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REPLICATION OF LENTIVIRUSES

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1. ABSTRACT

Lentiviruses belong to a subfamily of the retroviruses usually associated with persistent infections in animals and humans. They have complex replication cycles involving numerous regulatory and accessory proteins, which sets them apart from the oncoretroviruses and spumaviruses, the two other main subfamilies of the retroviruses. Studies over the years have elucidated the various molecular mechanisms involved in the replication of lentiviruses. The first step involves the fusion of the envelope glycoprotein (gp120) to the host cell membrane followed by entry of the virus into the host cell. Immediately following viral entry is reverse transcription, integration, gene expression, encapsidation, budding and lastly virus maturation. This review focuses on the molecular mechanisms involved in the lentiviral replication, using human immunodeficiency virus type I (HIV-1) as an example.

2. INTRODUCTION

The retrovirus family consists of three subfamilies: lentiviruses, oncoviruses, and spumaviruses (1). Lentiviruses differ from oncoretroviruses and spumaviruses by having a rather complex genomic organization, which consists of several regulatory genes and proteins not found in other retroviruses. Although all retroviruses have the *gag* and pro-*pol-env* genomic sequence in common, and their replication cycle is somewhat similar, the lentiviral replication cycle is very complex and involves multiple regulatory sequences and proteins (2). Lentiviruses are also termed “slow viruses“ because of the characteristic long latency period during which the virus, although infectious, does not produce any major symptoms in its host (3). Equine infectious anemia virus, isolated from horse was the first discovered lentivirus (4). Since then, several other lentivruses from felines, monkeys, and humans have been isolated (Table 1) (5,6,7). In particular, the identification HIV-1, as the causative agent of AIDS has led to the systematic investigation of the life cycle of this virus and the identification of many targets for intervention.

As in all retroviruses, lentiviruses carry two copies of their RNA genome. The RNA genome is converted (reverse transcribed) into a double stranded DNA

Table 1. The *Retroviridae* Family and Representative Virus Strains

Subfamily	Virus Strain	Vertebrale Host
Oncovirinae[a] (RNA tumor virus group)		
Group B	Mouse mammary tumor virus (MMTV)	Mice
Group C	Avian sarcoma virus (ASV)	Chicken
	Murine leukemia virus	Mice
	Feline leukemia virus	Cats
	Bovine leukemia virus	Cattle
	Human T-cell leukemia virus-I	Human
	Human T-cell leukemia virus-II	Human
Group D	Mason-Pfizer monkey virus	Monkey
Ungrouped	Guinea pig leukemia virus	Guinea pigs
Spumavirinae (foamy virus group)		
	Simian foamy virus	Monkey
	Human foamy virus	Human
	Feline syncytial virus	Cats
	Bovine syncytial virus	Cattle
Lentivirinae (slow virus group)		
	Visna/maedi virus	Sheep
	Equine infectious anemia virus	Horse
	Caprine arthritis encephalitis virus	Goat
	Human immunodeficiency virus-1 (HIV-1)	
	Lymphadenopathy-associated virus (LAV-1)	Human
	Human T-lymphotropic retrovirus III (HTLV-III)	Human
	AIDS-associated retrovirus (ARV)	Human
	Human immunodeficiency virus-2 (HIV-2)	
	Lymphadenopathy-associated virus (LAV-2)	Human
	Simian T-cell lymphotropic virus-III (STLV-III)	Monkey
	Human T-cell lymphotropic virus IV	Human

[a] Morphologically, "A" type virus particles are commonly found intracellularly and are considered to be immature virus particles. Adapted from Reference 7.

copy, which is integrated into the genome of the host cell (8). Thus, the life-cycle is similar to that of all other retroviruses investigated. However, in contrast to simple oncoretroviruses, lentiviruses have the ability to actively penetrate the nucleus of non-dividing cells. This feature makes these viruses attractive candidates for the development of gene transfer vectors.

Since HIV-1 is not only a lentivirus but also the most studied virus ever, it will be used as the prototype virus in this description of "Replication of Lentiviruses". In this review, different stages of the replication of the lentiviruses will be discussed. We will begin with the interaction and entrance of the virus into the host cell, during which the envelope protein plays an important role in selecting its host cell by the process of tropism. This will be followed by the reverse transcription and provirus integration processes, which are critical in the retroviral life-cycle. The processing of the viral mRNA will also be discussed, since in lentiviruses this activity has special features that are worthy of note, including their more complex regulation of the long terminal repeat (LTR) domains. Special attention will be given to the viral accessory genes, as those are found mainly in lentiviruses and are responsible for providing these viruses with the unique features that set them apart from the rest of the retroviruses. Finally, the pathway leading to the assembly and formation of the newly synthesized viral particle will be discussed. All of this will provide a clear picture of the entire replication cycle in lentiviruses, beginning with the infection of the host by the original viral particle, to the formation of the newly synthesized viral particles.

3. MORPHOLOGY

Like all retroviruses, lentiviruses are enveloped viruses containing a lipid bilayer surrounding the viral core. Embedded in the lipid bilayer are the viral envelope proteins, which consist of a transmembrane peptide (TM), also termed glycoprotein 41 (gp41). Gp41 is associated with the surface unit (SU) peptide, which mediates receptor binding (glycoprotein 120, gp120) (7). (The number indicates the relative molecular mass of each protein in thousands of daltons (kDa)). Underneath the retroviral envelope is a protein coat consisting of the matrix proteins (MA, p17). The matrix coat is associated with the viral capsid, which contains the viral RNA genome, other structural proteins (MA, CA, NC, p6), and the viral enzymes involved in particle maturation and replication of the RNA genome (e.g., protease, {PR}, reverse transcriptase, {RT}, and integrase {IN}) (1). The envelope confers the characteristic icosahedral morphology to the enveloped virus {Figure 1} (9,10,11).

4. GENOME ORGANIZATION

All retroviruses contain at least three gene units coding for proteins essential for particle formation: *gag*, *pol*, and *env*. The Gag proteins and Gag-Pol are synthesized as single precursor proteins from genomic viral

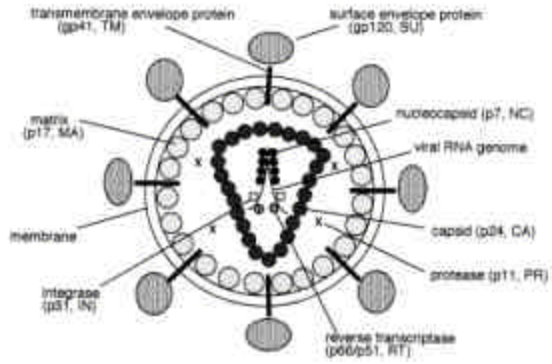

Figure 1. A schematic diagram of the HIV-1 virion organization showing the core proteins MA, CA, NC, pol encoded enzymes IN, RT, and PR and the surface envelope protein (gp120, SU) and the transmembrane envelope protein (gp41, TM).

