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The Role of Tat in HIV-1 Replication: an Activator and/or a Suppressor?

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Abstract

Tat is a key *trans*-activator of HIV-1 gene transcription and major progress has been accomplished in recent years in regard to understanding its mechanism of action. An important breakthrough was the identification of the TAR-Tat-Cyclin (Cyc) T1-Cyclin-dependent kinase 9 (CDK9) complex, in which CDK9 can hyperphosphorylate the carboxyl-terminus domain (CTD) of the RNA polymerase (RNAP) II complex. A different activity of Tat has recently been identified in reverse transcription. Notably, mutated HIV-1 that lacks a functional Tat protein cannot efficiently generate reverse transcription products following infection of permissive cells. Furthermore, Tat can also inhibit reverse transcriptase activity in cell-free assays and can act as a suppressor of reverse transcription at late stages in the viral life cycle. This suppressor activity of Tat can restrict the premature reverse transcription of viral RNA in the cytoplasm and allows the viral genome to be packaged as intact RNA molecules.

Key words

HIV-1 Tat. Reverse transcription. Gene transcription.

The human immunodeficiency virus type 1 (HIV-1) carries six regulatory genes, including *tat, rev, vpr, vif, vpu,* and *nef*[1]. Of these genes, *tat* encodes a 101-amino acid protein that plays key roles in controlling productive and processive viral gene transcription[2,3]. In recent years, a novel activity of Tat in reverse transcription has been identified that provides new insights into the multifaceted roles of Tat in the virus life cycle[4]. In this review, we focus on findings that explain the regulatory roles of Tat in reverse transcription.

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Structure of the Tat protein

Tat is made from a multiply spliced viral mRNA and is expressed during early stages of viral infection[5,6]. Tat consists of two exons; amino acids 1-72 are found in the first exon and amino acids 73-101 in the second[7]. Although several laboratory-passaged virus strains (such as LAI, NL4-3, and HXB2) harbor a 86-amino acid version of Tat, most *in vivo* isolates of HIV-1 possess the 101-amino acid full-length Tat[8]. The second-exon has been shown to be non-essential for Tat transactivation of HIV-1 gene transcription[2]; however, retention of the 101-version of Tat in clinical HIV-1 isolates underlies the importance of the second exon in viral propagation and pathogenicity[9-15].

Tat is a highly basic nuclear protein that contains a nuclear localization signal (NLS) with an amino acid sequence of RKKRRQRRR. On the basis of structural and functional studies, Tat can be divided into five domains (Fig. 1)[7]. Domain I (residues 1 to

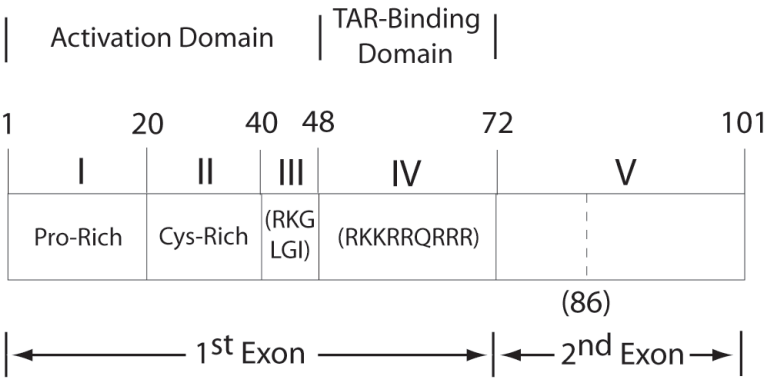

Figure 1. *Functional domains of the HIV-1 Tat protein. The full-length two-exon Tat contains 101 amino acids. A number of HIV-1 strains (e.g. LAI, DXB2, and NL4-3) encode a 86-amino acid version of Tat. The Tat sequence can be divided into five domains, i.e. I (amino acids 1 to 20), II (amino acids 21 to 40), III (amino acids 41 to 48), IV (amino acids 49 to 72), and V (amino acids 73 to 101). The first three domains, including amino acids 1 to 48, comprise the activation domain; domain IV is highly basic and serves as the TAR-binding domain.*

