



Beyond retrovirus infection: HIV meets gene therapy

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Abstract

The human immunodeficiency virus (HIV) is classified as a retrovirus because of its RNA genome and the fact that it requires reverse transcriptase to convert it into DNA. This virus belongs to the *lentivirinae* subfamily and is able to infect quiescent cells but is better known for its association with acquired immunodeficiency syndrome (AIDS) and can be described as one of the most effective vectors for gene transfer. Biosafety concerns are present whenever viral vectors are employed but are particularly pertinent to the development of HIV-based vectors. Insertional mutagenesis and the production of new replication-competent viruses (RCV) have been pointed to as major problems, but experimental data have shown that safe protocols can be developed for their production and application. Virological, evolutionary, immunological and cell biology studies must be conducted jointly to allow the clinical use of HIV vectors. This review will focus on the general properties, production and applications of retrovectors in gene therapy, with particular emphasis on those based on HIV systems.

Key words: HIV, MLV, retrovirus, lentivirus, gene therapy.

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Introduction

The basic principle of gene therapy is the introduction of exogenous genetic material to correct or modify the cell functions. The development of methods for the delivery of therapeutic genes to specific target cells has been under intense investigation for the last decade (reviewed in Romano *et al.*, 2000; Amalfitano and Parks, 2002). Although the identification of the appropriate therapeutic gene as well as the target cell are essential for successful gene therapy, identifying the most suitable gene transfer method for a determined group of target cells is also of fundamental importance.

The vectors used to transfer genes are basically classified as viral or non-viral, each presenting its own benefits and drawbacks. Retroviruses are largely used as stable gene transfer systems (reviewed in Chu *et al.*, 1998), and among them lentiviruses have been increasingly used due to their greater ability to transduce quiescent cells. This review will focus on the general properties, production and applications of retrovectors, with emphasis on HIV-based vectors.

General Properties of Retroviruses

All members of the family Retroviridae (oncovirus, lentivirus and spumavirus) present a RNA genome which is retro-transcribed into DNA by the enzyme reverse transcriptase. When this process is concluded (if all the proteins, cellular and viral factors required for reverse transcription are present) the provirus is able to integrate into the genome of the host cell. This is possible because regulatory signals are present in terminal regions of the provirus genome, named long terminal repeats (LTRs) (Figure 1). The viral genome also presents enhancers and other elements that regulate the expression of viral genes. Such enhancers can be localized within LTRs and other regulatory functions can be provided by accessory and regulatory genes such as *tat* and *rev* as well as *vif*, *vpr*, *vpu* and *nef*. The *tat* gene is a transactivator of the 5' LTR that acts as a viral promoter while the Rev protein is necessary to transport the viral transcripts from the nucleus to the cytoplasm mediated by the *rev* responsive element (RRE). Other accessory genes can be related to virulence and immune system gene modulation (Lanciotti, 2001).

In the genome of all retroviruses there are at least three open reading frames (ORFs) each of which encodes for a different viral element, e.g. the capsid and matrix (*gag* gene), enzymes (*pol* gene) and envelope (*env* gene)

(Figure 1). The capsid encases the genome and protects the viral genetic material, while enzymes are needed for viral replication and integration of the provirus and the envelope consists of glycoproteins that recognize specific cell receptors and determine the tropism of infection. Members of the *Lentivirinae* subfamily present a more complex genome than members of the *Oncovirinae* subfamily because the former contain additional regulatory and accessory genes (Swanstrom and Wills, 1997).

Integration of the viral genome into the host genome depends on its ability to enter the cell nucleus. In oncoviruses this is only possible when the nuclear membrane disintegrates during cell division but lentiviruses have an active

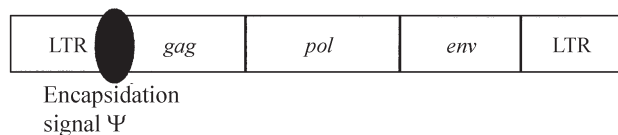


Figure 1 - Genomic organization of a typical retrovirus genome. Additional genes are present in lentiviruses such as HIV.

transport mechanism which allows the provirus to enter the nucleus through the pores in the nuclear membrane (Bukrinsky *et al.*, 1992). The active transport of lentivirus provirus is in part mediated by nuclear localization sequences (NLS) and the formation of proteic transportation complexes dependent on accessory genes such as HIV *vpr* (Naldini *et al.*, 1996a and b; Vodicka *et al.*, 1998) or on mechanisms regulating retrotranscription, such as strand displacement (resulting from the synthesis of double strand DNA from viral RNA) present in the pre-integration complex (PIC) (Klarmann *et al.*, 2003) (Figure 2). These are very important features in the context of gene therapy because such properties mean that HIV can be used in a different group of target cells in comparison to MLV-based vectors. For example, to transduce human hematopoietic progenitors in the absence of cytokine stimulation it may be better to use HIV-based vectors than murine leukemia virus (MLV) vectors (Salmon *et al.*, 2000).