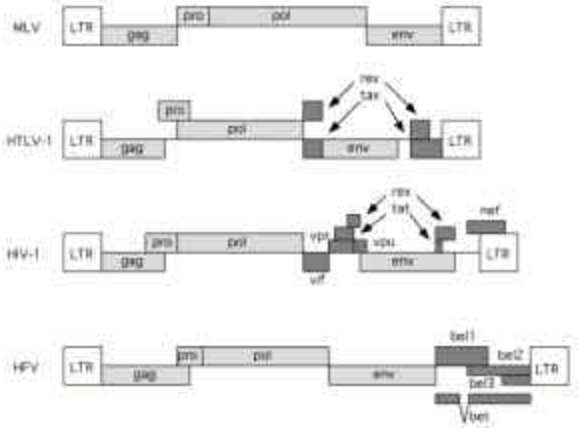

Figure 2. Schematic representation of the genome of several lentiviruses. All the lentiviruses shown here contain the *env*, *gag* and *pol* genes that encode the envelope glycoproteins gp120 and gp41 and the enzymes RT, IN and PR, respectively. The prototype human immunodeficiency virus type 1 (HIV-1) encodes six additional genes tat, rev, vif, vpu, vpr and nef.

RNA and are cleaved into mature virion proteins (MA, CA, NC, RT, IN, PR) by the viral protease during particle maturation (Figure 2). The envelope protein is translated from a single spliced RNA as one precursor protein (gp160), which is subsequently cleaved in the endoplasmic reticulum by a cellular protease. HIV-1 contains six additional genes, termed accessory or regulatory genes (tat, rev, vif, vpu, vpr, and nef), which are translated from multiple spliced RNAs {Figure 2} (12,13). Of note, HIV-2 contains Vpx instead of Vpu (14,15).

5. VIRUS LIFE CYCLE

As described above, the lentiviral life-cycle is similar to that of other retroviruses, except that several regulatory proteins are involved. Furthermore, while oncoretroviruses do not kill the infected host cell, several lentiviral proteins are cytotoxic and lead to the death of the infected cell.

CD4 is a primary receptor for HIV-1, and thus HIV-1 infection of host cell begins with interaction between gp120 of the envelope and host's cell-surface CD4 molecules. Both CD4 and gp120 are produced in the endoplasmic reticulum (ER) and these two proteins can bind prematurely to each other leading to the retention and destruction of gp120 in the ER. The formation of Env-CD4 complex in the ER is minimized by targeting CD4 for removal by the accessory proteins Vpu and Nef. Vpu binds to CD4 molecules leading to the dissociation of the Env-CD4 complex and the degradation of CD4 (16,17). Similarly Nef down-regulates CD4 by binding to the cell surface molecules and targeting them for endosomal degradation. These actions of Vpu and Nef result in the depletion of CD4 and enhancement of viral replication (18, 19).

Nef also induces apoptosis in bystander cells by activating the expression of FasL (20). The HIV-1 Tat and the envelope protein, gp120, have similarly been reported to induce apoptosis in T cells, either *in vivo* or *in vitro* through the activation of the Fas/FasL pathway (21), and in neurons (22,23). Tat when expressed endogenously or applied to cells have been reported to induce apoptosis in cultured T-cells and peripheral blood mononuclear cells (PBMCs) obtained from individuals not infected with HIV-1 (21).

5.1. Virus entry

As in all retroviruses, the initial step in the replication cycle of HIV-1 begins with the interaction of the viral envelope glycoproteins with specific membrane proteins (receptors) of the target cell (9). The HIV-1 gp120 contains several variable regions (V1-V5), several conserved domains, and a highly conserved region, C1-C4 that is sandwiched between the V4 and V5 loops (24). Of the five variable regions, the V3 loop is particularly important in that variations in its sequences determine the chemokine receptor specificity and, hence, the host's cell range and tropism of a particular HIV isolate (25).

Entry of HIV-1 into host cell is dependent on the binding of the gp120 envelope protein to the high affinity host's ligand CD4 (26). It has been postulated that this binding produces conformational changes in both CD4 and HIV-1 gp120 exposing the chemokine's binding surface which enables the glycoprotein to bind to the chemokine co-receptors. This interaction between gp120 and the co-receptor generates additional conformational changes that expose gp41, which is responsible for activating the processes of fusion between the virus and the cell, and therefore the direct entrance of the viral genome into the cell. The V3 loop of gp120 is critical for this interaction (27).

Although the cellular protein CD4 has been identified as the main receptor for initial binding, different chemokines receptors have been shown to be indispensable molecules for viral infection. These molecules have been

Table2. Chemokine Receptor Family Members' Known Function as Receptors in HIV and SIV Entry

Receptor	Ligand	Expression Pattern	Viral Usage
Major Receptors			
CCR3	Eotaxin, MCP-3, MCP-4, RANTES	Eosinophils, Microglia T_h2 cells	HIV-1 (minor for HIV-2)
CCR5	MIP-1_a, MIP-1β, RANTES	Monocytes, T cells	HIV-1, HIV-2, SIV
CXCR4	SDF-1	Lymphocytes, Macrophages, Brain	HIV-1 (minor for HIV-2)
BOB/GPR15	?	T cells, Colon	SIV, HIV-2 (minor for HIV-1)
Bonzo/STR33/TYSMTR	?	T cells, Monocytes, Placenta	SIV, HIV-2 (minor for HIV-1)
Minor Receptors			
CCR2	MCP-1, MCP-3	Monocytes, T cells	HIV-1, HIV-2
CCR8	1-309	Monocytes, Thymocytes	HIV-1, SIV
CX_3CR1 (V28)	Fractalkine/Neurotactin	Lymphocytes, Brain	HIV-1, HIV-2
GPR1	?	Macrophages	SIV

The "major" receptors have all been tested in viral infectivity assays with multiple strains. Some of the "minor" receptors have been tested mainly in cell fusion assays, which may not always be representative of viral infectivity. Adapted from Reference 194.

designated as co-receptors for HIV-1 infection, because of the role that they play in conjunction with the CD4 receptor for viral entry, infection, replication and the determination of the tropism of different virus strains (28,29,30).

