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4 Lentiviral Vectors

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4.1 INTRODUCTION

Lentiviruses are a subfamily of retroviruses infecting both primate and non-primate hosts (for reviews on lentivirus biology and molecular biology, see [1]). They are characterised by tropism for cells of the monocyte/macrophage series. The non-primate viruses infect domestic animals, maedi visna virus (MVV) infecting sheep, caprine arthritis encephalitis virus (CAEV), infecting goats, and equine infectious anaemia virus (EIAV) infecting horses. More recently described are bovine immunodeficiency virus (BIV) and feline immunodeficiency virus (FIV) infecting cattle and cats, respectively. The primate lentiviruses human immunodeficiency virus types 1 and 2 (HIV-1, HIV-2) and simian immunodeficiency virus (SIV) infect humans and other simians, respectively, where they are, in addition, tropic for lymphocytes. HIV-1 and HIV-2 cause AIDS in humans. The closely related SIV is subdivided into different strains, which infect different primate species and which, according to the strain and the host, may cause anything from asymptomatic carriage through to an AIDS-like immunodeficiency syndrome. Although attempts are being made to develop a number of non-primate lentiviruses such as FIV and EIAV as retroviral vectors, as yet there is little progress with viruses other than HIV-1, HIV-2 and SIV. This chapter describes current knowledge of vectors based on HIV with details on SIV and viral chimeras where appropriate.

The retroviral life cycle is illustrated in Figure 4.1 [2]. Lentiviruses are complex retroviruses with multiple spliced RNA species and a number of small regulatory proteins that allow sophisticated control of the viral replicative process with a distinguishable early and late phase to their replicative life cycle [3,4].

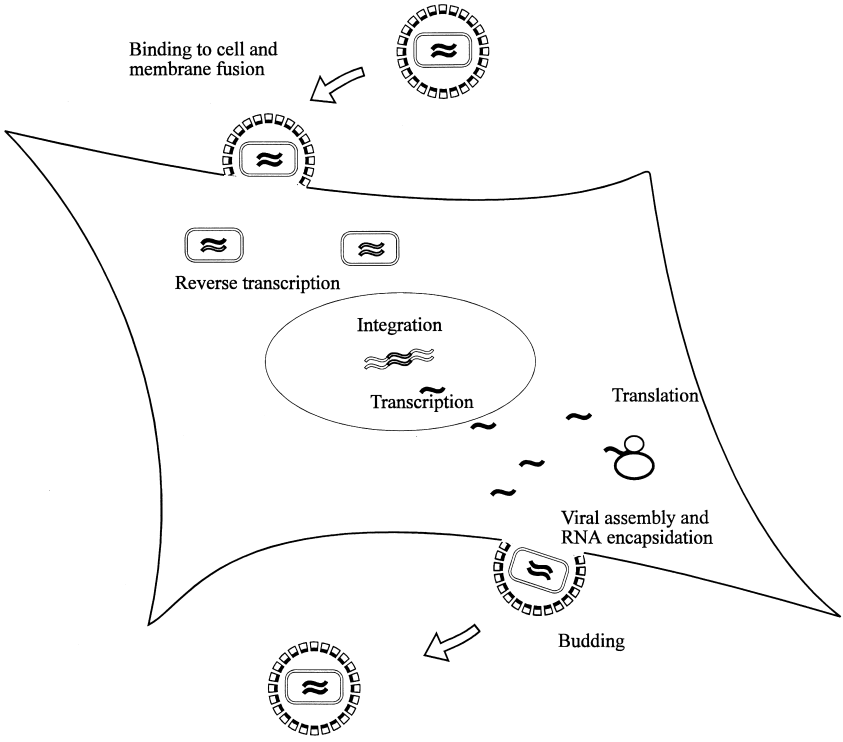


Figure 4.1. typical retroviral life cycle demonstrating infection of cell, conversion of genomic RNA to double-stranded DNA and integration into chromosome of cell, followed by transcription, viral protein production assembly encapsidation and budding of virus.

4.2 GENETIC STRUCTURE OF LENTIVIRUSES

The gene complement and RNA splicing pattern of HIV-1 as the lentiviral prototype are shown in Figures 4.2 and 4.3. In the provirus, the 5′ and 3′ ends are identical long terminal repeat (LTR), non-coding regions containing important *cis*-acting sequences. There are three major open reading frames: *gag*, coding for the viral capsid proteins, *pol*, coding for the virus enzymes, and *env*, coding for the envelope glycoproteins. Using HIV as the prototype, the *gag* and *pol* genes are encoded on the unspliced viral message. The Gag polyprotein is initially synthesised as a Pr55 precursor, which is then cleaved by the virus's own protease into at least four component segments. This cleavage occurs during or after budding. The most *N* terminal fragment is the matrix protein (MA) which, in the virus particle, occupies a peripheral site

