THE JOURNAL OF GENE MEDICINE
J Gene Med 2004; **6**: S67–S82.
Published online in Wiley InterScience (www.interscience.wiley.com). **DOI:** 10.1002/jgm.498

REVIEW ARTICLE

Toolbox for retrovectorologists

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Summary

Retroviral vectors have actively contributed to the advent of gene therapy as a realistic approach in human therapeutics. At the beginning, the use of retroviral vectors was thought to be as simple as the collection of a viral supernatant that was applied to the desired cell. Rapidly, target resistance to transduction appeared in various conditions, *ex vivo* as well as *in vivo*. At that time, retrovectorologists entered an active "back to the bench" era. This phase was thought to have reached its conclusion with the generation of theoretically safe lentiviral vectors and when, in 2000, a first clinical trial using retroviral vectors proved to be successful. Unfortunately, recent developments have shown that we still need to improve our knowledge of several steps in the retroviral life cycle before we can accurately adapt vectors to target specific cells.

In this review we will first briefly detail key features of the life cycle of wild-type retroviruses. Thereafter, an overview of the minimal requirements needed to generate retroviral vectors will be followed by the relevant developments in this rapidly moving field. Of note, we have highlighted the crucial biosafety issues in a specific section. Copyright © 2004 John Wiley & Sons, Ltd.

Keywords retrovirus; vector; gene therapy; biosafety

Starters

Results from the X-SCID clinical trial emphasised the Janus face of retroviral gene transfer. On one side the successful correction of the deficit highlighted the relevance of the Moloney murine leukemia virus (MoMuLV) derived vector [1]. In contrast, the emergence of "leukemia"-like cells, in two treated patients, sadly reminded us that the risk of insertional mutagenesis was not theoretical. For the two patients, a molecular analysis of the transformed cells showed a unique integration event close to *LMO 2*, a gene involved in the onset of T-cell leukaemia [2].

Retroviral vectors emerged when, 20 years ago, engineered defective viruses were proved to be able to stably transfer a reporter gene [3,4]. Rapidly, MoMuLV and avian retroviruses appeared to represent suitable vehicles for a new class of therapeutic agent, genes. These pioneer experiments led to an attractive and simple definition of what is needed to generate a retroviral vector: "the elaboration of a transportable element harbouring no viral coding sequences, mobilised via the furniture, in trans, of structural and enzymatic information essential for active retroviral particle formation and recombinant genome mobilisation". Beyond this basic definition, the use of retroviral vectors rapidly faced delivery problems. Considering MoMuLV-based retrovectors, the crucial role of mitosis, which controls preintegration complex access to the nucleus, has been revealed [5]. While lentiviral vectors escape this blockade, other post-entry check points that control their life cycle have been identified and could have deleterious effects for gene

transfer [6–9]. Furthermore, moving from the laboratory bench to the desired nucleus, *in vivo*, implies that lots of other pitfalls need to be avoided. Human anatomy and histology of organs, as well as activation of the immune system, act against transduction. More generally, immunity, as it concerns both the vector and the transgene, remains a major issue in the field of gene transfer.

It is noteworthy that almost every disease will require the finding of an appropriate and specific route for vector delivery. However, unless they influence vector design, these aspects will not be considered in the present review. Even though a specific section will deal with safety issues, we will concentrate here on vector structure. This field has been extensively revisited in recent years. Improvements that have addressed the generation of safer vectors to allow specific long-lasting expression of the transduced genes should now confirm their clinical relevance. In addition, the use of retroviruses from various species, including the pathogenic AIDS agent HIV 1, has enriched the field of retroviral vectors by opening the opportunity to achieve effective *in vivo* delivery [10]. Lentiviral vectors will be detailed in a companion paper. Scale-up management and industrial developments will not be detailed [11].

Main course: retrovirus and cell

From RNA to RNA

Structural players

Retroviral genetic information exists in two different forms, a genomic RNA within the viral particle, and a proviral double-stranded DNA integrated into host cell chromosomes [12]. Retroviruses are diploid: their genome is a capped dimerised mRNA of positive polarity (Figure 1A). Taxonomic classification of retroviruses distinguishes seven genera and three groups. It is also convenient to discern simple retroviruses from the complex [12]. While the latter display a sophisticated regulation of their expression, through a subset of accessory genes, the canonical genetic organisation of retroviruses was originally described according to the former. Functionally, it is advantageous to isolate two types of retroviral sequences. Trans-acting sequences, basically encompassing viral coding sequence, are required for particle formation, while cis sequences are involved in genome mobilisation. Cis sequences are mandatory for reverse transcription, packaging and integration. Of note, despite a common retroviral replication pathway, sequences are poorly conserved among the seven genera of retroviruses.

The three structural proteins, matrix (MA), capsid (CA), nucleocapsid (NC), are common to all retroviruses. They originate from a precursor poly-protein, which could be posttranslationally modified. Along with these proteins the Gag open-reading frame (orf) also encodes proteins governing particle budding. Another viral orf encodes proteins with enzyme activity. Pro codes an aspartic protease involved in the cleavage of the Gag precursor and is essential for the release of infectious viruses. The reverse transcriptase (RT) and the integrase (IN) are encoded by Pol. The Pol proteins are produced as fusion proteins with the Gag-Pro precursor. The ratio of Gag versus Gag-Pol is maintained by virus-specific mechanisms of messenger translational control, readthrough or frameshift. Finally, Env encodes a membrane glycoprotein, composed of a surface glycoprotein (SU) bound to a membrane-spanning domain (TM), anchored in a lipid bilayer of cellular origin. Env forms trimers at the viral surface.

The coding information detailed above is coupled together with non-coding sequences required to allow genome spread and the molecular transition from RNA to

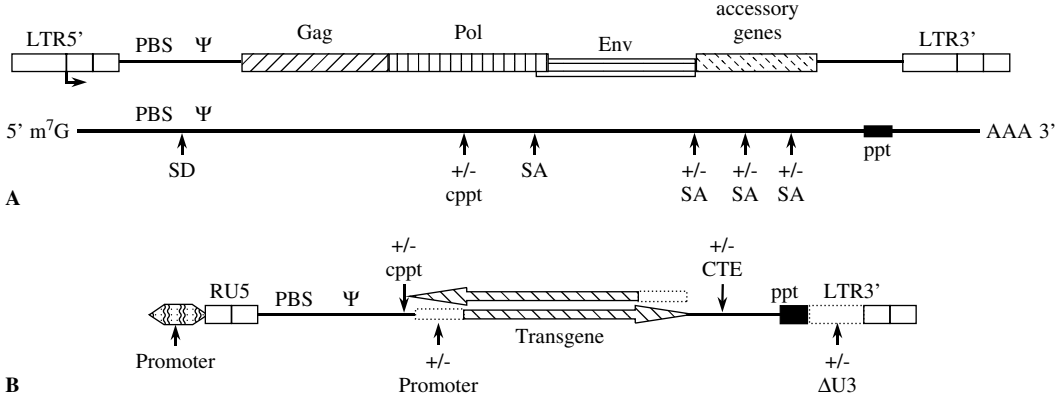

Figure 1. Canonical genomic organisation of retroviruses and retroviral vectors. (A) Wild-type proviral and RNA structures. LTR: long terminal repeat; PBS: primer binding site; Y: packaging signal; ppt: polypurine track; SD: splice donor; SA: splice acceptor; cppt: central polypurine track. Accessory genes are frequently observed on the 3′ side and could overlap the ppt and U3. An additional ppt (cppt) is present within lentiviruses. Extra splice acceptor sites are frequently used for the expression of accessory genes. Foamy viruses harbour an internal promoter. (B) Structure of a retroviral vector. Within the recombinant provirus the genomic RNA is transcribed using the 5′ U3 or a heterologous promoter. Optional features are: an internal promoter; a cppt; the deletion of the transcriptional sequences within the 3′ LTR and the addition of an heterologous RNA export signal, CTE (constitutive export element). Of note, the transgene could be cloned in either orientation