Some HIV-1 strains will infect cells of the monocyte-macrophage lineage. Those cells express CCR5 (R5) chemokine receptor, and the viruses that infect them are designated M-tropic viruses (31,32,33,34,35,36). On the other hand, some viruses mainly infect $CD4^+$ T-cell-line that express the CXCR4 (X4) chemokine receptor and these viruses are referred to as T-tropic viruses (2937) . Dual-tropic viruses (R5X4) use both CCR5 and CXCR4 co-receptors. Several studies have demonstrated the presence of other viruses that can use other co-receptors besides CCR5 and CXCR4. These alternate co-receptors include CCR3, CCR2b, CCR8, GPR15, GPR1, CX3CR1 and STRL33 (2,31,33,33,34,35,36,37,38,39). The various chemokine receptor family members and their known functions are depicted in Table 2.

Binding of gp120 to CD4 elicits virus-neutralizing antibodies from the host but HIV-1 is able to elude host's immune response. The surface of gp120 is extensively glycosylated and, as alluded to earlier, contains several variable loops, V1-V5. The occurrence of these glycosylations and the variable loops limits the surface area exposed to antibodies elicited from the host (40). Conventional wisdom is that the only two sites that are recognized by neutralizing antibodies: one near the CD4 binding region, and the other which is near the chemokine-receptor binding site, are made inaccessible by the V1/V2 and V2 and V3 loops, respectively. As a result, the virus is shielded from the host's immune response. Additional protection from immune response may also be provided by conformational changes resulting from the binding of HIV-1 gp120 to CD4 (41).

As with many other retroviruses, once the virus is effectively fused with the cell, the viral core is released into the cell and viral uncoating begins, liberating the viral RNA into the cytoplasm where it will begin to be copied by means of the viral reverse transcriptase. This process will allow a double stranded DNA to be copied from the genomic single stranded viral RNA.

5.2. Reverse transcription

After the viral core of HIV-1 enters the cell, the virus gains access to the host cell's nucleotides and reverse transcription of the viral genome is initiated (1). Synthesis of the viral DNA molecule is performed in several steps **(**Figure 3**)**. DNA synthesis starts at a specific region near the 5' end of the viral RNA using a the 5' end of the positive strand of viral genomic RNA as a template, and transfer RNA (tRNA) bound to a specific region (primer binding site, PBS) as a primer. This step in the process generates a short minus strand DNA, which is generally termed minus-strand strong stop DNA (-sssDNA) (42). The strong stop DNA includes the U5 and R regions of the viral genome with the tRNA still attached (43). The RNA that serves as the template is then degraded by the RNase H activity of the reverse transcriptase (RT) leaving only a single stranded DNA. Different retroviruses use different tRNAs as primers, and for most mammalian viruses, tRNAPro, tRNALys3 or tRNA Lys1,2 are used as primers. For HIV-1, tRNALys3 is used specifically as a primer for the initiation of viral DNA synthesis (44).

To continue synthesis of the minus strand DNA, the minus-strand strong stop DNA is translocated and hybridized to complementary sequences at the 3' end of the viral genomic RNA, which serves as a template for the continued synthesis and elongation of minus strand DNA (8,45). This is followed by RNase H degradation of the template strand, except a designated segment, the polypurine tract (PPT) (46,47), which is in position 5 ' of U3 (48,49,50). This PPT segment serves as the primer for the initiation of the synthesis of the plus strand DNA (51).

Synthesis of the plus strand DNA begins with copying of a portion of the tRNA to yield a small DNA called plus-strand strong DNA (+sssDNA). Once the synthesis of this segment of DNA is completed, the strand will be located in the analogous sequence at the 3' end of

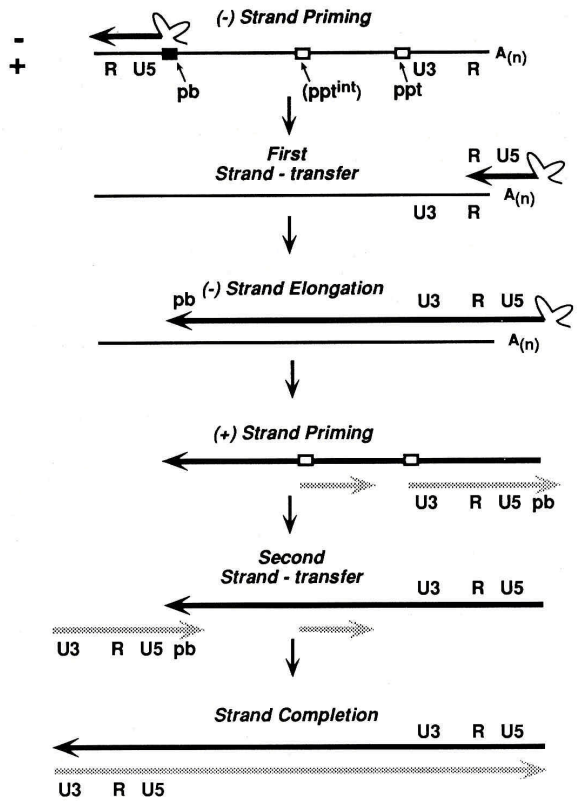

Figure 3. The process of reverse transcription. Synthesis of the first strand DNA is initiated by partial binding of tRNA, which serves as a primer, to the primer binding site (PBS). The second strand DNA synthesis is primed by the polypurine tract (PPT), which is generated through the RNase-H activity of RT.Adapted from Reference 193.

he minus-strand DNA (52). The tRNA is degraded by RNase H, and the PBS sequences in the strand anneals to the complementary sequences at the 3' end of –sssDNA. DNA syntheses and elongation of both the plus and minus strands continue with each strand using the other as a template (53). A modified base that is found in the tRNA located at the 5' end of minus-strand DNA is responsible for stopping this elongation process. Finally, a double stranded DNA molecule will be formed after the removal of the tRNAs located at the 5' ends (52).

