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Review

Pseudotyping Lentiviral Vectors: When the Clothes Make the Virus

Alexis Duvergé [1,2] and Matteo Negroni [1,2,*]

[1] Université de Strasbourg, CNRS, Architecture et Réactivité de l'ARN, UPR9002, 67000 Strasbourg, France
[2] Interdisciplinary Thematic Institute (ITI) InnoVec, Université de Strasbourg, 67000 Strasbourg, France
* Correspondence: m.negroni@unistra.fr; Tel.: +33-388-417-006

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Abstract: Delivering transgenes to human cells through transduction with viral vectors constitutes one of the most encouraging approaches in gene therapy. Lentivirus-derived vectors are among the most promising vectors for these approaches. When the genetic modification of the cell must be performed in vivo, efficient specific transduction of the cell targets of the therapy in the absence of off-targeting constitutes the Holy Grail of gene therapy. For viral therapy, this is largely determined by the characteristics of the surface proteins carried by the vector. In this regard, an important property of lentiviral vectors is the possibility of being pseudotyped by envelopes of other viruses, widening the panel of proteins with which they can be armed. Here, we discuss how this is achieved at the molecular level and what the properties and the potentialities of the different envelope proteins that can be used for pseudotyping these vectors are.

Keywords: pseudotyping; lentiviral vectors; envelope proteins; gene therapy

1. Gene Therapy Using Viral Vectors

According to the definition provided by the NIH Genetics Home Reference, gene therapy is an experimental technique aimed at treating or preventing a disease by using genes [1]. This can be achieved by various means. When the disease is of genetic origin and, particularly, when it is caused by a single defective gene, the ultimate goal is replacing the defective gene with a wild-type one. This has been possible only recently with the development of powerful genome editing techniques [2–4]. Although, these are not applicable routinely and alternative approaches are followed, the most common of which is the introduction of a gene conferring a dominant wild-type phenotype to the modified cell [5]. Whatever the approach followed, gene therapy relies on the use of vectors that allow the efficient genetic modification of cells, or tissues, combined with a high specificity for the target cells to reduce adverse effects [6]. Introducing exogenous genetic material in cells is efficiently performed by cellular "parasites"—phages for bacteria or viruses for eukaryotic cells. In particular, the vast range of human viruses provides a large panel of promising tools for vectorization (by transduction) in sight of intervention on human cells. How to reprogram human viruses for the purposes mentioned above is a major challenge in molecular medicine.

A main watershed in gene therapy is whether the genetic modification of the cell must be carried out ex vivo or in vivo. If the cells' target for the therapy can be isolated from the patient, modified ex vivo, and reinfused in the patient, essentially no specific tropism is required for the vector since the cells to modify are the only ones it comes into contact with [7–10]. In this case, the vectors can therefore carry pan-tropic envelope proteins such as, for example, the vesicular stomatitis virus (VSV) envelope protein G (see below). If, in contrast, the modification of the cells must be carried out in vivo, a high specificity for the target cells is required to avoid off-target transduction. The nature of the envelope proteins carried by the viral vector is the major determinant for the specificity of transduction.

Most gene therapy clinical trials carried out to date have relied on the use of adeno-associated vectors (AAVs) or retroviral vectors, which might be derived from γ-retroviruses or lentiviruses [11]. Modification of cells in vivo (liver, muscles, central nervous system and retina) has been restricted to the use of AAV-derived vectors, while ex vivo approaches (for the genetic modification of T cells and of human hematopoietic stem and progenitor cells) have relied on the use of vectors derived from murine γ-retroviruses and human lentiviruses. The neat division between clinical trials where AAV vectors have been used and those involving retroviral vectors is in part explained by the natural tropism of the viruses from which these vectors have been constructed.

