are incorporated into viral particles and can enhance the immunogenicity of vectors. It has therefore been important to demonstrate that LV vectors can be efficiently produced in the absence of these non-essential genes. In the gene transfer context, all accessory proteins (*vpr, vpu, vif* and *nef*) have been omitted in the production design without any negative effect on vector recovery yield [18,19]. However, some laboratories have noticed that *vif* and *vpu* may be required for optimal transduction of the liver [20] and of resting lymphocytes [21]. Moreover, Costello *et al*. [22] have found that production of vectors in the absence of all HIV-1 accessory proteins was accompanied by a 50% decrease in transduction efficiency in activated T cells. A recent report has, however, confirmed that *nef, vif, vpr* and *vpu* are not required for the transduction of both resident macrophages and activated B lymphoblasts [23].

In a similar way, SIV-derived LV particles have also been successfully generated in the absence of accessory proteins (*vpr, vpx, vif* and *nef*) [24]. Nevertheless, the transduction of human dendritic cells (DCs) may require the presence of *vpx* [25].

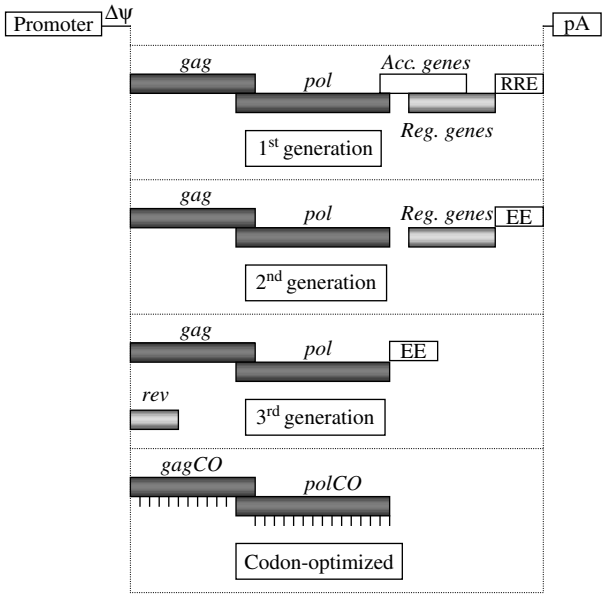

Figure 2. Schematic representation of the different LV packaging generations. The commonly maintained structures in all LV packaging elements are shown by the *cis*-acting sequences (the promoter and polyadenylation regions that replace the original 5′- and 3′-LTR elements, respectively, as well as the deletion of the encapsidation site (Δψ) and the trans-viral helper genes (*gag-pol*). Depending on the generation, the accessory (Acc.) and regulatory (Reg. or *rev* alone) genes are conserved in the packaging constructs. The dashed bars that are added in the *gag-pol* codon-optimized (*gagCO* and *polCO*) genes represent mutations in the third nucleotide of each codon

In the context of FIV-derived LV vectors, Johnston *et al*. [26] have removed the accessory genes encoding *vif* and orf2 from the packaging construct without observing any decrease in the particle production. However, the authors found that transduction with particles derived in this way was hampered in some feline cell types.

Regarding the EIAV-based approach, LV particles have been successfully produced in the absence of the S2 gene [8]. In addition, the authors have removed the dUTPase activity from the *pol* gene and found that a double packaging mutant (Δ *vif* ΔdUTPase) fulfilled their packaging and transduction criteria.

Studies applying BIV-derived vectors have shown that the inactivation and/or truncation of two accessory proteins, i.e. *vif* and *vpw*, did not alter the packaging efficiency [27].

Removal of regulatory genes

Recent studies indicate that the transactivator *tat* is also dispensable for the generation of fully efficient LV vector particles. In this design, the *tat*-dependent U3 sequence from the 5′-LTR which drives the transcription of the transfer vector genomic RNA in producer cells has been replaced by strong heterologous promoter sequences [19,28,29].

An additional improvement in safety is achieved by further splitting the original viral genome to express *rev* from a separate construct [30]. This so-called third generation of vectors displays only a marginal reduction in the packaging activity. It should be noted that the *rev* gene has also been expressed upon the design of a bicistronic *gag-pol*.IRES.*rev* packaging expression construct [24].

Optimal vector production has required the presence of *rev*, which interacts with the *rev* responsive element (RRE) and positively affects the nuclear export of both the unspliced *gag-pol* mRNAs and the transfer vector genomic RNA. Nevertheless, different solutions have been proposed for the design of *rev*-independent production systems.

The RRE sequences have been replaced by heterologous viral sequences known to enhance export and/or stability of unspliced transcripts. These include the constitutive transport elements (CTEs) from the Mason-Pfizer monkey virus (MPMV) [30–32] and the simian retrovirus type 1 (SRV-1) [33] or the RNA transport element (RTE) from the intracisternal A particle retroelement [34]. With the exception of the FIV-based system [32], the substitution of RRE/*rev* with such heterologous export sequences has resulted in some decreased titers in HIV-1- or SIV-based vectors.

Otherwise, host cell factor(s) have been shown to participate in the nuclear export of RRE-containing RNAs. In this regard, it has been reported that a cellular nuclear protein, Sam68 (Src-associated protein in mitosis), specifically interacts with the RRE and could partially substitute for *rev* function in supporting RRE-mediated gene expression [37].

In the absence of *rev, gag-pol* mRNAs are targeted for degradation through *cis*-repressive sequences (CRS), also called inhibitory sequences (INS), present in the coding region. The codon-optimization of HIV-1 [38,39] and SIV [39,40] *gag-pol* genes, which has been performed by inserting wobble mutations in each open reading frame (ORF), has led to the inactivation of CRS/INS and to an enhanced protein production. Moreover, mutations in the third nucleotide of each codon of the *gag-pol* ORFs also permit reduction of the sequence homology between the packaging construct and remaining viral sequences from the transfer vector backbone, such as the 5′ *gag* region necessary for encapsidation or the *cis*-regulatory central polypurine tract (cPPT) sequence derived from the *pol* ORF.

More recently, Pandya *et al*. [35] have used the 5′-LTR region from the spleen necrosis virus (SNV) to drive the expression of SIV *gag-pol* primary transcripts in the absence of an export sequence. In fact, the SNV 5′-LTR contains a post-transcriptional control element (PCE) that functions independently of the *cis*-repressive INS. This PCE element corresponds to the R-U5 region of SNV and contains stimulatory sequences that act in a 5′-proximal position to enhance initiation of translation of retroviral and non-viral reporter gene RNAs [36].

Splitting of the *gag-pol* genome and capsid modification

The risk of recombination can also be reduced by further splitting the *gag-pol* packaging construct into separate counterparts. One report has described HIV-1-derived vector production with split packaging elements, one expressing the *gag-pro* genome and the other expressing the *pol* gene [41]. In addition, another laboratory has used HIV-1 integrase-expressing cells to transiently deliver only *gag, pro* and RT genes [42].

The post-entry restriction of HIV-1-derived vectors to certain cellular types has recently been attributed to the interaction of an inhibitory cellular factor with the viral capsid protein [43]. To abrogate the inhibition in some refractory simian cells, Kootstra *et al*. [44] substituted the cyclophilin A binding region of an HIV-1-based LV vector with that of an autologous simian tropic domain.

Cross-packaging configurations

Another strategy to overcome the problem of susceptible points for homologous recombination between the packaging and the transfer vector constructs relies on the development of chimeric LV variants by using separate organisms for each of these elements.

Surprisingly, SIV- and HIV-2-derived transfer vector RNAs have been successfully encapsidated within HIV-1 cores [24,45–47]. Whereas Negre *et al*. [48] have shown the incapacity to generate the reverse HIV/SIV hybrid strategy, a recent paper has however described the possibility to encapsidate HIV-1 RNAs within SIV cores [35]. This contradiction may be explained by the use of different SIV isolates, e.g. SIVmac1A11 as compared with SIVmac251 that could potentially differ in their efficiency of HIV-1 RNA uptake. Moreover, one report has also demonstrated the cross-packaging efficacy of HIV-1 vector RNAs into HIV-2 cores [49].

In addition, FIV-derived transfer vector RNAs can be cross-packaged into primate LV particles such as HIV-1 and SIV, and vice versa [50]. The authors have confirmed that a non-LV particle such as MPMV was unable to package FIV RNA. Interestingly, FIV particles can package MPMV RNA but cannot propagate the vector RNA further for other steps of the retroviral life cycle. However, another report has not confirmed the cross-packaging capability between the FIV RNA and the HIV-1 core [32].

Optimization of transfer vectors

Transfer vector genomes contain all the *cis*-active sequences needed for packaging (ψ), reverse transcription (primer binding site, LTRs), integration (attL and attR integration sites) and transcription (5′-LTR or internal heterologous promoter), as well as the transgene of interest. Several laboratories have contributed to the knowledge of the minimal vector sequence requirements by deleting non-necessary viral sequences and/or replacing them by exogenous *cis*-acting genetic elements (Figure 3).

Incorporation of additional *cis*-acting regulatory sequences

In order to increase the efficiency of gene transfer, some additional *cis*-acting regulatory sequences have been incorporated into LV transfer vector backbones.

The polypurine tract located in the central position (cPPT) in all LV genomes has been shown to facilitate nuclear translocation of pre-integration complexes and to enhance vector efficiency in both dividing and non-dividing cells. Associated with the central termination site (CTS) which dictates the reverse transcriptase (RT) ejection in the specific context of strand displacement synthesis, the cPPT *cis*-acting benefit for proviral nuclear facilitation has been clearly demonstrated in the HIV-1 [51,52], SIV [53], EIAV [54] and FIV [55] contexts. A putative cPPT/CTS element has also been maintained in BIV-derived vectors [27]. Therefore, the introduction of such autologous sequences into LV vector constructs has led to an increase in the percentage of transduced cells.