20) is proline-rich. The next 20 amino acids (21-40) form the second domain which contains seven highly conserved cysteine residues at positions 22, 25, 27, 30, 31, 34, and 37. The third domain, i.e. amino acids 41 to 48, harbors a RKGLGI motif that is found in Tat molecules derived from both HIV and SIV. Amino acids 1-48 are implicated in interactions with a number of cellular proteins involving Cyclin T1 (Cyc T1)[16,17], Sp1[18,19], histone acetyltransferase (HAT)[20-23], and protein kinase R (PKR)[24]. These 48 amino acids comprise the activation domain. Next is a highly basic domain that spans amino acids 49-72 that is involved in several different activities; these include the binding of Tat to TAR RNA[25-28], Tat nuclear localization[29,30] and plasma membrane permeability of Tat[31]. The second exon (amino acids 73-101) defines a separate domain, the roles of which in Tat function are largely unknown.

The three-dimensional structure of the Tat protein has been resolved by nuclear magnetic resonance (NMR), but only at low resolution, and reveals that the basic region of Tat is well extended while the cysteine-rich region adopts loop structures[32,33]. Ordered α-helix and β-sheet structures are not revealed by these structures and a detailed understanding of Tat configuration must await higher resolution of a more accurate structure.

Trans-activation of the HIV-1 LTR promoter by Tat

Tat has long been understood to function as a special transcriptional regulator[2,3]. It is able to increase the transcription level of the HIV-1 LTR promoter by several thousand-fold. In general, the strength of a promoter is regulated by several key events: 1) recruitment of an RNA polymerase II (PNAP II) complex to the promoter; 2) the initiation rate of transcription; 3) clearance of the transcription complex from the promoter concomitant with transition from an initiation to elongation phase. It has been shown that Tat does not participate in the recruitment of the TBP/RNAP II complex to the promoter[34]. Since Tat needs to bind to a TAR (*trans*-activation responsive element) structure that is formed by nascent viral RNA in order to exert its stimulatory effects on transcription[35-37], it is proposed that Tat may play its roles in transcription by regulating the processivity of RNAP II.

The elongation activity of RNAP II is controlled by phosphorylation of heptapeptide repeats located within the carboxy-terminal domain (CTD)[38,39]. Upon CTD phosphorylation, RNAP II changes its conformation and, as a consequence, dissociates from negative elongation factors (N-ELF). This phosphorylation event is executed by cyclin T dependent kinase (CDK) that is associated with several positive elongation factors (P-TEF)[39,40]. Recent studies have demonstrated that Tat can recruit P-TEFb to the RNAP II complex[41-44]. It is also known that Tat directly binds to cyclin T1 (Cyc T1), a protein that is associated with CDK9[16,17,45]. Once the first 57-nucleotides of nascent viral RNA are synthesized, the TAR structure is formed. Following binding to TAR, Tat brings the Cyc T1-CDK9 complex close to the RNAP II complex. Since the nascent RNA exit channel is located adjacent to CTD, CDK9 is then positioned close to the CTD of RNAP II and can thus hyperphosphorylate this critical domain (Fig. 2)[46].