The most famous lentivirus is the human immunodeficiency virus (HIV) which is historically related to the ac-

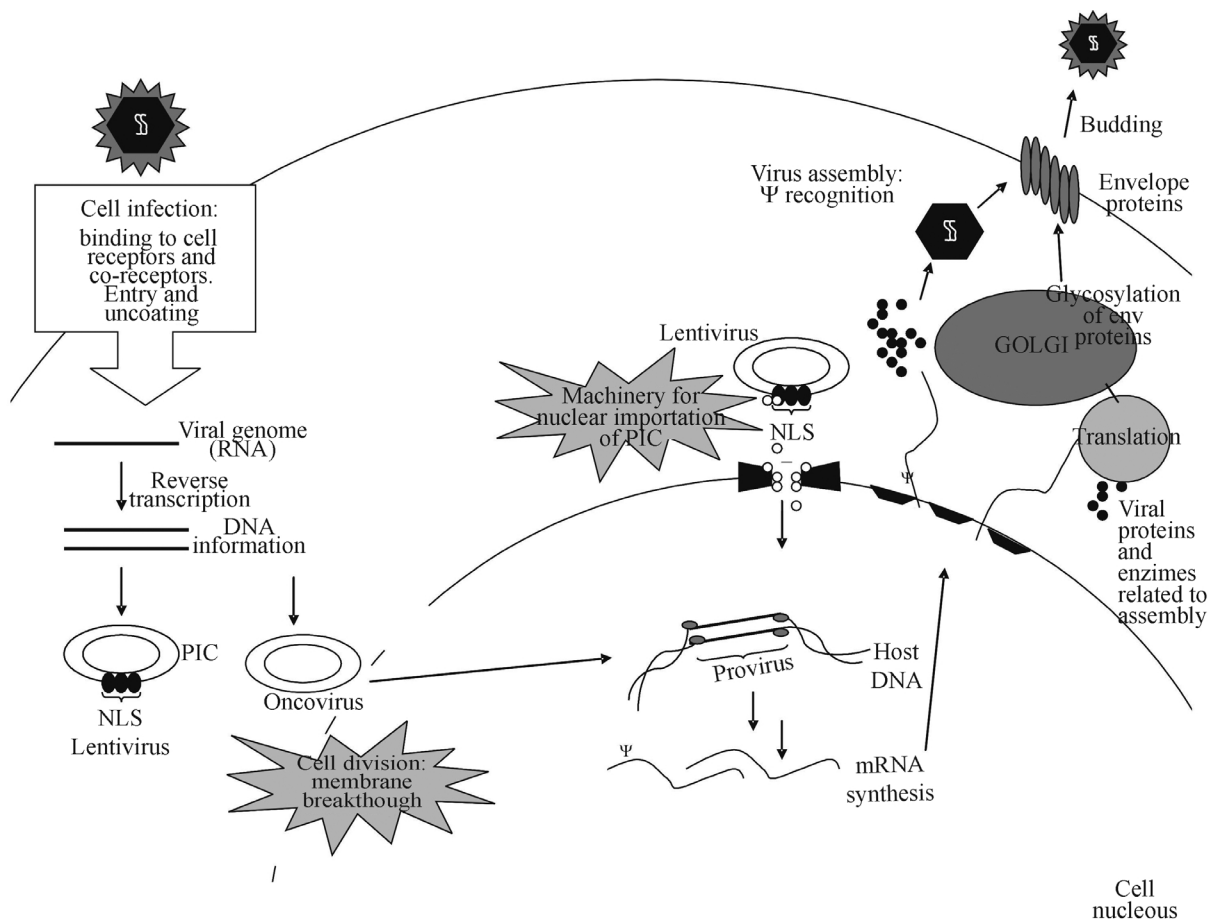


Figure 2 - Schematic diagram of cell infection by retrovirus. Infection starts by binding to cell receptors, which leads to virus entry and uncoating. Retrotranscription occurs in the cytoplasm. Lentiviruses have a nuclear localization signal (NLS) in their preintegration complex (PIC) that interacts with an active nuclear importation machinery dependent on viral and host factors. Oncoviruses do not present this system, and thus depend on membrane breakthrough to access the nucleus. In the nucleus, proviruses will integrate into the host DNA genome. Viral assembly occurs in the cytoplasm, and viruses are liberated from the infected cell by budding.

quired immune deficiency syndrome (AIDS) pandemic and still poorly understood despite the large body of knowledge resulting from intense research over the last twenty years (reviewed in Lever *et al.*, 2004). HIV preferentially infects cells possessing the CD4 surface marker, such as macrophages and helper T lymphocytes, although it may also bind to the CCR5 and CXCR4 co-receptors (Holmes *et al.*, 2001). In order to expand the possible applications of HIV vectors in gene therapy, it is necessary to use different envelope constructions that may enable the infection of different target cells. This process, called pseudotyping (Burns *et al.*, 1993), was initially designed for vectors based on MLV (Yee *et al.*, 1994) but has now been applied to several other viral vectors and has allowed the manipulation and concentration of viral stocks.

Production of Retrovectors

The construction of viral vectors implies the removal of some of the genes present in the wild type virus, since the vectors should be able to infect cells, retrotranscribe, integrate its genome into the host DNA and express the therapeutic gene without causing any disease. Specific genes and sequences related to virulence or unnecessary to transgene expression must be deleted and interesting genes may fill the available space. These minimal helper-packaging elements (or constructs) are the basis for the genetic engineering of virus-based vectors and the procedure itself is called transient transfection (Pear *et al.*, 1993). Transient transfection relies on the independent delivery of such constructs, each expressing a few primary elements for the assembly of viable viral particles (structural and envelope genes, for example). The construct that represents the encapsidated genome is the therapeutic vector, containing all the sequences required for the expression of the transgene in the target cells (reviewed in Delenda, 2004). The use of separated constructs is said to reduce the formation of helper-virus (replication-competent viruses or RCV) because more recombinational events would be necessary to restore the wild-type phenotype. (Soneoka *et al.*, 1995) (Figure 3).