5.3. Nuclear transport and integration

The newly synthesized double stranded DNA stays associated with viral proteins to form a pre-integration complex (PIC). This complex consists of the linear viral DNA, the matrix protein (MA), Vpr, integrase (IN), and reverse transcriptase (RT) (54). This pre-integration complex is transported to the nucleus, and the viral DNA is integrated into the cellular genome. The process involved in the transport of the PIC into the nucleus is not completely understood. Some studies suggest that the process is mediated by interaction of nuclear localization signals (NLSs) on the HIV-1 Gag matrix protein (MA, p17), the HIV-1 accessory protein, Vpr, and integrase (55,56,57).

The HIV-1 MA encodes a basic type NLS which is recognized by karyopherin / heterodimers which are components of the nuclear import pathway. In contrast, Vpr and IN do not contain the classical canonical NLS. In the case of Vpr, it has been suggested that the six arginine residues that occur at its carboxyl terminus may act as an NLS (58). It has been reported that phosphorylation of the C-terminal tyrosine residue of MA facilitates its binding to IN which triggers the incorporation of the MA/IN complex into the viral core (57,59). Karyopherin-alpha facilitates translocation and the docking of MA/IN complex at the nuclear pore by binding to the protein to form karyopherin-alpha-NLS protein complex (57,59,60,61,62). Karyopherin-beta on the other hand, increases the affinity of karyopherin-alpha for NLS and also regulates the docking of karyopherin-NLS complex at the nuclear pore (59).

In a study to determine the role of MA NLS in nuclear import of PIC, Bukrinsky et al., (57) reported that mutations in the highly basic domain of MA abrogated the infection of non-dividing cells. Also bovine serum albumin-conjugated with peptides obtained from the highly basic MA domain were found to localize to the nucleus. These observations suggest that MA NLS is involved in the nuclear localization of the HIV-1 PIC (57). However, Fouchier and co-workers (63), in a study analyzing the involvement of an MA NLS peptide in nuclear import of PIC, concluded that the NLS of MA is involved in Gag processing but not in the transport of PIC to the nucleus. A similar opinion has been echoed by Freed and Martin (64), and Freed et al. (65). However, in a comprehensive review of the HIV-1 nuclear import, Bukrinsky and Haffar (66) concluded that MA NLS is involved in nuclear translocation of PIC, but provides a weak signal that acts in concert with other proteins, such as Vpr and integrase, to effectively translocate the PIC to the nucleus. This weakness in activity is also believed to be compensated for by the presence of multiple copies of HIV-1 MA NLS (66,67).

Vpr mediates the docking of PIC at the nuclear pore by binding to karyopherin-alpha. This increases the affinity of this protein for MA NLS and, as a result, facilitates the docking and passage of the PIC across the nuclear pore complex (56,68,69).

Once the PIC has been transported into the nucleus, viral DNA integration is initiated. Like the transport into the nucleus, the process of viral DNA integration is also performed in several steps. First, IN cleaves portions of the 3' terminals of the viral DNA (70), generating recessed termini with free 3' hydroxyl groups (3'-OH). IN then makes a staggered cleavage in the host DNA to produce short overhangs, and simultaneously joins the 3'-OH ends to the cellular DNA by a strand transfer reaction (53,71). Finally, cellular DNA repair machinery fills in the gaps in the integrated molecule. Once inserted in the cellular genome, the viral DNA is referred to as a provirus (Figure 4). The provirus can either hold onto latency or begin the process of transcription with the

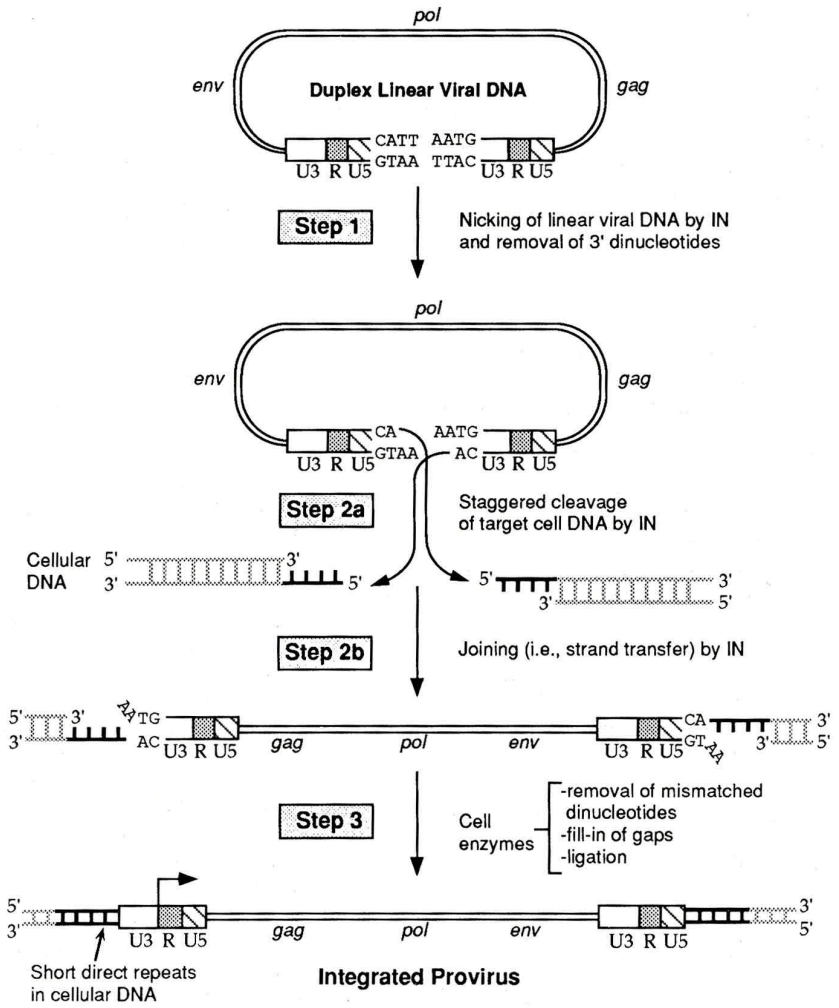

Figure 4. Schematic of the various steps involved in the process of integration. Following entry of virions into the host cell, the viral RNA is reverse transcribed into double-stranded DNA which is then imported and integrated into the host cell chromosomes. The integration of the viral DNA is performed in several steps. First, IN cleaves parts of the 3' terminals of the viral DNA, generating the hydroxyl groups (3'-OH), which is simultaneously joined to the target cell DNA by a strand transfer reaction. Finally, cellular DNA repair machinery fills in the gaps in the integrated molecule. Adapted from Reference 193.

purpose of generating new viral particles. The LTR sequences play a major role in this regard.

