REVIEW ARTICLE

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Retrovirus Vectors: Toward the Plentivirus?

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Recombinant retroviral vectors based upon simple gammaretroviruses, complex lentiviruses, or potentially nonpathogenic spumaviruses represent relatively well characterized tools that are widely used for stable gene transfer. Different members of the *Retroviridae* family have developed distinct and potentially useful features related to their life cycle. These natural differences can be exploited for specialized applications in gene therapy and could conceivably be combined to create future retroviral hybrid vectors, ideally incorporating the following features: an efficient, noncytopathic packaging system with low likelihood of recombination; serum resistance; an ability to pseudotype with cell-specific envelopes; high-fidelity reverse transcription before cell entry; unrestricted cytoplasmic transport and nuclear import; an insulated expression cassette; specific chromosomal targeting; and physiologic or regulated levels of transgene expression. We envisage that, compared to contemporary vectors, a hybrid vector combining these properties would have increased therapeutic efficacy and an enhanced biosafety profile. Many of the above goals will require the inclusion of nonretroviral components into vector particles or transgenes.

Key Words: reverse transcription, integrase, mouse leukemia virus, human immunodeficiency virus, spumavirus

Contents

INTRODUCTION

The family *Retroviridae* shares three common features that are of major interest for gene delivery [1–6]:

(1) receptor-mediated uptake of a membrane-coated viral particle into target cells,
(2) reverse transcription of a plus-stranded RNA genome into a double-stranded DNA that is integrated into cellular chromosomes to establish active or latent infection, and
(3) cytoplasmic assembly of particles with incorporation of the full-length retroviral mRNA as the mobile form of genetic information.

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Three genes are required in this process and can thus be found in all replicating retroviruses: *gag* (encoding viral matrix, capsid, and nucleocapsid proteins), *pol* (encoding a protease, reverse transcriptase, and integrase), and *env* (encoding a bipartite membrane-anchored surface protein mediating target cell recognition and particle uptake). The proteins encoded by these open reading frames are processed into several subunits, which require cleavage by the retroviral protease following budding of the immature particle. Replication-competent retrovirus vectors contain genes in addition to the canonical *gag–pol–env* genome [7]. Splitting *gag–pol* and *env* into two separate retroviral genomes may allow the generation of a complementary replicating vector system [8]. The more widely used replication-deficient retroviral vectors are generated by coexpressing the basic retroviral *trans*-acting genes from transcripts that are not intended to be incorporated into retroviral particles. The transgene is encoded within a transcript that contains all *cis*-regulatory sequences required for its retroviral packaging (Fig. 1).

Three forms of replication-defective retroviral vectors can thus be generated (Fig. 2; Table 1):

(1) If only mRNA is to be transduced, *cis*-acting sequences need to lack crucial elements necessary for interaction with reverse transcriptase (RT), the vector particle needs to be devoid of this enzyme, or its function needs to be blocked by treatment of target cells with RT inhibitors [9].
(2) If episomal delivery of circular transgene DNA is to be achieved in target cells, *cis*-acting sequences interacting with integrase (IN) must be destroyed or IN needs to be mutated or inhibited [10].
(3) Stable integration of a replication-deficient provirus into chromosomes is the archetypal application of retrovirus vectors and occurs with an efficiency that has not yet been reached with any alternative integrating vector system.

For future vector design, two avenues can be followed: the creation of specialized vectors that profit from the naturally evolved tropism of a given retrovirus for a cell type of interest or the development of innovative hybrid vectors that combine advantageous features of different *Retroviridae*. The present review deals primarily with the classical use of retroviral vectors for stable transgene insertion; the final section also addresses the advent of retrovirus-derived vectors for delivery of mRNA or episomal DNA.

Retroviruses Typically Used for Vector Design and Desirable Features

Distinct retroviral genera have been classified based upon the processing of the fused *gag–pol* transcription unit, the

