Suppression of an intrinsic strand transfer activity of HIV-1 Tat protein by its second-exon sequences

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Abstract

The Tat protein of human immunodeficiency virus type 1 (HIV-1) has been shown to restrict premature reverse transcription at late stages of virus infection and to thus ensure the integrity of the viral RNA genome for packaging. To gain further insights into the roles of Tat in HIV-1 reverse transcription, we have assessed its effects on the first-strand transfer during the synthesis of minus-strand DNA through use of a reconstituted cell-free system. The results demonstrated that a form of Tat, containing only the first exon (Tat72), was able to enhance the first-strand transfer as efficiently as did the viral nucleocapsid protein. Coincidentally, this form of Tat was unable to inhibit the production of minus-strand strong-stop DNA. Further studies with various mutated forms of Tat showed that its Cys-rich region, rather than the core and Arg-rich domains, was essential for this strand transfer activity. Moreover, this activity of Tat is largely independent of the TAR RNA structure. Although full-length Tat protein (Tat86) was also able to promote strand transfer, this activity was limited by a strong overall inhibition of reverse transcription because of the presence of the second Tat exon. Other nucleic-acid-binding proteins (e.g., single-strand DNA-binding protein) were employed as negative controls and were unable to promote strand transfer in these assays. We propose that Tat possesses nucleic acid chaperone activity and can promote the first-strand transfer during HIV-1 reverse transcription; however, these activities are restricted by the second Tat exon, and the roles of these Tat activities in viral replication remain to be elucidated.
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Introduction

The reverse transcription of human immunodeficiency virus type 1 (HIV-1) genomic RNA involves four critical steps (Telesnitsky and Goff, 1997). The first involves placement of a cellular tRNA[Lys.3] onto a viral RNA region of 18 nucleotides (nt), termed the primer-binding site (PBS). When synthesis of minus-strand DNA reaches the 5′ end of the genome, a first-strand transfer occurs that is mediated by the 5′R and 3′R regions as well as by trans-acting factors such as the viral nucleocapsid (NC) protein. Next is the generation of a polypurine tract of RNA that is needed to prime the synthesis of plus-strand DNA. Finally, a second-strand transfer is required to complete the synthesis of viral cDNA, and this is mediated by the PBS sequence.

In addition to the reverse transcriptase (RT) enzyme, a number of viral and cellular proteins have been shown to participate in reverse transcription, among which NC has been the most extensively investigated (Darlix et al., 1995). HIV-1 NC is a small basic protein and contains two CCHC zinc finger motifs. Because largely of its nucleic acid chaperone activity (Rein et al., 1998; Tsuchihashi and Brown, 1994), NC is able to promote the annealing of the tRNA[Lys.3] primer onto the PBS, to increase the processivity of RT, and

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to assist with the first- and second-strand transfers (Darlix et al., 1995). Aside from NC, viral proteins such as Nef (Aiken and Trono, 1995; Schwartz et al., 1995), Vif (Von Schwedler et al., 1993), Vpr (Heinzinger et al., 1994), and matrix (Bukrinsky et al., 1993; Von Schwedler et al., 1994), as well as several cellular proteins including cyclophilin A (Franke et al., 1994; Thali et al., 1994), DNA topoisomerase I (Priel et al., 1990), and ERK2 (Jacque et al., 1998), have also been reported to modulate reverse transcription through distinct mechanisms.

In recent years, the Tat protein has also been reported to play key roles in HIV-1 reverse transcription. Tat was first recognized as a transactivator of HIV-1 gene transcription. It recruits the cellular factors cyclin-dependent kinase 9 (CDK9) and cyclin T1 (CynT1) to the transcription elongation complex and enables CDK9 to hyperphosphorylate the carboxyl terminus domain of RNA polymerase II (Wei et al., 1998). It was recently shown that mutations either in Tat or in the Tat transactivation-responsive element (TAR) were able to ablate the generation of viral cDNA during infection (Harrich et al., 1997, 2000; Ulich et al., 1999). Although it is unknown whether the roles of Tat and TAR in reverse transcription are related, these studies suggest a novel activity of Tat in generation of viral cDNA. Our lab has developed a reconstituted cell-free assay system to directly assess the activities of Tat in reverse transcription and showed that the wild-type two-exon form of the Tat protein could significantly diminish the generation of minus-strand strong-stop DNA ((−)ssDNA) (Kameoka et al., 2001). At the same time, we have shown that Tat possesses RNA chaperone activity and can mimic the role of NC in the placement of the primer of HIV reverse transcription, that is, tRNA^{lys3}, onto the viral PBS; importantly, different regions of Tat seem to be responsible for these different activities (Kameoka et al., 2002).