Tat may also enhance transcription through interactions with other components within the RNAP II complex. For instance, Tat may recruit other kinases to phosphorylate CTD[41]; Tat may regulate the dephosphorylation of CTD by inhibiting the CTD phosphatase activity of FCP1[47]. It is also possible that Tat may stimulate re-initiation from HIV-1 LTR. This notion is supported by findings of TAR-independent transactivation by Tat. In this context, Tat can directly interact with a DNA-bound Sp1 factor1[8,19,48,49]. Another important role of Tat in transcription relates to the regulation of gene expression from proviral DNA which becomes integrated into host chromosomal DNA and is protected by histone proteins in nucleosome structures. Tat is able to increase the accessibility of the RNAP II complex to

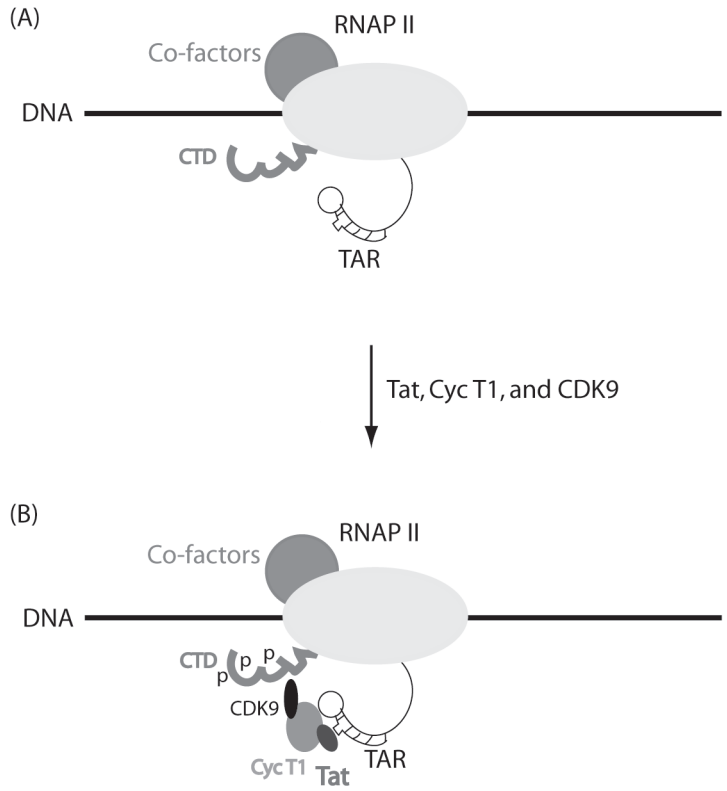

Figure 2. *Tat transactivation of HIV-1 LTR promoter. (A) RNAP II complex, including a number of co-factors, initiates RNA transcription from the HIV-1 LTR promoter. The TAR structure is formed when the first 57 nucleotides are synthesized. (B) Tat, together with Cyc T1, binds to TAR and positions CDK9 close to the CTD (carboxyl-terminal domain) of RNAP II. CTDK9 then causes hyperphosphorylation of CTD, and, as a consequence, transcription from LTR is switched from initiation to productive elongation.*