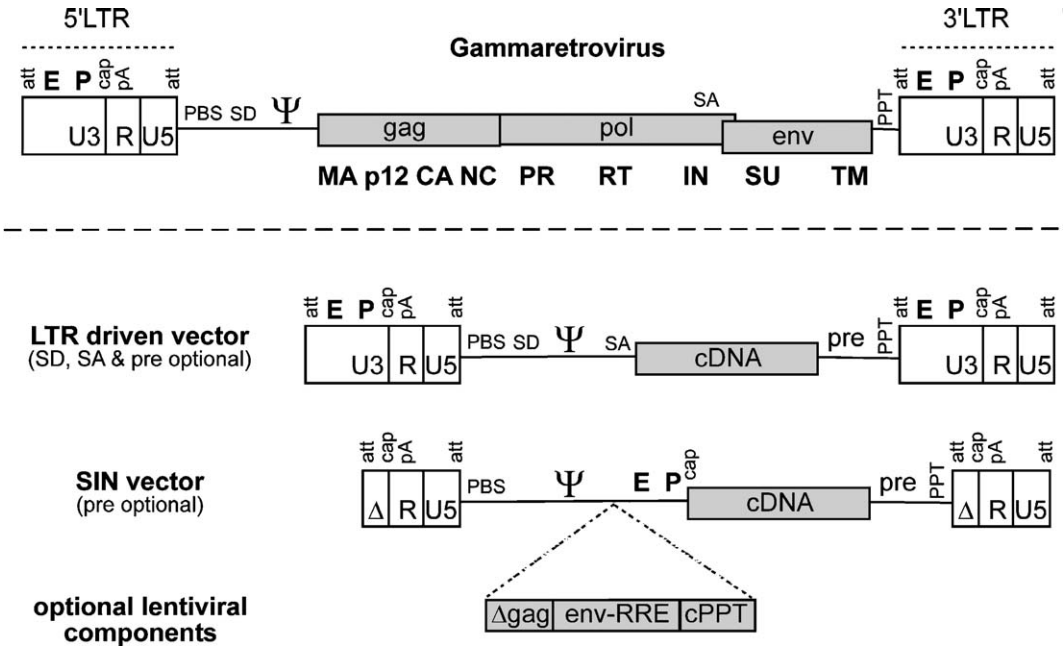

FIG. 1. Schematic representation of a gammaretrovirus with its simple genomic architecture. Coding sequences (encoding the protein subunits indicated below the viral genome) are separated from cis-regulatory sequences. Two types of vectors can be generated, either LTR-driven or self-inactivating (SIN). The latter are obtained by deleting (Δ) the enhancer-promoter (EP) from the U3 region of the long terminal repeat (LTR). pre, post-transcriptional regulatory element; att, attachment site; pA, polyadenylation signal; PBS, primer binding site, SD, splice donor; SA, splice acceptor; PPT, polypurine tract. Optional lentiviral vector components are also indicated (see text).

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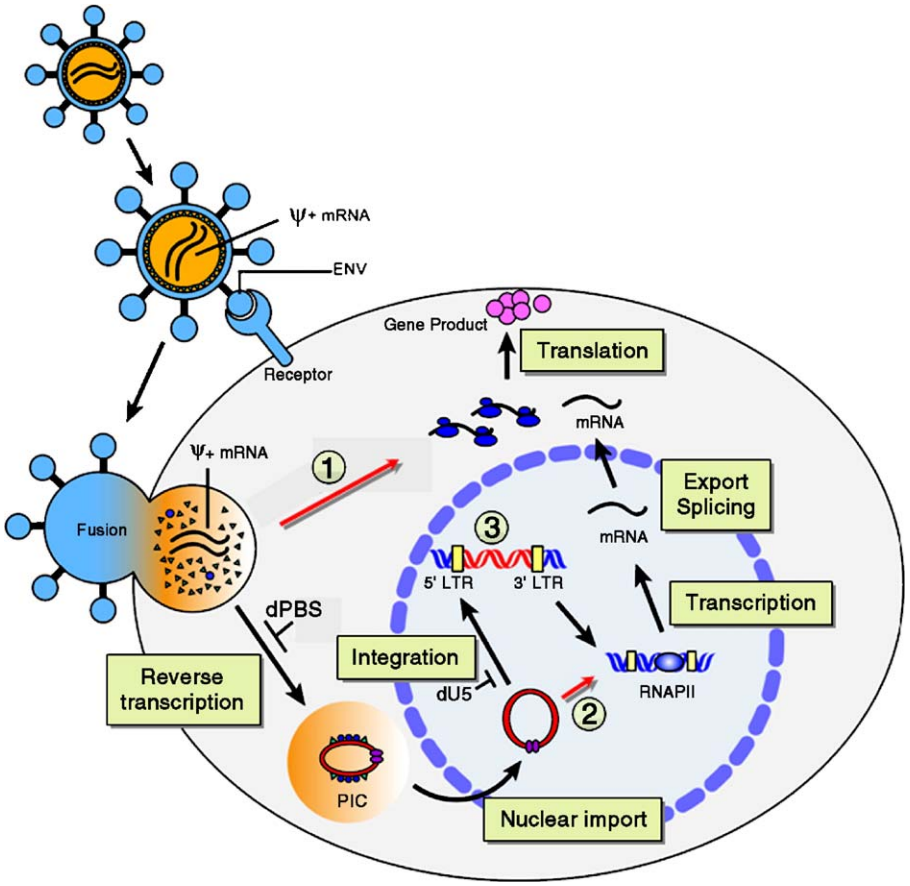

FIG. 2. Delivery of nucleic acids by retroviral particles. Following receptor-mediated uptake (by fusion or via endosomes, depending on the envelope protein), retroviral particles can deliver three forms of genetic information: (1) if reverse transcription does not occur, the mRNA may be subject to immediate translation; (2) if integration is blocked, episomal circles can be generated that may persist in non-dividing cells; (3) if all steps of the retroviral transduction process are completed, a double-stranded DNA integrates in cellular chromosomes. ΔPBS, deletion/mutation of the PBS, Δatt, deletion/mutation of the att sites; RNAPII, RNA polymerase II. Reprinted with minor modifications from [9], with permission from Elsevier.

acquisition of accessory proteins, increasing genomic complexity, and site of particle assembly (Table 2). Although interesting properties relevant for vector design can be found in all types of retroviruses, so far the focus has almost exclusively been on the following three genera (Table 2):

(1) Simple gammaretroviruses with the paradigmatic vectors derived from murine leukemia virus (MLV). These vectors possess the most advanced packaging system.

(2) Complex lentiviruses with the paradigmatic vectors derived from the human immunodeficiency virus type 1 (HIV). These vectors may circumvent aberrant splicing of their transcript in producer cells and are able to transduce many types of nondividing cells.

(3) The more distantly related spumaviruses with the paradigmatic vectors derived from "human" foamy virus (HFV). This virus is considered to be apathogenic in humans and has the unique property of completing reverse transcription prior to cell entry.

TABLE 1: Retroviral vectors may deliver three forms of nucleic acids

	Viral proteins required	*Trans*-acting sequences to be mutated in packaging cells	*Cis*-acting sequences to be deleted in vector	Pharmacological inhibitors to be applied to target cells
Delivery of Integrated DNA	Env, Gag, PR, RT, IN	None	None	None
Episomal DNA	Env, Gag, PR, RT	IN	Attachment sites in U5 and U3	IN inhibitors
mRNA	Env, Gag, PR	RT	Primer binding site	RT inhibitors

Env, envelope; Gag, group-specific antigen; PR, protease; RT, reverse transcriptase; IN, integrase.