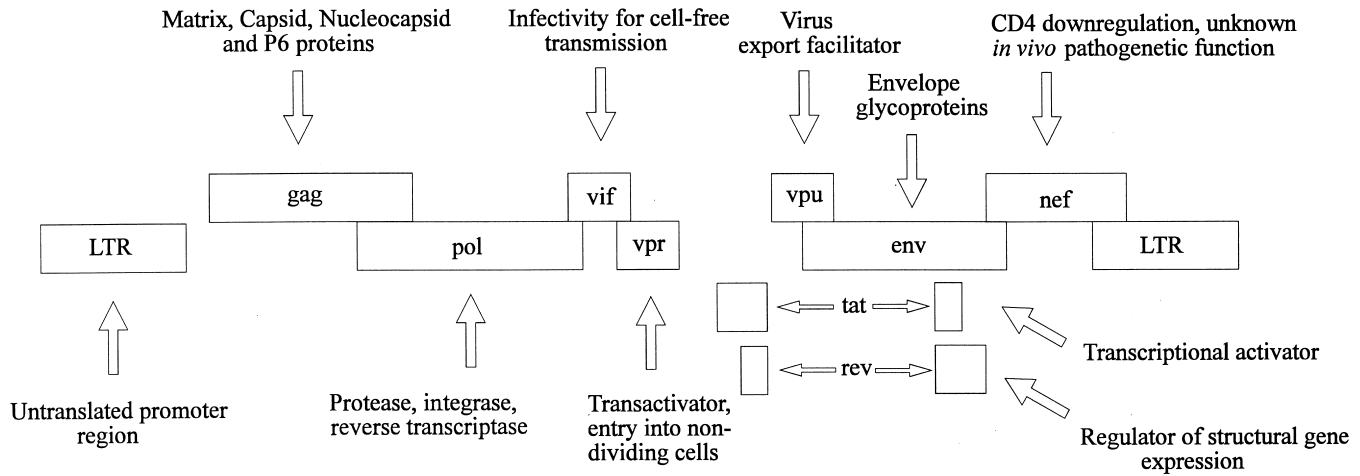
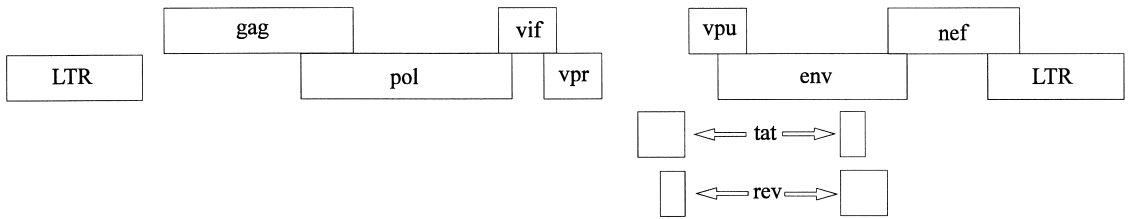


Figure 4.2. Gene complement of HIV as prototype lentivirus showing major open reading frames and function of gene products.



mRNA species

Unspliced	———————————————————————	gag, pol
Singly spliced	——— ———————————————————	env, nef
Multiply spliced	——— —— ———————————	tat, rev, etc.

Figure 4.3. Schematic of the family of mRNA molecules derived from the provirus, falling into three groups: unspliced, singly spliced and multiply spliced.

inside the envelope and is involved in anchoring the envelope proteins into the virion [5,6]. Following virus entry into the cell, MA also appears to be involved in targeting the integration complex to the nucleus and permitting integration of the provirus into a non-dividing cell [7]. A putative nuclear localisation signal has been identified within its amino acid sequence which may be responsible for this. C terminal to MA is the capsid (CA), which is the major structural protein of the virus core. It is also the protein recognised in some commercial ELISA assays for virus particles (p24 in HIV-1). It is responsible for the structural integrity of the virus core [8]. The p9 or nucleocapsid (NC) protein is the third segment of *gag*. This has two zinc finger-like CCHC motifs and is involved in coating the genomic RNA [9] within the virus to protect it from degradation. It also has functions after viral entry facilitating the reverse transcription of the viral RNA [10]. Recent work suggests that the 'spacer' peptide P2 between CA and NC may influence RNA encapsidation [11]. The most C terminal fragment of *gag* is p6, mutation of which leads to a defect in the terminal events of viral budding from the cell [12,13]. The exact mechanism by which p6 influences the final stages of virus assembly and budding is unclear although it may involve interactions with cellular proteins.

The *pol* gene products are produced initially as a Gag/Pol fusion protein. In the case of HIV this occurs during translation where a −1 frameshift occurs towards the end of the *gag* open reading frame, resulting in the ribosome beginning to translate in the *pol* open reading frame [14]. The fusion protein produced is a Pr160 Gag/Pol precursor. The *pol* gene products include, from the *N* terminal, protease (PR), which cleaves the Gag and Gag/Pol proteins into their component parts, reverse transcriptase (RT), responsible for converting the RNA genome into a double-stranded DNA molecule, and integrase (INT), which is then able to insert the double-stranded provirus into the cellular chromatin, dependent on specific recognition sequences in the viral LTRs.

Env protein is translated from a singly spliced RNA. The major splice donor is in the untranslated region 5′ of *gag*, and the *env* splice acceptor is upstream of the *env* initiation codon. A singly spliced transcript also codes for Nef. The Env protein is translated to produce a gp160 precursor on endoplasmic reticulum (ER) bound ribosomes. Gp160 is translocated into the ER lumen, cleaved by cellular proteases into a transmembrane gp41 (TM) and a surface gp120 (SU) protein. These associate non-covalently, undergo complex glycosylation and are exported to the cell surface, probably as a trimeric or possibly a tetrameric complex.

In addition, HIV has a number of small open reading frames for which there are homologues in most lentiviruses [15]. Tat is a powerful transactivator of transcription encoded on a doubly spliced message which acts in

trans to enhance processivity of RNA polymerase through an RNA stem loop structure formed in the region downstream of the transcriptional start site in the LTR. This is known as the Tat-responsive (TAR) stem loop [16]. The Tat/LTR combination is one of the most powerful promoters known and enhances production of all viral mRNA species. In early infection due to instability sequences (INS) found within the *env* intron (i.e. the *gag* and *pol* sequence) and in the *env* gene itself, full-length messenger RNA is rapidly processed to doubly spliced forms [17]. Tat is thus the major gene product early in the life cycle and feeds back positively, enhancing its own synthesis.

The Rev protein is encoded by two exons which overlap those of Tat. This protein is also produced in abundance early in the life cycle and as it accumulates it is able to interact with a Rev-responsive element (RRE) in the *env* coding region to facilitate export of singly spliced and unspliced messenger RNAs from the nucleus to the cytoplasm. The Rev/RRE interaction appears to overcome the effect of the INS, which favours splicing. This gives a temporal control to viral RNA expression, in which large amounts of RNA coding for regulatory proteins are produced in the early phase followed by a Rev-mediated switch to production of singly spliced and full-length messenger RNAs coding for the structural and enzymatic proteins of the virus. Four other small open reading frames merit mention. The *nef* gene situated at the 3′ end of the virus has a number of functions. Although initially identified as a negative regulator of transcription [18], it appears to have other more important functions *in vivo*, including downregulation of CD4 [19] from the infected cell surface and as yet unidentified essential functions which lead to small mutations in *nef* (in SIV) being repaired by the virus on passage *in vivo* yet reappearing on passage *in vitro* [20].