The industrial scale-up production of viral vectors through transient transfection is still an issue, since this procedure is not as homogenous as it should be. The titers achieved are somewhat variable and the concentration of viral stocks is still to be standardized (Pagès and Bru, 2004). These limitations explain why there is a great effort from the scientific community towards the improvement of viral vector production in a standardized manner, possibly mediated by stable packaging cell lines (PCLs). Table 1 presents examples showing that transiently transfected cells may lead to stable PCLs if cell clones that stably produce viral vectors are selected.

Stable PCLs constitutively express some of the chosen genes which may be subjected to the regulation of inducible systems. To activate virus production, the inductor

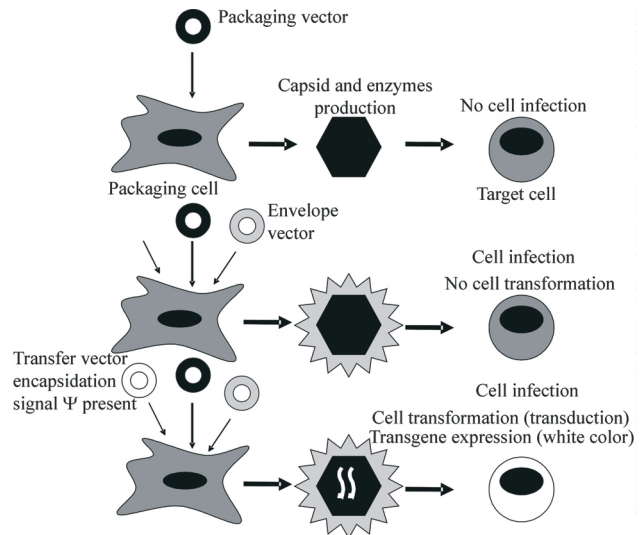


Figure 3 - Production of retrovectors by transient transfection (adapted from Silva, 2002). Packaging cell lines are transiently transfected with the vectors responsible for the assembly of recombinant viruses. In cases (A) and (B), retrovectors are not functional. Complete vectors able to infect and transduce target cells are produced only when the packaging cells are transfected with the three plasmids (C).

element must be present in the cultures, as is the case of the tetracycline-induced systems described by Chen *et al.* (1996), Farson *et al.* (2001) and Xu *et al.* (2001). Since PCLs can be previously characterized and tested for RCV production, the production of viral vector becomes safer when stable transfection is combined with regulated expression systems. If the titers achieved are not high enough, or if RCV is detected, the PCL can be eliminated and another stable clone tested. This is a time-consuming method but worth performing since yield and quality may differ widely among stable and non-stable PCLs (Corbeau *et al.*, 1996; Klages *et al.*, 2000). Comparative studies involving vectors produced on a large scale are important in validating these arguments, particularly if the transfection methods can be compared in the same cell lineage.

Cell lines present different susceptibility to transfection (reviewed by Pagès and Bru, 2004). The most frequently used PCL is currently 293, but other cell types are being investigated. We have recently developed an interesting alternative PCL for the production of HIV-based vectors (Silva *et al.*, unpublished results) using a murine mesenchymal stem cell line previously established in our laboratory (Meirelles and Nardi, 2003).

The approval of a retroviral PCL, whether transient or stable, depends also on the pattern of insertional mutagenesis presented by the viruses produced and potential recombination with human endogenous retroviral sequences or wild type viruses (Cornetta *et al.*, 1991). These concerns are increased when the wild type virus involved is related to a human severe pathology such as AIDS but the reduction of overlapping sequences between therapeutic vectors and packaging components has been proved to reduce RCV for-

Table 1 - Characteristics of some of the protocols available for retrovector production.