DNA. Among these cis-acting sequences are those involved in this last step: reverse transcription. The primer binding site (PBS), an anchoring sequence for a strain-specific tRNA (MoMuLV: $tRNA^{pro}$), is crucial to initiate reverse transcription. The polypurine track (ppt), a purine-rich sequence 3′ to Env, less sensitive to the Rnase H activity of RT, triggers the second-strand DNA synthesis. Some retroviruses harbour an additional ppt (e.g. lentiviruses) [13]. At each end of the genomic RNA, two repeats (R) allow the RT to jump from one side to the other during reverse transcription. R contains a polyadenylation signal and thus determines the genomic RNA size. Others cis sequences are needed for expression at the stage of proviral DNA. U3, unique in 3′ within the RNA genome, is duplicated in the provirus. The 5′ U3 drives proviral transcription: it contains various binding sites for the transcription factor. The RNA unique 5′ sequence (U5) is GU rich, permitting an efficient recognition of the R polyadenylation signal. Lastly, a set of several stem loops, interacting with viral proteins, forms the packaging signal, Ψ. It allows the specific recruitment of viral genomic mRNA within the particle. The packaging signal overlaps a genome dimerisation signal (DIS). For some retroviral species, an R secondary structure cooperates in encapsidation and transcription. Of note, viral particles also randomly package small cellular molecules.

Retroviral life cycle

Starting with the entry of the virus into the target cell, the retroviral life cycle is completed in four steps.

Entry Particles interact with their target cell through exposed trimers of SU which recognise a specific receptor at the cell surface. The SU–receptor interaction induces complex conformational changes within the former, uncovering a fusion peptide at the amino terminus of the TM [14,15]. As the core enters the cytoplasm, a poorly characterised uncoating process delivers the genomic RNA into the cytoplasm. Cellular partners that allow the viral cytoplasmic transport are poorly characterised. Recent data suggest that incoming HIV particles use the microtubule network to reach the nucleus [16–18]. The route from the membrane to the nucleus could be hazardous for retroviruses. Several centuries of co-evolution between retroviruses and their hosts have allowed the emergence of molecular mechanisms aimed at protecting the latter from a retroviral infection. This phenomenon, part of a broader process termed *restriction*, relies on the production of cellular factors affecting the retroviral life cycle. Not all the mechanisms underlying trafficking restriction are fully understood [19]. Of note, restriction, initially described for simple viruses, also exists for complex viruses [6,7,9]. As discussed below, this has implications in gene transfer.

Reverse transcription Conversion of the genomic RNA into a double-stranded DNA is completed in the cytoplasm. Nevertheless, it is well established that reverse transcription starts within the particle [20]. We should add that foamy viruses have a peculiar cycle, as reverse transcription is almost complete when the particles bud out of the producing cell [21]. The enzyme driving reverse transcription, RT, is a dimer with three distinct biochemical activities, RNA-dependent DNA synthesis, DNA-dependent DNA synthesis and Rnase H. A striking feature of reverse transcription is the generation of duplicated sequences at each extremity of the genome. This leads to the long terminal repeat (LTR: U3 R U5). U5 and U3, unique at the 5′ and 3′ ends of the genomic RNA, respectively, are the templates for the two homologous sequences contained within the LTRs. Therefore, any modifications introduced, into either the 3′ U3 or the 5′ U5 of a provirus, will be duplicated in both LTRs of any offspring provirus. Availability of free nucleotides in the cytoplasm could influence the efficacy of reverse transcription, and a low nucleotide concentration could explain why HIV infection of quiescent lymphocytes is impaired at an early step following entry [22,23]. Finally, the proviral DNA linked to a mixture of viral and cellular proteins form the so-called pre-integration complex (PIC).

Crossing the nuclear pore A key step in the retroviral life cycle is access into the nucleus. MoMuLV and most of the oncoretroviruses are unable to replicate in non-dividing cells. Roe *et al*. have shown that the PICs of MoMLV are not able to be translocated across the nuclear membrane [5]. Conversely, lentiviruses and possibly avian retroviruses have evolved specific functions allowing access to the nucleus.

PICs are heterogeneous structures comprising the proviral genome, associated with viral and cellular proteins (e.g. HMG1/Y for HIV) [24,25]. Like others, the PICs of HIV are over 45 kDa and cannot pass across the nuclear envelope by simple diffusion. Transport through the nuclear pore involves specialised cargo proteins [26]. The process is energy-dependent and karyophilic proteins share specific motifs (nuclear-localising sequences, NLS) that interact with cargoes. For avian viruses, PIC translocation has been explained by the presence of an NLS in the integrase [27,28]. However, nuclear localisation appears necessary but not sufficient for virus replication to be successful. Rous sarcoma viruses have been shown to integrate into resting cells but appear to be dependent on mitosis for viral expression [29]. Surprisingly, recent data suggest that vectors derived from the avian virus ASV can transduce resting cells [30]. For HIV, the observation of an active infection in resting macrophages triggered a significant effort to decipher the pathways allowing entry into growth-arrested cells [31]. Molecular determinants controlling the nuclear entry appear rather complex and have been ascribed to different HIV proteins, none of which seems dominant over the others [32–36]. Various nucleophilic signals could participate in the process. Of note, the experimental conditions, such as the target cell and the

multiplicity of infection, used to evaluate the influence of each signal are subject to controversy and diminish data significance. Within the HIV matrix (MA p15), karyophilic properties have been ascribed to the phosphorylation of a carboxy-terminal tyrosine [33,34]. Vpr has been proposed to promote docking of the PIC by direct interaction with karyopherin β [35]. Three NLS motifs have been described in HIV integrase but the exact localisation of these sequences remains controversial [36]. Lastly, nuclear localisation of HIV has been attributed to the central Flap sequence, a triplex region formed by strand displacement initiated in the central ppt during reverse transcription and finishing at the central termination signal (cts) 100 bases further on [37]. Two recent studies challenged the role of the central Flap, although it was shown that HIV does not only rely on this structure to access the nucleus. It has been suggested that at low multiplicity of infection and in lentiviral vectors, the Flap could give an advantage for transduction of quiescent cells [38,39]. Importantly, HIV is not fully independent of the cell cycle since reverse transcription is not completed in T cells until they reach the G1b phase of the cycle [23,40]. The nucleus is also a complex organelle with specific compartments in which chromatin is functionally different. Late domains within the CA of retroviruses influence fine nuclear localisation that could explain some features of the retrovirus cycle [41,42].

Integration and expression Once the PIC has reached the nucleus, the provirus has the opportunity to integrate into the host genome. Within the nucleus, the proviral DNA is subject to molecular rearrangements leading to three different forms of DNA. This process is triggered by the non-homologous DNA end-joining (NHEJ) pathway of DNA repair [43]. Among the proviral forms observed within the nucleus only the 'linear two LTR provirus' is a substrate for integration. Integrase directs the three steps of integration. Integrase specifically cleaves and binds the proviral ends at the attachment (att) sites of the LTR. Cleavage and ligation to the host genome uses a strand exchange process leaving two single-stranded repeats that are filled by the host DNA repair machinery [44].