AAV are non-enveloped non-integrative single-stranded DNA viruses of the *Parvoviridae* family. They require coinfection by adenoviruses to replicate and are non-pathogenic for humans. They infect replicating as well as quiescent cells and enter into the target cells by interaction with sialic acid, heparan sulfate, or galactose present on their surface, and therefore possess a large tropism. Differences in the capsid protein of AAV determine cell type-specific preferences and define the existence of the eleven serotypes of this virus. For gene therapy, according to the type of target tissue, serotypes that naturally target that type of tissue, when such serotypes exist, are the preferred choice for building a viral vector. To date, in gene therapy, eight serotypes (1–2 and 4–9) have been used to orient viral transduction toward the tissue of interest [12].

In sharp contrast to AAV, γ-retroviruses and lentiviruses do not present different serotypes and no variation in tissue specificity is found for these viruses, which both target blood cells. For example, in human immunodeficiency virus (HIV), despite its impressive genetic diversity, which is particularly high at the level of its envelope proteins, infection remains essentially restricted either to CD4+/CCR5+ or CD4+/CXCR4+ cells. However, an interest of retroviral-derived vectors (and therefore of lentiviral-derived vectors as well) comes from the possibility of replacing the original envelope proteins with those of other viruses, a process called pseudotyping. In this review article, we focus on the perspectives on which pseudotyping lentiviral-derived vectors (LV vectors) open and how this is achieved.

2. Lentiviruses and Gene Therapy

Retroviruses are enveloped viruses that integrate in the infected cell. This property has made of these viruses the preferred choice for developing vectors when the expression of the transgene must be stable or when the transgene must be inherited by the progeny of the transduced cell. For these reasons, retroviral vectors have been chosen for the expression of transgenes in hematopoietic stem and progenitor cells (HSPCs) and, more recently, they have been used for the transduction of peripheral blood cells for the generation of CAR-T cells [13]. Gammaretroviral vectors derived from Moloney murine leukemia were used for the earliest gene therapy assays using retroviral vectors. They have been successful in the treatment of several primary immunodeficiencies, such as the X-linked severe combined immunodeficiency (SCID) or the adenosine deaminase deficiency-induced SCID [14–16], and they have been employed in the treatment of the Wiskott–Aldrich syndrome and of X-linked chronic granulomatous disease [17–19]. However, γ-retroviral vectors have been progressively replaced by the lentiviral vectors (LV vectors), mostly due to the lower levels of induction of the innate immune response they trigger [20,21] and, in particular, for biosafety reasons. Indeed, LV vectors predominantly integrate in transcription units [22], rather than in regions controlling gene expression as promoters and enhancers that are, instead, the preferential sites of integration for gammaretroviral vectors [23,24]. This difference has been shown to lead to a lower probability for lentiviruses to cause insertional oncogenesis [25,26]. LV vectors have thus been used in most recent trials, always for the treatment of blood diseases. Besides treating the same diseases with these new vectors as are treated with γ-retroviral vectors mentioned above [27–31], β-thalassemia [32], Fanconi anemia [33], metachromatic leukodystrophy [34,35], mucopolysaccharidosis type I [36], adrenoleukodystrophy [37] and sickle cell disease [38] have also been made the object of clinical trials using LV vectors.

3. Molecular Biology of Lentiviruses

Lentiviruses belong to the subfamily *Lentivirinae* of retroviruses [39]. They are considered as "complex" retroviruses, due to the presence of additional genes, compared to other retroviruses. As all retroviruses, they are enveloped integrative viruses. The viral particle is constituted by a spheric matrix shell that lies immediately underneath the lipid bilayer, which consists of a patch of the cell membrane that is carried over during viral budding from the infected cell [40]. More internally, a fullerene-shaped core [41] contains the genomic RNA that is constituted by a single-stranded positive-sense molecule, present in two copies in the viral particle, in a dimeric form. Upon infection of the host cell (that occurs after recognition of a specific receptor on the surface of the cell) the viral capsid enters the cytoplasm. The availability of the nucleotides, to which the capsid is permeable, allows the initiation of reverse transcription. This results in the conversion of the genomic RNA into double-stranded DNA which is then integrated in the cell genome [42–45].