The improvement of transgene expression has also been facilitated by the addition of elements that act post-transcriptionally. The insertion of the post-transcriptional regulatory element (PRE) of the woodchuck (WPRE) or human (HPRE) hepatitis B viruses (HBVs) has substantially increased the levels of expression from HIV-1-derived vectors in a transgene-, promoter- and vector-independent manner [56]. Interestingly, they have shown that the WPRE was significantly more active than its HBV counterpart; this increased activity may correlate with the presence of an additional *cis*-acting sequence in the WPRE which is not found in the HPRE. Surprisingly, while the insertion of the WPRE in SIV-derived vectors has also been shown to increase transgene expression in 293T cells, its presence seemed to be detrimental to gene transfer in human DCs [25]. Relative to the EIAV-based system, the addition of the WPRE *cis*-acting sequence to vectors markedly increased gene expression in mouse skeletal muscle cells [57].

Interestingly, further enhancements were obtained when WPRE was combined with sequences corresponding to the untranslated regions (UTRs) of several eukaryotic mRNAs such as fragments of the rat tau and tyrosine hydroxylase 3′-UTRs and of the 5′-UTR of the human Alzheimer amyloid precursor [58].

LTR modifications

As already mentioned above, the U3 region of the 5′-LTR, which drives the expression of the primary transcripts in producer cells, has been replaced by other heterologous

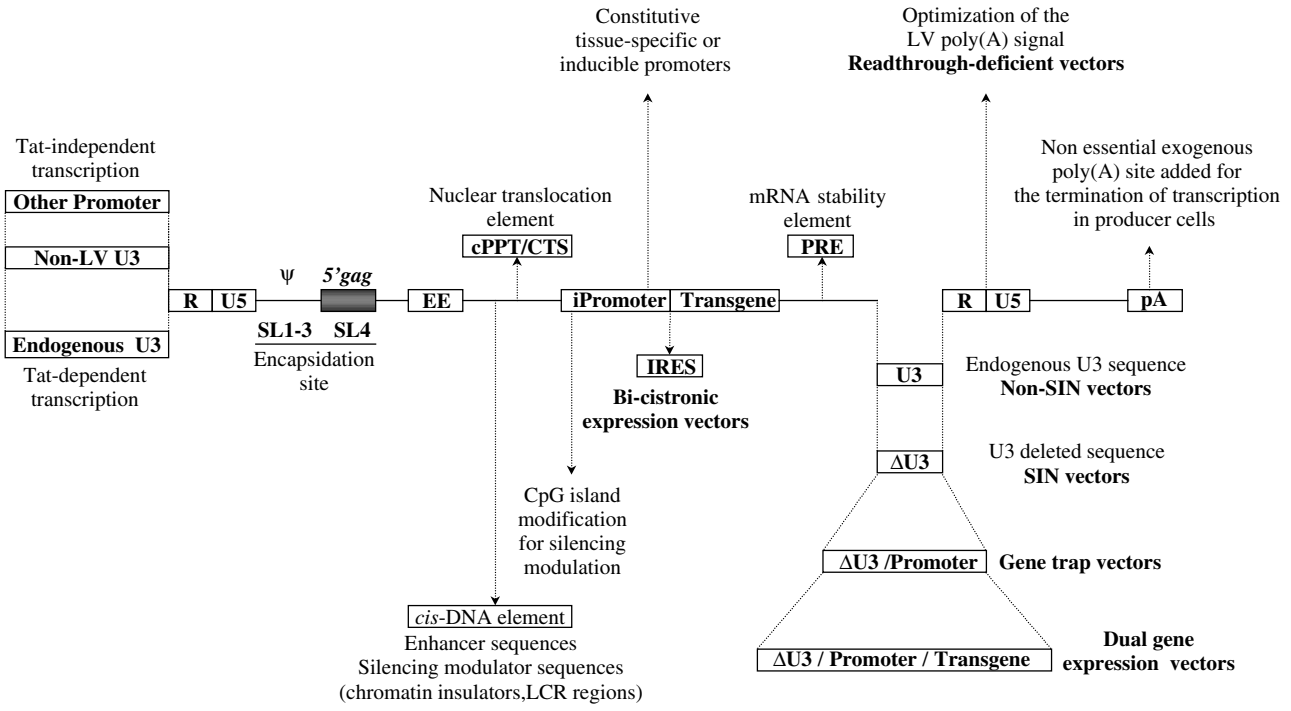

Figure 3. Illustration of the essential and optimizing elements contained in the LV transfer vector backbone. The presence, location and optimal activity of the various *cis*-acting regulatory elements [the post-transcriptional responsive element (PRE), the *rev*-responsive element (RRE) or any other nuclear export element (EE)] are displayed. Interestingly, to satisfy the nuclear import enhancement, it has been hypothesized that the central polypurine tract associated with the central termination site (cPPT/CTS) has to be inserted in the center of the proviral genome. The *cis*-acting DNA elements, comprising promoters, enhancers or silencing modulators, can also be inserted into the U3-deleted region of the 3′-LTR

promoting sequences in order to drive a *tat*-independent primary transcription.

In addition, the U3 region of the 3′-LTR has also been deleted in order to eliminate, in target cells, the risk of promoter interference and susceptible oncogenic derivations. Such LTR-inactive LV vectors, termed self-inactivating (SIN), have largely been generated in HIV-1 [28,29,59,60], SIV [3,61] and EIAV [57] contexts. Although the absence of promoting sequences in the 3′-LTR of the integrated proviral genomes should prevent the potential transcriptional activation of a downstream gene, a recent study has shown high frequencies of transcriptional readthrough of the 3′-polyadenylation signals from the internal promoter of SIN-derived HIV-1 and MLV vectors [62]. One way of tempering read-through, which is mostly due to a deficient cleavage and polyadenylation of vector transcripts within the 3′-LTR [63,64], has consisted of adding to the retroviral polyadenylation signal other exogenous elements (e.g. β-globin or SV40) [60].

To drive the transcription of the transgene cDNA in the absence of an internal promoter, some laboratories have inserted heterologous promoting elements in place of the LV U3-deleted sequence. As an example, the level of transgenic transcription deriving from the U3-promoting sequence of the murine stem cell virus (MSCV) has been increased in the human hematopoietic stem cell (HSC) type as compared with the classically used internal constitutive promoters [65,66]. The incorporation of a heterologous promoter element in the 3′-LTR has also been designed in LV vectors for use in gene-trapping experiments where the screening of susceptible downstream genes was a concern [67]. Moreover, the insertion of transgene expression cassettes (promoter and transgene) in replacement of the LV U3 region has permitted dual gene expression strategies in the SIV-based expression system [68]. In addition to these U3 modifications, it should be possible to reduce the length of the LTR region as HIV-1 mutants with deletions in the 3′-R sequences still replicate efficiently [69].

Minimal requirements for the encapsidation site

The majority of LV encapsidation sites require the presence in the transfer vector RNA of a minimal 5′-fragment of the *gag* gene that contains stem loop structure(s) necessary for the *cis*-packaging activity [1,8,9,24,26]. In most LV systems, the *gag* ORF contained in this N-terminal fragment, which is around 300 to 400 base pairs (bp) long, has been truncated by frameshift mutations so that transcription/translation of the susceptibly emerging *gag* peptide does not interfere with that of the transgene. In the context of FIV, sequences both within the 5′-UTR and the 5′-end of *gag* are required for efficient packaging and transduction [70]. The authors have further demonstrated that the 5′-distal 100 bp region

of *gag* sequences in conjunction with the 5′-UTR was sufficient for efficient RNA packaging [71]. In a similar way, the first 104 bp of BIV *gag* also contains a functional part of the packaging signal [27].

Otherwise, an *in vitro* selected RNA with high affinity for the HIV-1 nucleocapsid (NC) protein has been shown to mediate packaging into HIV-1 virions and could then be substituted for the viral ψ encapsidation site [72,73].

Studies implicating oncoretroviruses have shown that the ψ packaging sequence is efficiently removed from the vector genome during reverse transcription when placed between direct repeats [74]. Extending this to LV vectors would enhance safety by preventing vector mobilization and the spread of susceptible generation of recombinant-competent retroviruses (RCRs) that may involve the ψ sequence rescue.

Splicing signal concern

It has been suggested that the spliceosome assembly complex may participate in *rev*-mediated export, since optimal *rev* activity requires the presence of functional splice sites on the target RNA [75]. More recently, it has been proposed that *rev* could bind to other viral RNA sequences called exon splicing enhancers (ESE) increasing the RNA nuclear export [76,77]. The ESE elements are described as purine-rich sequences recruiting arginine-rich proteins responsible for exon recognition [78]. Such purine-rich regions which have been described downstream of the *tat/rev* splice acceptor sites of the HIV-1 [79] and SIV [25] genomes have been found to increase the transduction efficiency of LV-derived transfer vectors.

Otherwise, the removal of the donor splice site in the HIV-1 transfer vector has been shown to yield enhanced levels of unspliced cytoplasmic mRNAs [80]. Moreover, titers of vectors that no more contain the splice donor site $(SD)^-$ were not decreased with respect to SD^+-vectors when packaged with an HIV-1 helper that provides *rev*. This observation has been confirmed by Cui *et al*. [81] who showed that elimination of the splicing signals flanking the RRE did not affect vector titers.

Modulation of the transcriptional silencing

One of the major obstacles to gene transfer is the gap between gene expression of cellular genes from their genomic loci, which usually occurs at adequate levels, and the relatively poor expression levels obtained when the same gene is expressed from integrative viral vectors, in many cases from heterologous promoters.