Vif is an accessory protein needed in some systems for cell-free infection. Cell to cell transmission of virus occurs efficiently in Vif positive or negative viruses but in a number of cell lines, cell-free infection can only occur if Vif is present [18]. Recent evidence suggests Vif is packaged into the virus particle and may affect an early step in virus uncoating [21].

Vpu has the function of increasing the efficiency of viral export. This appears to have two components. First, it disaggregates complexes between the envelope protein and the CD4 protein of an infected cell, which occur in the endoplasmic reticulum during export of both proteins, allowing envelope export. Second, it has a more generalised export function evidenced by the inability of HeLa cells to export viruses or envelope-free Gag particles in the absence of Vpu [22,23,18].

Vpr is a protein with a number of suggested and identified functions [24]. It is incorporated into the virus particle [25] (unlike Vpu). It has a transactivating capability which is weaker than Tat [18]. It appears to contribute to the ability of HIV to integrate its genome into a non-dividing cell [26]. It has been shown to induce cell cycle arrest in G2 in some cell lines

[27]. Thus, from a vector perspective, it has both desirable and undesirable properties.

4.2.1 ADVANTAGES OF LENTIVIRAL VECTORS

Like all retroviral vectors, lentiviruses offer the potential for stable single-copy gene insertion into a host cell chromosome and targeted gene transfer into cells bearing an appropriate viral receptor. The major reason for considering lentiviruses over other retroviruses is their ability to target cells that are in G_0 [28,29]. Many of the cell populations to which therapeutic gene delivery is targeted, such as liver, muscle and brain, spend most or all of their time in G_0.

The second advantage is the feasibility of high-level gene expression using the powerful Tat/LTR promoter combination.

Thirdly, the accessory genes provide additional regulatory functions such that genes can be made inducible.

4.2.2 DISADVANTAGES OF LENTIVIRAL VECTORS

The obvious disadvantage of an HIV-based vector lies in the danger of recombination producing a wild-type virus. This can only occur if all the components necessary to re-create a complete viral genome are present, and the system should ideally be designed so that this cannot happen. A second disadvantage encountered to date is the relatively low titre of lentiviral vector systems. It is not yet clear why this is so. However, the perception of the problem is somewhat artificially exaggerated by direct comparisons of lentiviral vectors with murine vectors on transformed (often murine) cell lines *in vitro*. In general, researchers have found that when assessed for their transducing ability for primary cells *in vitro*, the difference between lentiviruses and murine-based vectors is much less marked, i.e. the murine vectors are less efficient as well.

4.3 LENTIVIRUS-BASED VECTORS

A retroviral vector system has two fundamental components [30]: (i) a modified viral genome containing the foreign gene of interest with *cis*-acting sequences necessary for its encapsidation into the virus particle and stable insertion into target cells – the vector – and (ii) a helper virus or viral construct(s) providing in *trans* all the structural and enzymatic proteins necessary to create an intact virion into which the vector can be packaged for delivery.

The minimal components for a vector are: packaging signal(s), which allows selective encapsidation of viral RNA into a particle, a primer binding

site (PBS) for initiation of reverse transcription, a polypurine tract (PPT) for initiation of proviral plus strand synthesis, and LTR sequences which supply both promotor and enhancer functions, polyadenylation signals and the recognition sequences required for provirus integration. All of these can be omitted from the packaging system constructs. In lentiviruses, the 5′ LTR and primer binding site are almost contiguous and are usually included as one continuous sequence in the vector. The position of the encapsidation signal ψ in HIV is still a matter of some debate. Initial studies identified a sequence important for encapsidation between the 5′ splice donor and the *gag* initiation codon [31–33]. Mutations in this region led to a variable packaging defect ranging from 10- to 1000-fold less than the wild-type virus partly dependent on the cell type. Subsequent studies have identified different additional regions as apparently able to enhance packaging, but with each study performed in a different system, drawing conclusions is difficult. The 3′ end of the *env* gene, including the RRE (but not dependent on it), appears to enhance packaging of some constructs [34,35]. The 5′ region of the *gag* gene has been identified as containing a packaging enhancing signal [36]. The TAR structure has also been suggested as being an important component of the packaging signal [37]. The packaging signal has been suggested to be discontinuous and made up of several different motifs [38]. The minimum sequence or sequences required for packaging have not yet been identified. However, under some circumstances, the 3′ end of *env*, all of *gag* and the TAR region are dispensible for packaging whereas there is still general agreement that the region between splice donor and *gag* ATG has the most influence. A pentanucleotide sequence GGNGR appears to be a commonly occurring motif in packaging signal regions of primate lentiviruses [39]. In practical terms, many vectors include the complete 5′ region from the transcription start site through to the beginning or into the first part of the *gag* gene together with the 3′ end of *env.* The latter is included either on the basis of it being a separate packaging enhancing signal or to overcome the instability sequences present on incorporating the 5′ end of the *gag* gene into the construct (see Figure 4.6).