Only when integrated is the provirus expressed. Retroviral transcription depends on the host RNA polymerase II. A close relationship exists between cellular transcription and the new gene that represents the retrovirus. Some viruses have evolved through trans-activating accessory proteins, a system to improve and control their level of expression. A sophisticated example of this is the Tat-dependent HIV gene expression. It is noteworthy that each strain of the virus has evolved a defined pattern of expression, relying on the presence of tissue-specific binding sites for transcription factors, in the LTR. In the case of the MMTV, this transcription control can even behave as a hormone-dependent inducible system. Unravelling and combining the existing combination could be helpful in deriving chimeric LTRs fully adapted to a target cell [45]. For this long-lasting pathogen, an equilibrium exists between expression and latency. The virus or the infected cell can modulate the latency. Foamy viruses control their expression and also their latency by a modulated expression of an accessory protein Tas [21]. On the other hand, infected cells can modulate viral gene expression by promoter shut-off. For example, promoter methylation is an efficient mechanism to achieve extinction [46].

Another very important feature which has a major influence on retroviral expression is the selection of the integration site. The theory that retroviral integration was a random process has now vanished. Furthermore, it has been possible, for HIV and ASLV, to define a weak consensus target sequence [47,48]. However, the requirement for these sequences is not absolute and other sequences are able to host a provirus. Recently, a genome-wide analysis, as well as more specific studies, gave indications about the preferred integration regions. Converging data indicate that there is a bias favouring the selection of expressed chromatin region for integration [49,50]. Of note, Schroder *et al*. have also shown that the virus itself can influence the expression of genes in the vicinity of the integration site. This had been known for some time and supported the enhancer trap strategies and the recently developed technique aimed at detecting genes involved in cellular transformation, by integration-tethered oncogene inactivation [51,52].

Particles formation The viral RNA product resulting from proviral expression can contain a complex set of splice sites controlling subgenomic RNA production [53]. Spliced and unspliced mRNAs give rise to viral proteins. Importantly, the latter is also the support for the genomic RNA. This explains why the ratio of each RNA species is of major importance. The proportion of each viral RNA is tightly controlled by modulation of RNA trafficking [54]. In addition to splicing, intrinsic viral RNA unstability can reduce the cytoplasmic availability of full-length genomic RNA. Two mechanisms can exert a protective effect on viral RNA processing and degradation. Retroviruses use either a sequence that directly binds nuclear RNA export factors, or produces an accessory protein that bridges a defined viral sequence to the nuclear export machinery [55–57]. Complex retroviruses featuring several splice acceptor sites use special pathways in order to provide the budding particle with sufficient amounts of full-length RNA species. HIV produces a viral protein (REV) which bridges a secondary structure of the genomic RNA (RRE) to the CRM1-dependent cellular RNA export pathway [58]. For the same purpose, the MPMV constitutive transport element (CTE) directly binds a cellular export protein [59].

C-Type retroviruses assemble and bud at the cell surface. For these viruses, the Gag and Gag-Pol precursors are targeted to the inner face of the membrane by a motif promoting myristilation of the Gag protein. Viral core proteins are produced in two different forms. The genomic mRNA codes for both a Gag and a Gag-Pol precursor.

Two different mechanisms lead to the formation of the latter. For MoMuLV, suppression of the Gag stop codon using a degenerate tRNA allows a readthrough leading to Pol translation. For this, HIV and other viruses use a mechanism of frameshift suppression conditioned by a RNA stem loop. Both mechanisms are poorly efficient, accounting for the correct ratio of Gag versus Gag-Pol, which is critical for infectious particle formation. Clusters of mixed Gag/Gag-Pol precursors are assembled at the inner face of the cell membrane. Gag proteins contain an L domain necessary for particle budding. Recently, protein–protein interaction domains, such as WW motifs, and others promoting the interaction of Gag with the ubiquitine machinery, have been characterised in small L-domain-containing Gag proteins, p12 for MoMuLV and p6 for HIV [60,61]. These sequences are mandatory for a correct budding and it is noteworthy that any domain can be replaced by another [62]. The host cell protein content can also affect the quality of the produced virus. For HIV, the Vif accessory protein participates in the infectiveness of newly formed virus. However, the phenotype of Vif-deficient viruses is dependent on the producing cell. Recently, the expression of a cellular gene, CEM15, has been shown to control the virus Vif dependence in CEM15-deficient cells [8]. Of note, except for the foamy viruses, Env is dispensable for budding of A-type particles with no L domain characterised so far. Any abundant membrane-bound protein could, passively, be incorporated into the viral envelope [63]. Env glycoprotein follows a regular pathway for maturation and glycosylation. During their traffic through the Golgi apparatus, oligomerised Env gene products are cleaved by a luminal furin protease leading to SU and TM monomers. Only cleaved proteins will exhibit fusion abilities essential for viral infectivity [64]. A second cleavage, performed by the viral protease, is necessary to activate fusion of MoMuLV and GaLV envelopes [64,65]. This cleavage takes place during particle maturation, an essential process to obtain infectious viruses. Maturation induces a complete reorganisation of the viral proteins leading to the modification of particle morphology. It results from the cleavage of precursor proteins by the viral Pro [66]. Importantly, protease-mediated cleavage is also required to process the reverse transcriptase and integrase proteins to their active form. Being non-lytic, retroviruses are released and, for cultured cells, can therefore be collected in the supernatant. This is a convenient way to harvest recombinant vectors.

Retroviral vector production

What you need in trans

Viral vectors are morphologically indistinguishable from their parental counterparts. Conversely, vector genomic organisation shows major modifications (Figure 1B). The above description allows us to recapitulate the requirements for the production of recombinant particles. Essential viral proteins are structural (Gag), enzymatic (Pol) and targeting (Env). Ideally, in order to minimise the risk of generating replication-competent viruses by recombination, all these components must be separated on different transcriptional units (Figure 2A).

Tropism As Pol proteins are translated using the Gag mRNA, and as the ratio of Gag versus Gag-Pol is important, all Gag-Pol-producing constructs are together on the same transcription unit. Nevertheless, splitting Pol from Gag has been possible for the production of lentivectors [67]. Conversely, the ratio of Env to other viral proteins is not critical for generating infectious particles. Furthermore, Env is translated from a spliced subgenomic viral RNA. Thus, expressing Env from a separate unit is easy and offers the advantage of the choice of the promoter driving Env expression. Retroviral envelopes such as ecotropic, xenotropic, amphotropic for MoMLV or gp120 for HIV are of obvious relevance but have proved to be fragile [68]. Heterotropic incorporation of Env, producing pseudotypes, could be a way to escape this limitation. For HIV and other lentiviruses, the size of the intra-cytoplasmic tail of the TM hampers their incorporation into MLV particles. Nevertheless, upon minor modifications, MoMLV and HIV vectors are easily pseudotyped using either retroviral envelopes, e.g. gibbon ape leukemia virus (GaLV), or proteins from other enveloped viruses; the G protein from vesicular stomatitis virus (VSV-G), the HA from the flu virus and even the envelope from Ebola virus [69–71]. This allows a better adaptation of the Env protein to the target cell. Most retroviral receptors have now been cloned and it is thus easy to test their expression and to choose the most suitable envelope for the target cell. Nevertheless, more than one envelope should be tested as transduction efficiencies do not always parallel level of receptor expression [72]. When using the VSV-G, its cytotoxicity imposes to either transiently transfect an expression vector or to use a controllable system [73] (Figure 2B).