Where and when this conversion occurs and is achieved remains a matter of debate. The traditional view according to which reverse transcription was completed in the cytoplasm or at the nuclear pore, followed by the dismantling of the capsid core and the import of the preintegration nucleoprotein complex [46–49], has recently been challenged by the observation of intact or almost-intact cores, as well as the detection of ongoing reverse transcription in the nucleus [50]. However, irrespective of the form under which the genetic material is imported into the nucleus, the import occurs in an active manner, through the interaction of the viral capsid protein p24 with the cellular protein cyclophillin A and the cellular splicing factor CSPF6 [51–53]. This interaction leads to the use of the nuclear import pathway relying on the pair of nuclear pore proteins Nup153/Nup358 and transportin 3 (TNPO3) [54]. This complex system allows lentiviruses (in the specific case detailed above, human immunodeficiency virus type 1 (HIV-1)) to infect non-replicating cells. This not only allows LV vectors to deliver transgenes to cells that naturally do not replicate, but also can be exploited for transducing cells, such as HSPCs, that must be kept in a quiescent state to avoid their differentiation and loss of pluripotency. Retroviruses as γ-retroviruses are instead unable to enter the nucleus of the infected cell and require the disassembly of the nuclear membrane at mitosis for reaching the genome of the infected cell for integration.

Integration is carried out by the viral enzyme integrase with poor sequence specificity for the selection of the integration sites, although preferential types of genomic regions (as, for example, regions where actively transcribed genes are located, or the proximity with respect to transcription start sites) can be defined for the different types of retroviruses [55,56]. The reverse transcription product, integrated in the genomic RNA of the infected cell, is called a provirus. The provirus is flanked by the terminal repeated regions (LTRs) that contain the viral promoter sequence (see below). Transcription from the LTR in 5′ will lead to the synthesis of the new genomic RNA as well as the viral proteins required for infection to be continued. At the moment of assembly of the viral particle, the dimers of viral genomic RNA will be packaged in the budding particle [57]. The particle will also incorporate the envelope proteins at their surface, as detailed below, and be released in the extracellular space as an immature particle. Activation of the viral protease in the immature particle, will then lead to viral maturation and the production of an infectious virus [40].

4. Molecular Bases for the Making of LV Vectors

4.1. Structure of the Genomic RNA

LV vectors are generally derived from the best characterized lentivirus—human immunodeficiency virus type 1 (HIV-1). Lentiviral infection, detailed above, is conceptually composed of two phases. Entry and the conversion of the genomic RNA (gRNA) into DNA that will be integrated in the cell's chromosomes are considered as the "early phase" of the infectious cycle. With the exception of entry, which depends on the nature of the envelope employed in the viral vector, all the steps of the early phase of HIV-1 infection are carried out essentially in the same manner during LV vector-mediated

transduction. The late phase, constituted by the production of the gRNA and of the viral proteins, is instead absent in the case of LV vector transduction.

The viral gRNA of HIV-1 is characterized, proceeding from 5′ to 3′, by the terminal repeated sequence R, the unique sequence in 5′ U5; then, contiguous to U5, are found the 18 nucleotides that constitute the sequence to which the tRNA Lys^3 anneals for priming reverse transcription (primer binding sequence (PBS)) [58] followed by an untranslated 5′ region that is responsible for the dimerization of the gRNA and its packaging in the viral particle [57]. Then, the main three genes (*gag*, *pol* and *env*) follow, overlapping the sequences for the auxiliary proteins Vif, Vpr and Vpu, as well as the proteins Tat and Rev (Figure 1). Finally, partially overlapping with the 3′ portion of *env*, the Nef coding sequence is found, followed by the unique sequence in 3′ U3, the repeated sequence R and the polyA tail [59]. The sequences required for priming the synthesis of the second strand of DNA (3′ and central polypurine tracts, -3′ PPT and cPPT, respectively) are located immediately upstream of the U3 sequence and in the 3′ end portion of *pol*, respectively [60,61]. The Rev Responsive Element (RRE) sequence that, when bound by the Rev protein allows the export of partially unspliced RNAs from the nucleus, is located in the portion of *env* encoding the gp41 protein [62].