The cause of retroviral vector silencing has been attributed to *de novo* cytosine methylation of CpG dinucleotide sequences and subsequent histone deacetylation leading to chromatin condensation [82]. However, data from several studies suggest that methylation acts only as a secondary or associated step in the retroviral silencing pathway [83,84]. From retroviral gene transfer analyses in stem cells, Pannell *et al*. [84,85] have speculated on the presence of cell-specific silencing factors that may induce a dormant state of gene activity prior to long-term silencing by methylation. The exogenous retrovirus in stem cells may be detected by a putative 'somno-complex' inactivating the LTR-driven transcription.

Attempts to increase expression from retrovirally transduced genes have focused on the deletion of known retroviral silencer elements. As an example, the MSCV virus [86] and human stem cell (HSC1) vectors [87] containing mutations in LTRs and in the primer binding site (PBS) regions, respectively, have been developed in an effort to relieve the silencing effects. Although LV genomes contain an extremely low level of CpG dinucleotides [88], the ablation of these remaining sequences may favor a potential elevation of transcription. As an example, the use of SIN-derived LV vectors that do not contain the U3 LTR region has allowed sustained expression of the transgene [89,90]. A recent report has shown that the transcriptional silencing in murine cells of HIV-1-derived vectors was limited to non-SIN transfer backbones that still generate LTR-mediated transcription and therefore participate in promoter interference [91]. Interestingly, O'Rourke *et al*. [92] have compared the duration of expression from HIV-1- or EIAV-based SIN vectors in a variety of human cell types, including growth-arrested cells, and found that the EIAV vectors were more subjected to silencing, probably due to the instability of vector-derived RNA transcripts.

Because SIN-based vectors require the expression from a heterologous promoter, it should be relevant to take into account the potential transcriptional inactivation from these additional promoter loci. The removal of CpG islands from cellular promoter elements should probably be attempted for the design of vectors harboring an evident sustained expression activity. In addition, the choice of the ideal promoter should be seriously envisaged for optimal long-term expression performance. As an example, the benefit in terms of sustainable expression activity of the human phosphoglycerate kinase promoter (hPGK) over the *cis*-active promoting region of the cytomegalovirus (CMV) has been clearly demonstrated in the hepatocyte transduction model [89]. Additional heterologous promoter elements such as those derived from virus genomes have participated in an enhancement of the long-term transgene expression. As an example, an HIV-1 LV vector containing an internal promoter derived from the spleen focus forming virus has delivered a sustained transgene expression in primary HSCs *in vivo* [93].

Another strategy to regulate transgene silencing relies on the addition of positive regulatory elements to the expression cassette, such as locus control regions (LCRs), chromatin insulators or matrix attachment sites.

The discovery of DNaseI hypersensitive sites (HS) far upstream of the human β-globin cluster [94,95] has improved the prospects for gene therapy for human hemoglobinopathies. These HS sites are nucleosome-free

regions of open chromatin that are highly accessible to *trans*-acting factors [96,97]. Moreover, this *cis*-acting DNA element, termed the β-LCR, has been reported to confer position-independent and copy number-dependent expression. This region, which is 20 kbp long, has been reduced to shorter forms of a few hundred base pairs long that still confer the silencing modulation activity [98,99]. The insertion of the β-LCR sequence within HIV-1 LV vectors has been useful for increasing erythroid-specific synthesis of the β-globin protein in transgenic mice [84,100,101]. In heterotypic expression situations, the insertion into the 3′-LTR of a SIN HIV-1 vector of another erythroid-specific LCR, e.g. the HS2 site from the GATA-1 gene, has also made it possible to prevent the down-modulation of the eGFP reporter gene in the erythroid lineage [102]. In a similar way, the LCR locus deriving from the human CD2 gene has conferred a clear expression advantage in the T cell lineage on HIV-1-based vectors [103].

Several chromatin insulator boundary elements have also been described to protect expression cassettes from position effects. These include the HS4 core from the chicken β-globin LCR [104,105], the specialized chromatin structures scs and scs' which flank the *hsp70* genes of the 87A7 heat shock locus in *Drosophila melanogaster* [106] or the less-characterized vertebrate insulators BEAD-1 from the human T-cell receptor gene locus [107] and from the intergenic spacer of the ribosomal RNA genes of *Xenopus* [108]. In the context of non-SIN Moloney leukemia-derived (MLV) vectors, Modin *et al*. [109] have, however, shown that none of the insulators, scs, BEAD-1 or HS4, was able to shield an internal promoter from the repressive effect of the silencer at the PBS region. Nevertheless, results obtained from SIN HIV-1-mediated transduction have confirmed that the chicken β-globin HS4 insulator was able to confer a sustainable expression in human HSCs [110].

Structural studies of chromosome organization have led to the discovery of matrix or scaffold attachment regions (S/MARs) which are DNA sequences that mediate the attachment of individual chromatin loops to a proteinaceous matrix or scaffold in both interphase nuclei [111,112] and mitotic chromosomes [113]. One putative function of these sequences, particularly those that flank individual genes or gene clusters, is to act as insulator elements. The insertion of a S/MAR from the immunoglobulin-kappa gene into HIV-1-based SIN transfer vectors has significantly increased the liver and hepatocyte transduction efficiency [114,115]. Moreover, the human interferon-β S/MAR element inserted in the same kind of LV vector also resulted in an increase in the duration time of transgene expression in human HSCs [110]. These authors also observed a synergistic effect when this S/MAR element was associated in LV constructs with an HS4 insulator. This synergy of action with these two silencing elements has also been described in HIV-1-transduced human embryonic stem cells where a reduction in the variability of transgene expression was clearly observed [116].

Transcriptional targeting

To specifically direct and/or substantially enhance the LV transgenic expression in a particular cell type or organ, some laboratories have used tissue-specific promoter and enhancer elements.

Concerning the already-described erythroid-lineage specific gene expression, Moreau-Gaudry *et al*. [117] compared the efficiency of transgene expression with HIV-1 SIN vectors containing different erythroid-specific promoters (ankyrin-1, α-spectrin, β-globin or ζ-globin) or enhancers (GATA-1 autoregulatory element, β-globin LCR, intron I8 from the 5-aminolevulinate synthase gene or α-globin HS40) in human and mouse hematopoietic stem cells as well as in engrafted mice. Highest levels of expression were obtained when the ankyrin-1 promoter was used in combination with two enhancers in tandem (the GATA-1/HS40 or the I8/HS40 enhancer pairs). The addition of the γ-globin intron sequences to the ankyrin-1 promoter has further augmented vector expression [118]. Moreover, the enhancement for LV-mediated erythroid gene expression, driven by another chimeric promoter/enhancer element (ankyrin-1/α-globin HS40), has also been reported in a model of erythropoietic protoporphyria where the ferrochelatase (FECH) cDNA was used in gene repair experiments [119].

To achieve antigen presenting cell (APC)-specific transgene expression, Cui *et al*. [120] took advantage of the fact that major histocompatibility complex class II (MHCII) genes are expressed selectively in APCs and highly in DCs after differentiation and maturation. Using the NOD/SCID mouse engraftment model, they demonstrated selective expression of transgene in MHCII$^+$ human cells with an HIV-1 SIN vector harboring the human MHCII-specific HLA-DRα promoter.

Specific expression in CD4+ cells has been recently achieved by inserting in an HIV-1-derived vector the CD4 gene-silencing element [121]. The addition of this regulatory element to the CD4-mediated transcriptional unit in the LV construct has resulted in the restriction of expression into CD4+ T cells in reconstituted mice and in *ex vivo* transduced human T cells.

In order to specifically target exogenous genes to endothelial cells (ECs) of tumor vessels, De Palma *et al*. [122] have engineered HIV-1 SIN vectors with promoter and enhancer sequences from genes preferentially expressed in ECs, such as the vascular endothelial growth factor receptor Flk-1, the angiopoietin receptor Tie2, the endothelial receptor tyrosine kinase Tie1, as well as the adhesion molecules vascular endothelial cadherin and intercellular adhesion molecule 2. They found that vectors carrying transcriptional elements from the Tie2 gene achieved remarkable specificity of eGFP expression in ECs *in vitro* and *in vivo*.

HIV-1-based LV vectors containing specific neuron promoters have also been evaluated for specific transgene expression in the central nervous system (CNS). Using the neuron-specific enolase promoter, 30% more eGFP-positive cells were observed in the mouse brain striatum

and hippocampus than with the CMV promoter [123]. Moreover, 90% of the CNS cells transduced by the LV vector controlled by the neuronal promoter are neurons.

Regarding gene transfer in retinal cells, the use of the rhodopsin promoter in an HIV-1 vector has led not only to photoreceptor-specific expression, but also to higher expression levels than with constitutive promoters such as CMV [124] or those deriving from the mouse PGK and the human elongation factor-1 (hEF1) genes [125]. *In vivo* gene delivery to photoreceptor cells is important for gene therapy of inherited retinal degenerative diseases because most of the identified genes responsible for retinal degeneration so far are expressed specifically in photoreceptor cells [126].

Whereas the systemic delivery of HIV-1 vectors that contain constitutive promoter elements has resulted in the transduction of several mouse tissues, e.g. liver, spleen and bone marrow, the use of the albumin promoter has restricted the transgenic expression to hepatocytes [127]. Moreover, Oertel *et al*. [128] observed that long-term expression in rat liver was achieved when the transgene was driven by the liver-specific albumin enhancer/promoter but was silenced when the CMV promoter was used. In addition, recent results have demonstrated the possibility of using a LV-transduced expression unit containing the rat α-fetoprotein promoter to restrict suicide gene sensitivity to human hepatocarcinoma cells [129].