TABLE 2: Taxonomy of the retroviral family (*Retroviridae*) and commonly used vector systems discussed in this article

Genus	Species (examples)	Paradigmatic vector system discussed here	Key properties relevant for vector design
Alpharetrovirus	Avian leukosis sarcoma virus	—	Relatively unbiased integration pattern.
Betaretrovirus	Mouse mammary tumor virus (MMTV), Mason–Pfizer monkey virus (MPMV), human endogenous retroviruses K (HERV-K)	—	Tissue-specific promoter (MMTV); presence of constitutive (MPMV) or inducible (HERV-K) RNA export elements.
Deltaretrovirus	Human T cell leukemia virus, bovine leukemia virus	—	Poorly explored for vector design (replicates primarily as a provirus along with cellular DNA replication).
Epsilonretrovirus	Walleye dermal sarcoma virus	—	Poorly explored for vector design (infects fish).
Gammaretrovirus	Gibbon ape leukemia virus, feline leukemia virus	Mouse leukemia virus	Simple genome architecture, ''clean'' packaging system; powerful constitutive promoters; nontoxic Env proteins.
Lentivirus	Equine infectious anemia virus, feline immunodeficiency virus, simian immunodeficiency virus, bovine immunodeficiency virus	Human immunodeficiency virus type 1	Stabilization of genomic vector RNA in packaging cells; transduction of nondividing cells.
Spumavirus	Chimpanzee foamy virus	''Human'' foamy virus	Completion of reverse transcription prior to entry; relatively low preference for integration in active genes.

All the retroviruses shown belong to the subfamily of *Orthoretrovirinae,* with the exception of spumaviruses, which are separated into their own subfamily (*Spumaretrovirinae*) (http://www.ncbi.nlm.nih.gov/ICTVdb/Ictv/index.htm).

These three vector systems show important differences in their life cycle, biological properties of their *trans*-acting proteins, and the minimal *cis*-elements required to generate high-titer vectors. It is of significance that none of the current recombinant retroviral vectors fulfills the criteria of an ideal vector system for human gene therapy, which should combine the following features:

- efficient, noncytopathic packaging system with low likelihood of recombination,
- serum-resistant particles pseudotyped with cell-specific envelopes,
- reverse transcription prior to cell entry,
- unrestricted cytoplasmic transport and nuclear import,
- insulated expression cassette and/or specific chromosomal targeting,
- physiologic or regulated levels of transgene expression,
- efficiency in relevant animal models, and
- avoidance of horizontal or vertical transmission (high biosafety).

Toward the generation of such an ideal hybrid "super-retrovirus" vector, we should contemplate the different types of vectors available to date and additionally consider other members of the *Retroviridae* family as a source of potentially useful features. In addition, many of the above characteristics cannot be achieved without adopting nonretroviral components.

Efficient, Noncytopathic Packaging System and Low Likelihood of Recombination

The risk of generating replication-competent retroviral vector particles through homologous recombination of viral packaging genes is reduced by avoiding sequence identity between the vector's *cis*-elements and *trans*-acting components [11,12]. The *trans*-acting genes should be encoded by at least two separate plasmid constructs with minimal sequence homology. In addition, the particles should not be composed of cytopathic elements, to guarantee their sustained production from stable packaging cell clones and to avoid toxicity when applied to target cells. However, despite these safety modifications, crude vector stocks need to contain high titers of infectious particles to facilitate transduction of primary cells.

Distinct retroviral *cis*-acting sequences regulate packaging of proviral RNA, reverse transcription, genomic insertion, and transgene expression. A major advantage

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TABLE 3: *Cis*-sequences that overlap with coding regions and might be required for production of high-titer retroviral vector stocks

Gene remnant	Mouse leukemia virus (gammaretroviral)	Human immunodeficiency virus type 1 (lentiviral)	''Human'' foamy virus (spumaviral)
Gag	Not required	40–400 bp	645 bp
Pol	53 bp (SA)[a]	0–130 bp (cPPT)	1850 bp
Env	Not required	0–850 bp (RRE plus SA)	165 bp
Sum	0–53 bp	40–1380 bp	2660 bp

SA, splice acceptor.
[a] Sequence degeneration or deletion in the packing construct possible; residual coding sequences contained in vectors are typically deleted of their AUG start codons.

of MLV is that these elements do not overlap with retroviral coding regions [13–15] (Table 3). The only *cis*-element showing residual overlap with coding sequences is the splice acceptor site located downstream of the packaging and dimerization (Psi, Ψ) motif. This splice acceptor site is of major interest in the design of LTR-driven vectors because the export of the unspliced LTR-derived transcript to the cytoplasm is inefficient [13,14, 16,17]. It can be condensed to a partially degenerate oligonucleotide [13], completely wobbled, or replaced by alternative cellular sequences [18]. Thus, improved gammaretroviral packaging systems will not only avoid the presentation of potentially immunogenic virus-derived open reading frames [13,19] but also completely eradicate residual sequence overlap between the transfer vector and the expression plasmids for *gag–pol* and *env* [12]. It should be noted that early generations of MLV vectors that are currently used in clinical studies may contain large portions of *gag–pol* [20–22].

When generating lentiviral vectors, optimal titers are achieved by incorporating several hundred base pairs derived from *gag–pol* and *env* genes (Table 3). The packaging signal of lentiviral vectors extends up to 300 bp into the N-terminus of *gag* [15]. To date, the defined minimal packaging sequence for HIV-based vectors still contains about 40 bp of *gag* [23,24]. In our hands, sufficient titers of such minimal lentiviral vectors require the use of relatively high amounts of plasmid DNA for transfection of packaging cells. The more widely distributed versions of lentiviral vectors harbor larger *gag* sequences (up to 400 bp) [25].