5.4. Viral RNA transcription and splicing

The genome of each of the integrated proviruses is flanked at each 5' and 3' end by the long terminal repeat (LTR), which consists of repeated segments termed U3, R and U5 (Figure 5). These domains play important roles in the regulation of the retrovirus life cycle. The HIV-1 5' LTR contains elements for transcriptional regulation as well as RNA syntheses. The transcriptional regulatory elements called the enhancer and the upstream and downstream promoters are located in the U3 region, whereas the CAP site and the start site for mRNA synthesis and polyadenylation are found in the R region. The promoter elements include the initiator element, the TATA Box and binding sites for the host's NF-kappa B and SP-1 cellular transcription factors (38,72,73,74,75), which are responsible for up-regulating the level of transcription performed by the cellular RNA polymerase (71). The TATA box recruits host cellular RNA Polymerase II transcription complexes and is also located in the U3 region. In addition, the U3 region contains the binding sites for other cellular transcription factors, such as AP-1, LEF, NF-AT, USF and Ets-1 (76,77).

In contrast to HIV-1, other macrophage-tropic viruses such as visna virus and CAEV contain sequences recognized by the cellular factors AP-1 and AP-4, and these are also located in the U3 region of the LTR (78,79). Furthermore, the pol gene of non-primate lentiviruses, such as FIV, EIAV, CAEV, and visna virus contains a sequence that encodes a protein that has UTPase activity (80). This virus-encoded UTPase could have an important function in efficient viral replication in non-dividing cells, and possibly increases the pool of available nucleotides for viral reverse transcription. This hypothesis is supported by the fact that the cellular UTPases are cell-cycle regulated, in order to

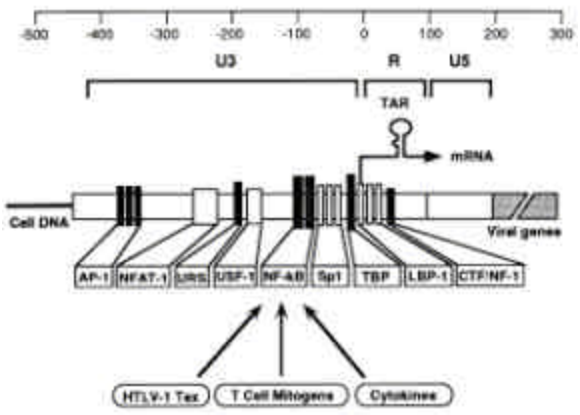

Figure 5. Schematic representation of the HIV-1 Long terminal Repeats (LTR). The LTR contains three regions; U3, R and U5. The upstream promoter and enhancer elements, which include the TATA-binding protein (TBP), the NF-B binding region and the three Sp1 binding sites are found in the U3 region. Slightly upstream of the promoter and the enhancer regions are binding sites for cellular factor such as AP-1, NFAT-1 and USF-1. The R region contains the cap site, the start of mRNA synthesis, and the polyadenylation signal. Adapted from Reference 193.

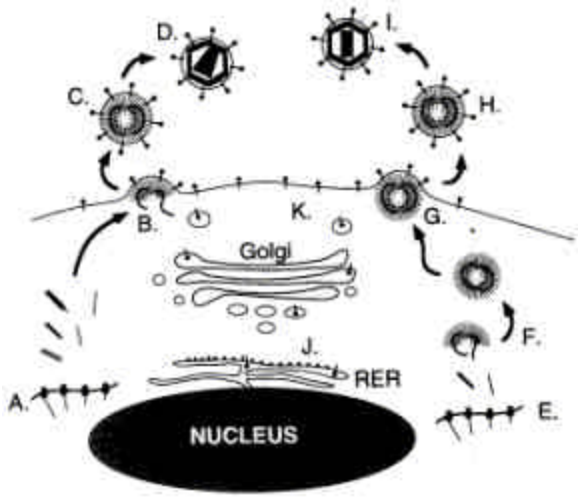

Figure 6. A schematic diagram depicting the assembly of both the C-type (A-D) and B/D-type retroviruses. Adapted from Reference 92.

avoid any unwanted incorporation of uracyl into the newly synthesized DNA strand (72).

RNA transcription of the provirus and splicing of the viral RNA of simple oncoretroviruses appear to be solely dependent on proteins provided by the host cell (1). However, in lentiviruses, this process is vastly more complicated and the viral regulatory proteins Tat and Rev play vital roles in this process.

During the early stages of the RNA expression of HIV-1, only fully or multiply spliced mRNA is found in the cytoplasm. At this stage, only the regulatory proteins Tat, Rev and Nef are produced with no structural or enzymatic proteins. These proteins accumulate and Tat begins to activate viral transcription. This regulatory protein acts by activating cellular factors, thus increasing the processivity of viral transcription by the activation of the cellular RNA polymerase II. This effect will dramatically increase the genetic expression of HIV-1 (5,12), as compared to the expression levels obtained with just the cellular components.

Tat acts through sequences down-stream of the transcriptional start site generating a stable stem-loop structure. These sequences, found only in the RNA of the primate lentiviruses and referred to as TAR (Tat activating region), are critical for viral regulation by Tat. TAR will enable the virus to be *trans*-activated by several factors: T-cell activators, proteins from other viruses, and by components from other infected cells (81,82,83). Therefore, Tat acts not only as a transcriptional activator on the proviral DNA, but also has an important effect on the TAR sequence found on the nascent RNA molecules, increasing the genetic expression of lentiviruses at the transcriptional and post-transcriptional levels. Nevertheless these sequences have not been identified in visna virus, CAEV, or FIV (84,85).

In contrast to primate lentiviruses, Tat-mediated enhancement of the transcription process in viruses such as FIV, CAEV, and visna virus, is conducted through a direct interaction with cellular proteins, because of the absence of the TAR element in those viruses (78). In the case of visna virus, the Tat protein activates the processes of transcription via the AP-1 site near the TATA box, an effect that also activates the transcription of viral and cellular promoters that contain the AP-1 site (78).

The late phase of the RNA expression of HIV-1 is characterized by the optimum production and accumulation of the regulatory protein, Rev. During the late-phase, Rev activates the export of unspliced and partially-spliced viral mRNA to the cytoplasm by binding to the Rev-responsive element (RRE) on the viral mRNA (86,87,88,89,90). At this late stage, the transport of unspliced and partially spliced viral mRNAs to the cytoplasm will usher in the syntheses of the structural and enzymatic proteins of HIV-1 (encoded by spliced and unspliced mRNA), while the synthesis of regulatory transactivators is inhibited (91).