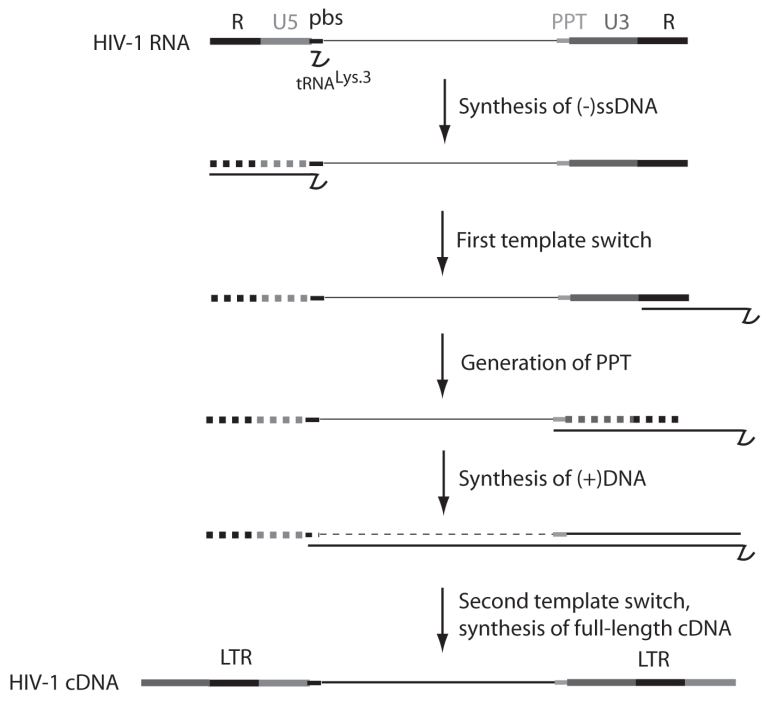

Figure 3. *Synthesis of HIV-1 cDNA. HIV-1 reverse transcription is initiated from a cellular $tRN^{Lys,3}$ that binds to an 18 nucleotide viral RNA fragment termed the primer binding site (PBS). Following the synthesis of minus-strand strong-stop DNA ((–)ssDNA), the first strand transfer occurs, which is mediated by the R sequences. A polypurine tract (PPT) serves as primer for the synthesis of plus (+) strand DNA. The full-length HIV-1 cDNA, which is flanked by the 5' and 3' LTR (long terminal repeats), is generated following the second template switch.*

HIV-1 LTR by acetylation of histone proteins through Tat-associated histone acetyltransferase (TAH)[20-23].

Roles of Tat in HIV-1 reverse transcription.

In addition to its roles in transcription, Tat also regulates a number of other viral activities, one of which is reverse transcription. HIV-1 reverse transcription is initiated from a cellular $tRNA^{Lys.3}$ that binds to an 18-nucleotide viral RNA sequence located proximal to the 5' end of the viral RNA genome. Subsequent reactions involve the first strand transfer, the synthesis of plus-strand DNA, and a second strand transfer event (Fig. 3). Essential roles in these processes are played by *cis*-acting viral RNA elements, such the primer binding site (PBS), the polypurine tract (PPT), the 5' and 3' R regions, and the central cDNA flap sequence[50]. In addition, a number of viral and cellular proteins are required for efficient reverse transcription. These include the viral nucleocapsid (NC) protein as well as Nef, Vif, Vpr, and matrix (MA)[51-56]. Cellular proteins that are involved are cyclophilin A (Cyp A) and DNA topoisomerase I[57-59]. Tat can also impact on HIV-1 reverse transcription[4].

The first clue to these roles of Tat came from revertants of TAR mutations that were able to support efficient viral gene transcription, although the relevant mutated viruses still exhibited an attenuated replication phenotype[60]. Further analysis revealed that defective reverse transcription was associated with these TAR mutations. Since TAR serves as the binding site for Tat, it was then speculated that the mutated TAR may have failed to recruit Tat to the initiation complex for reverse transcription. On this basis, it was assumed that Tat plays a role in reverse transcription.

However, direct studies of HIV-1 variants carrying mutated Tat were hampered by the need to generate sufficient quantities of the mutant virus particles for infection of permissive cells. This technical difficulty was solved by transfection of HIV-1 DNA that had been deleted of the *tat* gene into 293 cells that express both the E1A and E1B proteins of adenovirus[4]. These two proteins allow the yield of mutant HIV-1 at levels of 1-2 ng/ml. When these mutant HIV-1 viruses were used to infect peripheral blood mononuclear cells (PBMC), substantially lower levels of reverse transcription products were detected in comparison to wild-type infection, and this includes each of minus-strand strong stop DNA ((–)ssDNA), viral DNA synthesized after the first template switch, as well as full-length viral DNA. These results constitute the first evidence linking Tat and HIV-1 reverse transcription[4].