To increase the transduction efficiency, lentiviral vectors may contain a so-called central polypurine tract (cPPT) in a fragment comprising 130 bp derived from *pol*. The cPPT supports reverse transcription and potentially also nuclear translocation [26]. Lentiviral vectors also require the presence of the Rev-responsive element (RRE), up to 850 bp in size including surrounding *env* remnants [25] (Fig. 1). The Rev/RRE interaction overcomes nuclear retention of the lentiviral genomic RNA, which is mediated by inhibitory sequences involving the splice donor and *gag*. Interestingly, RNA export systems equivalent to Rev/RRE are found not only in lentiviruses, but also in the endogenous human retroviral family HERV-K and mouse mammary tumor virus, both representing betaretroviruses (Table 2) [27–30]. Although the "core" RRE of HIV-1 consists of only 230 bp, the flanking sequences cannot be omitted without a significant loss in titer [23]. This implies that sequences surrounding the RRE are important for proper folding and Rev function [31–33].

Rev/RRE-independent lentiviral vectors have been constructed using the following three approaches:

(1) A vector designed on the basis of simian immunodeficiency virus (SIV) uses the R/U5 region of spleen necrosis virus to export vector RNA [34].
(2) The constitutive transport element (CTE) of Mason–Pfizer monkey virus, a betaretrovirus (Table 2), has been incorporated in HIV vectors. Depending on the splice donor site in the HIV leader, the CTE mediated superior titers compared to the standard construct [35,36]. Similarly, a Rev-independent packing construct was developed using four copies of the CTE [37] (A.S., unpublished results).
(3) Codon-optimized, Rev-independent *gag–pol* genes that have the additional benefit of reducing overlapping sequences between packaging construct and transfer vector have been developed [38]. This concept is applicable to various forms of lentiviral vectors [39,40]. However, the specific infectivity of HIV-derived vector particles produced using a codon-optimized *gag–pol* construct in hematopoietic cells was lower than that of a previous vector generation [41]. This might be explained by the unexpected link between nuclear fate of the HIV *gag–pol* mRNA and capsid assembly [42].

Gag remnants, cPPT, and RRE are all optional components of lentiviral vectors that typically are used when attempting to generate optimal titers. If the Rev protein is coexpressed in packaging cells, the nascent lentiviral transcript containing the RRE will be recruited into a CRM-1-dependent nuclear export pathway [43], potentially competing with splicing. Although this mechanism does not show complete fidelity, it might

promote the incorporation of unspliced genomic RNA into vector particles. Hence, this may facilitate the generation of vector particles containing complex expression cassettes that otherwise would be subject to "pregenome splicing" [44,45]. However, reliable suppression of a downstream intron by Rev/RRE requires that the splice sites are suboptimal [46]. Both gammaretroviruses and spumaviruses lack the Rev/RRE interplay, and it will therefore be interesting to explore its potential utility in these nonlentiviral vectors. Of note, it has been observed that MLV vectors may also preserve introns, although to a lesser extent compared with RRE-containing lentiviral vectors packaged in the presence of Rev [47].

Of the three major vector types under consideration, HFV apparently needs the greatest overlap between coding sequences of vector RNA to generate high-titer stocks, yet still show the lowest yields. The sequence requirements for efficient packaging of the transfer vector comprise a bipartite element that resides in the leader region extending into *gag* and a second element found in the 3′ half of *pol* extending into the cPPT and the *env* open reading frame (ORF). This partially explains the need for residual coding sequences in the transfer vector (Table 3) [48–50]. The sum of these sequences is 2.6 kb (Table 3). The latest versions of HFV vectors generate titers exceeding 10^5 infectious units/ml in unconcentrated supernatant [50,51], which is 10- to 50-fold lower than the output of optimized lentiviral or gammaretroviral production systems. Moreover, relatively large amounts of transfer and helper plasmid are typically used in transient transfection systems for production of HFV vectors. Considering both high expenses and the potential contamination of retroviral supernatants with plasmid [52], the high quantity of GMP-grade plasmid DNA required for large-scale production systems represents a potential drawback.

So-called self-inactivating (SIN) vectors are obtained by deleting enhancer–promoter sequences from the U3 region of the long terminal repeats (LTRs). Initial MLV-based SIN vectors had poor titers [53], but recent data suggest that modifications of the 5′ promoter and the 3′ untranslated region allow the production of MLV SIN vectors with titers that are equivalent to those of their LTR-driven counterparts [54] (A.S., J.B., C.B., unpublished data). SIN design of spumaviral and lentiviral vectors has previously been achieved without major reduction in vector yields [25,51,55].

An open question is which SIN vector system will be useful for the generation of high-titer vector stocks from stable, cloned packaging cells. Using an innovative cassette exchange technology, a first success has been achieved with MLV SIN vectors [56]. However, crucial components of lentiviral and spumaviral packaging systems may be cell toxic, including the lentiviral protease [57] and certain Env proteins used for lentivirus vector pseudotyping, such as the glycoprotein of vesicular stomatitis virus (VSV-g) [58,59], and the RT and Env proteins of HFV. Hence, this cellular toxicity presents a significant hindrance to the development of stable packaging lines. Nevertheless, significant progress has also been achieved for HIV-based vectors [60–62]. The rationale for alternative glycoproteins is discussed below.

Irrespective of the type of *gag–pol* and *env* sequences, use of packaging cells that are of human origin appears to be optimal to reduce the danger of transducing nonhuman endogenous retroviral sequences [62,63].

Serum Resistance and Pseudotyping with Cell-Specific Envelopes

Appropriate pseudotyping of retroviral particles may increase serum resistance, confer cell-specific transduction properties, and avoid target cell toxicity.

Inactivation of retroviral or lentiviral particles by human complement can be circumvented when using packaging cells of human origin for vector production [64]. However, certain envelope proteins such as VSV-g are still subject to serum inactivation. This can be overcome by PEGylation of viral particles [65]. Downstream processing of retroviral supernatants may also reduce potential target cell toxicity of VSV-g, which may result from cell debris copurifying with VSV-g-pseudotyped gammaretroviral or lentiviral particles [66].