Stable production Retroviruses offer the opportunity to either use stable producing cells or naive cells transiently transfected with plasmids containing all the necessary coding sequences for recombinant retroviral particle formation (Figures 2A and 2C). The simplest way to generate a stable producing cell line is to isolate cells which have stably integrated plasmids encoding Gag-Pol and Env after transfection and antibiotics selection [74]. Particles released in the supernatant were thought to be empty unless a recombinant vector was expressed in the cell. In fact, as RNA is needed for particle shaping in the absence of Ψ-containing RNA, any abundant RNA can be recruited [75].

An important goal for manufacturing retroviral vectors is the production of recombinant particles at very high titre. The origin of the cell on which the packaging system is based has a great influence on this. However, before considering this, we need to define relevant criteria for the choice of a packaging cell line. We must consider security,

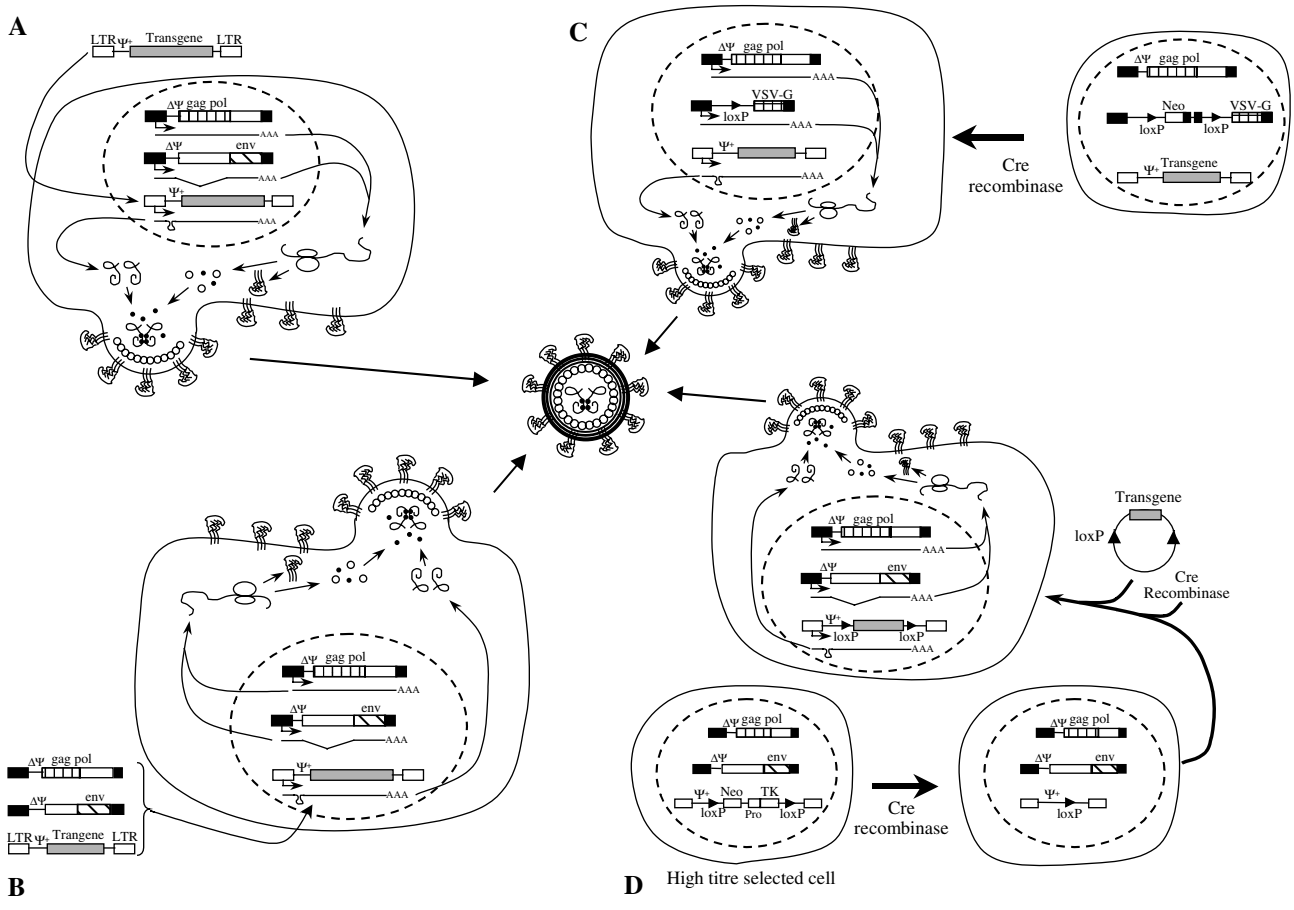

Figure 2. Packaging cell lines. (A) Stable producing cells: packaging cells contain two different Ψ-vectors stably integrated into the host genome. One drives the expression of structural (gag) and enzymatic (pol) genes for MoMLV particles production. Another contains the env gene essential for viral targeting. For biosafety issues, these genes are split in two vectors. Recombinant vector production can be obtained either by transient or stable transfection using a retroviral vector of interest. Only RNAs derived from the vector contain the packaging signal allowing their efficient and specific recruitments within viral particles. (B) Transient production: trans expression plasmids (Gag-Pol and Env) are transfected together with the recombinant retroviral vector. Of note, strong promoters, such as the CMV IE, are preferred. (C) Stable VSVG expressing cells: as the VSV-G is cytotoxic, generation of stable packaging cell lines harbouring this pseudotype is only possible through conditional VSV-G expression. Arai *et al*. [73] have constructed a packaging cell line constitutively expressing gag and pol genes and containing a silenced VSV-G gene. The promoter driving the VSV-G is followed by two lox surrounding a neomycine cassette that impairs VSV-G translation. Expression of the cre recombinase induces the deletion of the neomycine resistance gene and allows the production of the VSV-G protein. (D) Controlled vector expression: a stable producing packaging cell line is infected by a specific neo expression retroviral vector. Neomycine allows the selection of infected cells with high-level transgene expression. Within the vector the neomycine gene is surrounded by two lox sites. Transient transfection of a cre recombinase expressing vector drives neomycine excision, leaving the retroviral expression cassette intact. The remaining lox sequence, within the vector, offers a simple and efficient way to introduce any transgene at a site optimal for vector production

absence of pathogens, traceability of the parental cell, and a low rate of homologous recombination. Furthermore, the fact that endogenous retroviruses can compete for packaging imposes careful selection of the candidate producing cells [76–78]. NIH 3T3 were the first cells and, along with the gibbon-derived PG13, are the only ones that have received a licence to produce virus for clinical trial [79]. A huge amount of work is still ongoing in the search for good producing cells. A serious candidate that we will mention is the HEK 293 cell line and its derivative, 293 T. It has the advantage of being efficiently transfected using the classical and cost-effective calcium phosphate procedure, and have also been approved for clinical grade recombinant adenovirus production [80]. TE 671, a human rhabdomyosarcoma derived cell line, which recently appeared for retroviral vector production, also gives high titre [81]. Where several cell lines have been tested for their ability to produce recombinant retroviruses it has been observed that factors produced by these cells can negatively influence viral entry into the target cell and consequently the infectious titre [82,83].