To better understand the roles of Tat in reverse transcription, we have now studied the effects of Tat on the first-strand transfer during the synthesis of minus-strand DNA. To pursue this subject, a viral RNA template was generated that contained the R regions at both the 5′ and the 3′ ends; hence, this RNA template can support strand transfer in both an intra- and an intermolecular fashion. Since both intra- and intermolecule transfers occur with similar frequency during reverse transcription within infected cells (Hong et al., 1998; Jones et al., 1994; Van Wamel and Berkhout, 1998), experiments performed with this RNA template may approximate the in vivo situation. Our results show that a truncated form of Tat, containing only the first exon, that is, one-exon Tat (Tat72), was able to promote the first-strand transfer event in reverse transcription as efficiently as the NC protein. However, this activity was severely limited if the second-exon of Tat was also present in the context of full-length Tat protein (Tat86).

Results

Stimulation of the first-strand transfer by HIV-1 Tat72 protein

HIV-1 genomic RNA possesses two identical R regions at its 5′ and 3′ ends that mediate both the intra- and the intermolecule strand transfer events during reverse transcription. The cell-free systems that have been developed to study strand transfer usually contain two RNA templates that serve as donors and acceptors and thus permit only the evaluation of interstrand transfer. To better mimic the in vivo situation, we have constructed an RNA template, termed Tp-2, that includes both the 5′R and 3′R regions as well as each of the U5 region, the PBS, part of the first-exon leader sequence, and part of the U3 region (Fig. 1A). This Tp-2 RNA template allows us to monitor both intra- and interstrand transfers in a single reaction. Moreover, reverse transcription performed with this Tp-2 RNA template yielded higher levels of strand transfer products than that performed with two other donor and acceptor RNA templates (data not shown).

Reverse transcription was first initiated from an 18-nt DNA oligonucleotide that was placed onto the PBS by heat annealing. The Tp-1 RNA template contains the 5′ R, U5, and PBS and thus only supports the synthesis of (−)ssDNA (Fig. 1A). When reverse transcription was performed with this RNA template, self-primed cDNA products were seen when high levels of RT, that is, 200 ng, were employed (Fig. 1B). In the presence of the Tp-2 RNA template, cDNA products of 296 nt were observed at the top of the gels when RT was used at amounts ≥200 ng, irrespective of the presence of the self-primed cDNA products (Fig. 1B); this indicates that strand transfer had occurred. The yield of strand transfer products did not change with the increase of RT levels from 200 to 400 ng.

To verify that the DNA band of 296 nt represented a strand transfer product, reverse transcription was performed with an RNase H-negative form of HIV-1 RT termed E478Q (Gotte et al., 1995). Since RNase H degradation of the RNA template is essential for successful strand transfer to occur, no strand transfer should take place with use of the E478Q enzyme. Indeed, only (−)ssDNA was synthesized by E478Q from the Tp-2 RNA template (Fig. 1C). Even under conditions in which exogenous NC protein was added to the reactions, no cDNA products longer than (−)ssDNA were seen on the gels. However, when the RNase H enzyme of *Escherichia coli* was provided in trans, reverse transcription proceeded beyond the stage of (−)ssDNA synthesis and led to the generation of cDNA products longer than (−)ssDNA (Fig. 1C). Thus, it is further validated that Tp-2 RNA template was able to support strand transfer.