5.5. Encapsidation and virus particle assembly

The final step after the synthesis of the genomic viral RNA is encapsidation and liberation of the viral particles (Figures 6 and 7). Two basic processes for viral assembly have been described, depending on the type of virus (Figure 6) (92).

In the first type, the viral capsid is assembled within the cytoplasm and is subsequently transported to the plasma membrane where envelope formation and budding into new virions take place (93,94,95). Examples of

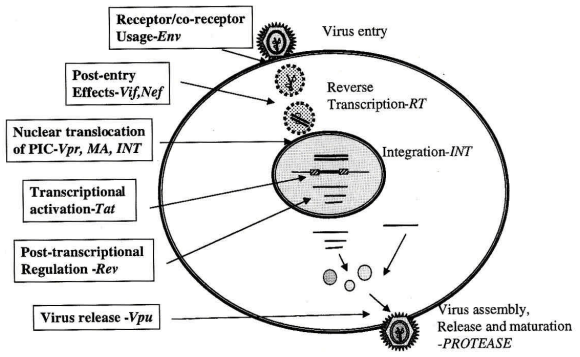

Figure 7. Replication cycle of HIV-1. After an infectious virion has entered a host, the viral gp120 binds to the CD4 of a suitable host cell, such as macrophages, dendritic cells or CD4+ T-lymphocytes. The viral membrane then fuses with the target cell membrane through the mediation of gp41. This is followed by entry and uncoating of the virus to release the viral core. RT then reverse transcribes the viral RNA into a double stranded DNA. The core DNA-complex which is referred to as the pre-integration complex (PIC), is translocated to the nucleus of the host cell where it is integrated into the host genome. The integration of the viral DNA into the host cell is followed by viral gene expression, assembly, release, and virion maturation. The names in italics refer to the various viral genes involved in the HIV-1 life-cycle. Adapted from Reference 2.

iruses using this type of assembly and maturation are B-type virus such as mouse mammary tumor virus (MMTV), type-D - the Mason-Pfizer monkey virus (M -PMV) and related simian retroviruses (SRV), and several members of the spuma viruses (96). In the second morphogenetic pathway, normally found in C-type retroviruses, the Gag and Gag-Pol precursors are transported to the plasma membrane where the capsid is formed, followed by budding to generate new virions (92). Examples of viruses using this type of assembly include the avian sarcoma/leukosis virus (ASLV) and murine leukemia virus (MLV).

The assembly of virions, such as HIV-1 as well as in other lentiviruses, is by means of the Type-C route. Therefore, the membrane-bound viral glycoproteins of HIV-1, as in any other type-C retroviral morphogenetic pathway, are transported through cellular secondary secretory routes to the plasma membrane, where the viral proteins are located to produce the salient capsid (96,97,98,99).

As in all replication competent retroviruses, HIV-1 contains the gag, pro, pol and env genes that encode the structural and enzymatic components used to initiate the process of encapsidation (packaging) and assembly into virions. The Gag proteins are translated from an unspliced genome-length viral RNA on free polyribosomes in the cytoplasm as polyprotein precursors (92,100,101,102). The polyprotein precursor has been shown to be adequate for the encapsidation of RNA in the absence of any other viral protein {see reviews by Wills and Craven (100), Hunter (92)}. Shortly after its synthesis, the polyprotein is cleaved into MA, NC, and CA by viral protease enzyme, PR. After cleavage, MA remains attached to the viral membrane whereas CA forms a core around the RNA/NC complex (92,100).

The viral enzymes PR, RT and IN are encoded by the pol gene. The function of PR is to cleave the Gag-Pro-Pol polyprotein, whereas RT, an RNA-dependent DNA polymerase, synthesizes DNA from an RNA template. IN, on the other hand, is responsible for the integration of linear double-stranded HIV-1 DNA into the host cell genome. Like the Gag gene products, these enzymes are produced by the cleavage of a larger precursor polyprotein, the Gag-Pro-Pol polyprotein. The generation of the Gag-Pro-Pol precursors occurs via translational suppression, a mechanism utilized by retroviruses to regulate the relative amounts of Gag and enzymatic proteins incorporated into virions (103). There are two distinct types of translational suppression: suppression of the Gag termination (read-through) codon and ribosomal frameshifting.

In some retroviruses, such as the mammalian C-type retroviruses that utilize the Gag termination (read-through) codon, the gag and pol coding sequences are in frame with respect to each other, with a single UAG stop codon sandwiched between them. This arrangement allows the termination codon to be occasionally misread as a sense codon, thereby allowing the ribosomes to continue translation of the pol sequences beyond the termination codon. In the other retroviruses including HIV-1, the Gag-Pro-Pol precursors are processed by ribosomal frameshifting. Here, the gag and pol coding sequences are in different frames and during translation of gag, the ribosomes may slip backward one nucleotide and then continue to translate the pol gene (104,105,106).

The *env* gene encodes two products, the surface envelope glycoprotein (SU or gp120) and the transmembrane glycoprotein (TM or gp41). As with Gag and Pol, these two proteins are initially synthesized as a larger precursor polyprotein (gp160, SU-TM) from spliced genomic RNA, which is devoid of *gag-*, *pro-* and *pol-*coding sequences. The polyprotein is subsequently cleaved by cellular protease into the respective subunits.

Once the Gag, Gag-Pro-Pol and Env proteins are synthesized, they begin to aggregate at a site specified by the MA Gag protein on the cell membrane, with two copies of viral RNA and tRNA primers to initiate encapsidation and assembly of viral particles (104,105,106). The encapsidation process favors full length, unspliced RNA over spliced RNA, and viral RNA over cellular RNA (107). The HIV-1 genomic RNA is selected for encapsidation and assembly through an interaction between the *cis*-acting region of the viral RNA, designated the psi RNA packaging sequence, and the NC protein of the HIV-1 Gag (27). This psi site is located between the 5' long terminal repeat and the Gag initiation codon (108). The region contains four hair-loop structures, denoted stem-loops 1-4 (SL1, SL2, SL3 and SL4) (108). Although some studies have shown that SL1, SL3 and SL4 are required for the efficient

encapsidation of HIV-1 (108,109,110), it appears that only the SL3 interacts with NC to direct recognition and packaging into viral particles. Mutational analysis of the NC domain of HIV-1 Gag has also demonstrated that the CCHC-type zinc binding domain which is made up of one or two copies of the sequences Cys-X2-Cys-X4-His-Cys (where X can be any amino acid), is essential for efficient viral assembly (111-116).