Virus (envelope) ¹	PCL cell line (Titer) ²	PCL ³	RCV content ⁴	Conc ⁵	Observations and Reference
MLV (WT)	293 (10 ⁹ CFU mL ⁻¹)	T	NM	Y	Rendered a stable PCL (BOSC 23). Burns <i>et al.</i> , 1993
F-MLV (WT)	293T (> 10 ⁶ beta-gal+ cells mL ⁻¹)	T	HF	N	Pear <i>et al.</i> , 1993
MLV (VSV-g)	293GP, PA317, rat 208F (10 ⁵ -10 ⁶ CFU mL ⁻¹)	T	HF	Y	Yee <i>et al.</i> , 1994
MLV (WT & RD114)	HT1080 (> 10 ⁷ IU mL ⁻¹)	T	NM	N	Rendered a stable PCL. Cosset <i>et al.</i> , 1995
MLV (WT)	NIH 3T3, 293T (> 10 ⁷ IU mL ⁻¹)	T	HF	N	Sodium butyrate induction to improve titers. Soneoka <i>et al.</i> , 1995
RV (VSV-g)	Rat 208F, 293GP, HT1080 (10 ⁴ -10 ⁵ CFU mL ⁻¹)	T	HF	N	VSV-g expression is regulated by tetracycline; rendered stable PCL. Chen <i>et al.</i> , 1996
MLV (VSV-g)	293GPG (10 ⁶ - 10 ⁷ CFU mL ⁻¹)	T	Evaluated; HF	N	VSV-g expression is regulated by of tetracycline; rendered stable PCL. Ory <i>et al.</i> , 1996
RV (VSV-g)	DA, 2A, HX, 2X (10 ⁵ -10 ⁸ CFU mL ⁻¹)	T	12%, from 77 lots	N	Rendered stable PCL. Sheridan <i>et al.</i> , 2000
MLV (WT)	Suspension of CEM and Namalwa cells (10 ⁶ -10 ⁷ IU mL ⁻¹)	T	Evaluated; HF	N	Rendered stable PCL. Pizzato <i>et al.</i> , 2001
HIV (WT)	Fibroblast (> 10 ⁵ transduced cells mL ⁻¹)	S	NM	N	Stable PCL; transfer vector transiently transfected to produce viral particles. Corbeau <i>et al.</i> , 1993
HIV (VSV-g)	293 (10 ⁹ IU mL ⁻¹)	T	Evaluated; HF	Y	Tetracycline-regulated PCL. Kafri <i>et al.</i> , 1999
HIV (VSV-g)	293 (1-20 HeLa transducing units per cell per day)	S	NM	N	Stable; doxycycline-repressible expression of HIV-1 Rev/Gag/Pol and VSV-g envelope. Klages <i>et al.</i> , 2000
HIV (VSV-g, MLV & Rabies)	293 (10 ⁹ TU mL ⁻¹)	T	NM	Y	Evaluates cell culture medium composition and concentration of stocks. Reiser, 2000
HIV (VSV-g)	293T (> 10 ⁶ IU mL ⁻¹)	T	NM	Y	Rendered stable PCL; regulated by tetracycline system. Xu <i>et al.</i> , 2001
HIV (WT)	293 (High titers)	T	NM	N	Regulated by doxycycline; it is employed to investigate HIV vaccines. Verhoef <i>et al.</i> , 2001
HIV (VSV-g)	293G (50 ng mL ⁻¹ 24h ⁻¹ Gag p24)	S, I	Evaluated; HF	N	Minimal packaging construct of HIV-1 gag-pol, tat and rev genes was used. Farson <i>et al.</i> , 2001
HIV (VSV-g)	293T (High titers)	T	HF	N	PCL was supplied with purified Rev protein; rev gene is dispensable. Kowolik <i>et al.</i> , 2003
HIV (Alphavirus)	293T (10 ⁷ IU mL ⁻¹)	T, S	NM	Y	Expression of alphavirus receptors may be limited in certain cell types. Strang <i>et al.</i> , 2005

¹MLV = murine leukemia virus; RV= retrovirus; HIV = human immunodeficiency virus; VSV = vesicular stomatitis virus; WT = wild type. ²PCL = packaging cell line; CFU = colony-forming units; IU= infection units; PFU = plaque-forming units; TU = transducing units; pgGagp24 = picograms of Gag p24 protein. ³I = inducible; S = stable; T = Transient. ⁴RCV = replication-competent viruses: HF = Helper-free; NM = Not mentioned. ⁵Conc = concentrated stocks available: Y= yes; N = no.

mation and may be helpful in overcoming these problems (Otto *et al.*, 1994; Sheridan *et al.*, 2000). These aspects of vector production need to be properly measured, but as shown in Table 1 there are different ways of quantifying viruses and detecting RCVs. Padronization is also needed to compare the results from different research groups.

The first clinical-grade HIV lentiviral vector has already been tested in gene therapy for transgene *ex vivo* delivery, but still needs to be evaluated in a phase three clinical trial confronting a greater number of individuals (Manilla *et al.*, 2005). Three clinical protocols are being developed to evaluate the application of this specific HIV

construct in anti-AIDS therapy, through different approaches (Gene Therapy Clinical Protocols Worldwide, 2005). These trials are a hallmark in the establishment of lentivector gene transfer protocols, and may be helpful in consolidating HIV vectors as safe and efficient tools for clinical cell transduction

Because recombination events between therapeutic vectors and endogenous retroviral sequences have been reported (Chong *et al.*, 1998) the development of sensitive assays to specifically detect recombinant lentiviral DNA mobilization would be of great value for clinical application (Wu *et al.*, 2000; Sastry *et al.*, 2003). Contamination of

vector supernatants with packaging cell line DNA can also be a source of false positive results in tests for RCV detection if no functional tests are simultaneously done (Chen *et al.*, 2001). These techniques can also help to standardize virus production, not only providing a reproducible method for viral titration (Sastry *et al.*, 2002) but also improving safety tests. It is expected that new assays for the determination of safety level will lead to the improvement of viral vectors, particularly HIV-based vectors. Recently, a specific methodology was described to evaluate the RCV content in HIV vectors produced on a large scale (Escarpe *et al.*, 2003).

Features of the HIV Virus

The development of HIV vectors for anti-HIV gene therapy is the most logical application of this system, but the ability of HIV to specifically target CD4+ non-cycling cells makes it a promising candidate for many other situations in which *in vivo* gene delivery is important (Poeschla *et al.*, 1996). In fact, this possibility has led to experimental evidence for its application in the transduction of a wide variety of cell types, such as hematopoietic progenitor and stem cells (Reiser *et al.*, 1996; Case *et al.*, 1999), post-mitotic neurons (Naldini *et al.*, 1996a; Reiser *et al.*, 1996), fibroblasts and human primary macrophages (Naldini *et al.*, 1996b), and primary human marrow stroma (Bahner *et al.*, 1997).