Effects of Tat on the construction of a functional reverse transcription complex

Although Tat has not been shown to be present within virus particles, it is possible that the levels of Tat within virions may be too low to be detected by currently available techniques. For example, it is conceivable that as few as two molecules of Tat may be bound to the two copies of viral genomic RNA within each virion. Hence, Tat can conceivably be part of the reverse transcription complex and directly participate in RT reactions following infection of target cells. In support of this hypothesis, wild-type Tat protein that was provided *in trans* in producer cells, but not in target cells, was able to overcome defects in reverse transcription associated with Tat-mutated viruses[4]. Furthermore, in endogenous reverse transcription assays performed in the presence of dNTPs, low concentrations of detergent, and appropriate buffer conditions, the nucleoprotein complexes within virus particles were able to undergo reverse transcription. However, lysed Tat-mutated virus particles yielded lower amounts of viral cDNA products in these endogenous reverse transcription experiments than did wild-type viruses[4]. Moreover, *trans*-supplemented recombinant wild-type Tat protein suppressed, rather than augmented, endogenous reverse transcription performed with wild-type viruses[61].

In which way are the Tat-mutated virus particles defective? Biochemical analysis of these viruses showed wild-type levels of each of reverse transcriptase activity, viral genomic RNA, *Gag* and Env proteins[4]. However, the viral RNA of the mutated Tat viruses appeared as partially degraded molecules on native agarose gels and not in dimeric form as found within wild-type virus particles[61]. It has been reported that the conformation of virion RNA is essential for reverse transcription, especially for the first strand transfer[62]. The degraded nature of the RNA found in the absence of Tat protein accounts, at least in part, for the failure of this RNA to undergo efficient reverse transcription in infected cells.

What causes this degradation of viral RNA within virus particles? Two copies of full-length HIV-1 RNA molecules are normally packaged by each virion[63]. However, only limited reverse transcription occurs within mature virus particles themselves[64-68]. In contrast, substantial levels of viral cDNA molecules were shown to be associated with Tat-mutated viruses[61]. Consistent with this observation, Tat-mutated virus particles gave rise to lower levels of nascent endogeneous reverse transcription DNA products than did wild-type viruses[61]. Therefore, premature reverse transcription must have occurred in the absence of Tat protein before mature virus particles were made, and a direct consequence of this premature reaction may be the partial degradation of the viral RNA template, due to RT-associated RNase H activity.

It is unknown whether these Tat mutations also affect other structural aspects of HIV-1 particle. For example, an immature and non-infectious particle is first made when Gag proteins are assembled along the plasma membrane. Following protease-mediated cleavage, the structure of the virus particle is reorganized and an infectious particle is formed[1]. Structural differences between these mature and immature virus particles can be distinguished by electron microscopy (EM) and EM also permits the study of how the absence of Tat protein may affect the transition of virus particles from immature to mature forms. Another example is that after virus

entry into target cells, the uncoating of the ribonucleoprotein complex is necessary for the viral reverse transcription machinery to gain access to cellular dNTP pools and other cellular factors[50]. It is possible that mutations in Tat may affect the stability of the core structure and thus jeopardize uncoating.

Suppression of reverse transcription by Tat protein

The increased levels of viral cDNA products found within Tat-mutated virus particles suggest that Tat may act as a suppressor of reverse transcription in the cytoplasm[61]. Furthermore, when Tat is mutated, higher levels of viral cDNA are also detected in infected cells[61]. In further support of this concept, a recombinant two-exon form of Tat was shown to strongly inhibit reverse transcription in cell-free assays performed with synthetic viral RNA templates, $tRNA^{Lys.3}$ primer and recombinant HIV-1 RT (p66/51)[61,69]. Figure 4 illustrates this suppressor activity of Tat in reverse transcription in the context of the HIV-1 life cycle.

Reverse transcription from the HIV-1 RNA:$tRNA^{Lys.3}$ complex involves distinct initiation and elongation stages. The initiation stage is associated with strong pausing events after the extension of 1, 3, or 5 nucleotides[70-76]. This stage is distinct from the subsequent elongation stage as measured by distinct RT binding and kinetic values. In the presence of recombinant Tat protein, the elongation, but not the initiation stage of reverse transcription is suppressed[69]. Consistent with this, the majority of the $tRNA^{Lys.3}$ primer that is annealed onto the viral RNA template within wild-type virions is extended by two nucleotides in the case of viruses that encode wild-type Tat protein[68,77].