Once the cell line has been defined, we need to consider the trans-acting retroviral sequences. It appears obvious that none of the Gag-Pol or Env constructs should contain a packaging signal. To minimise homology between vector and trans-acting sequences, the retroviral polyadenylation signal, 3′ to Gag-Pol or Env, is substituted for a heterologous sequence. Interestingly, as discussed by Miller, producing cells expressing Gag, Pol and Env from the same unit can be as safe as cells with split genomes

[79]. Issues of retroviral protein expression levels have focused mostly on modifications of the Gag-Pol and Env, with an aim of improving the titre. The 5′ LTR can be substituted for a stronger promoter such as those from the cytomegalovirus immediate early promoter or the human elongation factor 1α [81,84]. Linking the expression of the viral trans-acting sequences to the selection marker has proven to be a good system to assure a high expression of the former by increasing that of the latter. In one such system the sequence of the selectable marker is placed 72 base pairs (bp) downstream of the viral product without any IRES or splice site [81,84]. Here, translation of the marker relies on ribosomes re-entry, which is more likely if translation of the viral gene is high. This co-expression also makes it possible to maintain the stability of viral protein production hampered by several chromatin modifications and also by promoter DNA methylation.

Homologous recombination is the main mechanism by which replication-competent viruses are generated. Codon wobbling is a smart technique to reduce the homology between trans- and cis-acting sequences that could lead to the recombination [85]. Another very interesting result obtained with MA-deleted HIV suggests that it should be possible to generate minimal trans-complementing sequences [86]. If this is conceivable in the context of an MLV, these constructs would lead to a reduced risk of recombination with cis vector elements. Lastly, shedding light on the molecular events governing particle budding could help to improve particle release and, doing so, would increase viral titre. Some cellular co-factors have been shown to be helpful for increasing viral release: overexpression of BUL1 protein is known to enhance MPMV budding [87].

Choosing the right producer Among the possible techniques to produce retroviral vectors we will examine the selection of stable producer clones, or the transient transfection [88–90]. Transient transfection can rapidly be adapted to any transgene, but the titre is not guaranteed. Stable clone selection offers advantages of known titre that can be obtained reliably and is well adapted to industrial scale-up. Nevertheless, the generation of a stable high-titre producer clone remains time-consuming. Vanin *et al*. described a strategy to facilitate rapid generation of high-titre producer cells [91] (Figure 2D). It is based on the selection of a highly efficient genomic transcription site where a provirus containing a lox sequence is introduced. This lox sequence offers a simple way to introduce any transgene of interest. Nevertheless, the existent lox vehicle construction has to be modified to allow the acceptance of different sets of retroviral constructs. Alternatively, the use of 'high-throughput' screening of transfected producer clones would help the isolation of high-titre clones from a large population. Several techniques of accurate viral titre estimation are based on either dot blot screening or competitive PCR [92–94]. Direct blotting of crude supernatant has also been reported although interpretation of the results is ambiguous. Using real-time PCR and exploiting retroviral strong stop synthesis it is possible to develop a rapid PCR-based assay for viral titration [95]. As time goes by, stable clones frequently show a drop in recombinant viral production. As described above, the design of packaging constructs can help minimise this phenomenon. It is also possible to chemically wake up transcription of packaging constructs [96,97].

Other industrial techniques aimed at improving viral titres have been described. Culture conditions could ameliorate viral stability before supernatant collection and procedures for viral concentration and purification are also under technical development [98]. Bioreactors, using fibres or beads, are also under evaluation. It is important to bear in mind that the half-life of a vector is short. Viral instability could result from envelope shedding or other modifications. Furthermore, some producing cells simultaneously release inhibitors and recombinant virus [99]. Retroviral titration depends on several parameters including the target cell, the volume used for transduction, viral half-life and time of target cells exposure [99,100]. These factors greatly influence the transposition of published results. As it was proposed for adenoviruses, retroviral titration has to be standardised. Andreadis and co-workers settled on a convenient formula, based on physical parameters, which should be useful in standardisation of retroviral titres [100]. It is also important to mention that a low density of Env proteins at the cell surface can result in a low transduction efficiency. It is often worth stimulating stable producing cells with the selection agent used to generate them.

Transient production Transient production mainly relies on transfection using commercially available reagents such as calcium phosphate or positively charged liposomes. To improve the process authors have proposed more efficient techniques to introduce the trans and cis elements into a cell. Packaging constructs have been successfully inserted into recombinant adenoviruses. The system needs three recombinant viruses, one driving Gag-Pol production, one providing Env and the third carrying the recombinant construct [82,101]. As adenoviral vectors efficiently transduce cells *in vitro* as well as *in vivo*, it is possible to achieve retroviral production *in vivo* after direct injection of the different adenoviruses. This local retroviral production could theoretically lead to highly efficient *in vivo* transduction. As recombinant adenoviruses are immunogenic, infected producing cells should rapidly be cleared out leaving the retrovirally transduced cells. We should be aware that repeated target cell transduction could augment the risk of insertional mutagenesis to levels observed with replication-competent viruses. As a consequence, this approach should be restricted to cancer therapy for which replication-competent viruses have been proposed [102]. This procedure is complex and time-consuming. Furthermore, in addition to the risks related to the retrovirus, we have to consider those related to recombinant

adenoviruses. The use of other potent expression systems, such as Semliki-derived vectors, to produce recombinant retroviruses has been suggested [103,104]. This system offers two kinds of advantages, the very high titres that are obtained, and the opportunity to produce intron-containing retroviral vectors. Nevertheless, this strategy is very risky: SFV are highly recombinant and it has been shown that SFV vectors could be packaged within the recombinant retroviral particle [75].

Also cis to use

As discussed above, cis-acting sequences are required for two essential steps: reverse transcription and packaging. Reverse transcription involves two primers, the PBS anchored tRNA and the ppt, and one jumping sequence, R. The organisation of these three elements must be correct within the virus. Thus, the transgene must not contain a polyadenylation signal which could lead to a shorter RNA, ppt and R deleted, not able to be reverse transcribed. Figure 3 depicts the more frequently used recombinant structures.

Packaging The minimal packaging signal, defined by deletion experiments, allows an efficient recruitment of recombinant RNA. Nevertheless, extending the packaging signal, up to 400 bp into *gag*, improves titres [105]. This produces an overlap between the vector and the trans sequences increasing the risk of homologous recombination leading to RCR. It has been suggested that the 400-bp gag sequences could behave as a nuclear export signal [57,106]. Indeed, this gag stretch is site-independent and could be replaced by a constitutive transport element (CTE) from Mason-Pfizer monkey virus (MPMV). It is now common to add an export sequence from a variety of origins within retroviral vectors [106,107]. Surprisingly, Kim *et al*. have shown that these gag sequences can be removed with a minor reduction of the viral titre [84]. It is also possible to replace the Ψ by a sequence derived from an endogenous retrovirus (i.e. VL30) [108].

Transgene limitations Transgene size might be a limiting factor. Cloning capacity of retroviral vectors depends on the parental virus, but 8 kb appears to be the maximal transgene size for most vectors. Packaging, however, is not the limiting step: a recent study has shown that it was possible to package up to 20 kb in a MoMLV-derived vector [109]. Transduction efficiency was limited by a poorly efficient reverse transcription, especially for vectors over 15 kb.