To produce viral vectors it is possible to employ first, second and third generation systems based on the number of plasmids to be transfected to the PCL (Figure 4). First generation vectors present only two plasmids, so that two combinations are possible: envelope and packaging vector or packaging with intact envelope sequence and the therapeutic vector. In terms of HIV vectors, two-plasmid systems have been described in the past, but few reports present *env* deletion in the structural vector (Page *et al.*, 1990; Landau *et al.*, 1991; Reiser *et al.*, 1996).

A three-plasmid system, which includes one therapeutic vector, one structural vector without the wild type HIV *env* gene and one additional VSV-G envelope vector, was described by Naldini *et al.* (1996a) as an efficient system to achieve gene transfer to the central nervous system - a logical application if we consider that neurons are quiescent or poor mitotic cells and that HIV is a lentivector. Blömer *et al.* (1997) employed this HIV-based system in the murine central nervous during a comparative study including oncoviral (based on MLV), adenoviral and adeno-associated viral systems but only the lentiviral vector was able to efficiently and stably infect quiescent cells, allowing *b-galactosidase* transgene expression for over six months. Mordelet *et al.* (2002) were able to modify the protocol used by Blömer *et al.* (1997) to reinforce the efficiency of HIV-based vectors in transferring genes to the central nervous system. It is important to state, however, that adeno-associated viral vectors can also infect this tissue, so the transduction efficiency (but not long term stable transgene expression) of these two types of viral vector may be similar.

Second generation vectors include three plasmid systems, which have been important in the design of safer HIV vectors because they are less prone to interplasmid recombination leading to restoration of the wild-type phenotype and also do not transfer packaging and wild-type envelope sequences to target cells.

Third generation vectors are now considered the safest HIV vectors available. These vectors are produced from four different plasmids, *i.e.* therapeutic vector, envelope vector, packaging vector (containing *gag*, *pol* and the responsive element of *rev* gene) and the *rev* vector (Dull *et al.*, 1998). The presence of *tat* in packaging vectors is a controversial issue because it is not interesting that the 5' LTR of HIV-based vectors is transactivated by *tat*, since interference of the LTR promoter with the internal promoter may reduce the level of expression of the transgene (Cui *et al.*, 1999; Zaiss *et al.*, 2002). *Rev* gene, however, is consid-

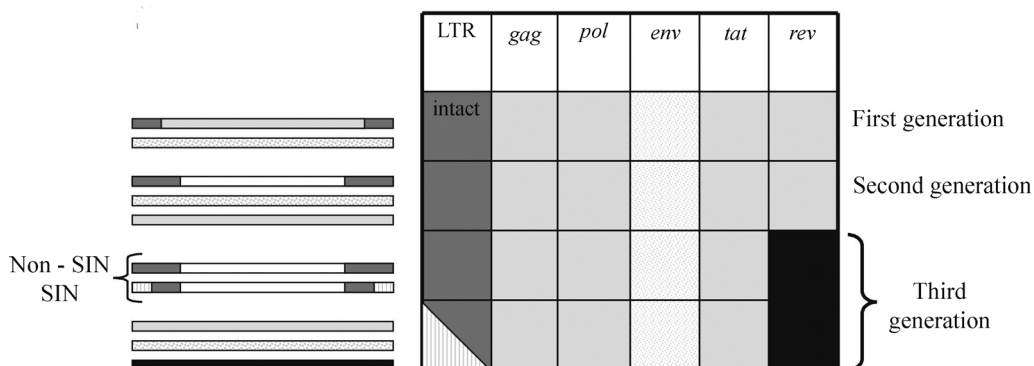


Figure 4 - Schematic diagram of first, second and third generation vectors. Long terminal repeats (LTR, dark gray), structural and accessory genes from packaging constructs (gray) and envelope construct (light gray). Self-inactivating (SIN) vectors are represented by altering the LTR shade (gray and light gray). First generation vectors are derived from only two constructs (transfer and envelope). Second generation vectors present three constructs (transfer, packaging and envelope). Third generation vectors present the *rev* gene in a different construct (black) with a total of four plasmids: transfer (SIN or non-SIN), packaging (*gag*, *pol* and *tat* genes only), *rev* and envelope vectors.

ered necessary for the production of high titers and higher transduction efficiency, when appropriate *rev* responsive elements are present (Parolin *et al.*, 1994).

Another important feature of retroviral vectors that has contributed positively to the design of HIV-based vectors is the development of self-inactivating (SIN) vectors (Figure 5), characterized by one-way infection of target cells - an important advance for safer retroviral gene therapy protocols. Deletions of specific regions of viral LTR can impair the replication potential of a virus without altering gene transfer efficiency (Miyoshi *et al.*, 1998).

Long terminal repeats are very important for virus integration and replication and are composed of three parts (U3, R and U5), the LTR only being complete at the provirus level. The U3 region is partial or totally deleted at the 3' LTR and after reverse transcription this deletion can be transferred to the 5' LTR of the provirus maintaining the integration of regulatory signals but impairing the ability of the provirus to replicate so that the LTR is transcriptionally inactive in the provirus (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998; Iwakuma *et al.*, 1999). However, Logan *et al.* (2004) recently reported integrated self-inactivating lentiviral vectors capable of producing full-length genomic transcripts competent for encapsidation and integration, showing that refined molecular studies are needed to assess the safety of SIN HIV vectors.