Research regarding alternative envelope proteins has led to satisfactory transduction rates of several clinically relevant cell types and potentially allows cell-type-specific targeting (reviewed in [67]). HFV as the prototypic spumavirus is the least flexible among the known retroviruses with respect to pseudotyping. So far, this genus has been resistant to packaging by heterologous envelope proteins [51]. In contrast, numerous pseudotypes have been made available for both MLV and lentiviral vectors, using glycoproteins that originate from other retroviruses (e.g., GALV, RD114), other membrane-coated viruses (e.g., VSV, LCMV, Ebola), or recombinant design (typically based on a viral glycoprotein equipped with artificial targeting domains) [68–71]. Importantly, Env protein incorporation involves an interaction of its cytoplasmic tail with Gag proteins. In addition, appropriate protease-mediated cleavage of Env into its surface and transmembrane subunits (SU and TM in Fig. 1) is a prerequisite for efficient uptake of retroviral particles by the target cells. Modifying cytoplasmic tails and protease consensus motifs can greatly improve both production and infectivity of pseudotyped MLV or lentiviral vectors [61,69]. Moreover, the surface domain of Env proteins can be spiked with growth factor domains to induce target cell prolifer-

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ation, although particle infectivity in this case depends upon the copresentation of unmodified Env proteins [71–73]. Comparative studies of gammaretroviral and lentiviral vectors pseudotyped with related Env proteins may provide further insights into potential limitations occurring at the level of assembly and envelope stability, receptor interactions, or associated postreceptor pathways [54,74–76].

HIGH-FIDELITY REVERSE TRANSCRIPTION, IF POSSIBLE PRIOR TO CELL ENTRY

Improving the fidelity of reverse transcription is a major challenge in retroviral vector technology. The different vectors currently available all suffer from a relatively high mutation frequency (introducing one point mutation in about 10 kb processed RNA). To our knowledge, successful ways to engineer versions of RT that reduce the incidence of point mutations have not been reported. In addition, the error rate of cellular RNA polymerase II generating the viral genomic RNA might also contribute to point mutations.

Genetic instability during reverse transcription might also be triggered by transfer of RT between sequence repeats or between the two strands of genomic RNA that are simultaneously incorporated as a noncovalently linked dimer into a retroviral particle. Interestingly, HIV appears to be more susceptible to recombination at this step of the life cycle than MLV, potentially owing to differences in the dimerization process of the respective genomic RNAs [77].

Larger stretches of C-to-U hypermutations can result from the copackaging of a cellular protein named APOBEC3G, a cytidine deaminase that may block the replication of both endogenous and exogenous retroviruses [78–80]. Incorporation of this cellular defense principle into any type of retroviral particle can be avoided by choosing packaging cells that do not express this editing enzyme.

HFV differs from other types of retrovirus in that its RT has a higher processivity [81]. Another desirable feature is that the reverse transcription process may be complete prior to cell entry [82]. This capacity is linked to the unique ability of HFV to package active RT [48,82,83]. In contrast, both gammaretroviruses and lentiviruses process RT to its active form only at a late stage of particle maturation. Thus, a hallmark of infectious HFV particles is that their genome may contain double-stranded DNA (Table 2), very similar to the situation found in hepadnaviruses [82]. In contrast, the genome of both gammaretroviruses and lentiviruses consists of two strands of single-stranded mRNA that have just initiated reverse transcription. Consequently, HFV vectors seem to be less dependent on the nucleotide supply of the target cell to form double-stranded DNA, potentially contributing to their ability to transduce metabolically inactive cells such as unstimulated hematopoietic stem cells [84,85].

UNRESTRICTED CYTOPLASMIC TRANSPORT AND NUCLEAR IMPORT

The mechanisms underlying cytoplasmic transport of retroviral particles are not well understood. This most dynamic interphase of the retroviral life cycle comprises particle uncoating, reverse transcription, formation of the preintegration complex, and trafficking to the nucleus (reviewed by [76]). Gag proteins are a major viral determinant involved in host interactions at these steps. Accordingly, modifications of *gag* sequences may overcome postentry defense mechanisms affecting cytoplasmic fate and nuclear translocation. Over recent years, several restriction factors have been identified that operate mainly at the level of uncoating, i.e., the disassembly of the viral core into capsid monomers, and later stages of cytoplasmic trafficking (reviewed in [76,79,86,87]).

A prominent example of a cellular restriction factor is TRIM5α which exerts a block before RT [88], most likely during the complex steps that rearrange the viral capsid before formation of the preintegration complex. TRIM5α targets a domain in the capsid protein of Gag. The block is saturated by an excess of particles and can be circumvented by mutations in the capsid domain. TRIM5α operates in a species-dependent manner, leading to major phylogenetic differences in the susceptibility to certain lentiviral and gammaretroviral strains. Currently used gammaretroviral vectors based on *gag* sequences of the Moloney MLV escape this block, whereas HIV-derived vector infectivity in rhesus cells is inhibited by this mechanism (reviewed in [86,87]). For this reason HIV-based vectors cannot be tested directly in rhesus species (see below).

Conversely, there are important factors that block MLV but not HIV. One example is the ZAP protein that degrades retroviral RNA (reviewed in [86]). Moreover, both MLV and HFV encounter a block in nuclear import of the preintegration complex in quiescent cells [85,89], while lentiviral particles are typically capable of transducing nondividing cells or at least those in the G1 phase of the cell cycle [90–92]. The important consequence of the ability to transduce many nondividing cells is that lentiviral vectors became the preferred delivery system for stable transgene delivery in many tissues *in vivo* [24,55,93]. Moreover, for cell types such as hematopoietic stem cells, which are difficult to maintain *in vitro*, it is possible to develop *ex vivo* transduction protocols that largely preserve their biological features [94]. Recently, a novel SIV-derived vector system that is even capable of transducing some cell types that reside in the G_0 phase of the cell cycle has been described [95]. However, it remains to be seen whether this vector will maintain this property in the absence of all lentiviral

accessory proteins. Early studies demonstrated that the capacity of lentiviral particles to transduce nondividing cells could be mapped to the nuclear import signals in HIV IN, the Gag matrix, or features of the cPPT. However, these reports have all been called into question ([96–98] and references therein). The discovery of the underlying mechanisms remains an important topic, potentially allowing the transfer of this property to other types of retroviral vectors.