As stated above, NC has been shown to promote strand transfer in reverse transcription (Allain et al., 1994; Brulé et al., 2000; Darlix et al., 1993; Davis et al., 1998; DeStefano, 1995; Guo et al., 1997, 2000; Kim et al., 1997; Peliska et al.,
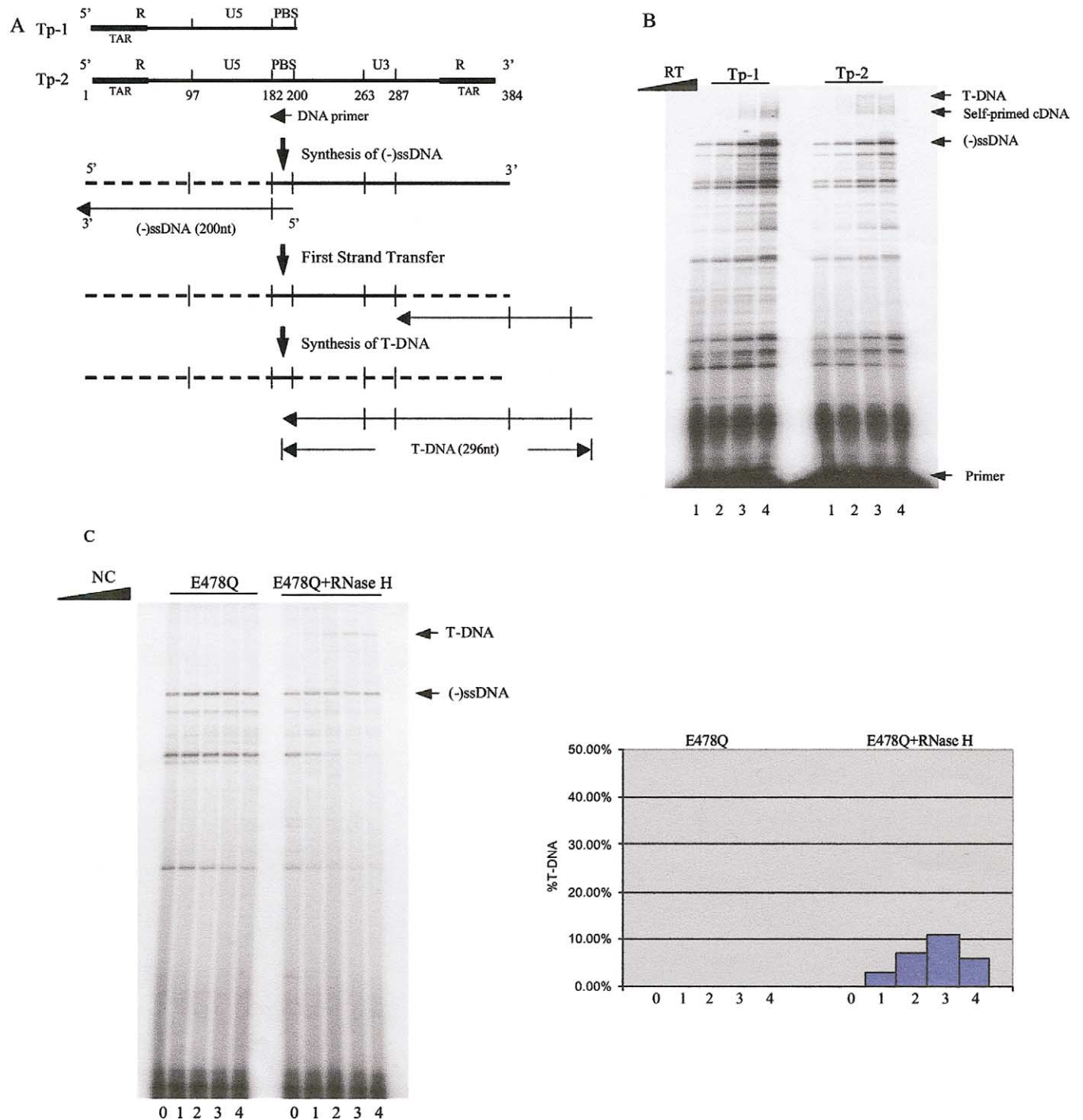
Fig. 1. (A) Depiction of the first-strand transfer during synthesis of minus-strand DNA. The Tp-2 RNA template contains the 5′R and 3′R regions and is thus able to support strand transfer in both an intra- and an intermolecular fashion. In addition, this RNA template possesses intact U5 and PBS sequences, a portion of the noncoding leader region of exon 1 (nt 200 to 263), as well as 24 nt of the U3 sequence. The dashed lines represent RNA fragments that result from digestion by RT-associated RNase H activity. (B) Synthesis of (−)ssDNA and strand transfer DNA (T-DNA) in the presence of various concentrations of HIV-1 RT. Reverse transcription was primed with DNA oligonucleotides that were annealed onto the PBS. Synthesis of (−)DNA was performed at 37°C for 30 min. The Tp-1 RNA template contains a portion of Tp-2 RNA spanning nt 1 to 262 and thus only supports the generation of (−)ssDNA. Synthesis of T-DNA was generated through use of Tp-2 RNA. Self-priming events occurred in reactions performed with