4.3.1 OTHER INFLUENCES ON PACKAGING

RNA packaging in HIV appears to be more promiscuous and less specific than in murine and avian retroviral systems. Several publications attest to the ability of HIV to encapsidate spliced RNA, either virus derived or from vectors, at a reasonably high efficiency although lower than that of the full-length unspliced message [40,41]. It is not clear whether this results from heterodimer formation between spliced and full-length species. A number of factors appear to influence this. In certain cell types used for transient transfection, such as Cos cells, there appears to be significant packaging of

the spliced RNA, possibly as a result of an extremely high cellular concentration of this species. In T lymphocytes the packaging is more specific although spliced RNA can still be detected within the virion particles. No cell line has been shown to be able to discriminate completely full-length RNA for encapsidation when transfection techniques have been employed. It is possible that high-level expression, which is the aim of transfection, over a short period of time, leads to a saturation of the system through the sheer quantity of RNA available in the cell. In experiments designed to identify packaging signals, *infection* procedures have been associated with a higher specificity of packaging than transfection [42]. Concurrent with this has been the observation that the packaging defect associated with small deletions in the 5′ leader is much greater in infection-based experiments. In HIV-2 we have demonstrated a hierarchy of packaging specificity in which packaging in Cos cell transfections is less specific than in T cell transfections, which is again less specific than in T cell infections [42].

Even in packaging cell lines based on HIV, there is evidence that spliced RNA derived from a stably integrated proviral construct may still enter the packaging pathway and be incorporated into the virions. If this is the situation *in vivo* it would at first appear distinctly disadvantageous for the virus in that spliced RNAs would compete for packaging with the desired full-length genome. However, a small degree of promiscuity would also have the effect of enhancing heterodimer encapsidation, recombination, and with it the possibility for increasing viral diversity. It is also possible that the system is being assessed under artifical conditions and that the specificity may depend largely on the qualitative difference in RNA available for encapsidation when the viral capsids are assembling. Lentiviruses undergo a switch from early to late gene expression, and it may be this coincidence of increased unspliced RNA appearing in the cytoplasm together with structural proteins being produced which tips the balance against packaging of multiply spliced RNAs, which are more abundant earlier in the life cycle.

In practical terms, there is as yet no satisfactory cell line which will eliminate encapsidation of spliced RNAs. Those based on lymphocytes appear in our hands to be somewhat better than those based on epithelial or fibroblast-like cell lines. Sufficient cell lines have not yet been studied to be able to give categorical recommendations.

A further twist to this comes from the recent observation that HIV-1 can package HIV-1 or HIV-2 based vectors but that HIV-2 can only package HIV-2 vectors [11]. This appears to be due to a preference by HIV-2 for packaging RNA co-translationally, thus limiting its ability to pick up heterologous vector RNA in *trans*. The ability of HIV-1 to package vectors may be due to its decreased dependence on packaging in *cis*. Hence, its ability to act as a packaging system for other RNAs may depend on this promiscuity of encapsidation (Kaye and Lever, personal observations).

Certain heterologous genes appear to be associated with an unexplained difficulty in packaging under some conditions. The chloramphenicol acetyl transferase (CAT) gene appears to be encapsidated extremely well into a virion particle when it is included within the genome as a substitute for the *nef* [11] gene or in the middle of a deleted envelope gene. Inclusion of the CAT gene within the *gag* region [34] appears to have a negative effect on encapsidation, possibly due to the proximity of the heterologous sequence to the packaging signal where it might interfere with the secondary structure of the packaging signal.

The aim of a vector system is to deliver a heterologous gene to a target cell line. A variety of methods involving HIV have been tried. These range from gene substitution in a replication-competent helper virus through to conventional separated constructs designed to minimise or abolish the possibility of generating replication-competent virus. The major gene transfer papers using lentiviruses with native virus envelopes are summarised in Table 4.1.

4.3.2 DIRECT GENE TRANSFER

The first demonstration of this was performed by substituting a heterologous gene (CAT) for the *nef* open reading frame of a full-length HIV (Figure 4.4). A series [43] of CAT constructs of different lengths were inserted into the 3′ end of the virus and some were found not to affect replication competence, delivering the full-length genome with the CAT gene intact to target cells, the CAT gene most likely being expressed from a spliced transcript. *In vitro*, the replication competence of the virus decreased as the size of the insert increased. This was postulated to be due to the increased size of the full-length transcript imposing an extra packaging constraint. Although there is evidence that greater than genome length RNA is packageable (McCann and Lever, personal observations), the maximum size of an RNA that can 'fit' into a lentivirus has never in fact been formally analysed. With the smallest CAT gene insertion, the competence of the virus to replicate *in vitro* was in fact slightly enhanced over the wild-type, probably because *nef*, which had been deleted, itself has a minor inhibitory effect on virus replication *in vitro*. Thus, for delivery of marker genes this is probably the most efficient system. However, none of the virulence of HIV is attenuated.

4.3.3 HELPER VIRUS MEDIATED

In earlier packaging studies using HIV where the exact *cis*-acting sequences were still being analysed, stable expression of test vectors in a CD4 positive cell line was used [34]. These cells were infected with wild-type HIV and the supernatant virus from these cultures was analysed by RNA slot blot and also by gene delivery to a second population of CD4 positive cells to calculate

the vector titre (Figure 4.5). Using such a system, gene transfer of vectors with titres of 10^3 and 10^4 cfu/ml (colony forming units per millilitre) was achieved. Because of the nature of this system, wild-type helper virus was present.

4.3.4 CO-TRANSFECTION SYSTEMS

4.3.4.1 Envelope Complementation

A simple vector methodology employed by a number of groups is that of *env* complementation. In this, a heterologous gene is introduced into the central portion of a deleted *env* open reading frame (Figure 4.4). The open reading frames for the accessory proteins Tat and Rev are left intact. The heterologous gene introduced is a marker gene such as CAT or β-galactosidase [44]. This plasmid is then co-transfected into a cell together with an envelope expressing gene and between them they produce viral particles in which the full-length RNA containing the heterologous gene in the envelope region is encapsidated. This has particular strengths when studying the envelope protein itself and has been used extensively for functional analysis of envelope mutants, where it has given a quantitative read-out of virus entry into a target cell population [45]. It also has applications in pathogenesis studies in that the system has single-round replication kinetics and can deliver a suitably detectable heterologous gene. It is able to provide information on subjects such as the first cell population susceptible to viral infection through various different routes of infection. The large degree of overlap between the envelope gene sequences in the two constructs raises the spectre of recombination and regeneration of full-length wild-type virus. This system, therefore, is not applicable for *in vivo* use in humans. However, in animal models using other lentiviruses, such as visna and SIV, it is valuable for studies of infectivity and pathogenesis.