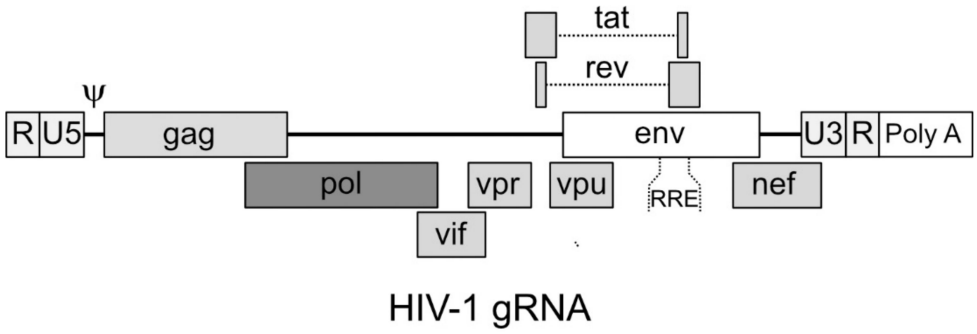

Figure 1. Organization of the human immunodeficiency virus type 1 (HIV-1) genomic RNA. U3, unique sequence 3′; R, repeated sequence; U5, unique sequence 5′; Ψ, indicates the packaging and dimerization sequences; RRE, Rev responsive element. The PPT sequences as well as the primer binding sequence (PBS) region are not shown.

To generate the gRNA of the LV vector, the viral gRNA is modified by removing all the coding sequences for the viral proteins and leaving the elements required in *cis* for genomic RNA packaging, reverse transcription and integration. Specifically, the gRNA of the vector must contain: the PBS sequence; the 3′ PPT and cPPT sequences; the region (located in the 5′ untranslated portion of the genome) responsible for the packaging and dimerization of the genomic RNA; the RRE sequence; the repeated terminal sequence R and the sequence U5, which are required for achieving reverse transcription and integration [63]. The U3 sequence, instead, is only partially preserved, since a large deletion (approximately half of its total length) is made in this sequence [64]. The deletion is essential for inactivating, in LV vectors, the promoter activity of U3, generating what are known as self-inactivating (SIN) vectors [64]. In natural infections, the U3 sequence is located inside the LTR sequences, present at both ends of the proviral DNA (Figure 2A). The U3 sequence located in the 5′ LTR contains the promoter that is used to drive the transcription of the genomic RNA. The genomic RNA contains only the U3 sequence of the 3′ LTR (Figure 2A). After reverse transcription of this genomic RNA, the LTRs are again generated (Figure 2A). In the case of SIN LV vectors, the U3 sequence of the 3′ LTR carries the deletion (in black in Figure 2B) and it will be this sequence that will be present in the genomic RNA. After reverse transcription, this deleted version of U3 will be present in both LTR, the 5′ and the 3′ regions (Figure 2B). Transcription is thereby no longer possible from this proviral DNA, since the promoter in the U3 sequence in the 5′ LTR is not functional. Taking into account these requirements, the gRNA of the LV vector can accommodate up to 8 kb of exogenous sequences.