both RNA templates. Lanes 1–4, experiments performed with 50, 100, 200, and 400 ng of HIV-1 RT. (C) Reverse transcription performed with the Tp-2 RNA template and RNase H-negative HIV-1 RT E478Q (Gotte et al., 1995). Various amounts of NC protein (i.e., 15, 30, 60, and 90 pmol) were added to stimulate the strand transfer. The RNase H enzyme (0.2 unit per reaction) from *E. coli* was provided *in trans* to compensate for the lost RNase H activity of E478Q. The lengths of the (−)ssDNA and T-DNA products were determined by running DNA sequencing samples in parallel (not shown).
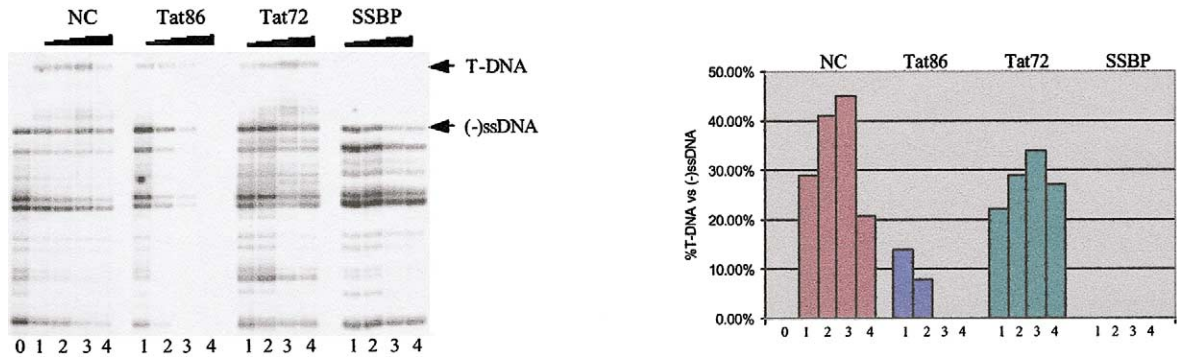
Fig. 2. Effects of NC, Tat86, Tat72, and SSBP on the first-strand transfer. Reverse transcription was performed with DNA primer, 50 ng of HIV-1 RT, and various concentrations of the aforementioned proteins at 37°C for 30 min. Strand transfer efficiency in each reaction was determined by calculating the percentage of T-DNA generated relative to total levels of both (−)ssDNA and T-DNA. Lane 0, reaction performed with 50 ng of RT alone as a control. Lanes 1–4, experiments conducted with 15, 30, 60, and 90 pmol of NC, Tat86, Tat72, or SSBP. The data of strand transfer were plotted. The gels shown are from one representative experiment of three experiments that were performed.