The finding that Tat acts as a reverse transcription suppressor also helps to explain the deficient reverse transcription of Tat-mutant viruses in target cells. In producer cells, wild-type Tat may limit RT activity associated with either *Gag-Pol* or free RT and thus ensures the integrity of viral RNA for incorporation into virus particles. In contrast, an absence of Tat in producer cells may cause aberrant viral RNA to be present within virions; this defect may not be reparable by Tat proteins that are newly expressed in target cells.

The biological importance of the suppressor activity of Tat in reverse transcription awaits further verification and several issues need to be clarified in this regard. First, Tat is a nuclear protein, and, although it shuttles between the nucleus and cytoplasm, its cytoplasmic concentration has never been determined. Therefore, it is unclear whether the amount of Tat available within the cytoplasm is sufficient for inhibition of RT activity. A second issue relates to HIV-1 assembly and the cleavage of *Gag* and *Gag-Pol* precursors by protease during or shortly after budding. Although the levels of RT activity within infected cells are low, it needs to be determined to what extent cytoplasm RT activity at post-integrational stages of the viral life cycle is deleterious to viral replication. This suppressor role of Tat has been mapped to the second Tat exon, the cysteine-rich and the core regions in studies per-

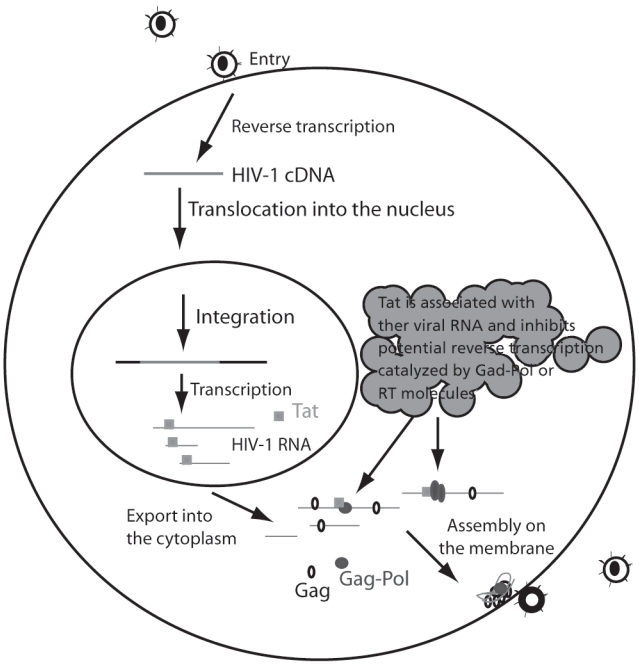

Figure 4. *Suppressor activity of Tat at the post-integration stage of the HIV-1 life cycle. The HIV-1 life cycle includes a number of essential steps, these are: entry into host cells via interactions with receptor (CD4) and co-receptor (CXCR4, CCR5) molecules on the surface of host cells; reverse transcription; translocation of viral cDNA into the nucleus; integration; transcription of viral RNA molecules; export of viral RNA molecules into the cytoplasm; translation; assembly and budding. The Gag-Pol and free reverse transcriptase (RT) molecules may reverse transcribe viral RNA within the cytoplasm. This, in turn, results in degradation of viral RNA. Tat is proposed to inhibit these reactions that are deleterious to virus assembly.*

formed with synthetic Tat molecules[69]. In contrast, the amino-terminal sequence, which is largely dispensable for *trans*-activation of gene expression, was required for Tat to execute its positive activity in reverse transcription within cells newly infected by HIV-1[78]. This apparent discrepancy suggests that Tat may regulate reverse transcription through more than one mechanism.