The HIV-1 genomic RNAs are co-packaged as loosely-linked dimers within the virus particle. During maturation, these genomic viral RNA are believed to form a more tightly-linked association in a process referred to as dimerization. The purpose of this dimerization is unknown but the general consensus is that it may be involved in viral assembly (109,117). Evidence for this came from the observation that the NC domain facilitates the dimerization of viral RNA, and that mutations in the SL1 affects the process of dimerization and encapsidation in the same way (109,117).

6. ROLE OF OTHER ACCESSORY PROTEINS

6.1. VIF

The viral infectivity factor (Vif) is a 192 amino-acid protein with a mass of 23-kDa (118) and has been reported to be essential for the viral replication *in vivo* (119,120), although not in all cultured cell-types (121-124). Numerous studies have shown that the amount of Vif packaged into virions changes with the level of its expression, however those variations do not yield any change in the infectivity of the newly produced virus. These data suggest that Vif is not specifically packaged into virions, and its packaging is not essential for virus infectivity (125-127).

Vif appears to stabilize the preintegration complex (125,128,129,130,131), plays an important role in the synthesis of the proviral DNA (118), and also in maintaining the morphological integrity of the virus, suggesting a possible role in viral assembly (125,128-131). The aberrant viral morphology that is produced in viral particles in non-permissive CEM cells (132) infected with Vif-negative virus suggests that Vif might have a role in virion maturation (133), by acting on the Env protein (134), and regulates the establishment of the provirus (128). It has been observed that Vif-deleted viral particles formed in non-permissive cells are deficient in envelope glycoproteins suggesting that Vif regulates the maturation of infectious viral particles (128).

Vif is an RNA-binding protein and specifically binds to HIV-1 genomic RNA *in vitro*. Thus, Vif may be involved in the folding and packaging of viral RNA (135). The protein may also act as a stabilizer of the viral mRNA, protecting it from cellular degradation, and also mediating the interaction of RNA with the HIV-1 Gag precursors (135). The association of Vif with viral mRNA, and the impairment of reverse transcription in Vif-defective viruses suggests that Vif may play a potential role in the process of reverse transcription of viral RNA (136). Furthermore, in non-permissive cell-lines, the expression of Vif influences the uncoating and/or internalization of the virus, allowing a complete process of reverse transcription to be performed, and generating complete molecules of viral DNA (136,137).

Vif is phosphorylated (138) at several residues as: Ser^{144}, Thr^{155}, Thr^{188}, Thr^{96}, and $Ser.^{165,68}$ The last two residues have been shown to be phosphorylated by the p44/42 mitogen-activated protein kinase (MAPK). This indicates that Vif may play a role in the mitogen-mediated viral regulation (139).

The role of Vif in the regulation of nuclear localization was studied after it was observed that the Vif protein has a basic domain with a sequence similar to a nuclear localization signal. Interestingly, Vif has a basic region containing ^{154}RKKR^{155}, which is similar to a nuclear localization signal (NLS) (140). However, several studies using synthetic peptides that overlap this and other sequences of Vif indicate that it actually inhibits nuclear transport, after determining that the molecule SV40-NLS-BSA was unable to be nuclearly localized in the presence of the Vif peptide (140). Similarly, these Vif peptides were unable to promote the nuclear localization of other bovine serum albumin (BSA)-conjugated peptides (140). It is possible that this signal actually regulates these processes by inhibiting rather than mediating nuclear import (140). Hence, Vif might have an important role in the regulation of the nuclear import of several cytosolic proteins (140).

6.2. Nef

The HIV-1 negative factor, Nef, is a 206 amino-acid (141) and 25 to 29-kDa mass protein (142). Initially, Nef was considered as a negative regulator of viral transcription (143,144,145,146), but it is now accepted as a stimulator of viral infectivity (145). Nef deletion mutants have been shown to be considerably less infective than wild-type, indicating that this protein is important for the stimulation of viral infectivity (145). Furthermore, Nef protein increases viral replication in non-stimulated cells (145), an effect that could be important in viral infectivity *in vivo*, since most CD4+ T-lymphocytes *in vivo* are in the resting stage (147,148).

Several studies have shown that Nef plays an important role in the down-regulation of the CD4 receptors on the surface of infected cells (142,149-153). Down-regulation of the CD4 receptors might decrease the rates of super infection by the virus (154), prevent virus aggregation during the budding process, and regulate T-cell activation processes (152,155). Nef-mediated down-regulation of the CD4 receptor occurs at the early phase of the virus life-cycle (156), as opposed to the gp160- and Vpu-mediated down-regulation of CD4 receptors, which occur at later phases of the virus life-cycle (157). Considering that the CD4 receptors and the Env protein are both synthesized in the endoplasmic reticulum (ER), the early removal of the CD4 receptors by Nef might decrease the possibility of Env neutralization by the CD4 at an early stage. Vpu also has an important role in keeping a low level of CD4 receptors (see below).

Nef-deleted viruses are able to enter target cells, producing viral particles with normal RNA content, however the reverse transcription process is compromised in cells infected by these particles. This suggests a role for

Nef in the cellular regulation of the reverse transcription process, and therefore in the stimulation of proviral DNA synthesis (158).

It has also been shown that Nef is able to inhibit the mitogenic induction of NF-kappaB and AP-1 (159). This suggests a role for Nef in the regulation of the cellular and viral processes of transcription and the replication cycle of the virus (160), by influencing the genetic expression directed by the 5' LTR (144,161). This property of the Nef protein has been associated with the capacity of HIV-1 to maintain latency (143), as opposed to inhibiting its infectivity. The demonstration of the association of Nef with several signal transduction cascades, such as intracellular calcium mobilization (Ca^{2+}), suggests a role of this protein in the regulation of cellular activation processes (162,163). Of note, only primate lentiviruses possess the nef gene (158).