4.3.4.2 Co-transfection Using Independent Vector and Packaging Constructs

Several groups have published data describing the practicability and efficiency of this system [46–48]. The number of separate DNA constructs co-transfected into cells is either two (packaging construct plus vector) or three (two complementary packaging constructs plus vector) (Figure 4.6). In the first case, systems have been designed to minimise commonality between sequences in the vector and the packaging construct by removal from the latter of LTRs and by deletion or mutation of the 5′ leader packaging signal sequence, leaving intact only the open reading frames and appropriate splicing signals. Using this system, helper-free transduction of CD4 positive cells was demonstrated with titres of less than 10^2 cfu/ml.

Table 4.1 Summary of major gene transfer papers using lentiviral-based vectors

Reference	Co-transfection/ infection/stable cell line	Particle producing cells	Target cell	Packaging construct	Vector	Titre (cfu/ml)	Comments
[43]	Transfection	Jurkat	Jurkat		WT Gene in place of nef	> WT	Heterologous gene in place of nef in replication-competent virus
[44]	Co	Cos-7	HeLa T4	FL Δ env and env expressor	Gene in env ORF	Up to 3×10^5	Envelope complementation
[49]	Co	Cos-1	Jurkat	(1) FL ΔΨ SV40pA (2) FLΔ([env SV40pA and env expressor	T – R –	Up to 10^5	Highest titre published. Later publication from same group suggested this was difficult to reproduce
[47]	Co	Cos-1	HeLa T4 CEM HPB-ALL	FL ΔΨ CMV promoter SV40pA	T – R –	$< 10^2$	Titre equivalent to amphotropic Moloney virus
[62]	Co	Cos-1	HeLa HeLa T4	FL Δ env and murine amphotropic env expressor	T – R –	10^2	722 nt at ′ end of gag enhanced packaging

[63]	Co	Cos M6	HeLa T4	FL CMV promoter SV40pA	T – R – HIV and SIV	HIV < 500 SIV < 50	Cross-packaging of HIV and SIV RRE dispensible
[48]	Co	Cos-1	Jurkat	CMV promoter ΔΨ Δ env SV40pA and env expressor	Various T –	10^2	653 ntT at 5′ end of gag enhanced transfer, RRE dependent. Otherwise RRE dispensible
[34]	Co	Cos-1	HeLa T4 HeLa T8 Jurkat-tat	WT WT ΔΨ WT ΔΨ Δ env and env expressor	T + R + T – R –	Up to 24 < 50	High titres with Cos defective helper but with recombinant virus. No helper virus generated with two-plasmid packaging system – CD4 specific
[46]	Stable vector line WT virus infection	Jurkat (± Tat)	HeLa T4 Jurkat (± Tat)	WT	Various	Up to 10^4	3′ end of env enhanced packaging Genes in gag region inhibit packaging
[50]	Stable producer cell line	Vero	SupT1	FL ΔΨ Δ 3′ LTR	T – R –	10^2	CD4 specific Evidence for poor gag processing
[51]	Stable packaging cell line	HeLa T4	FL ΔΨ	Δ env Δ 3′ LTR tet rev + env	T – R – T + R + (FL Δ env)	10^3 10^4	TET inducible system 5 + days to induce

Table 4.1 (*cont.*)

Reference	Co-transfection/ infection/stable cell line	Particle producing cells	Target cell	Packaging construct	Vector	Titre (cfu/ml)	Comments
[53]	Stable packaging cell line	CMT3-Cos	HeLa T4	CMV promoter ΔΨ gag pol and either CMV promoter rev CMV promoter env or CMV promoter env CTE	T – R –	Up to 10^4	Nef enhances titre
[52]	Stable packaging cell line or incomplete producer line	SW480 MDS	HeLa T4	ΔΨΔ env Δ 3′ LTR and LTR env or ΔΨΔ env Δ 3′ LTR and vector	T + R + or T – R –	10^2	THP-1 and HeLa Not useful as packaging cells

Co, co-transfection; FL, full length virus; Δ env, deletion in envelope gene; ΔΨ, deletion in 5′ leader packaging signal sequence; SV40pA, polyadenylation signal from SV40; WT, wild type virus; 3′ LTR, deleted 3′ long terminal repeat; tet, tetracycline repressor based inducible system; CTE, Mason Pfizer monkey virus constitutive transport element; T, tat (T – , absent tat); R, rev (R – , absent rev); RRE, Rev-responsive element.