Prostate cancer cells express a well-characterized antigen, prostate-specific antigen (PSA). PSA has been demonstrated to be a sensitive and specific tumor marker for cancer screening and assessment [130] and is used as an indicator of disease and response to prostate cancer therapy [131]. A patient-derived PSA promoter inserted into an HIV-1-based vector has driven efficient transgenic activity in prostate cells with satisfactory efficacy and specificity in *in vitro* as well as *in vivo* experiments [132]. More recently, a PSA promoter-based HIV-1 vector has been used to deliver the diphtheria toxin A gene into prostate cancer cells, and has shown promising tissue-specific eradication of prostate cancer cells in cell culture and in a mouse tumor model [133].

Single-cell mouse embryos have been transduced *in vitro* with recombinant HIV-1 SIN vectors to generate transgenic mice carrying the eGFP reporter gene in a specific tissue compartment [134]. Mice generated using LV vectors with muscle (myogenin) and T lymphocyte (proximal *lck*) specific promoters expressed high levels of eGFP only in the appropriate cell type.

Transcriptional regulation

An important feature of all vectors for human gene transfer should be the possibility to regulate the transgene expression. Among the most widely used induction systems, the tetracycline-based expression approach has recently been adapted for transcriptional regulation of HIV-1-derived transfer vectors [135–140]. For gene therapy applications, the situation for an induction based on the delivery of the antibiotic (tet-on) will, of course, be preferred over one based on the silencing of patients constantly treated with antibiotics in whom the induction would have to be performed by tetracycline-withdrawal (tet-off). With the exception of two reports [136,140], all the tetracycline-inducible HIV-1 vectors listed in the literature were constructed using the tet-on induction system. The authors have shown that the tetracycline-based expression system applied to HIV-1 gene transfer vectors allowed inducible expression over at least two orders of magnitude. Moreover, they found a stable dose-dependent level of transgene expression that is rapidly switched on and switched off.

The delivery of the *trans*-activating inductor (tTA or rtTA for the tet-on and tet-off systems, respectively) and the *cis*-inducible operator (tet-responsive element, TRE) that control the transgene transcription has been designed through single or binary expression vectors that contain these two genetic inducible elements separately or not. From comparison analyses, some laboratories have demonstrated a clear advantage of the binary vector system over the one-piece transduction approach. In fact, although the binary strategy imposes the necessity that each target cell will have to be efficiently transduced by two vectors, it seems, however, to be less leaky by comparison with the single-based system, which therefore is better suited to the desired induction parameters. A more advanced tetracycline regulation design has recently been applied in the LV field context where the use of a codon-optimized tetracycline transactivator has been shown to significantly reduce the leakiness in different cell types [138,141].

Another way to regulate the transgene signal is to use excisable proviruses in order to switch off the expression at the time required. Recombinatorial systems such as the Cre-*loxP* nuclease design have already been attempted in the LV-based gene transfer model, where *loxP cis*-active sequences were generally introduced into the U3-deleted region of the 3′-LTR [142–144]. In a similar way, the Flp-FRT site-specific recombination system from *Saccharomyces cerevisiae* has proved a powerful and efficient tool for the generation of multiple MLV-derived producer cell lines [145].

Multiple gene expression design

In addition to the inducible expression systems already described, there are several situations in the LV gene transfer design that have necessitated the expression of more than one transgene within the same target cell. Most commonly, such applications have been attempted with vectors harboring bicistronic expression cassettes.

One of these situations consists of co-expressing the transgene of interest with a reporter gene in order to follow the expression in bulk cell population and/or to

enrich positively transduced cells. As an example, to set up *in vitro* complementation assays in patient cells suffering from the carbohydrate-deficient glycoprotein syndrome (CDGS), we have constructed bicistronic HIV-1 expression vectors, with the eGFP gene being expressed downstream of the internal ribosome entry site (IRES) of the encephalopathy myocarditis virus. Through these studies, aimed at confirming genetic and biochemistric upstream characterizations generated in the laboratory of Dr S. Moore, we have followed in primary and transformed patient cells the expression of two human cDNAs encoding enzymes participating in the N-glycosylation process [146,147].

Another strategy to enrich a transduced cell population lays in the expression of drug-resistant mutant genes. For erythropoietic protoporphiria *ex vivo* applications, Richard *et al*. [148] have co-expressed the transgene of interest, i.e. the human FECH cDNA, with a human alkylating drug resistance mutant O(6)-methylguanine DNA methyltransferase. They observed a clear enrichment in transduced hematopoietic cells and confirmed that these cells were efficiently protected from drug toxicity and corrected for enzymatic deficiency.

Finally, other situations for the use of a dual gene expression system could emerge from the necessity for some gene therapy applications to express more than one gene. As an example, a dopamine-replacement strategy has been investigated as a treatment in a Parkinson's disease animal model where three different catecholaminergic synthetic enzymes were delivered from a single EIAV-based vector [149]. In addition, Mitta *et al*. [143] have constructed HIV-1-derived transfer vectors that contain tricistronic expression cassettes for coordinated expression of up to three transgenes.

As already mentioned above, expression cassettes inserted in the U3-deleted region of the 3′-LTR can be duplicated after proviral genesis and therefore may allow an improvement in the transgene expression [68]. However, such a system is no longer self-inactivating.

By taking advantage of the natural splicing signals of HIV-1, one laboratory has designed autologous multigene vectors by which multiple mRNAs are generated from a single transcriptional unit [150,151].

Another possible alternative consists of expressing hybrid fusion proteins that will contain two separate transgenes. Such a design has already been described in the context of LV-mediated gene transfer. Murthy *et al*. [152] have expressed from an HIV-1-derived vector a fusion protein composed of the biologically active domains of human endostatin 18 and kringle-5 proteins for inhibiting both endothelial cell proliferation and migration, respectively. In addition, the delivery by LV-based vectors of the fusion eGFP/VP22 protein into the CNS has allowed the transport between neuronal cells via axons [123], as the herpes simplex virus type I VP22 protein has been shown to facilitate intercellular protein transport.

RNA polymerase III-dependent transcription

RNA interference (RNAi) is becoming an important tool for the study of biological processes through reverse genetics. In that evolutionary conserved process of gene silencing, an RNA-induced silencing complex uses small interfering RNAs (siRNAs) to cleave the target mRNA at the homologous sequence, resulting in a decrease in the steady state levels of transcripts [153]. DNA expression vectors have been developed to express hairpin or duplex siRNAs, which employ the type III class of RNA polymerase promoters to drive the expression of siRNA molecules [154–156].

Short hairpin RNAs (shRNAs) have been delivered to cells using a LV-mediated transduction and where their transcription originated from the human U6 polymerase III transcriptional unit [157,158]. In their reporter gene expression models (eGFP and luciferase), the authors observed a clear reduction in gene expression in transformed or primary human cells. Another report has also shown that LV-delivered shRNAs are capable of specific, highly stable and functional silencing of gene expression in a variety of human cell types and in transgenic mice [159]. Using a LV-mediated siRNA delivery, one laboratory is also attempting to generate 'knock down' mice in which the expression of a specific gene is down-regulated substantially [160]. In an additional LV-based expression design, the transcription of eGFP-derived shRNAs by another polymerase III-dependent promoter, i.e. the H1-RNA promoter, is also effective for the delivery of interfering RNAs [161]. These authors have shown in this paper that the silencing was dose-dependent, occurred as early as 72 h post-transduction, and persisted for at least 25 days.

Scherr *et al*. [162] have analyzed kinetic and quantitative aspects of mammalian RNAi in different cell lines using LV constructs with shRNA expression cassettes located in the U3 region of the LTR. Using the eGFP as a target gene for RNAi and the red fluorescence protein as a surrogate marker for intracellular siRNA expression, they have shown that long-term siRNA expression mediated stable RNAi.

Dedicated to AIDS vaccine strategies, an anti-*rev* siRNA construct has also been introduced into an HIV-1-based LV vector [163]. *In vitro* HIV-1 challenge of siRNA-expressing macrophages and T cells with macrophage-tropic and T-cell-tropic HIV-1, respectively, showed marked viral resistance. Another report has also expressed from HIV-1-derived vectors short HIV-1 RNA viral molecules, such as an anti-CCR5 ribozyme, a nucleolar localizing TAR RNA decoy as well as polymerase III-expressed siRNAs, which all clearly induced viral protection [164]. From a parallel work, blocking CCR5 expression by siRNAs via a LV-mediated transduction has provided a substantial protection for the lymphocyte populations from CCR5-tropic HIV-1 virus infection but only a minimal effect on infection by a CXCR4-tropic virus [165].

Dirac and Bernards [166] have developed a LV vector that directs the synthesis of a p53-specific shRNA. In mouse embryo fibroblasts, the induction of senescence, which normally requires the presence of p53, was hampered when cells were previously transduced, highlighting the efficacy of gene silencing of the LV-mediated RNAi process. The p53 gene silencing using siRNA-mediated LV transduction has also been described by another group in both dividing and non-dividing human cells with similar down-regulation of gene capability [167].

Concluding remarks

As mentioned in this review, many efforts have been undertaken in the context of LV-based vectors to optimize the production yields and the efficiency of gene transfer while minimizing the number of required viral sequences.

Concomitant with the biosafety requirements that will be imposed by the regulatory agencies, LV vectors are produced in such a way that no susceptible RCRs can arise in vector batches. In addition to the genetic modifications that have permitted a reduction in the number of viral sequences, another limitation in their use in clinical trials will be the requirement for production using standard operating procedures.