6.3. Vpu

The viral protein U (Vpu) is an 81 amino-acid protein only produced by HIV-1 (164). The protein has a molecular weight of 16 kDa (164) and has been reported to enhance the release of viral particles from the plasma membrane, through the formation of oligomeric complexes in the post-endoplasmic reticulum compartments (165). The capacity of Vpu to facilitate the release of viral particles from infected cells might have an effect on viral tropism, suggesting that Vpu has a role in the adaptation of *in vitro* M-tropic viral strains to T-tropic viral strains (166). Unlike T-cell cultures, which are actively dividing *in vitro*, infection of macrophages by HIV-1 requires other accessory genes (167,168). Absence of these might generate mutational variants of Vpu which, on one hand, will not affect the viral particle production in T-cells, but would considerably diminish the viral infectivity in macrophages (168).

Like HIV-1 Nef, several researchers have shown that Vpu plays an important role in the down-regulation of the CD4 receptor in the ER (169); a process that is mediated by the phosphorylation of Vpu by casein kinase II; and the down-regulation of the class I histocompatability complex (MHC-I) (165, 169-171). Both the Env protein and the CD4 receptors are simultaneously synthesized in the ER. Therefore, the Vpu-mediated CD4 degradation process does not only decrease the amount of CD4 receptors in the cellular membrane, but also abolishes any possible interference of the newly synthesized CD4 with the Env protein, facilitating the incorporation of the Env protein in the cellular membrane (156).

6.4. Vpr

The viral protein R (Vpr), is a 96 amino-acid protein with a M_r of 14 kDa (172), found in HIV-1, HIV-2, and simian immunodeficiency virus (SIV) (173). Vpr is packaged into virions through the mediation of p6 protein (173-175). Vpr also plays an important role in the nuclear localization of proviral DNA in growth-arrested cells, as suggested by an impairment of Vpr-defective viruses in synthesizing and translocating proviral DNA to the nucleus (176).

The cellular distribution and activity of Vpr is the result of a well-orchestrated mechanism between Vpr and various proteins and factors (177,178). The co-expression of Vpr with the Gag protein with deleted p6 sequences impairs the export of Vpr (179), suggesting that the interaction of Vpr with the protein p6 plays an important role in the packaging of Vpr into virions (173-175,179). Similarly, the interaction of Vpr with the MA protein has been reported to mediate the nuclear transport of the pre-integration complex in infected cells (56,68,176,178). In this regard, interaction between MA, Vpr and the karyophyrin pathways of the host play a major role in the nuclear localization of the preintegration complex (PIC) (56,66,68).

Vpr has an important role in G_2 cell-cycle arrest of HIV-1-infected cells (180-183), perhaps to establish a more robust infection by evading the immune system and optimizing viral production in infected cells (66). The association of Vpr with the protein phosphatase 2A (PP2A) might explain the mechanism by which this protein arrests the cell-cycle (172,180). Briefly, the progression of the cell-cycle is controlled by the complex $p34^{cdc25}$-cyclin B. In this mechanism, $p34^{cdc25}$ is inactivated after being phosphorylated by the protein ^{Wee1}, while the protein ^{cdc25} can dephosphorylate $p34^{cdc25'}$ returning this protein to the original active state. As protein phosphatases are considered upstream regulators of the ^{Wee1} and ^{cdc25} proteins, the activation of ^{Wee1} and the deactivation ^{cdc25} by PP2A might exert their function by preventing $p34^{cdc25}$ in inducing cell-cycle progression in conjunction with its partner cyclin B. It has been shown that cells expressing Vpr produce low and high amounts of p34cdc2-cyclinB and 2A (PP2A), respectively. This suggests that interaction of Vpr inhibits the transition of G2 to mitosis (172,180).

In addition to the role in G2-cell-cycle arrest, Vpr also functions in the modulation of the apoptotic response. It was shown recently that HIV-1-infected cells were resistant to several apoptotic stimuli (184,185). This might be a mechanism used by the virus to prevent cells from using the apoptotic machinery to rid themselves of the infection. Nevertheless, after a longer time post-infection, the infected cells initiated apoptosis mechanisms, a process that is suggested to be dependent on the level of the cellular Vpr expression (186).

Vpr has also been reported to play a role in the modulation of the HIV-1 mutation rate (187,188). Because RNA uses uracyl instead of thymidine in its structure, the misincorporation of uracyl in the DNA synthesis is an unusual event that might produce mutations in several organisms. These misincorporations are avoided by the uracyl DNA glycosylase (UNG) enzyme (189), which is responsible for the DNA repair system (187), a process that has also been studied in yeast systems (187,190,191). It has been found that not only is UNG incorporated into virions through the mediation of Vpr, but also that a higher number of mutations are found in Vpr-deleted virus as compared to the wild-type virus (188). However, some reports suggest that the rate of mutation is lower *in vivo* as compared to *in vitro* (192).

In a nutshell, lentiviruses are extraordinarily complex at the entry, pre-integration, transcription, post-transcription, post-transduction, and assembly levels. Nevertheless their complexity also allows fine-tuning of their genomes for potential use as novel vectors in gene therapeutic paradigms.

7. PERSPECTIVE

Since the discovery of HIV-1 and AIDS a little over two decades ago, numerous resources have been devoted to the study of lentiviruses and the wealth of knowledge developed, particularly about HIV-1, is perhaps unparalleled in the history of scientific research. Research into the general biology and replication of HIV-1 has helped not only in elucidating the complex pathogenetic pathways of the virus, but also in developing antiretroviral drugs that are effective in limiting HIV-1 replication and arresting further decline in the host's CD4+ T-lymphocytes. Consequently, there is improvement in the immune function, a slowing and reversal of clinical progression to AIDS, and protection of infected individuals from opportunistic infections.

Despite these biomedical advances, HIV-1 and AIDS continue to pose significant health risks to the world population and finding a cure for HIV-1 infection continue to elude scientists. Also, many unanswered questions remain regarding HIV-1 pathogenesis in general and the replication of the virus in particular. For example the mechanisms involved in the nuclear localization of PIC are not fully understood. Similarly, there are conflicting reports regarding the mechanisms involved in the trafficking of Gag precursor proteins to the site of assembly. In addition, complete understanding of the structural and functional analyses of such viral proteins as the Gag polyprotein, and complete encapsidation signals, are almost non-existent. Understanding the structural and functional details, encapsidation signals and the interaction between the viral and host's cellular proteins will provide us with new targets for chemotherapeutic intervention in the HIV-1 replication cycle and infection.

Thus, given the current state of knowledge and the amount of resources and significant efforts being devoted to HIV-1 research, it is envisioned that more viral targets will be identified for effective treatment of this devastating lentiviral infection.

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