As the genome of retroviruses is an mRNA, the presence of an intron within the vector could interfere with its packaging. Four strategies could be used to mobilise intron-containing transgenes. One would introduce an HIV RRE within the intron, the vector being produced in a Rev-expressing packaging cell. It is also possible to clone the transgene in a reverse orientation, relative to transcription of the recombinant virus. A smart strategy using the reverse transcription features is another possibility: here the SD site is cloned in the 3′ U3 and the SA is 5′ to the transgene [110]. In the transduced cell, the SD is copied into the 5′ U3 and allows excision of the intron. Lastly, the SFV production system is cytoplasmic and hence escapes from RNA splicing [103]. Introns within cis-acting retroviral sequences have a puzzling effect. Krall *et al*. have shown that the MFG of the intron-containing vectors has a higher titre compared with intron-less constructs [111]. The reason for this is unclear since splicing should remove the packaging signal. Attempting to get rid of viral sequences would command their removal. Yu *et al*. have shown that synthetic SA can advantageously replace pol SA, and that the usefulness of SA depends on the transgene [112].

Transgenes are classically cloned in place of the retroviral coding sequences, between the packaging signal and the ppt. Reverse transcription induces a duplication of the sequences comprised either between PBS and R in 5′ or between the ppt and R in 3′. This feature has been exploited to generate double copy vectors. Here, the transgene is introduced in the 3′ U3 of the proviral recombinant vector. In the transduced cell, the transgene is duplicated, which can be helpful in achieving a high level of transgene expression [113]. Likewise, application of the double copy system has been developed to obtain a 'clean' site of transgene insertion [114]. Choulika *et al*. have introduced, in the 3′ U3 of a cre-expressing recombinant retrovirus, a single loxP sequence placed 5′ to a promoter and transgene. After transduction and duplication of the U3, cre recombinase deletes all the sequences between the loxP sites. Therefore, a simple copy of the parental 3′ LTR, comprising the transgene, is the only stigmata of vector insertion. The need for a cre-expressing unit reduces the cloning capacity of the vector. Finally, it is possible to use the template switch observed during reverse transcription to remove a sequence. Delviks *et al*. have shown that surrounding the Ψ by direct repeats leads to its efficient (>90%) removal in the integrated provirus [115]. In preventing further vector mobilisation this system improves biosafety.

Expression Long-lasting transgene expression, through integration, is the most important characteristic offered by retroviral vectors. This is an indisputable requirement for a cure for a constitutive genetic disease. On the other hand, as we will discuss later, uncontrolled integration could be troubleshooting. Here, it is worth distinguishing vectors in which transgene expression is dependent on LTR transcription and those containing an internal promoter.

The use of the LTR is advantageous as it preserves the cloning capacity and does not give rise to promoter competition, leading to promoter occlusion. On the other hand, structural constraints imply that the mRNA containing the transgene also bears the packaging

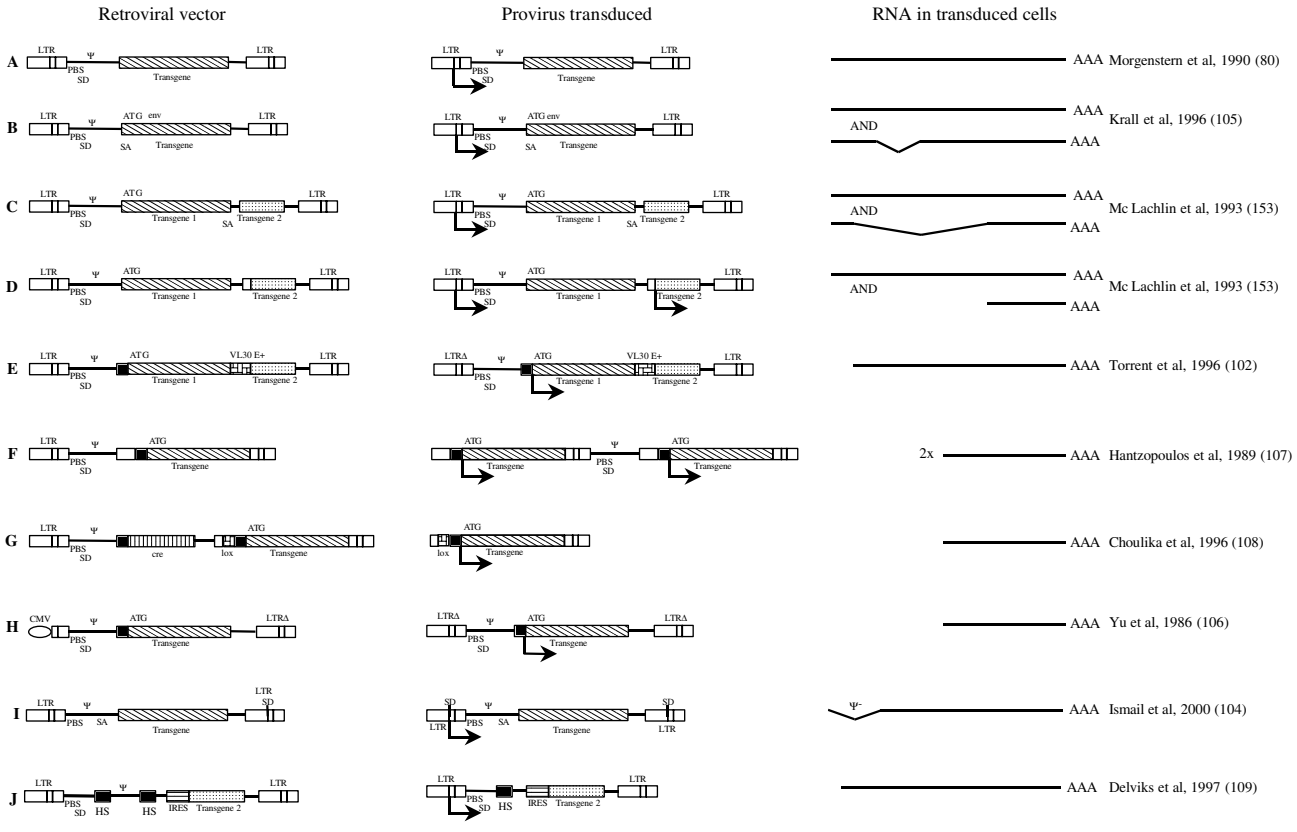

Figure 3. Possible structures for retroviral vectors. (A) First-generation recombinant retroviral vectors. (B) MFG vectors: an intron allows high viral titres. (C) Dicistronic vector with splicing sites for expression of the second gene [164]. (D) Dicistronic vector with internal promoter [164]. (E) Dicistronic vector with VL30 sequence which ensures packaging signal and IRES functions. (F) Double copies: the transgene is cloned within the 3′ U3 of the retroviral vector and is duplicated in the transduced cells. (G) Self-cleaning vectors: a LoxP sequence placed 5′ to the promoter and transgene in the 3′ U3 within the vector allows, in the transduced cells, the deletion of most viral sequences through Cre-driven recombination. (H) Self-inactivating vectors: deletion of the promoter within the U3 3′ LTR in the retroviral vector is transferred to the 5′ LTR after reverse transcription. (I) Split vectors: a splice donor site (SD) is cloned in the 3′ LTR of the vector which contains a splice acceptor (SA). After reverse transcription, the intron excision order is reconstituted allowing the removal of the packaging signal. (J) Psi-vectors: a homologous sequence (HS) surrounds the packaging signal. During reverse transcription homologous recombination in HS drives PSI removal

sequence. This could be avoided with the so-called split retroviruses [110]. LTR expression suffers from the chromatin context. Pioneering work by Jaenish and co-workers, who attempted to generate transgenic animals by retroviral transduction, showed that retroviruses can be shut off by transcription silencing. Methylation-dependent and methylation-independent molecular modifications of the LTR explain this shutoff [116,117]. Using transcriptional insulators from the β globin gene it has been possible to minimise this downregulation of transgene expression [118]. Using a cHS4 insulator, Yannaki *et al*. have shown that the position of this sequence, within the vector, can influence its effect on transgene expression [119]. CpG islands also appear to protect against methylation silencing [46]. As mentioned, the selection of a specific LTR adapted to the target cell is a convenient way to obtain viruses less sensitive to silencing [45]. Exploiting reverse transcription allows the introduction of a heterologous promoter within the 3′ LTR in the parental provirus. Finally, this promoter is duplicated in the transduced cells and could drive expression of a specific transgene [120]. No data are available concerning the duration of expression from these vectors. Of note, this strategy has been successfully adapted to lentiviral vectors with a reduced risk of mobilisation [121].