Alternative mechanisms for the regulatory activity of Tat in reverse transcription

Tat is an RNA-binding protein and also interacts with a number of cellular proteins[3]. It is unknown whether these inter-molecular interactions play roles in reverse transcription. Several studies have shown that the TAR binding activity of Tat is not required in order for Tat to exert its effects on reverse transcription. For instance, mutated Tat that lacks basic residues and TAR-binding ability can still efficiently support reverse transcription[78]; and mutant HIV-1 RNA that contains a TAR without the bulge sequence can be reverse transcribed at wild-type levels[79]. Although evidence is lacking regarding the roles of the Tat-Cyc T1-CDK9 complex in reverse transcription, studies have shown that over-expression of CDK9 did not augment reverse transcription from Tat-mutated viruses[78]. Together, these studies indicate that the direct involvement of this TAR-Tat-Cyc T1-CDK9 complex in reverse transcription is unlikely.

It was recently observed that Tat may play a role in the construction of the 5' cap structure of HIV-1 RNA[80]. Like most eukaryotic mRNA that is synthesized by RNAP II, HIV-1 RNA is also modified by a 5'-terminal m^7GpppG cap. This reaction is usually catalyzed by three enzymes, i.e. RNA triphosphatase, RNA guanylyltransferase, and RNA (guanine-7)-methyltransferase[81]. Capping occurs when nascent RNA is transcribed and released from RNAP II[82-84]. Therefore, transcription and capping are two temporally linked events. The capping reaction may be regulated by physical interactions between capping enzymes and the CTD of RNAP II[85-91]. The mammalian capping enzyme, Mce 1, binds to the phosphorylated but not the non-phosphorylated CTD of RNAP II, and this binding event stimulates the guanylytransferase activity of Mce I[85,87-89]. It is known that Tat can recruit CDK9 to RNA P II and that CDK9 can then catalyze hyperphosphorylation of CTD[3,16,17]. As a result, Tat may, at least indirectly, stimulate Mce I activity. In addition, it has been reported that Tat can directly interact with Mce I and thus promote the capping of HIV-1 RNA[80]. Furthermore, the TAR structure that is present at the 5' end of viral RNA can substantially ablate the capping efficiency of HIV-1 RNA by Mce I. In light of these findings, it is possible that the 5' capping of HIV-1 RNA may occur at low efficiency in the absence of Tat protein. These uncapped RNA molecules are not only unstable but may also be poorly reverse transcribed upon infection of target cells.

Although the roles of the 5' cap structure in reverse transcription of viral RNA remain to be