Even though the transient transfection procedure allows for efficient and safe HIV-1 vector production, the generation of clinically acceptable vectors will ultimately require stable producer cell lines. This will eliminate the risk of homologous recombination between the transfected plasmids, as well as the problem of carrying over plasmid DNA in the vector batches. Additionally, it will facilitate the standardization and scaling-up of vector production. Several groups have described the isolation and characterization of cell lines producing HIV-1 vectors with either the original *env* proteins [31,168–171], VSV-G-pseudotypes [172–177], or glycoproteins derived from oncoretroviruses [178]. The fact that the protease and *rev* LV proteins and the VSV/G glycoprotein are cytotoxic or cytostatic when constitutively expressed [179,180] has mandated the use of inducible expression systems. Two different regulated systems responding to tetracycline [170–175] or ecdysone [176,177] have thus been used for this purpose with satisfactory vector production.

References

1. Naldini L, Blomer U, Gallay P, *et al*. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector [see comments]. *Science* 1996; **272**: 263–267.
2. Poeschla E, Gilbert J, Li X, Huang S, Ho A, Wong-Staal F. Identification of a human immunodeficiency virus type 2 (HIV-2) encapsidation determinant and transduction of nondividing human cells by HIV-2-based lentivirus vectors. *J Virol* 1998; **72**: 6527–6536.
3. Mangeot PE, Negre D, Dubois B, *et al*. Development of minimal lentivirus vectors derived from simian immunodeficiency virus (SIVmac251) and their use for gene transfer into human dendritic cells. *J Virol* 2000; **74**: 8307–8315.
4. Poeschla EM, Wong-Staal F, Looney DJ. Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat Med* 1998; **4**: 354–357.
5. Berkowitz R, Ilves H, Lin WY, *et al*. Construction and molecular analysis of gene transfer systems derived from bovine immunodeficiency virus. *J Virol* 2001; **75**: 3371–3382.
6. Mselli-Lakhal L, Favier C, Da Silva Teixeira MF, *et al*. Defective RNA packaging is responsible for low transduction efficiency of CAEV-based vectors. *Arch Virol* 1998; **143**: 681–695.
7. Olsen JC. Gene transfer vectors derived from equine infectious anemia virus. *Gene Ther* 1998; **5**: 1481–1487.
8. Mitrophanous K, Yoon S, Rohll J, *et al*. Stable gene transfer to the nervous system using a non-primate lentiviral vector. *Gene Ther* 1999; **6**: 1808–1818.
9. Metharom P, Takyar S, Xia HH, *et al*. Novel bovine lentiviral vectors based on Jembrana disease virus. *J Gene Med* 2000; **2**: 176–185.
10. Fletcher TM III, Brichacek B, Sharova N, *et al*. Nuclear import and cell cycle arrest functions of the HIV-1 Vpr protein are encoded by two separate genes in HIV-2/SIV(SM). *EMBO J* 1996; **15**: 6155–6165.
11. Bukrinsky M, Adzhubei A. Viral protein R of HIV-1. *Rev Med Virol* 1999; **9**: 39–49.
12. Luo W, Peterlin BM. Activation of the T-cell receptor signaling pathway by Nef from an aggressive strain of simian immunodeficiency virus. *J Virol* 1997; **71**: 9531–9537.
13. Hanna Z, Kay DG, Rebai N, Guimond A, Jothy S, Jolicoeur P. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. *Cell* 1998; **95**: 163–175.
14. Piguet V, Schwartz O, Le Gall S, Trono D. The downregulation of CD4 and MHC-I by primate lentiviruses: a paradigm for the modulation of cell surface receptors. *Immunol Rev* 1999; **168**: 51–63.
15. Pandori MW, Fitch NJ, Craig HM, Richman DD, Spina CA, Guatelli JC. Producer-cell modification of human immunodeficiency virus type 1: Nef is a virion protein. *J Virol* 1996; **70**: 4283–4290.
16. Camaur D, Trono D. Characterization of human immunodeficiency virus type 1 Vif particle incorporation. *J Virol* 1996; **70**: 6106–6111.
17. Sato A, Yoshimoto J, Isaka Y, *et al*. Evidence for direct association of Vpr and matrix protein p17 within the HIV-1 virion. *Virology* 1996; **220**: 208–212.
18. Zufferey R, Nagy D, Mandel RJ, Naldini L, Trono D. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. *Nat Biotechnol* 1997; **15**: 871–875.
19. Dull T, Zufferey R, Kelly M, *et al*. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998; **72**: 8463–8471.
20. Kafri T, Blomer U, Peterson DA, Gage FH, Verma IM. Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. *Nat Genet* 1997; **17**: 314–317.
21. Chinnasamy D, Chinnasamy N, Enriquez MJ, Otsu M, Morgan RA, Candotti F. Lentiviral-mediated gene transfer into human lymphocytes: role of HIV-1 accessory proteins. *Blood* 2000; **96**: 1309–1316.
22. Costello E, Munoz M, Buetti E, Meylan PR, Diggelmann H, Thali M. Gene transfer into stimulated and unstimulated T lymphocytes by HIV-1-derived lentiviral vectors. *Gene Ther* 2000; **7**: 596–604.
23. Rossi GR, Mautino MR, Morgan RA. High-efficiency lentiviral vector-mediated gene transfer into murine macrophages and activated splenic B lymphocytes. *Hum Gene Ther* 2003; **14**: 385–391.
24. Negre D, Mangeot PE, Duisit G, *et al*. Characterization of novel safe lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) that efficiently transduce mature human dendritic cells [in process citation]. *Gene Ther* 2000; **7**: 1613–1623.
25. Mangeot PE, Duperrier K, Negre D, *et al*. High levels of transduction of human dendritic cells with optimized SIV vectors. *Mol Ther* 2002; **5**: 283–290.
26. Johnston JC, Gasmi M, Lim LE, *et al*. Minimum requirements for efficient transduction of dividing and nondividing cells by feline immunodeficiency virus vectors. *J Virol* 1999; **73**: 4991–5000.