SPECIFIC CHROMOSOMAL TARGETING

All of the currently used retrovirus vectors integrate into chromosomal DNA in a semirandom manner. The functional consequences of this are twofold:

(1) The potential deregulation of cellular gene expression might induce a selective disadvantage or advantage, depending on the integration site [99]. In the worst case, malignant outgrowth might be triggered by the activation of some proto-oncogenes [100–102]. The implications of these severe adverse events, which clearly represent dose-limiting toxicities but are highly context-dependent in manifestation, have been the subject of several recent reviews [103–109].
(2) The clonal variability of transgene expression and silencing dependent upon the genomic architecture adjacent to the proviral integration site.

Silencing of transgene expression has been observed with both gammaretroviral and lentiviral vectors and is strongly dependent on transgene sequence, integration site, and cellular differentiation conditions (reviewed in [110]). Randomly integrated HIV vectors may be more refractory to transgene silencing than conventional LTR-controlled MLV vectors, which might reflect differences in sequences of the vector backbone, transgene, and integration patterns [110–116]. SIN vector design reduces the incidence of gene silencing in both gammaretroviral and lentiviral backbones [110]. Importantly, lentiviral vectors can be used to create transgenic animals [117,118], and it will be interesting to see whether SIN vectors designed on the basis of other *Retroviridae* achieve similar results in this setting. Genetic and epigenetic variability due to differences in transgene copy number and integration sites remains a challenge in the creation of transgenic animals using viral gene transfer. In human gene therapy, numerous somatic cells are subject to gene delivery. Due to the stochastic nature of the transduction process, a profound inconsistency with respect to transgene copy numbers and expression levels may result, especially when transducing cells with multiple vector copies [119,120]. For all of these reasons, increasing the specificity of chromosomal targeting is of major importance.

Tethering of the preintegration complex (PIC) to certain areas of chromatin and local structural features are believed to be the major determinants of target recognition (reviewed in [121,122]). The precise components of the PIC that mediate tethering remain to be identified. These could be cellular proteins such as transcription factors that bind the transgene DNA and/or viral proteins derived from the incoming particles. Since both genomic sequence and viral proteins vary dependent upon the type of retrovirus used, the integration patterns of different retroviruses are not identical. HIV and derived vectors tend to integrate into transcribed gene regions [123], whereas MLV shows a preference for integration within a few kilobases upstream of the transcriptional start site [124]. Among the retroviruses studied to date (avian sarcoma-leukosis virus, MLV, HFV, HIV, SIV), HFV shows the least preference for integration within genes [125–127]. Although the reported degree of differences appears rather small, this might still have biological consequences. One recent striking observation is that lentivirally transduced hematopoietic cells transplanted into rhesus monkeys have not shown insertions upstream of the primate *EVI1* proto-oncogene, while numerous such clones were detected following transduction with MLV vectors [128]. The *Evi1* allele is also a potent inducer of clonal dominance in murine hematopoietic cells [99,129]. Future studies may resolve whether the differences observed between lentiviral and gammaretroviral vectors depend upon transgene sequence, particle origin, or transduction conditions.

Epigenetic factors regulating chromatin accessibility may well be involved in target site selection. This is one of the major variables to be considered when addressing cell-type dependence of insertional side effects. It remains to be seen whether stimulation of cells with cytokines influences the risk of integration in the vicinity of crucial proto-oncogenes. This may be another area where the choice of retroviral vector is particularly influential as cytokine treatment is essential for successful transduction of many primary cells with MLV but is required to a lesser degree for HIV-based vectors.

Alterations of the IN enzyme encoded by *pol* may possibly target transgenes to specific sites within the genome. Fusion of IN with other DNA-binding domains has been shown to modify sequence specificity in naked DNA, but convincing *in vivo* results remain to be obtained (reviewed in [121]). Given that PIC and chromatin determine target site selection in a multifactorial fashion, such modifications are certainly not trivial. Even site-specific recombinases still show a significant off-target rate, especially when being overexpressed [130].

Eventually, the impact of insertional mutations will have to be determined using functional assays. We have described murine models that appear to be useful to

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reveal clonal dominance or malignant transformation as side effects of vector-mediated insertional mutagenesis [99,102]. Themis et al. recently reported that certain lentiviral vectors may transform primary hepatocytes when injected at a high dosage into fetal mice [131], although formal evidence of insertional mutagenesis as a driving force of oncogenic progression remains to be demonstrated in this model [132]. Another recent study has reported an *in vitro* culture system that reveals transforming events elicited by MLV vectors [129]. Alternative models will likely be established in the near future, providing clear insights into the transforming potential as a function of transgene architecture, particle composition, and transduction conditions.

Insulating the Expression Cassette, Considering both DNA and RNA Processing

An elegant concept to reduce the risk of insertional mutagenesis is to flank the transgene with *cis*-acting sequences that reduce the likelihood of functional interactions with neighboring cellular alleles. Such attempts should reflect two basic pathways [103]: enhancer-mediated interactions that may occur over tens to hundreds of kilobases [99,129] and transcriptional readthrough, which is a notorious problem of retroviral vectors since they typically contain suboptimal termination motifs [47].