If the 5' U3 is also deleted, the space left can be used to clone a heterologous promoter, resulting in the provirus being a SIN vector without LTR promoter activity. This molecular alteration results in a *tat*-independent transcriptional mechanism, so the viral promoter does not interfere with the heterologous promoter and the possibility of activation of cellular genes flanking the vector integration site is reduced (Yam *et al.*, 2002).

Regardless of which U3 is altered, SIN and non-SIN vectors are capable of integrating the provirus into the host genome, although this may result in insertional mutagenesis (Cornetta *et al.*, 1991). The first clinical protocol was performed with infusion of autologous T-lymphocytes ex-

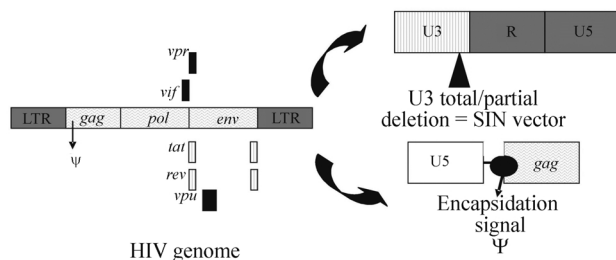


Figure 5 - Alterations of the HIV genome. Genes such as *vpr*, *vif* and *vpu* that are deleted from the transfer vector (black). Structural genes such as *gag*, *pol* and *env* (light gray). Long terminal repeat composed of the U3, R and U5 segments (gray). When the U3 segment is partially or almost completely deleted a self inactivating (SIN) vector is formed. Besides U3 modification, it is important to maintain the encapsidation signal ψ in the transfer vector. Because the ψ signal presents an overlapping of sequences with *gag* gene, *gag* deletions must be carefully analyzed.

panded and transduced *in vitro* by a MLV vector with the normal adenosine deaminase gene (Blaese *et al.*, 1995; Bordignon *et al.*, 1995). The follow-up study involved two years of treatment and two years of assessment, after which the retroviral gene therapy protocol was considered successful despite the need for simultaneous exogenous enzyme replacement therapy (Blaese *et al.*, 1995).

Cavazzana-Calvo *et al.* (2000) proposed a retroviral gene therapy protocol to deliver a copy of normal γ c cytokine receptor subunit of interleukine-2, -4, -7, -9 and -15 receptors to correct X-linked human severe combined immunodeficiency (SCID-X1). As the adenosine deaminase protocol, target cells (CD34+ cells) were transduced *in vitro* and reinfused into the patients. However Hacein-Bey-Abina *et al.* (2003) reported that two of the treated patients presented pre-malignant cell proliferation as a consequence of MLV vector integration close to the LMO2 gene promoter (a proto-oncogene). These findings had a negative impact on viral gene therapy (Check, 2002a and b), the same having happened after the death of Jesse Gelsinger in 1999 due to gene therapy treatment (Belgian Biosafety Server, 2005). The risks of developing long-term cancer because of insertional mutagenesis associated with retroviral vectors is still an issue to be solved, but some findings have helped to estimate the risks for onco and lentivectors.

The pattern of retroviral integration has always been considered variable and random, but recent reports described some level of preference in terms of integration hotspots. Very interestingly, these reports are different for HIV and MLV, because the former integrates into active genes while the latter preferentially integrates into transcription start regions (Schröder *et al.*, 2002; Wu *et al.*, 2003). This agrees with the findings of Hacein-Bey-Abina *et al.* (2003) for LMO2 gene activation due to integration of MLV near the LMO2 proto-oncogene promoter. These results indicate that besides having different integration patterns, vectors based on these viruses could present different theoretical risks for insertional mutagenesis.

It is true, however, that these important studies have been done with wild type HIV and MLV viruses and so far no similar study has been conducted with the recombinant pseudo-typed viruses commonly used in retroviral gene therapy, although the SCID-X1 case cited above supports the conclusions of Schröder *et al.* (2002) and Wu *et al.* (2003). It is important to remember that HIV may be less prone to insertional mutagenesis than MLV, but how the observations discussed above can be transposed to viral vectors still needs to be properly evaluated. For instance, in a murine model of β -thalassemia the frequency of proviral integration within genes regulating hematopoiesis has been considered to be too high (Imren *et al.*, 2004), but if these integrations are far from the transcriptional start sites the risk of transcribing flanking genes can be considered small (Wu *et al.*, 2003).

A comparative study of HIV and MLV vectors has reported different implications for vector safety, since MLV vectors presented higher readthrough frequencies for the 3' polyadenylation signal than HIV vectors. Interestingly, a SIN HIV vector presented results similar to the MLV vector, suggesting that SIN HIV and MLV vectors may present similar risks of activation of flanking genes if no modifications are made in the 3' poly(A) signals, such as changing the wild-type poly(A) signal for a stronger one (Zaiss *et al.*, 2002). Studies like these are very important in helping to define boundaries between retrovectors and to properly evaluate the safety of such constructs.