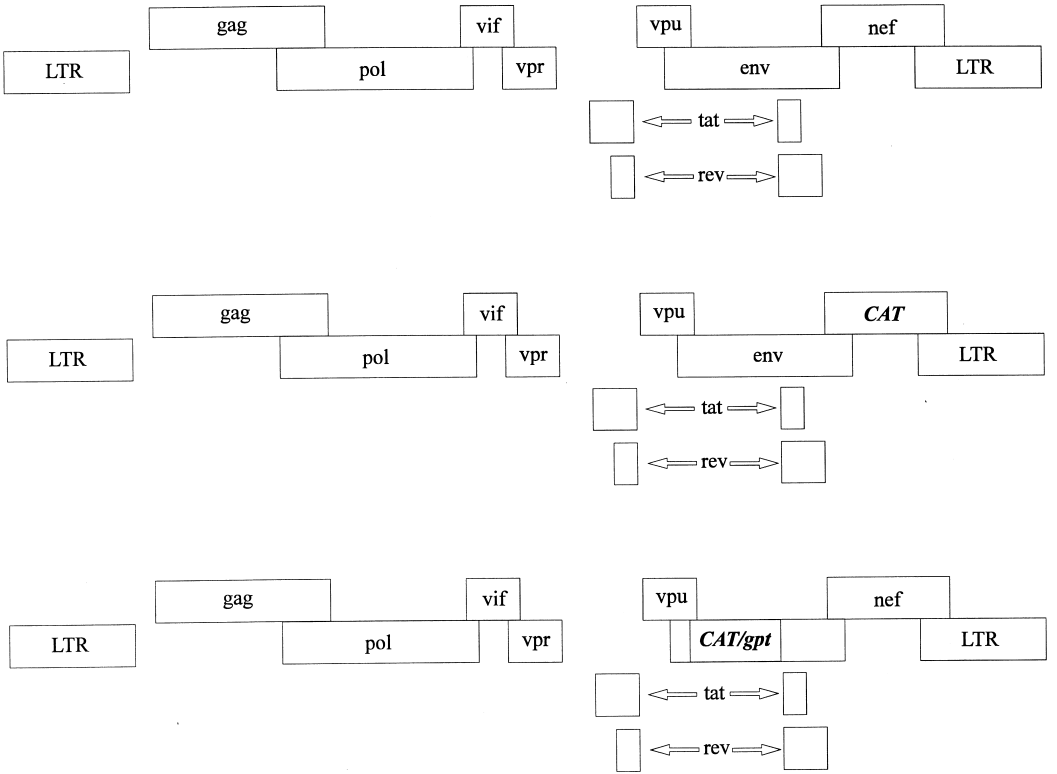


Figure 4.4. Alternatives for insertion of heterologous genes into a proviral construct. The upper panel shows the intact provirus, and the central panel demonstrates insertion of CAT gene in place of *nef*. This is replication competent. The third panel shows CAT or *gpt* inserted in place of the envelope gene. This requires complementation with an envelope expressor.

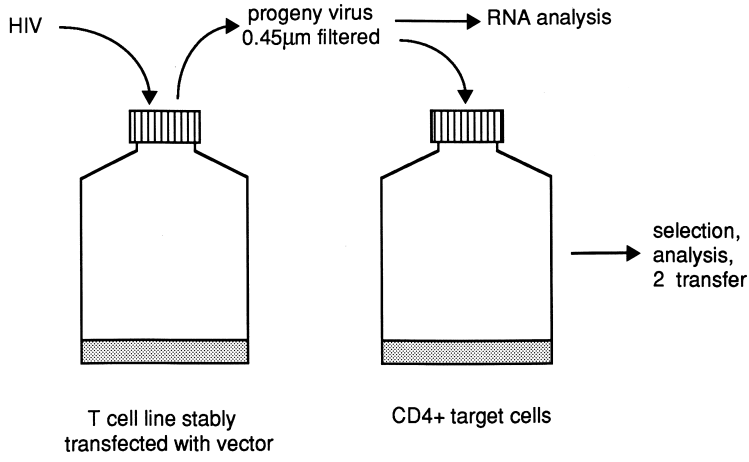


Figure 4.5. Helper virus mediated gene transfer. Useful for studies of encapsidation.

Importantly, this was shown to be no worse than a murine vector-based system assessed in parallel (personal observations). Where two-plasmid co-transfection has been used with extensive overlap between vector and helper virus, replication-competent virus is almost inevitably generated, even when packaging signal sequences have been deleted from the helper virus. Even a deletion of 35 base pairs in the packaging construct/helper, which has a major inhibitory effect on packaging in T cells, was insufficient to prevent recombination [46]. The weakness of co-transfection systems are that they are, for reasons of efficiency of expression, used in cells that have been shown in packaging studies to be relatively promiscuous and will package, at reasonable efficiency, RNA with deletions in otherwise important *cis*-acting packaging sequences. Recombination between heterodimers during reverse transcription after infection of the target cell is probably the source of replication competent virus.

4.3.4.3 Three-plasmid Co-transfections

Transient transfection assays using three plasmids have to date been found not to generate replication-competent virus, probably because this would require at least two recombination events between three constructs. The envelope construct is commonly driven from a heterologous promoter or a deleted LTR and has a heterologous polyadenylation signal. To date, both the HIV LTR and heterologous promoters have been used to generate the Gag/Pol proteins. The envelope expressor has usually had a heterologous promoter. The maximum titre so far documented for co-transfection delivery is in