27. Molina RP, Matukonis M, Paszkiet B, Zhang J, Kaleko M, Luo T. Mapping of the bovine immunodeficiency virus packaging signal and RRE and incorporation into a minimal gene transfer vector. *Virology* 2002; **304**: 10–23.
28. Kim VN, Mitrophanous K, Kingsman SM, Kingsman AJ. Minimal requirement for a lentivirus vector based on human immunodeficiency virus type 1. *J Virol* 1998; **72**: 811–816.
29. Miyoshi H, Blomer U, Takahashi M, Gage FH, Verma IM. Development of a self-inactivating lentivirus vector. *J Virol* 1998; **72**: 8150–8157.
30. Gasmi M, Glynn J, Jin MJ, Jolly DJ, Yee JK, Chen ST. Requirements for efficient production and transduction of human immunodeficiency virus type 1-based vectors. *J Virol* 1999; **73**: 1828–1834.
31. Srinivasakumar N, Schuening FG. A lentivirus packaging system based on alternative RNA transport mechanisms to express helper and gene transfer vector RNAs and its use to study the requirement of accessory proteins for particle formation and gene delivery. *J Virol* 1999; **73**: 9589–9598.
32. Curran MA, Kaiser SM, Achacoso PL, Nolan GP. Efficient transduction of nondividing cells by optimized feline immunodeficiency virus vectors. *Mol Ther* 2000; **1**: 31–38.
33. Mautino MR, Keiser N, Morgan RA. Improved titers of HIV-based lentiviral vectors using the SRV-1 constitutive transport element. *Gene Ther* 2000; **7**: 1421–1424.
34. Nappi F, Schneider R, Zolotukhin A, *et al*. Identification of a novel posttranscriptional regulatory element by using a rev- and RRE-mutated human immunodeficiency virus type 1 DNA proviral clone as a molecular trap. *J Virol* 2001; **75**: 4558–4569.
35. Pandya S, Boris-Lawrie K, Leung NJ, Akkina R, Planelles V. Development of an Rev-independent, minimal simian immunodeficiency virus-derived vector system. *Hum Gene Ther* 2001; **12**: 847–857.
36. Roberts TM, Boris-Lawrie K. The 5′ RNA terminus of spleen necrosis virus stimulates translation of nonviral mRNA. *J Virol* 2000; **74**: 8111–8118.
37. Reddy TR, Xu W, Mau JK, *et al*. Inhibition of HIV replication by dominant negative mutants of Sam68, a functional homolog of HIV-1 Rev. *Nat Med* 1999; **5**: 635–642.
38. Kotsopoulou E, Kim VN, Kingsman AJ, Kingsman SM, Mitrophanous KA. A Rev-independent human immunodeficiency virus type 1 (HIV-1)-based vector that exploits a codon-optimized HIV-1 gag-pol gene. *J Virol* 2000; **74**: 4839–4852.
39. Wagner R, Graf M, Bieler K, *et al*. Rev-independent expression of synthetic gag-pol genes of human immunodeficiency virus type 1 and simian immunodeficiency virus: implications for the safety of lentiviral vectors [in process citation]. *Hum Gene Ther* 2000; **11**: 2403–2413.
40. Kuate S, Wagner R, Uberla K. Development and characterization of a minimal inducible packaging cell line for simian immunodeficiency virus-based lentiviral vectors. *J Gene Med* 2002; **4**: 347–355.
41. Wu X, Wakefield JK, Liu H, *et al*. Development of a novel trans-lentiviral vector that affords predictable safety. *Mol Ther* 2000; **2**: 47–55.
42. Cherepanov P, Pluymers W, Claeys A, Proost P, De Clercq E, Debyser Z. High-level expression of active HIV-1 integrase from a synthetic gene in human cells. *FASEB J* 2000; **14**: 1389–1399.
43. Towers GJ, Hatziioannou T, Cowan S, Goff SP, Luban J, Bieniasz PD. Cyclophilin A modulates the sensitivity of HIV-1 to host restriction factors. *Nat Med* 2003; **9**: 1138–1143.
44. Kootstra NA, Munk C, Tonnu N, Landau NR, Verma IM. Abrogation of postentry restriction of HIV-1-based lentiviral vector transduction in simian cells. *Proc Natl Acad Sci U S A* 2003; **100**: 1298–1303.
45. Corbeau P, Kraus G, Wong-Staal F. Transduction of human macrophages using a stable HIV-1/HIV-2-derived gene delivery system. *Gene Ther* 1998; **5**: 99–104.
46. Stitz J, Muhlebach MD, Blomer U, *et al*. A novel lentivirus vector derived from apathogenic simian immunodeficiency virus. *Virology* 2001; **291**: 191–197.
47. Goujon C, Jarrosson-Wuilleme L, Bernaud J, Rigal D, Darlix JL, Cimarelli A. Heterologous human immunodeficiency virus type 1 lentiviral vectors packaging a simian immunodeficiency virus-derived genome display a specific postentry transduction defect in dendritic cells. *J Virol* 2003; **77**: 9295–9304.
48. Negre D, Mangeot PE, Duisit G, *et al*. Characterization of novel safe lentiviral vectors derived from simian immunodeficiency virus (SIVmac251) that efficiently transduce mature human dendritic cells. *Gene Ther* 2000; **7**: 1613–1623.
49. D'Costa J, Harvey-White J, Qasba P, *et al*. HIV-2 derived lentiviral vectors: gene transfer in Parkinson's and Fabry disease models in vitro. *J Med Virol* 2003; **71**: 173–182.
50. Browning MT, Schmidt RD, Lew KA, Rizvi TA. Primate and feline lentivirus vector RNA packaging and propagation by heterologous lentivirus virions. *J Virol* 2001; **75**: 5129–5140.
51. Zennou V, Petit C, Guetard D, Nerhbass U, Montagnier L, Charneau P. HIV-1 genome nuclear import is mediated by a central DNA flap. *Cell* 2000; **101**: 173–185.
52. Follenzi A, Ailles LE, Bakovic S, Geuna M, Naldini L. Gene transfer by lentiviral vectors is limited by nuclear translocation and rescued by HIV-1 pol sequences. *Nat Genet* 2000; **25**: 217–222.
53. Ilyinskii PO, Desrosiers RC. Identification of a sequence element immediately upstream of the polypurine tract that is essential for replication of simian immunodeficiency virus. *EMBO J* 1998; **17**: 3766–3774.
54. Stetor SR, Rausch JW, Guo MJ, *et al*. Characterization of (+) strand initiation and termination sequences located at the center of the equine infectious anemia virus genome. *Biochemistry* 1999; **38**: 3656–3667.
55. Whitwam T, Peretz M, Poeschla E. Identification of a central DNA flap in feline immunodeficiency virus. *J Virol* 2001; **75**: 9407–9414.
56. Zufferey R, Donello JE, Trono D, Hope TJ. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 1999; **73**: 2886–2892.
57. O'Rourke JP, Hiraragi H, Urban K, Patel M, Olsen JC, Bunnell BA. Analysis of gene transfer and expression in skeletal muscle using enhanced EIAV lentivirus vectors. *Mol Ther* 2003; **7**: 632–639.
58. Brun S, Faucon-Biguet N, Mallet J. Optimization of transgene expression at the posttranscriptional level in neural cells: implications for gene therapy. *Mol Ther* 2003; **7**: 782–789.
59. Zufferey R, Dull T, Mandel RJ, *et al*. Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* 1998; **72**: 9873–9880.
60. Iwakuma T, Cui Y, Chang LJ. Self-inactivating lentiviral vectors with U3 and U5 modifications. *Virology* 1999; **261**: 120–132.
61. Schnell T, Foley P, Wirth M, Munch J, Uberla K. Development of a self-inactivating, minimal lentivirus vector based on simian immunodeficiency virus. *Hum Gene Ther* 2000; **11**: 439–447.
62. Zaiss AK, Son S, Chang LJ. RNA 3′ readthrough of oncoretrovirus and lentivirus: implications for vector safety and efficacy. *J Virol* 2002; **76**: 7209–7219.
63. Swain A, Coffin JM. Mechanism of transduction by retroviruses. *Science* 1992; **255**: 841–845.
64. Zhang QY, Clausen PA, Yatsula BA, Calothy G, Blair DG. Mutation of polyadenylation signals generates murine retroviruses that produce fused virus-cell RNA transcripts at high frequency. *Virology* 1998; **241**: 80–93.
65. Gao Z, Golob J, Tanavde VM, Civin CI, Hawley RG, Cheng L. High levels of transgene expression following transduction of long-term NOD/SCID-repopulating human cells with a modified lentiviral vector. *Stem Cells* 2001; **19**: 247–259.
66. Choi JK, Hoang N, Vilardi AM, Conrad P, Emerson SG, Gewirtz AM. Hybrid HIV/MSCV LTR enhances transgene expression of lentiviral vectors in human CD34(+) hematopoietic cells. *Stem Cells* 2001; **19**: 236–246.
67. Lai Z, Han I, Park M, Brady RO. Design of an HIV-1 lentiviral-based gene-trap vector to detect developmentally regulated genes in mammalian cells. *Proc Natl Acad Sci U S A* 2002; **99**: 3651–3656.
68. Nakajima T, Nakamaru K, Ido E, Terao K, Hayami M, Hasegawa M. Development of novel simian immunodeficiency virus vectors carrying a dual gene expression system. *Hum Gene Ther* 2000; **11**: 1863–1874.
69. Berkhout B, van Wamel J, Klaver B. Requirements for DNA strand transfer during reverse transcription in mutant HIV-1 virions. *J Mol Biol* 1995; **252**: 59–69.
70. Browning MT, Mustafa F, Schmidt RD, Lew KA, Rizvi TA. Delineation of sequences important for efficient packaging of feline immunodeficiency virus RNA. *J Gen Virol* 2003; **84**: 621–627.
71. Browning MT, Mustafa F, Schmidt RD, Lew KA, Rizvi TA. Sequences within the gag gene of feline immunodeficiency