Even though these data represent the first results towards the development of safer retroviral vectors - particularly HIV vectors - for the future *in vivo* use of lentiviral gene therapy, some modifications have been proposed in order to improve even more the HIV-based system. Many suggestions have been made based on knowledge of HIV biology acquired from different fields, including molecular virology, cell biology, immunology, etc. Almost all structural, accessory and regulatory HIV genes have been tested for possible advantages in gene therapy vectors and the minimal requirements for the construction of HIV-based vectors has started to be defined (Zufferey *et al.*, 1997; Sutton *et al.*, 1998; Wu *et al.*, 2000).

A minimal HIV-based vector has been developed presenting a lower number of viral sequences (Kim *et al.*, 1998) and which may represent a safer construct since the probability of recombination with wild-type viruses or endogenous retroviral sequences is diminished (Miyoshi *et al.*, 1998; Iwacuma *et al.*, 1999). The number of known *cis* regulatory elements is still increasing, allowing the improvement of SIN vectors (Déglon *et al.*, 2000; Ismail *et al.*, 2000; Ramezani *et al.*, 2000; Yam *et al.*, 2002). Sequential deletions of regulatory regions of the HIV genome have been tested and it was found that some elements were essential for efficient gene transfer (Parolin *et al.*, 1994; Hildinger *et al.*, 1999; Sirven *et al.* 2000; Manganini *et al.*, 2002) but, in contrast to previous results, some were unnecessary (Cui *et al.*, 1999). Corroboration of such observations *in vivo* has also been reported (Baekelandt *et al.*, 2002). The major aspects of these elements are reviewed below.

The promoter chosen to drive the expression of the transgene is probably the first element that should match the target cells. In a comparative study Bonamino *et al.* (2004) evaluated CMV, EF1 α and PGK promoters for their ability to transfer the CD40L gene to human B-cell precursors of acute lymphoblastic leukemia via second-generation SIN HIV vectors, the best transcription efficiency being achieved with the CMV promoter. However, Yam *et al.* (2002) reported that the CMV promoter showed low activity in CD34⁺, erythrocytic and myelomonocytic cells. In both cases, the promoter activity was constitutive and it seems that regulated lentiviral vectors better reflect

physiological transgene expression levels (Mitta *et al.*, 2004; Markusic *et al.*, 2005). Despite these observations, the molecular context in which these promoters were evaluated is of major importance because *cis* elements can have synergistic effects on cell transduction efficiency.

Special splicing signals, constitutive RNA transport elements and woodchuck hepatitis virus post-transcriptional regulatory elements (WPRE) have all been used to achieve effective expression of protein-coding transgenes (Schambach *et al.*, 2000). In contrast to what was previously thought (Gasmi *et al.*, 1999), WPRE elements can improve the stability of mRNA and RNA processing (Kraunus *et al.*, 2004) and when used in combination with other elements (e.g. such as spleen focus-forming virus promoter (SFFV) and HIV central flap) results in significantly higher transduction rates (Yam *et al.*, 2002; Demaison *et al.*, 2002).

The nuclear import of HIV pre-integration complex (PIC) depends on a strand displacement (called the central DNA flap) generated during reverse transcription (Stevenson, 2000) which is recognized by viral and host elements, suggesting that the nuclear importation of PIC depends on both viral and host elements (Bukrinsky *et al.*, 1992; Gally *et al.*, 1997). Contrary to previous reports (Dvorin *et al.*, 2002), it is now known that the HIV central flap improves transduction efficiency because of an increase in PIC nuclear translocation of HIV vectors (Van Maele *et al.*, 2003). For hepatocyte transduction, rates were higher when the central flap was present (Park and Kay, 2001; VandenDriessche *et al.*, 2002), the same being true for the transduction of human nondividing monocytes and T-lymphocytes (Manganini *et al.*, 2002).

Multi-expression HIV-based vectors have been reported as versatile vectors based on their ability to express multiple transgenes (Reiser *et al.*, 2000). Differential splicing and translational control depending on an internal ribosomal entry site (IRES) sequence did not decrease virus titers or the ability of virions to infect quiescent cells (Zhu *et al.*, 2001), and these vectors may be used to deliver a reporter gene plus a therapeutic gene or two therapeutic genes at the same time.

Applications of HIV-Based Vectors

The ability of lentivectors to infect nondividing cells has been shown in various target cells, such as primary cultures from different tissues and human progenitor cells. More specifically, the efficiency of HIV vectors has been shown in targets such as hematopoietic stem cells (HSC) (Akkin *et al.*, 1996; Case *et al.*, 1999; Miyoshi *et al.*, 1999; An *et al.*, 2000; Demaison *et al.*, 2002) and peripheral-blood monocyte-derived dendritic cells (Chinnasamy *et al.*, 2000). The best results concerning the HSC transduction have been obtained using HIV-vectors instead of MLV-vectors (Case *et al.*, 1999; An *et al.*, 2000). Other lentiviruses, such as the feline immunodeficiency virus (FIV),

have been shown to be unsuitable for delivering genes to this type of progenitor cell (Price *et al.*, 2002). Specific modifications of HIV-based vectors resulting in better transduction rates of CD34⁺-derived hematopoietic cells have already been reported (Sirven *et al.*, 2001).

Promising results have been found for the *in vivo* transduction of airway epithelia (Johnson *et al.*, 2000), as well as *in situ* application of HIV vectors in human and mice corneal tissue, showing that HIV-based vectors may be a promising tool for gene therapy of recessive forms of inherited retinal degradation (Takahashi *et al.*, 1999; Wang *et al.*, 2000; Bainbridge *et al.*, 2001; Kostic *et al.*, 2003). Absence of toxic effects or immune responses after the *in vivo* administration of HIV vectors have been reported (Miyoshi *et al.*, 1998; Naldini, 1998; Han *et al.*, 1999; Pan *et al.*, 2002) and recent experiments with heart grafts in mice support these findings (Kearns-Jonker *et al.*, 2004), showing that HIV vectors may present advantages other than the ability to infect quiescent cells.