Retroviral vectors are very compliant in accepting the use of an internal tissue-specific promoter. In this case, the parental construct is improved by a deletion of the 3′ U3 promoter. In such a vector, called self-inactivated (SIN), the transgene is expressed by an internal transcript devoid of the mobilisation sequence [122]. Its expression is controlled by the specificity of the promoter: it could be absent in the producing cell and elevated in the target cell. Furthermore, the absence of an enhancer within the LTR means there will be no transactivation effect in the vicinity of the integration site. Several tissue-specific retroviruses have been successfully generated [97,123]. A tighter control of transgene expression could be advantageous when using toxic transgenes or transgenes for which controlled expression is required. It has been possible to produced regulated retroviral vector expression using the tetracycline system [124].

Recent development
Modification of retroviral vector tropism through envelope engineering is detailed by Verhoeyen and Cosset in this issue.

Resting cells, the ultimate challenge? As mentioned above, it is now well established that MoMLV-derived vectors do not transduce quiescent cells [5]. Not only is this problematic for *in vivo* applications, but it also has dramatic consequences on *ex vivo* transduction. A naturally occurring system by which the virus triggers entry into the cell cycle target exists. Murine mammary tumor virus (MMTV) infects circulating B-cells, which are quiescent before stimulation. Within the MMTV genome, an open reading frame, coding a super antigen (sAg), is thought to facilitate viral infection. Incorporated within the viral envelope the sAg could stimulate a B-cell, which becomes permissive to viral infection [125]. Self-activating vectors either co-express an envelope-anchored cytokine (e.g. hepatocyte growth factor (HGF) or interleukin 2 (IL2)) together with a wild-type retroviral Env protein, or harbour an N-terminally modified Env expressing a peptide competent for receptor activation. Both systems proved to fulfil the expectancies when assayed *in vitro* [126]. Nevertheless, HGF-exposing viruses failed to improve *in vivo* transduction of c-met-expressing hepatocyte (N'Guyen, personal communication). Hematopoïetic stem cells are a major target in gene therapy. Several protocols using specific culture conditions have been designed in order to improve transduction efficiency [127]. Almost all rely on increasing the number of cycling cells susceptible to retroviral transduction. Yet, serial transplantation experiments in mice have shown that the stimulated stem cells lost their ability to repopulate irradiated animals [128]. In humans, where the pool of cells that actively contributes to the hematopïetic lineage is cycling, this could be deleterious in the long term. This strategy of cytokine stimulation of the target cells has been successfully used to increase hepatocyte transduction *ex vivo* as well as *in vivo* [129,130].

Several approaches have attempted to modify retroviral genomes in order to allow MoMLV transduction of arrested cells. Deminie and Emerman constructed a chimeric MoMLV virus expressing HIV_{MA} or HIV_{CA} in place of the wild-type proteins [131,132]. Both viruses were partially defective and neither virus was able to productively infect quiescent cells. More recently, Lieber and co-workers [133] generated a MoMLV-based vector bearing binding sites for an adenoviral protein involved in nuclear import (terminal protein, TP). This vector was used to transduce arrested TP-expressing cells. Despite nuclear import of the retroviral genome, integration was irrelevantly obtained [133]. As an NLS sequence has been described in the IN protein from HIV or ASLV, Seamon *et al*. [134] introduced the canonical SV40 NLS within the carboxy-terminal end of MLV IN. Such a modification had a dramatic effect on particle formation and was unsuccessful in allowing infection of quiescent cells [134]. Conversely, by introducing an NLS into the MA protein of a spleen necrosis virus (SNV), Parveen and collaborators were able to generate packaging constructs allowing transduction of quiescent cells [135]. Furthermore, another avian virus, ASV, appears to be able to transduce quiescent cells [30]. This emphasises life cycle differences between simple retroviruses. A companion paper will deal with the advantage of using HIV-based vectors to achieve quiescent cell transduction.

Risk evaluation

Insertional mutagenesis

As retroviral vectors are integrating vectors, biosafety importantly deals with integration as a source of both, mutation and inappropriate transactivation. Enhancer trap strategies and the use of murine retroviruses to efficiently trigger transformation, in a permissive genetic background, take advantage of that insertional mutagenesis risk [51,52]. Other experimental data include studies on chromatin preferences for retroviral integration [136]. However, it was considered that integration was a random process that led to theoretical calculation of an insertional mutagenesis risk [137]. Recent experimental data and one clinical trial have shown that this calculation should be re-examined.

The X1 HuSCID clinical trial had raised great hopes, being the first successful human gene therapy trial [1]. However, it has recently been stopped after a "leukemia-like" syndrome, affecting T cells, arose in two patients who received *ex vivo* transduced $CD34^+$ cells [2,138]. Molecular examination of the tumour cells has shown that the therapeutic vector was inserted close to the *LMO* 2 gene, a transcriptional factor involved in transformation events leading to T-cell leukaemia in humans [139].

Until recently little was known concerning MLV integration selectivity. PCR techniques and whole genome databases have been used to improve our knowledge of this event [140,141]. Genome-wide studies using human primary cells are an obligatory step to accurately estimate risks. In this context, the study by Wu *et al*. confirmed that gene vicinity appears to offer favoured sites for retroviral integration [142]. This bias must now be taken into account to design formulas for risk evaluation. Of note, no insertional mutagenesis has been reported in one other clinical trial involving retroviral vectors [143,144]. This suggests that the transgene or the target cell could have influenced the arising of the transformation. Li *et al*. noticed ten leukemias within ten mice that had received a bone marrow transduced *in vitro* with a recombinant retrovirus expressing a truncated cell surface receptor [145]. Analysis of the transformed cells revealed first that the retroviral vector was inserted within the *Evi* 1 gene, and second that the theoretically defective receptor was in fact able to transduce a growth signal upon stimulation by NGF [145]. The involvement of the

truncated NGF in the onset of transformation remains controversial, as noticed by Bonini *et al*., who could not detect any transformation events in a series of experiments in which this transgene was used [146]. Concerning Fischer's patients, the constitutive unregulated expression of the γc receptor could also be responsible for a permanent cell cycle deregulation of the transduced cell. Nevertheless, Hacein-Bey-Abina and collaborators were not able to detect any alteration of the γc-transducing pathway within patients' cells [2]. Lastly, the vector used in the trial contains two fully active LTRs that might be responsible for the two-fold increase in *LMO* 2 transcription. It is also important to consider the target cell itself: lymphocytes are prone to repeated cycling upon exogenous stimulation, such as a viral infection observed for one patient. Such a proliferation could participate in the transformation process.