virus (FIV) are important for efficient RNA encapsidation. *Virus Res* 2003; **93**: 199–209.
72. Berglund JA, Charpentier B, Rosbash M. A high affinity binding site for the HIV-1 nucleocapsid protein. *Nucleic Acids Res* 1997; **25**: 1042–1049.
73. Clever JL, Taplitz RA, Lochrie MA, Polisky B, Parslow TG. A heterologous, high-affinity RNA ligand for human immunodeficiency virus Gag protein has RNA packaging activity. *J Virol* 2000; **74**: 541–546.
74. Delviks KA, Hu WS, Pathak VK. Psi-vectors: murine leukemia virus-based self-inactivating and self-activating retroviral vectors. *J Virol* 1997; **71**: 6218–6224.
75. Stutz F, Rosbash M. A functional interaction between Rev and yeast pre-mRNA is related to splicing complex formation. *EMBO J* 1994; **13**: 4096–4104.
76. Gontarek RR, Derse D. Interactions among SR proteins, an exonic splicing enhancer, and a lentivirus Rev protein regulate alternative splicing. *Mol Cell Biol* 1996; **16**: 2325–2331.
77. Belshan M, Park GS, Bilodeau P, Stoltzfus CM, Carpenter S. Binding of equine infectious anemia virus rev to an exon splicing enhancer mediates alternative splicing and nuclear export of viral mRNAs. *Mol Cell Biol* 2000; **20**: 3550–3557.
78. Blencowe BJ. Exonic splicing enhancers: mechanism of action, diversity and role in human genetic diseases. *Trends Biochem Sci* 2000; **25**: 106–110.
79. Staffa A, Cochrane A. Identification of positive and negative splicing regulatory elements within the terminal tat-rev exon of human immunodeficiency virus type 1. *Mol Cell Biol* 1995; **15**: 4597–4605.
80. Mautino MR, Ramsey WJ, Reiser J, Morgan RA. Modified human immunodeficiency virus-based lentiviral vectors display decreased sensitivity to trans-dominant Rev. *Hum Gene Ther* 2000; **11**: 895–908.
81. Cui Y, Iwakuma T, Chang LJ. Contributions of viral splice sites and cis-regulatory elements to lentivirus vector function. *J Virol* 1999; **73**: 6171–6176.
82. Yoder JA, Walsh CP, Bestor TH. Cytosine methylation and the ecology of intragenomic parasites. *Trends Genet* 1997; **13**: 335–340.
83. Kempler G, Freitag B, Berwin B, Nanassy O, Barklis E. Characterization of the Moloney murine leukemia virus stem cell-specific repressor binding site. *Virology* 1993; **193**: 690–699.
84. Pannell D, Osborne CS, Yao S, *et al*. Retrovirus vector silencing is de novo methylase independent and marked by a repressive histone code. *EMBO J* 2000; **19**: 5884–5894.
85. Pannell D, Ellis J. Silencing of gene expression: implications for design of retrovirus vectors. *Rev Med Virol* 2001; **11**: 205–217.
86. Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. *Gene Ther* 1994; **1**: 136–138.
87. Osborne CS, Pasceri P, Singal R, Sukonnik T, Ginder GD, Ellis J. Amelioration of retroviral vector silencing in locus control region beta-globin-transgenic mice and transduced F9 embryonic cells. *J Virol* 1999; **73**: 5490–5496.
88. Berkhout B, van Hemert FJ. The unusual nucleotide content of the HIV RNA genome results in a biased amino acid composition of HIV proteins. *Nucleic Acids Res* 1994; **22**: 1705–1711.
89. Gerolami R, Uch R, Jordier F, *et al*. Gene transfer to hepatocellular carcinoma: transduction efficacy and transgene expression kinetics by using retroviral and lentiviral vectors. *Cancer Gene Ther* 2000; **7**: 1286–1292.
90. Pfeifer A, Ikawa M, Dayn Y, Verma IM. Transgenesis by lentiviral vectors: lack of gene silencing in mammalian embryonic stem cells and preimplantation embryos. *Proc Natl Acad Sci U S A* 2002; **99**: 2140–2145.
91. Ginn SL, Fleming J, Rowe PB, Alexander IE. Promoter interference mediated by the U3 region in early-generation HIV-1-derived lentivirus vectors can influence detection of transgene expression in a cell-type and species-specific manner. *Hum Gene Ther* 2003; **14**: 1127–1137.
92. O'Rourke JP, Newbound GC, Kohn DB, Olsen JC, Bunnell BA. Comparison of gene transfer efficiencies and gene expression levels achieved with equine infectious anemia virus- and human immunodeficiency virus type 1-derived lentivirus vectors. *J Virol* 2002; **76**: 1510–1515.
93. Demaison C, Parsley K, Brouns G, *et al*. High-level transduction and gene expression in hematopoietic repopulating cells using a human immunodeficiency [correction of immunodeficiency] virus type 1-based lentiviral vector containing an internal spleen focus forming virus promoter. *Hum Gene Ther* 2002; **13**: 803–813.
94. Tuan D, Solomon W, Li Q, London IM. The "beta-like-globin" gene domain in human erythroid cells. *Proc Natl Acad Sci U S A* 1985; **82**: 6384–6388.
95. Tuan DY, Solomon WB, London IM, Lee DP. An erythroid-specific, developmental-stage-independent enhancer far upstream of the human "beta-like globin" genes. *Proc Natl Acad Sci U S A* 1989; **86**: 2554–2558.
96. Tuan D, London IM. Mapping of DNase I-hypersensitive sites in the upstream DNA of human embryonic epsilon-globin gene in K562 leukemia cells. *Proc Natl Acad Sci U S A* 1984; **81**: 2718–2722.
97. Forrester WC, Thompson C, Elder JT, Groudine M. A developmentally stable chromatin structure in the human beta-globin gene cluster. *Proc Natl Acad Sci U S A* 1986; **83**: 1359–1363.
98. Forrester WC, Novak U, Gelinas R, Groudine M. Molecular analysis of the human beta-globin locus activation region. *Proc Natl Acad Sci U S A* 1989; **86**: 5439–5443.
99. Pasceri P, Pannell D, Wu X, Ellis J. Full activity from human beta-globin locus control region transgenes requires 5'HS1, distal beta-globin promoter, and 3′ beta-globin sequences. *Blood* 1998; **92**: 653–663.
100. May C, Rivella S, Callegari J, *et al*. Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin. *Nature* 2000; **406**: 82–86.
101. May C, Rivella S, Chadburn A, Sadelain M. Successful treatment of murine beta-thalassemia intermedia by transfer of the human beta-globin gene. *Blood* 2002; **99**: 1902–1908.
102. Lotti F, Menguzzato E, Rossi C, *et al*. Transcriptional targeting of lentiviral vectors by long terminal repeat enhancer replacement. *J Virol* 2002; **76**: 3996–4007.
103. Indraccolo S, Minuzzo S, Roccaforte F, *et al*. Effects of CD2 locus control region sequences on gene expression by retroviral and lentiviral vectors. *Blood* 2001; **98**: 3607–3617.
104. Bell AC, West AG, Felsenfeld G. The protein CTCF is required for the enhancer blocking activity of vertebrate insulators. *Cell* 1999; **98**: 387–396.
105. Recillas-Targa F, Pikaart MJ, Burgess-Beusse B, *et al*. Position-effect protection and enhancer blocking by the chicken beta-globin insulator are separable activities. *Proc Natl Acad Sci U S A* 2002; **99**: 6883–6888.
106. Udvardy A, Maine E, Schedl P. The 87A7 chromomere. Identification of novel chromatin structures flanking the heat shock locus that may define the boundaries of higher order domains. *J Mol Biol* 1985; **185**: 341–358.
107. Zhong XP, Krangel MS. An enhancer-blocking element between alpha and delta gene segments within the human T cell receptor alpha/delta locus. *Proc Natl Acad Sci U S A* 1997; **94**: 5219–5224.
108. Robinett CC, O'Connor A, Dunaway M. The repeat organizer, a specialized insulator element within the intergenic spacer of the Xenopus rRNA genes. *Mol Cell Biol* 1997; **17**: 2866–2875.
109. Modin C, Pedersen FS, Duch M. Lack of shielding of primer binding site silencer-mediated repression of an internal promoter in a retrovirus vector by the putative insulators scs, BEAD-1, and HS4. *J Virol* 2000; **74**: 11 697–11 707.
110. Ramezani A, Hawley TS, Hawley RG. Performance- and safety-enhanced lentiviral vectors containing the human interferon-beta scaffold attachment region and the chicken beta-globin insulator. *Blood* 2003; **101**: 4717–4724.
111. Gasser SM, Laemmli UK. Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of *D. melanogaster*. *Cell* 1986; **46**: 521–530.
112. Strissel PL, Espinosa R III, Rowley JD, Swift H. Scaffold attachment regions in centromere-associated DNA. *Chromosoma* 1996; **105**: 122–133.
113. Strick R, Laemmli UK. SARs are cis DNA elements of chromosome dynamics: synthesis of a SAR repressor protein. *Cell* 1995; **83**: 1137–1148.
114. Park F, Kay MA. Modified HIV-1 based lentiviral vectors have an effect on viral transduction efficiency and gene expression in vitro and in vivo. *Mol Ther* 2001; **4**: 164–173.