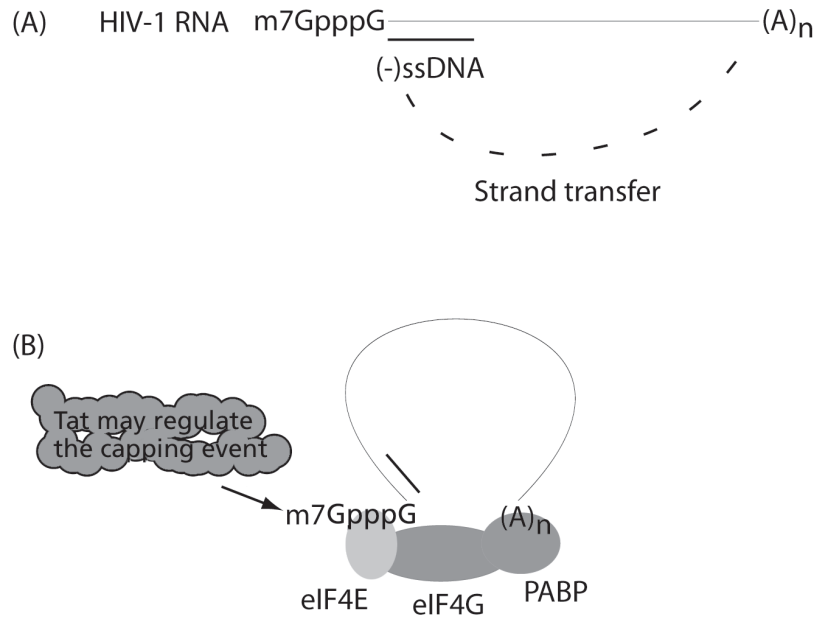

Figure 5. *Physical bridging of the 5' and 3' ends of HIV-1 RNA may facilitate the first strand transfer during reverse transcription. (A) HIV-1 RNA is modified at the 5' and 3' ends by addition of a cap structure (m7GpppG) and a poly(A) tail. When synthesis of (–)DNA reaches the 5' end of HIV-1 RNA, a strand transfer is needed in order for reverse transcription to proceed. (B) Translation initiation factors eIF4E and eIF4G, together with the poly(A) binding protein (PABP), bridge the 5' and 3' ends of HIV-1 RNA together; this event facilitates the transfer of (–)ssDNA from the 5' end to the 3' end of viral RNA. eIF4E binds to the 5' cap, PABP binds to the ploy(A) tail, and eIF4G interacts with eIF4E and PABP.*

determined, both the 5' cap and 3' poly(A) tail significantly increased the efficiency of the first strand transfer during reverse transcription in studies performed with synthetic MLV (murine leukemia virus) RNA[92]. Both of these modifications in HIV-1 RNA may be required for efficient RT reactions.

In support of this notion, the 5' cap and 3' poly(A) structures can stimulate the translation of mRNA through the recycling of the small ribosomal subunit[93-95]. This is achieved by bridging the 5' and 3' ends of mRNA via interactions between three proteins: eIF4E (eukaryotic initiation factor 4E), eIF4G and the PBP (poly(A) binding protein) (Fig. 5). eIF4E specifically binds to the 5' cap structure, PBP binds to the 3' poly(A) tail, and eIF4G binds to both eIF4E and PBP[96]. These RNA-protein interactions can physically bring the 5' and 3' ends of mRNA into close proximity, and, as a consequence, this mRNA can form a ring structure. Conceivably, close contact between these two ends may greatly enhance the efficiency of the first strand transfer during reverse transcription. The absence of Tat protein may lead to diminished levels of HIV-1 RNA that possess the 5' cap. As a consequence, the formation of mRNA ring may be jeopardized and reverse transcription adversely affected.

Therapeutic considerations

As a key protein in HIV-1 replication, Tat has long been a target for development of anti-viral compounds. Efforts have been made mainly on the basis of the *trans*-activation activity of Tat in gene transcription and, in particular, at disruption of Tat-TAR interactions[97,98]. For instance, short TAR RNA decoy molecules have been developed that can compete with wild-type TAR for binding to Tat[99-101]. Three small molecule TAR ligands were identified that specifically disrupt binding of Tat to TAR RNA[102]. Screening for anti-Tat compounds has primarily been based on two types of assays, including the use of Tat-dependent reporter genes in primate cells and cell-free Tat-TAR interactions. A series of compounds have been selected, e.g. the benzodiazepines Ro 5-3335 and Ro 24-7429[103], ALX-40C[104], carbocyclic adenosine analogs[105], nordihydroguaiaretic acids[106] and the bisbenzimidazole derivative Hoechst 33258[107]. Yet, only ALX-40C has reached clinical trials, and, paradoxically, ALX-40C has now been shown to inhibit HIV-1 replication by disrupting interactions between gp120 and its receptors rather than by compromising Tat transactivation activity. The limited output of these screens may be due to the fact that the strategies employed aimed only to antagonize Tat transactivation activity. Now, the role of Tat in reverse transcription provides a novel concept that may lead to screening of a much broader range of compounds for anti-viral activity.

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