The potential advantages of so-called insulators for reducing position-dependent silencing and enhancing vector safety have recently been reviewed [110]. Further studies are ongoing in several laboratories to exploit this important tool. Insulators would be expected to work with similar efficiency in any kind of retroviral vector, at least in the context of SIN vectors in which major wild-type enhancer motifs are deleted from the U3 regions. Introducing nuclear scaffold attachment regions may further increase the transcriptional autonomy of randomly integrating vectors. However, both insulators and scaffold attachment regions may be subject to differentiation-dependent regulation [110,133,134].

The termination motif of HIV has been shown to be somewhat stronger than that of MLV [135], but it also tends to be leaky when SIN vectors are formed by deleting wild-type U3 sequences [47]. Enhancers of termination and polyadenylation are known from several viruses and organisms. An important aim of current work is to incorporate such enhancers of 3′ RNA processing into SIN vectors.

A first step in this direction is the use of the posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE). This element increases retroviral titers and in some configurations also enhances retroviral transgene expression by improving nuclear export of unspliced RNA via CRM-1 and possibly also by improving 3′ termination and/or polyadenylation [136,137]. The safety of this element has been subject to some debate, because it overlaps with sequences that have been associated with the induction of liver cancers by woodchuck hepatitis virus [138,139]. Recent work based on insights into the WPRE's modular assembly [137] showed that the RNA enhancer function of the WPRE can be trimmed to avoid the coexpression of potentially oncogenic sequences [140].

Physiologic or Regulated Levels of Transgene Expression

Several studies addressed which of the various retroviral backbones (gammaretroviral, spumaviral, or lentiviral) provides the best platform for incorporation and ex pression of a given transgene cassette [47,54,84,111, 116,141]. However, not all of these expression studies have been conducted under conditions of a low and homogeneous transgene copy number, which is required to reduce experimental variability [119,142]. When transducing hematopoietic and fibroblast target cells with identical transgene copy number and identical internal expression cassettes, we observed no significant differences in the transcriptional potency of SIN vectors derived from MLV or HIV [54]. In contrast, an earlier study demonstrated superior transgene expression from HIV vectors, possibly linked to improved 3′ termination and polyadenylation [47]. However, it should be noted that improved 3′ processing was shown to depend on the presence of the intact HIV-1 U3 region and was lost in the corresponding SIN vectors [47]. Moreover, the MLV-based SIN vectors tested in this study still contained the extended basal promoter of the U3 region [47], in contrast to our more recent study in which both HIV- and MLV-based vectors had both enhancer and promoter regions of the U3 regions removed [54].

More complex expression cassettes, such as those required for lineage-specific expression of β-globin, tend to be very unstable in conventional MLV backbones while showing a remarkable performance when contained in HIV-derived SIN vectors [44,45,143–145]. It is likely, although not formally shown, that the stabilization of the genomic RNA is at least partly mediated by the Rev/RRE interaction that directs the vector RNA into an unusual RNA export pathway (see above) [24]. Using the recently developed Rev-independent lentiviral vectors, this important question can be addressed.

Other lineage-specific expression cassettes can be efficiently incorporated into SIN vectors of any origin. Examples are promoters specific for B cells or T cells [146–149]. Efficient inducible transgene expression has been obtained from artificial promoters that respond to drugs. Drug-inducible artificial transcription factor cassettes have been successfully incorporated into lentiviral vectors [150–152]. Similar inducible constructs might be possible using advanced generations of HFV or MLV vectors. As

residual enhancers and cryptic promoters of the vector backbone potentially modify the performance of the transgene cassette [153], comparative studies using different viral backbones might be helpful to develop improved vectors for inducible or tissue-specific gene expression.

Efficiency in Relevant Animal Models

The various cellular restriction factors discussed above (APOBEC3G, TRIM5α, etc.) operate in a very species-specific manner [79,86,87]. This has major consequences for the design of preclinical animal models. In the context of gene transfer into cultured hematopoietic cells, MLV vectors (that are derived from the relatively unrestricted NB-tropic Moloney strain) have been shown to work with reasonable efficiency in murine, canine, and nonhuman primate models [154]. However, culture conditions and pseudotypes typically need to be optimized to allow efficient use of MLV vectors in species other than mouse. Importantly, the efficiency of transduction reported from some "large" animal models could be reproduced in clinical trials [20–22,155].

When targeting minimally manipulated cells *in vivo* or *ex vivo*, lentiviral vectors typically are much more efficient than MLV vectors [94] (and references therein). However, conventional HIV-based vectors fail to transduce rhesus monkey cells with high efficiency, unless variants such as those derived from SIV are used to overcome the block related to TRIM5α [87,156,157]. HFV vectors are also suboptimal for studies in some nonhuman primates, which seems to be related to the existence of preexisting antibodies to related endogenous viruses. The potential ability of antibodies to eliminate transfused, gene-modified cells that were transduced *ex vivo* implies that residual antigens are formed even though replication-deficient vectors were used. If their expression cassettes are successfully deleted of residual viral ORFs, antibody-mediated rejection may be related to a rather short culture time, which increases the chance that infused cells continue to present particle-derived antigens [158]. Following the use of HIV-based lentiviral vectors, the resulting antibodies might be recognized by routine diagnostic tests originally designed to detect HIV infection. If such a "seroconversion" occurs, several approved diagnostic methods exist to demonstrate the absence of viral sequences. The use of vectors derived from nonprimate lentiviruses [159], gammaretroviral vectors, and spumaviral vectors largely or entirely prevents this "psychological" issue.

If sensitization of the host or immune rejection of engineered cells is to be avoided, one might have to reexplore the utility of prolonged culture prior to cell transfusion. If a patient has developed an acquired immune response to vector particles, alternative retroviral vector types may still be applicable.