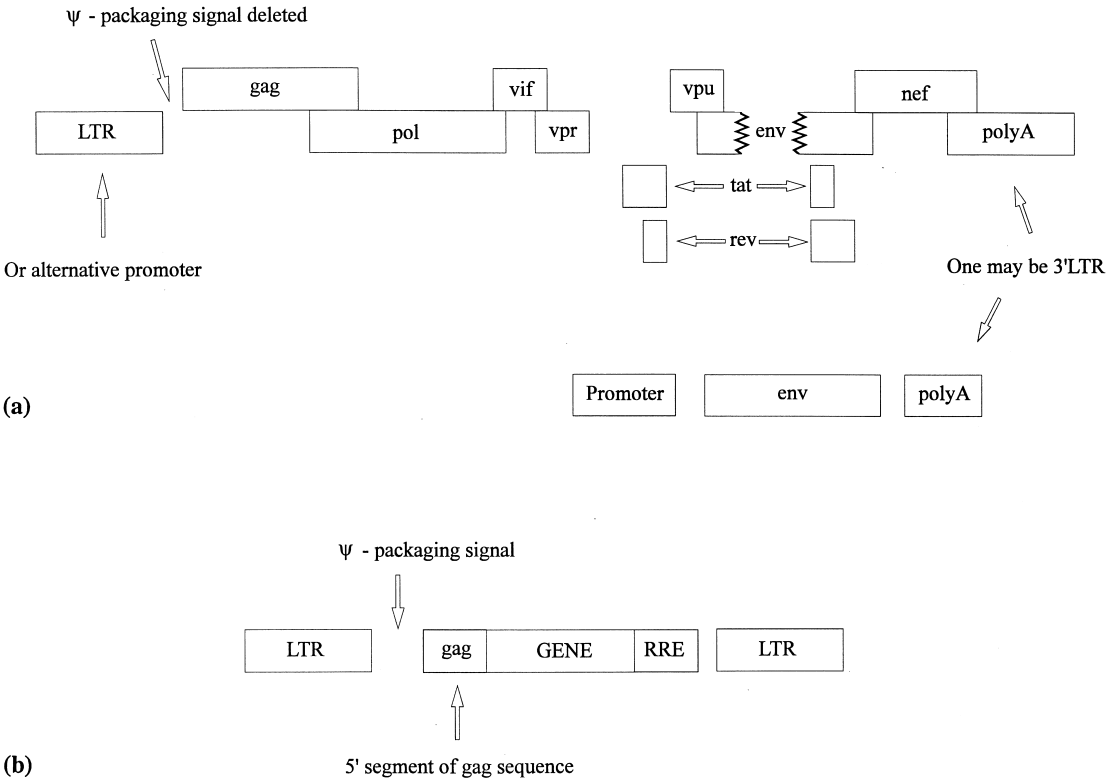


Figure 4.6. (a) Commonly used complementary constructs including all of the retroviral proteins. (b) Typical structure of HIV-based vector, where gene represents a heterologous gene which may be under control of its own promoter or, if in antisense, under the control of its own promoter and polyadenylation signal.

the order of 10^3 cfu/ml. The majority of publications suggest a lower titre of around 10^2 cfu/ml. An early paper suggesting extremely high efficiencies of 10^5 cfu/ml has not been replicated [49]. The system depends critically on having cells that are adept at taking up transfected DNA and that are tolerant of relatively large concentrations of transfected DNA, such as Cos-1. In theory there is no reason why cell lines stably expressing any one of the three plasmids should not be used to reduce the number of DNA constructs introduced at any one time. For example, stable expression of a vector containing a selectable marker in a cell line would ensure that every cell in the population that was transfected was expressing that vector so packaging constructs encoding only for the envelope and for the *gag/pol* regions would need to be introduced. There is a broad area of overlap between this and the next section on packaging cell lines but a general principle is that stable expression of the construct encoding the proteins that are most toxic to the packaging cell line might best be avoided and instead introduced by transient transfection into the system.

4.3.5 PACKAGING CELL LINES

For HIV there are now several publications [50–53] documenting packaging cell lines stably expressing structural proteins of the virus. In general these have been modelled on murine systems in which the envelope gene is expressed separately from the *gag/pol* construct. Ideally, these are generated using sequential steps in which limiting dilution of cells expressing one construct is performed. High-level expressors of that particular construct are then cloned out and used for the introduction of the next expressor and, again, limiting dilution is performed and a clonal population of high-level expression is selected. The very striking difference in the ability of different clones to express viral proteins justifies this approach and, for example, differences in p24 expression of 10^2–10^3-fold can be found in different clones derived from HIV *gag* expressing lines [52].

Most groups have created a packaging cell line in which the *gag/pol* construct is transfected into cells with the help of a stable selectable marker. Expression of *gag* is Rev-dependent, thus the *gag/pol* expressor must also include the RRE. This is a potential weakness in that the envelope expressor also will need to contain the RRE. Many groups also choose to include the same sequence in their vectors to optimise expression leading to all three of the constructs involved in production of infectious vectors containing the RRE, and this raises the risk of recombination. Point mutations have been introduced into the *gag* gene by Pavlakis's group [54] such that the nucleic acid sequence is changed but the amino acid coding sequence is unchanged. This apparently removes the *cis*-acting INS effect and it is claimed that *gag* is produced in a Rev-independent manner. This would be one possibility for

avoiding inclusion of the RRE. An alternative strategy would be to include another *cis*-acting sequence which could substitute for the RRE. In Mason Pfizer monkey virus (MPMV) a *cis*-acting sequence – the constitutive transporter element (CTE) – has been shown to be able to act independently of Rev, to allow RNA export from the nucleus [55]. Thus, a *gag/pol* expressor could include the CTE rather than the RRE. The envelope expressor already by definition includes the RRE as it is part of the *env* sequence. Recent work suggests that for the vector the RRE leads to greater efficiency of transfer than the CTE [56].

The genetic complexity of HIV-1 means that not only *env* and *gag/pol* genes need to be expressed but also the regulatory genes *tat* and *rev*. The accessory genes *vpr*, *vpu*, *vif* and *nef* are dispensible for vector transfer into T cells when Cos-1 cells are used for virus production but may be required in other circumstances. Vif, for example, is absolutely required for particle infectivity when the virus-producing cell is restrictive for Vif defective viruses. The Vpu protein considerably enhances the release of virus particles from various cell types and Vpr may be required for integration into G_0 cells. The HIV genome can be split into 5′ *gag/pol*, *vif* and 3′ *tat*, *rev*, *vpu* and *env* coding fragments. However, balanced expression of all these latter genes from the 3′ genome fragment may be problematic when they are placed outside the genetic context of the provirus. In addition, if one is beginning by selecting for a *gag/pol* expressor, Rev needs to be present in the system.

4.3.5.1 Problem Proteins

Lentiviral proteins pose a number of unique problems as some of these have proven difficult to express stably in certain cells. The viral protease enzyme, which is essential for processing of the Gag and Pol precursor proteins, appears to be toxic in some cell lines [57]. This may be due to the protease being able to cleave certain cytoskeletal proteins. Cell lines expressing protease were found only to grow when point mutants arose in the protease gene, rendering the enzyme defective. Other groups have found, usually using human-derived cell lines, that longer term protease expression is possible.

The *env* gene product is also potentially toxic, particularly to cells that are CD4 positive. A number of cell lines are described as being CD4 negative yet in some cases have detectable CD4 messenger RNA. It is probable that the level of CD4 on their surface is below the limit of detection by conventional histological staining techniques. In other cases, cells might be expressing receptors involved in CD4-independent viral entry. However, any expression of CD4 in a cell population also expressing gp120 can lead to cell fusion and death. Unfortunately, some of the best HIV particle producing cell lines are lymphocyte-derived and have CD4 on the surface. Again, in principle, it

is preferable to have a packaging cell line that does not have CD4 on the surface as this minimises the chances of re-infection of the cell line by the vectors that are being produced, a process which increases the likelihood of recombination.