In fact, the remarkable efficacy of HIV-based vectors for globin gene transfer observed in Cooley anemia shows other potential uses of this tool (Rivella *et al.*, 2003), which is also employed in studies concerning the gain of function of certain genes in early human development processes through the transduction of human embryonic stem cells (Ma *et al.*, 2003). Zhang *et al.* (2002) reported the transduction of mesenchymal stem cells (MSC) with the maintenance of their differentiation status. Carlotti *et al.* (2004) showed that adipocytes can also be efficiently transduced with HIV-based vectors without showing any side effects, which was not the case when adeno-associated vectors were employed.

A comparison between adeno-associated and HIV vectors for the same application and for the same target cells is necessary, since it has not yet been defined if immune response is a common consequence of *in vivo* application of these vectors. For instance, Auricchio *et al.* (2001) found no differences in the efficiency of adeno-associated and HIV vectors for retina transduction and no immunological responses observed, contrasting with the report by Carlotti *et al.* (2004). On the other hand, immune responses following multiple administration of adeno-associated vectors have been reported (Kok *et al.*, 2004), a problem that can be overcome through the use of novel AAV-serotypes (Louboutin *et al.*, 2004). Vectors based on HIV have been employed as *in vivo* gene transfer system in Parkinson's disease (Déglon *et al.*, 2000; Azzouz *et al.*, 2002) and no immune responses have been detected, but in a gene therapy protocol for murine mucopolysaccharidosis type I, Di Domenico *et al.* (2005) observed that clearance of transduced cells by the immune system limited long-term treatment. It thus seems clear that despite the good results presented by HIV vectors so far more comparative studies are needed for AAV, MLV and HIV vectors. In a recent review Blesch (2004) compared *in situ* and *in vivo* protocols

based on HIV and MLV vectors but no reviews comparing HIV and adeno-associated vectors are available.

In addition to their applications as therapeutic tools, HIV-based vectors can also be seen as a research tool. Due to the stability of HIV-mediated gene transfer it is possible to produce transgenic animals such as mice (Lois *et al.*, 2002) and pigs (Hofmann *et al.*, 2003), overcoming the microinjection problems which occur with superior mammals, such as primates.

Another promising application refers to the production of interference RNA (RNAi) inside target cells, RNAi being a very interesting alternative for silencing target genes, for basic research or for use in ablative gene therapy protocols (reviewed in Novina and Sharp, 2004). HIV-based systems have been shown to effectively silence genes in primary mammalian cells, stem cells and transgenic mice, representing cycling, non-cycling and differentiated cell progeny (Rubinson *et al.*, 2003). Actually, some target cells may be refractory for transfection systems so viral vectors may be more appropriate for achieving higher transduction efficiency and long-term silencing (Mangeot *et al.*, 2004).

In basic research, HIV-based vectors have been employed as RNAi delivery systems to knockout a specific gene and to evaluate its interaction with other genes (Sui *et al.*, 2004; Yang *et al.*, 2004). Scherr *et al.* (2005) used HIV vectors to investigate the potential therapeutic applications of RNAi as stable gene transfer systems for K562 cancer cell lines and for CD34⁺ cells from chronic myeloid leukemia patients and have shown that stable RNAi may represent an useful approach to cancer gene therapy. Not only HIV-based vectors have been evaluated in this context, with for instance, adeno-associated vectors having been used in intratumoral injection in order to promote antitumor response (Tsugawa *et al.*, 2004). However, it seems more appropriate to employ a viral vector able to induce immune responses which constitute by themselves a therapeutic approach for cancer. Even so, adenovirus vectors have been tested for short interference RNA, since cancer cells may not be easy to transfect with non viral systems (Uchida *et al.*, 2004). Which viral system to choose will depend on comparative studies. Wollmann *et al.* (2005) evaluated nine lytic viruses to transduce glioblastoma cells and observed that adeno-associated vectors were not as effective as vesicular stomatitis virus, murine minute virus and Sindbis alphavirus, although no lentiviruses were included in this study because members of this virus class do not present *per se* oncolytic potential.

Final remarks

In this review we have discussed some of the primary concerns involved in the use of HIV-based vectors as gene transfer tools. An open debate comparing the use of primate or non-primate lentivectors remains essential for the development of safety tests and the generation of more information about HIV vectors along with basic research on better

and safer agents for clinical trials. In fact, researchers involved in retrovector design are beginning to address such questions using comparative studies and new approaches to evaluate issues such as immune responses and the side effects of retrovectors, all of which will enrich retroviral-mediated gene therapy. In the near future we hope that phase three clinical trials will benefit from these discoveries and lead to biosafety protocols, particularly those related to the clinical use of HIV-based vectors.

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Electronic Resources

The URL for data presented here is as follows:

Gene Therapy Clinical Trials Worldwide, <http://82.182.180.141/trials/FMPro>.

Lee YJ, Belgian Biosafety Server, http://www.biosafety.be/GT/Regulatory/RapportEN27_06_2001.pdf.

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