Avoiding Horizontal or Vertical Transmission

The potential generation of replication-competent retroviruses (gammaretroviral, lentiviral, or spumaviral) with novel pseudotypes and unusual *cis*-elements represents a major safety concern. The range of potential diseases induced by such viruses includes tumors, immunodeficiency syndromes, and neurodegenerative disorders [160,161]. A theoretical advantage of HIV-based vectors is that those drugs designed to treat infection with the wild-type virus by targeting viral functions encoded by *gag–pol* are expected to also counteract spread of replication-competent vector recombinants [162,163]. Importantly, many of the clinically approved drugs (such as the nucleoside analogs and the nonnucleoside RT inhibitors) also inhibit replication of gammaretroviruses or spumaviruses.

Problematic scenarios also result from the potential mobilization of transgene sequences from somatic cells into the patient's germ line (vertical transmission) or into another patient (horizontal transmission). HIV-derived vectors can be packaged into wild-type viral particles, which are abundant in patients with an active HIV-infection. If copackaged into the same particle, recombination can occur between vector RNA and lentiviral RNA. In most of these cases defective mutants will arise, which has led to the suggestion that HIV vectors would be of particular interest for treating patients with HIV infection [164]. However, the outcome of such recombination events is not predictable. Lentiviral vectors that are closely related to HIV, such as those derived from SIV, would not escape this problem. In contrast, vectors based on HFV or MLV are not preferentially incorporated by HIV particles.

In general, SIN vectors strongly decrease the risk of mobilization. Lentiviral (and potentially also other retroviral) SIN vectors may still generate a small amount of full-length transcript in transduced cells. Detailed studies have shown that a cryptic transcriptional activator resides in the HIV leader region and that its modification reduces residual aberrant transcriptional initiation of SIN vectors [153]. Vectors with artificial primer binding sites that require cotransfection of a recombinant tRNA for complementation further decrease the potential for mobilization [165,166]. In addition there are attempts to replace the RRE with an artificial RNA stem–loop structure derived from the lambda phage MS2. Rev is then supplied as an Rev-MS2 fusion protein in the packaging cells [167].

mRNA Delivery and Nonintegrating Vector Mutants

Exploiting intermediate steps of the retroviral life cycle, such as the transfer of mRNA prior to reverse transcription or the presence of episomal nuclear DNA prior

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to integration, greatly enhances the potential utility of retroviral vectors (Fig. 2, pathways 1 and 2). If only mRNA is to be transduced, reverse transcription needs to be inhibited (Table 1) [9]. MLV appears to be a good choice for this approach as the genomic vector RNA can be deleted of aberrant reading frames that are typically contained within the 5′ untranslated region [13] and the inability of the PIC to transduce resting cells is no longer relevant. Particle-mediated mRNA transfer by retroviral mutants leads to relatively weak expression of the encoded proteins and may therefore be of particular interest for transient and low-level expression of the transgene cassette (i.e., for delivery of endonucleases; recombinases; signaling proteins affecting proliferation, survival, or differentiation; and receptors that modify cell homing) [9]. Lentiviral or spumaviral vectors contain small reading frames in their 5′ UTR that inhibit cap-dependent translation of a reading frame of interest. The use of internal ribosomal entry sites may circumvent this limitation for transient mRNA transduction. The lentiviral property of relatively "rapid uncoating" [168,169] may be of additional interest for mRNA delivery.

If reverse transcription is allowed to occur but integration is blocked, retroviral delivery systems can be adapted to form episomal circular DNA in target cells (Fig. 2, Table 1). Such nonintegrating vector mutants present mainly as so-called 2-LTR circles are formed as nuclear by-products by nonhomologous DNA repair if integration fails. Lentiviral vectors are of particular interest for the design of these episomal vectors, because of their ability to transduce nondividing cells in which episomal transgenes may persist over prolonged periods of time [10,170]. Episomal transgene delivery by lentiviral vectors is an emerging field, and studies that address the underlying mechanisms and utility of this approach in therapeutic models are under way. Introducing episomal maintenance signals may even lead to persistence of nonintegrated DNA in cycling cells [171–173]. However, residual integrase-independent integration activity needs to be addressed.

SUMMARY

A little more than 20 years after the first construction of retroviral vector packaging cells and the demonstration of retroviral gene transfer into somatic stem cells [1,2], the field has reached a stage of great diversity and productivity. The three major groups of retroviral vectors discussed here have important stories to tell, and further insights will result from detailed studies addressing the rich resource represented in the numerous additional members of the large family of retroviruses (Table 2).

Toward the development of the super-retrovirus or "plentivirus" vector, we reach the following conclusions: The various forms of retrovirus vectors are still by far the most efficient tool for stable transgene insertion and are also of increasing interest for the delivery of episomal DNA or mRNA. The best features of MLV vectors are the simple genome architecture with its clear separation of *cis*-acting sequences from the coding sequences, resulting in paradigmatic biosafety features. This is coupled with a clean packaging system that is amenable to industrial upscaling. However, MLV vectors only work well in proliferating target cells, which may complicate some applications unless efficient conditions triggering cellular self-renewal divisions are defined. The best features of lentiviral vectors include their capacity to transduce nondividing cells (possibly mediated by sequences in *gag–pol*) and the potential stabilization of the genomic transcript in packaging cells by the Rev/RRE interaction. One of the most attractive features of spumaviral vectors is the completion of reverse transcription prior to cell entry, which reduces target cell dependence in this most critical step of the retroviral life cycle. Other desired features related to Env-mediated cell targeting, site-specific integration, the inclusion of physiologic expression cassettes, insulation of enhancers, and improved transcriptional termination need to be derived from nonretroviral systems. The flexibility of retroviral genomes and particles is not tremendous. However, diligent work addressing basic mechanisms of retroviral replication and technical aspects of vector design will certainly promote the field. Hence, many of the above components may be combined in a single hybrid construct or exploited for specialized vectors designed to treat a disease of interest. Step by step, these efforts will increase the versatility and biosafety of this fascinating vector system.

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