In the future, as they offer an opportunity to transduce resting cells, lentivectors could be used in clinical trials. Thus, it appears important to study their integration pathway. Schröder *et al*. recently shed light on lentiviral insertion preferences in the human genome [50]. Taking advantage of the availability of the genome project data, this group genetically characterised the border from 524 retroviral insertions, after a unique HIV infection in a human lymphoid cell line. Among the 524 junctions analysed, 69% were localised within genes; this was confirmed by the study of Wu *et al*. [142]. Furthermore, using micro-array analysis, Schröder *et al*. elegantly showed that most of the targeted genes were expressed and that the insertion could give rise to a modified pattern of expression, thus confirming the need for enhancer-less vectors.

Could avian retroviruses be made safer by selecting an insertion region with a low transcription activity as suggested by Weidhaas *et al*. [147]? These authors observed an inverse relationship between gene expression and avian leukosis virus integration [147]. We express caution in the interpretation of this result, since the authors focused the study on a specific DNA region already modified by a transgene insertion. A more general approach might confirm these results, and the resulting modification of expression should be evaluated.

Finally, when examining modifications at the site of integration, readthrough is also an important feature to consider, as it could result in unsuitable gene expression. Notably, retroviruses have weak polyadenylation signals [148].

Recombination

There are other risks associated with the vector and the production process. When producing clinical-grade recombinant virus, one has to evaluate: the pathogenicity of the parental virus; the frequency at which replication competent retrovirus (RCR) emerges, during vector preparation or within the patient; and lastly, the toxicity of vector preparations, according to the presence of viral proteins, or of compounds from the production system.

Little is known, except with AIDS, about the pathological consequences resulting from the infection of a human by a virus with a nonhuman tropism. On one hand, the risk is low as MoMLV virus infects quiescent cells at very low efficiency. Conversely, immuno-suppressed monkeys infected with a preparation abundantly contaminated by a RCR developed leukemia [149]. The question is to evaluate the clinical relevance of this experiment with a high RCR contamination. We now have very sensitive procedures for RCR detection; however, we should keep in mind that cancer patients are often immuno-suppressed.

MoMLV-based vectors are now routinely produced under good manufacturing practice (GMP) conditions and have been validated for clinical use. Before Fischer's study, MoMLV vectors had been administered to tens of thousands of experimental animals and to more than 1200 patients with no reported toxicity. This emphasises a possible involvement of the transgene in HuSCID cell transformation. Recombination events at the origin of RCR have been addressed by careful design of vectors and packaging systems. This has drastically reduced this risk. At the DNA level, recombination events could occur between the mix of co-transfected plasmids or between a transfected plasmid or the proviral vector and a homologous chromosomal sequence in the target cell. This risk is low and, for retroviruses, recombination is more frequent at the RNA level. Template switches during reverse transcription are the major source of recombination. The rate of this 'copy choice' recombination in a single round of replication has been estimated to be about 4% per kilobase [150]. Therefore, biosafety essentially relies on minimising packaging of non-specific or helper RNAs. Of note, superinfection of the producer cell is known to augment the rate of recombination. This is essentially observed using VSV-G pseudotypes. The potential of endogenous retroviral sequences as recombination partners raises safety concerns; in particular, as human endogenous retroviruses (HERV) are related to either onco or spuma retroviruses and represent more than 1% of the genome [12]. No such homologous sequences have been identified so far for HIV. A functional equivalent, and putative ancestor of HIV Rev, is encoded by HERV-K sequences, but no sequence homology exists between the two genes [151]. Lentiviral vectors will take advantage of MoMLV experience. Using last-generation lentiviral vectors, none of the individual components can produce RCR [112,152].

Conventional toxicity

Toxicity could originate from vector preparation. The quality and safety of clinical-grade vectors are dependent on the production process. The need for effective purification procedures reliably separating the vector from potential contaminants is important. Strict requirements exist regarding the source and the traceability of serum.

Development and assessment of serum-free culture media are important goals [153]. Transient transfection vector production is predisposed to contamination with plasmid DNA. Extensive culture testing is required to provide reasonable assurance that producing cells are free of contaminating agents such as viruses, virus-like particles, bacteria, or fungi. The retroviral particles themselves incorporate a variety of components of cellular origin, their nature or abundance dependent on the producer cell type.

As with other vectors, humoral and cellular immunological responses against the vectors, the transgene or molecules co-injected with the vector (e.g. calf serum) are known to shorten the half-life of transduced cells [154]. Using lentiviral vectors, prolonged expression in muscle, brain, liver, eye and cochlea of rodents is obtained with no noticeable lymphocyte or macrophage infiltrates [152,155]. Early examination of the injection site has documented the presence of inflammatory cells, but since control animals have the same infiltrate, this was attributed to the surgical procedures [156–158]. Lentiviral vector gene transfer into the monkey nigrostriatal system has been shown to induce minor perivascular cuffing without apparent inflammatory response [159]. In the liver, after intraportal infusion of a lentiviral vector in Fischer rats, a dose-dependent increase in serum ALT, and a mortality rate of 74% for 8×10^8 transduction units, were observed [160]. The presence of contaminants that trigger a local inflammatory response shortly after injection can dramatically influence the onset of an immune response.

Will the story go further?

As a conclusion we will concentrate on biosafety, and biosafety again. Improved constructs well adapted to the target cell should minimise risks.

One important goal to assure the biosafety of a vector preparation is to avoid the co-production of RCR. In order to minimise homology between vector and packaging constructs, *in vitro* selected RNA with high affinity for HIV-1 nucleocapsid (NC) has proved to mediate HIV-1 RNA packaging into virions [161]. Also, further modifications could improve packaging constructs. Accola *et al*. have shown that it is possible to obtain particles with important deletions of viral structural proteins [86]. If such particles prove to be efficient at RNA packaging, this could be useful for the design of safer packaging constructs.

Integration studies have unequivocally shown that all retroviruses have a marked integration preference for gene-rich regions [49,50,52,142]. Weak consensus sequence and integration hot spots force us to reconsider retroviral vector design. We need to take into account gene disruption, but we should remember that retrovirally induced variegation can have dramatic effects on expression at the integration site. A possible explanation for this phenomenon is that a wild-type virus selects open chromatin for integration, and then expresses specific functions to keep it active for spreading [49]. This implies that we should greatly improve biosafety by using SIN vectors with tissue-specific promoters. Another amelioration could be obtained with vectors that contain flanking insulators. These sequences would isolate, from the influence of a strong internal promoter, the genes in the vicinity of the integration site [119].

Last, but not least, we have to evoke targeted integration of the provirus [162]. Site-specific integration vectors would dramatically increase biosafety, provided that the selected site is itself safe. However, it might be difficult to find a site where no adverse effect ever arises and that, at the same time, allows sustained transgene expression. Again engineering vectors, with both locus control regions and insulators, could permit this contradiction to be faced. Finally, we should perhaps try to obtain help from transposable elements, some of which having interesting features in site-specific integration [162,163].

Acknowledgements

We thank G. J. Towers for careful and constructive reading of the manuscript. The Vector Group is supported by grants from the AFM and the VLM. Thierry Bru received a fellowship from the Ministère de la Recherche et de l'Enseignement Supérieur.

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