115. Park F, Ohashi K, Kay MA. The effect of age on hepatic gene transfer with self-inactivating lentiviral vectors in vivo. *Mol Ther* 2003; **8**: 314–323.
116. Ma Y, Ramezani A, Lewis R, Hawley RG, Thomson JA. High-level sustained transgene expression in human embryonic stem cells using lentiviral vectors. *Stem Cells* 2003; **21**: 111–117.
117. Moreau-Gaudry F, Xia P, Jiang G, *et al*. High-level erythroid-specific gene expression in primary human and murine hematopoietic cells with self-inactivating lentiviral vectors. *Blood* 2001; **98**: 2664–2672.
118. Hanawa H, Persons DA, Nienhuis AW. High-level erythroid lineage-directed gene expression using globin gene regulatory elements after lentiviral vector-mediated gene transfer into primitive human and murine hematopoietic cells. *Hum Gene Ther* 2002; **13**: 2007–2016.
119. Richard E, Mendez M, Mazurier F, *et al*. Gene therapy of a mouse model of protoporphyria with a self-inactivating erythroid-specific lentiviral vector without preselection. *Mol Ther* 2001; **4**: 331–338.
120. Cui Y, Golob J, Kelleher E, Ye Z, Pardoll D, Cheng L. Targeting transgene expression to antigen-presenting cells derived from lentivirus-transduced engrafting human hematopoietic stem/progenitor cells. *Blood* 2002; **99**: 399–408.
121. Marodon G, Mouly E, Blair EJ, Frisen C, Lemoine FM, Klatzmann D. Specific transgene expression in human and mouse CD4+ cells using lentiviral vectors with regulatory sequences from the CD4 gene. *Blood* 2003; **101**: 3416–3423.
122. De Palma M, Venneri MA, Naldini L. In vivo targeting of tumor endothelial cells by systemic delivery of lentiviral vectors. *Hum Gene Ther* 2003; **14**: 1193–1206.
123. Lai Z, Brady RO. Gene transfer into the central nervous system in vivo using a recombinant lentivirus vector. *J Neurosci Res* 2002; **67**: 363–371.
124. Miyoshi H, Takahashi M, Gage FH, Verma IM. Stable and efficient gene transfer into the retina using an HIV-based lentiviral vector. *Proc Natl Acad Sci U S A* 1997; **94**: 10 319–10 323.
125. Kostic C, Chiodini F, Salmon P, *et al*. Activity analysis of housekeeping promoters using self-inactivating lentiviral vector delivery into the mouse retina. *Gene Ther* 2003; **10**: 818–821.
126. Dryja TP, Li T. Molecular genetics of retinitis pigmentosa. *Hum Mol Genet* 1995; **4**: **Spec No**: 1739–1743.
127. Follenzi A, Sabatino G, Lombardo A, Boccaccio C, Naldini L. Efficient gene delivery and targeted expression to hepatocytes in vivo by improved lentiviral vectors. *Hum Gene Ther* 2002; **13**: 243–260.
128. Oertel M, Rosencrantz R, Chen YQ, *et al*. Repopulation of rat liver by fetal hepatoblasts and adult hepatocytes transduced ex vivo with lentiviral vectors. *Hepatology* 2003; **37**: 994–1005.
129. Uch R, Gerolami R, Faivre J, *et al*. Hepatoma cell-specific ganciclovir-mediated toxicity of a lentivirally transduced HSV-TkEGFP fusion protein gene placed under the control of rat alpha-fetoprotein gene regulatory sequences. *Cancer Gene Ther* 2003; **10**: 689–695.
130. Muschenheim F, Omarbasha B, Kardjian PM, Mondou EN. Screening for carcinoma of the prostate with prostate specific antigen. *Ann Clin Lab Sci* 1991; **21**: 371–380.
131. Cadeddu JA, Pearson JD, Partin AW, Epstein JI, Carter HB. Relationship between changes in prostate-specific antigen and prognosis of prostate cancer. *Urology* 1993; **42**: 383–389.
132. Yu D, Chen D, Chiu C, Razmazma B, Chow YH, Pang S. Prostate-specific targeting using PSA promoter-based lentiviral vectors. *Cancer Gene Ther* 2001; **8**: 628–635.
133. Zheng JY, Chen D, Chan J, Yu D, Ko E, Pang S. Regression of prostate cancer xenografts by a lentiviral vector specifically expressing diphtheria toxin A. *Cancer Gene Ther* 2003; **10**: 764–770.
134. Lois C, Hong EJ, Pease S, Brown EJ, Baltimore D. Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 2002; **295**: 868–872.
135. Reiser J, Lai Z, Zhang XY, Brady RO. Development of multigene and regulated lentivirus vectors. *J Virol* 2000; **74**: 10 589–10 599.
136. Kafri T, van Praag H, Gage FH, Verma IM. Lentiviral vectors: regulated gene expression. *Mol Ther* 2000; **1**: 516–521.
137. Ogueta SB, Yao F, Marasco WA. Design and in vitro characterization of a single regulatory module for efficient control of gene expression in both plasmid DNA and a self-inactivating lentiviral vector. *Mol Med* 2001; **7**: 569–579.
138. Vigna E, Cavalieri S, Ailles L, *et al*. Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors. *Mol Ther* 2002; **5**: 252–261.
139. Regulier E, Pereira de Almeida L, Sommer B, Aebischer P, Deglon N. Dose-dependent neuroprotective effect of ciliary neurotrophic factor delivered via tetracycline-regulated lentiviral vectors in the quinolinic acid rat model of Huntington's disease. *Hum Gene Ther* 2002; **13**: 1981–1990.
140. Johansen J, Rosenblad C, Andsberg K, *et al*. Evaluation of Tet-on system to avoid transgene down-regulation in ex vivo gene transfer to the CNS. *Gene Ther* 2002; **9**: 1291–1301.
141. Koponen JK, Kankkonen H, Kannasto J, *et al*. Doxycycline-regulated lentiviral vector system with a novel reverse transactivator rtTA2S-M2 shows a tight control of gene expression in vitro and in vivo. *Gene Ther* 2003; **10**: 459–466.
142. Salmon P, Oberholzer J, Occhiodoro T, Morel P, Lou J, Trono D. Reversible immortalization of human primary cells by lentivector-mediated transfer of specific genes. *Mol Ther* 2000; **2**: 404–414.
143. Mitta B, Rimann M, Ehrengruber MU, *et al*. Advanced modular self-inactivating lentiviral expression vectors for multigene interventions in mammalian cells and in vivo transduction. *Nucleic Acids Res* 2002; **30**: e113.
144. Chang LJ, Zaiss AK. Self-inactivating lentiviral vectors and a sensitive Cre-loxP reporter system. *Methods Mol Med* 2003; **76**: 367–382.
145. Karreman S, Hauser H, Karreman C. On the use of double FLP recognition targets (FRTs) in the LTR of retroviruses for the construction of high producer cell lines. *Nucleic Acids Res* 1996; **24**: 1616–1624.
146. Chantret I, Dupre T, Delenda C, *et al*. Congenital disorders of glycosylation type Ig is defined by a deficiency in dolichyl-P-mannose:Man7GlcNAc2-PP-dolichyl mannosyltransferase. *J Biol Chem* 2002; **277**: 25 815–25 822.
147. Chantret I, Dancourt J, Dupre T, *et al*. A deficiency in dolichyl-P-glucose : Glc1Man9GlcNAc2-PP-dolichyl alpha3-glucosyltransferase defines a new subtype of congenital disorders of glycosylation. *J Biol Chem* 2003; **278**: 9962–9971.
148. Richard E, Geronimi F, Lalanne M, *et al*. A bicistronic SIN-lentiviral vector containing G156A MGMT allows selection and metabolic correction of hematopoietic protoporphyric cell lines. *J Gene Med* 2003; **5**: 737–747.
149. Azzouz M, Martin-Rendon E, Barber RD, *et al*. Multicistronic lentiviral vector-mediated striatal gene transfer of aromatic L-amino acid decarboxylase, tyrosine hydroxylase, and GTP cyclohydrolase I induces sustained transgene expression, dopamine production, and functional improvement in a rat model of Parkinson's disease. *J Neurosci* 2002; **22**: 10 302–10 312.
150. Zhu Y, Feuer G, Day SL, Wrzesinski S, Planelles V. Multigene lentiviral vectors based on differential splicing and translational control. *Mol Ther* 2001; **4**: 375–382.
151. Zhu Y, Planelles V. A multigene lentiviral vector system based on differential splicing. *Methods Mol Med* 2003; **76**: 433–448.
152. Murthy RC, McFarland TJ, Yoken J, *et al*. Corneal transduction to inhibit angiogenesis and graft failure. *Invest Ophthalmol Vis Sci* 2003; **44**: 1837–1842.
153. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 2000; **404**: 293–296.
154. McCaffrey AP, Meuse L, Pham TT, Conklin DS, Hannon GJ, Kay MA. RNA interference in adult mice. *Nature* 2002; **418**: 38–39.
155. Gitlin L, Karelsky S, Andino R. Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 2002; **418**: 430–434.
156. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. *Nature* 2002; **418**: 435–438.
157. An DS, Wersto RP, Agricola BA, *et al*. Marking and gene expression by a lentivirus vector in transplanted human and nonhuman primate CD34(+) cells. *J Virol* 2000; **74**: 1286–1295.
158. Stewart SA, Dykxhoorn DM, Palliser D, *et al*. Lentivirus-delivered stable gene silencing by RNAi in primary cells. *RNA* 2003; **9**: 493–501.

159. Rubinson DA, Dillon CP, Kwiatkowski AV, *et al*. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. *Nat Genet* 2003; **33**: 401–406.
160. Tiscornia G, Singer O, Ikawa M, Verma IM. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. *Proc Natl Acad Sci U S A* 2003; **100**: 1844–1848.
161. Abbas-Terki T, Blanco-Bose W, Deglon N, Pralong W, Aebischer P. Lentiviral-mediated RNA interference. *Hum Gene Ther* 2002; **13**: 2197–2201.
162. Scherr M, Battmer K, Ganser A, Eder M. Modulation of gene expression by lentiviral-mediated delivery of small interfering RNA. *Cell Cycle* 2003; **2**: 251–257.
163. Banerjea A, Li MJ, Bauer G, *et al*. Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. *Mol Ther* 2003; **8**: 62–71.
164. Li MJ, Bauer G, Michienzi A, *et al*. Inhibition of HIV-1 infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. *Mol Ther* 2003; **8**: 196–206.
165. Qin XF, An DS, Chen IS, Baltimore D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci U S A* 2003; **100**: 183–188.
166. Dirac AM, Bernards R. Reversal of senescence in mouse fibroblasts through lentiviral suppression of p53. *J Biol Chem* 2003; **278**: 11 731–11 734.
167. Matta H, Hozayev B, Tomar R, Chugh P, Chaudhary PM. Use of lentiviral vectors for delivery of small interfering RNA. *Cancer Biol Ther* 2003; **2**: 206–210.
168. Carroll R, Lin JT, Dacquel EJ, Mosca JD, Burke DS, St Louis DC. A human immunodeficiency virus type 1 (HIV-1)-based retroviral vector system utilizing stable HIV-1 packaging cell lines. *J Virol* 1994; **68**: 6047–6051.
169. Corbeau P, Kraus G, Wong-Staal F. Efficient gene transfer by a human immunodeficiency virus type 1 (HIV-1)-derived vector utilizing a stable HIV packaging cell line. *Proc Natl Acad Sci U S A* 1996; **93**: 14 070–14 075.
170. Yu H, Rabson AB, Kaul M, Ron Y, Dougherty JP. Inducible human immunodeficiency virus type 1 packaging cell lines. *J Virol* 1996; **70**: 4530–4537.
171. Kaul M, Yu H, Ron Y, Dougherty JP. Regulated lentiviral packaging cell line devoid of most viral cis-acting sequences. *Virology* 1998; **249**: 167–174.
172. Kafri T, van Praag H, Ouyang L, Gage FH, Verma IM. A packaging cell line for lentivirus vectors. *J Virol* 1999; **73**: 576–584.
173. Klages N, Zufferey R, Trono D. A stable system for the high-titer production of multiply attenuated lentiviral vectors. *Mol Ther* 2000; **2**: 170–176.
174. Farson D, Witt R, McGuinness R, *et al*. A new-generation stable inducible packaging cell line for lentiviral vectors. *Hum Gene Ther* 2001; **12**: 981–997.
175. Xu K, Ma H, McCown TJ, Verma IM, Kafri T. Generation of a stable cell line producing high-titer self-inactivating lentiviral vectors. *Mol Ther* 2001; **3**: 97–104.
176. Sparacio S, Pfeiffer T, Schaal H, Bosch V. Generation of a flexible cell line with regulatable, high-level expression of HIV Gag/Pol particles capable of packaging HIV-derived vectors. *Mol Ther* 2001; **3**: 602–612.
177. Pacchia AL, Adelson ME, Kaul M, Ron Y, Dougherty JP. An inducible packaging cell system for safe, efficient lentiviral vector production in the absence of HIV-1 accessory proteins. *Virology* 2001; **282**: 77–86.
178. Ikeda Y, Takeuchi Y, Martin F, Cosset FL, Mitrophanous K, Collins M. Continuous high-titer HIV-1 vector production. *Nat Biotechnol* 2003; **21**: 569–572.
179. Rolls MM, Webster P, Balba NH, Rose JK. Novel infectious particles generated by expression of the vesicular stomatitis virus glycoprotein from a self-replicating RNA. *Cell* 1994; **79**: 497–506.
180. Kaplan AH, Swanstrom R. The HIV-1 gag precursor is processed via two pathways: implications for cytotoxicity. *Biomed Biochim Acta* 1991; **50**: 647–653.