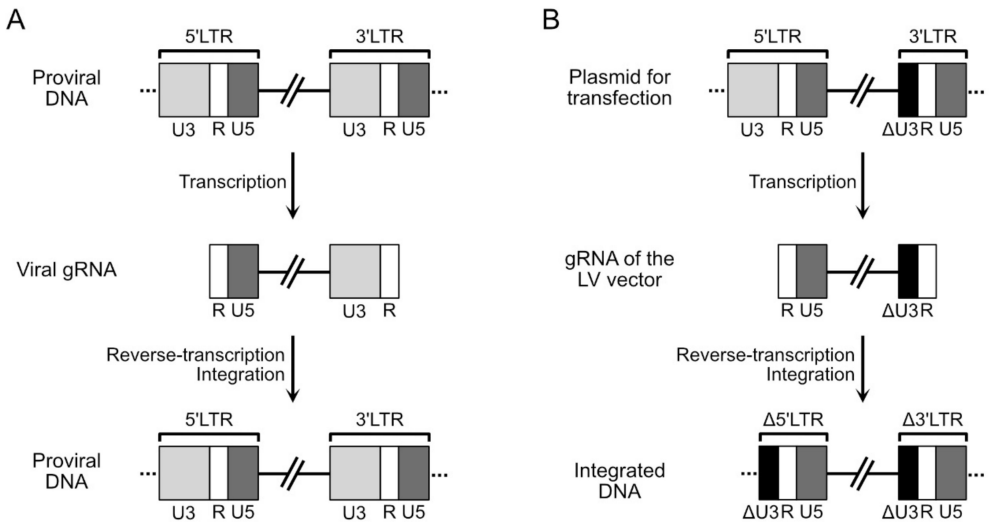

Figure 2. Schematic representation of the structure of the genomic forms of the viral DNA during natural infection (panel (**A**)) or during transfection and transduction by a self-inactivating (SIN) lentiviral-derived (LV) vector (panel (**B**)).

For the generation of the LV vector particle, the plasmid leading to the synthesis of the gRNA is cotransfected with transcomplementation plasmids leading to the synthesis of the viral proteins. Depending on which generation of LV vectors is considered, the structure of the plasmids varies as well as which viral proteins are provided (Figure 3). In this setting, in order to change the tropism of the viral vector through pseudotyping, the plasmid encoding the envelope proteins will be chosen to carry the desired, non-HIV, envelope protein coding sequences.

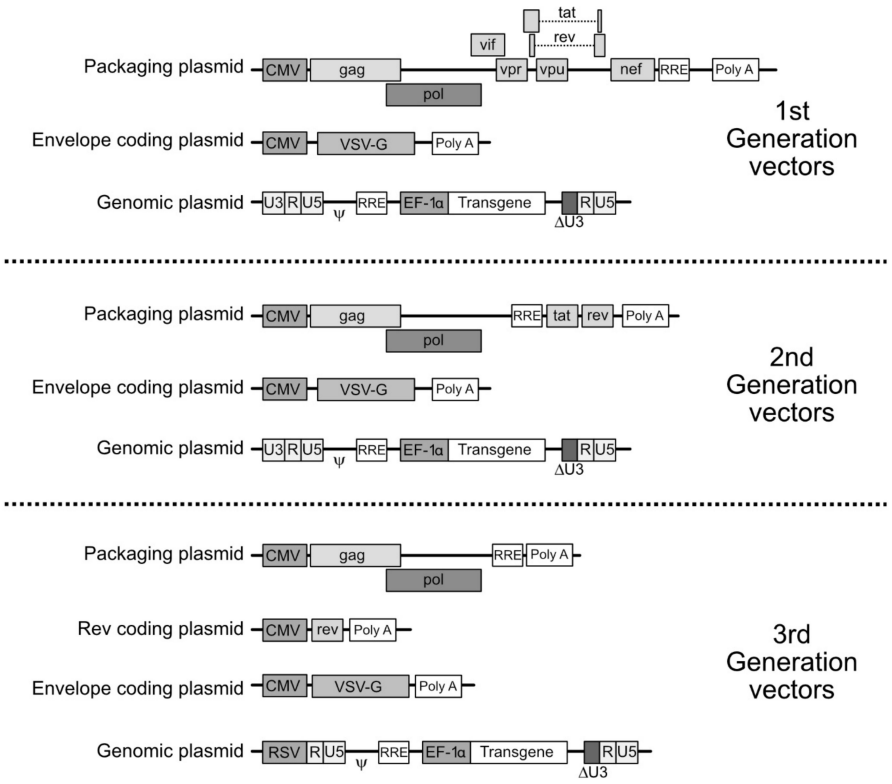

Figure 3. The various generations of lentiviral vectors. Top panel. Plasmids employed for constructing first generation lentiviral vectors. Three plasmids are employed. (1) A packaging (or transcomplementation)

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