The Vpr protein, important in entry into G_0 cells, may also be problematic. In a number of cell lines Vpr has been shown to induce cell cycle arrest. It has been shown to be necessary for Vpr to be produced in the cell line from which the virion is produced so that it can be encapsidated [18]. Experiments have clearly shown that providing Vpr in *trans* in the target cell line does not work. In a three-plasmid system (*gag/pol*, env, vector), the most practical approach is to have two of these constructs stably expressed and introduce the third one by transient transfection with the *vpr* open reading frame being included in this latter construct. Vpr has a degree of species specificity such that, for example, HIV-1 Vpr may have its greatest effect in human cell lines and SIV Vpr in simian cell lines, both having less effect in heterologous cells. This offers scope for using heterologous vectors to avoid growth arrest. A common observation in lentivirus packaging cell lines stably expressing viral proteins is a progressive decline in viral protein and, hence, viral particle production. The mechanism for this is not clear.

4.3.5.2 Inducible Constructs

To overcome some of these problems, a packaging cell line has been described in which components are inducible [51]. This was achieved using the powerful tetracycline repressor system (tet) to control Rev and Env products. This allows for short-term high-level expression of the desired proteins with genetic shut-off in between. The kinetics of production of structural protein in this system were unusually slow for a tet-based system, with induction taking over five days to maximise despite, in some cases, the presence of *rev* in *trans* from the vector. Although this system may be convenient for laboratory-based experiments, it adds an extra layer of complexity and difficulty in standardisation should these lines ever be produced on a commercial basis.

4.3.6 ENVELOPE PSEUDOTYPING

There have been many studies involving heterologous envelope pseudotyping of different viral cores. HIV envelope was shown to pseudotype murine vectors although with a significantly lower titre than the native murine amphotropic envelope. More recently, non-retroviral envelopes have been used, particularly the envelope (G protein) of vesicular stomatitis virus (VSV) [58,59]. These appear to pseudotype HIV cores readily and produce a particle that is stable on freezing [58] and that gives a high titre in a CD34

positive bone marrow stem cell population. The combination of a lentiviral core and a VSV envelope has also been shown to deliver genes efficiently to cells in G_0 when tested on growth-arrested cultures. In parallel experiments the titre of murine vectors dropped significantly to near zero. More impressive was the demonstration that highly concentrated (concentration unspecified) VSV-G pseudotyped HIV viral particles injected directly into the mammalian brain were able to effect stable gene transfer into both neurons and glial cells [59]. VSV pseudotyping appears to allow vector particle entry via an endosomal route, which may account for some of the increased efficacy [60]. This is now an area of great interest and gene delivery to a number of tissues *in vivo* has been demonstrated. It is clear, however, that although *in vitro* titres up to 10^9 cfu/ml are claimed there is still a relatively small number of cells transduced *in vivo*. There is also some controversy as to the efficacy of such vectors to deliver genes to important targets such as haemopoietic stem cells [61].

4.3.7 PROTEIN DELIVERY

HIV is one of the first viruses in which there has been a dissection of protein–protein interactions that lead to incorporation of accessory proteins into the virus particle. The protein Vpr, for example, is included in the virus particle due to a specific interaction with the P6 portion of Gag [24] and mutagenesis has identified amino acids involved in this interaction. Similarly, the cell-derived protein cyclophilin interacts directly with CA. Identification of these phenomena has been used to introduce P6 binding regions into heterologous proteins and to demonstrate incorporation of such tagged molecules into virus particles. Although gene therapy and vector systems are usually aimed at delivering nucleic acid sequences, there are clearly many applications for delivery of specific proteins to a cell.

4.3.8 VIRAL TITRES

When full-length viral RNAs incorporate into virus particles even with heterologous genes incorporated either in place of *nef* or substituting for part of the envelope sequence, the efficiency of encapsidation and delivery seems extremely high. This degree of efficiency has never been achieved with any of the known vector systems to date involving vectors that are less than genome length. Even with incorporation of all known packaging signal sequences into a vector including the TAR stem loop, the 5′ leader sequence, the *gag* gene, the 3′ end of envelope or the RRE alone rarely achieves titres of greater than 10^3 cfu/ml. The reason for this is not clear and it differentiates lentiviral vectors from murine systems, in which encapsidation of vector is achieved at levels equivalent to the wild-type viral RNA. This may reflect a

preference for lentiviruses to encapsidate RNA coding for Gag proteins cotranslationally.

4.4 RANGE OF APPLICATIONS

The potential uses of lentiviral-based vectors were alluded to earlier in the chapter. Obvious roles include the delivery of genes to CD4 positive cells for the gene therapy of HIV infection. This would most likely be directed first at those patients who are already HIV positive in an attempt to provide genetic protection to those CD4 cells in the individuals that were not yet infected. Other diseases affecting lymphocytes and cells of the monocyte/macrophage line might also be targeted.

The use of lentiviruses or lentiviral chimeras to enter non-dividing cells has been demonstrated and there is likely to be an expansion in this area of interest over the next few years. Most individuals involved in vector research have a conception that the perfect vector will be a mixture of components from different viruses, each contributing its own particular property, and that these may ultimately be incorporated into a completely non-viral particle.

4.5 SAFETY

In the United Kingdom, HIV and SIV are category III pathogens and all work involving live viruses or complementary constructs that could regenerate live virus has to be carried out under this level of protective isolation. Work with other lentiviruses (MVV, EIAV) is restricted only by the potential of these pathogens to come into contact with uninfected animals.

As yet, there have been no clinical trial protocols approved using lentiviruses in whole or in part in human gene transfer protocols. There would seem to be little difference in the level of safety required for a vector composed entirely of murine retroviral components compared to one which was chimeric in which part of the chimera involved a lentivirus.

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