1994; You and McHenry, 1994). This concept has now been further tested in experiments performed with our in vitro strand transfer system. The results of Fig. 2 show that strand transfer products were not seen in the presence of 50 ng of RT and absence of exogenous NC or Tat (lane 0). However, the addition of NC resulted in approximately 20% to 40% of the (−)ssDNA being converted to strand transfer products (Fig. 2). To test the specificity of this role of NC, single-strand DNA-binding protein (SSBP) from E. coli was used in the place of NC in these reactions. However, no strand transfer products were seen when even 90 pmol of SSBP was used (Fig. 2). Similarly, negative results were obtained using human Staufen protein, a well-characterized RNA-binding protein (Mouland et al., 2000), as an additional control (data not shown).

Next, we measured the effects of several different forms of the Tat protein on strand transfer. Strand transfer products were generated when the one-exon form of Tat, that is,

Tat72, was included in the reactions (Fig. 2). Moreover, it appears that Tat72 was able to stimulate the yield of strand transfer products to levels comparable to those achieved by NC protein on a molar basis.

We have previously shown that the two-exon form of the Tat protein (Tat86), equivalent to wild-type full-length Tat, was inhibitory to reverse transcription (Kameoka et al., 2001). Consistent with this finding, the results of Fig. 2 show that Tat86 markedly suppressed reverse transcription even at the low concentration of 30 pmol, a condition under which NC and Tat72 did not exert significant inhibitory effects. We then calculated the relative levels of the strand transfer products versus those of (−)ssDNA; the results show that Tat86 promoted strand transfer at levels similar to Tat72 when protein concentrations of 15 pmol were used (Fig. 2). Therefore, both Tat72 and Tat86 are able to stimulate strand transfer under appropriate conditions, but the abil-
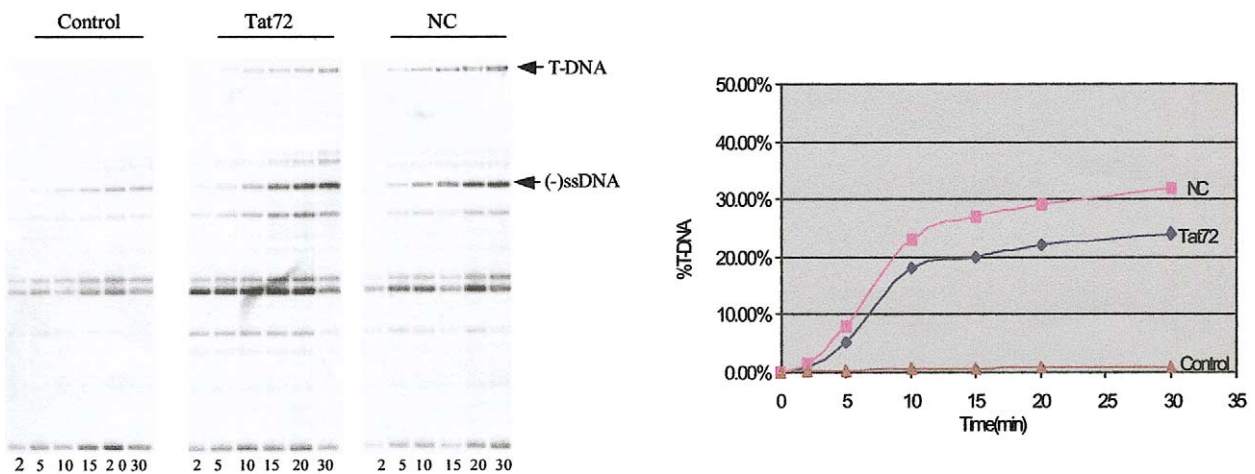

Fig. 3. Time-course experiments performed with the Tp-2 RNA template in the presence of either 30 pmol of NC or 30 pmol of Tat72. Reactions were conducted with 50 ng of HIV-1 RT at 37°C for various times. Control, reactions that were performed without addition of NC or Tat. Time points are labeled at the bottom of each gel panel. The percentage of T-DNA in each reaction was calculated and plotted as described in the legend to Fig. 2.

ity of Tat86 to play this role is limited because of its inhibitory effects on reverse transcription.

Both NC and Tat72 promote strand transfer with similar kinetics

Next, we compared the abilities of each of NC and Tat72 in time-course experiments to stimulate stand transfer. As mentioned, virtually no strand transfer occurred when RT was present alone at a concentration of 50 ng (Fig. 3). In contrast, strand transfer products were observed 10 min after the addition of NC or Tat72 to the reactions (Fig. 3). The efficiency of strand transfer in each case was determined by calculating the percentage of strand transfer products that were generated (T-DNA) relative to total levels of both ($-$)ssDNA and T-DNA. The data were then plotted and reveal that both NC and Tat72 promoted strand transfer with similar kinetics (Fig. 3).

Tat72 suppresses self-priming from ($-$)ssDNA

When full-length ($-$)ssDNA was generated in the above reactions, its nascent 3′ end was able to reinitiate DNA synthesis because of the presence of a TAR-like cDNA structure. This event is termed self-priming, and the resultant cDNA product is called self-primed cDNA (Guo et al., 1997). Self-priming has been shown to interfere with the first-strand transfer. The NC protein can both prevent self-priming and increase the efficiency of strand transfer (Guo et al., 1997). In view of the fact that the Tat72 protein can also promote strand transfer, we wanted to assess whether this might be due in part, at least, to a suppression of self-priming. This issue was first approached through use of the Tp-1 RNA template that supports only the generation of ($-$)ssDNA. The results show that self-primed cDNA products were seen in reactions performed with 200 ng of HIV-1 RT in the absence of exogenous NC or Tat proteins (Fig. 4, lane 0). The additional presence of NC protein, even at low quantities (e.g., 15 pmol), resulted in a sharp decrease in the amounts of self-primed cDNA products that were generated (Fig. 4). Tat72 was also able to suppress self-priming but at higher concentrations in comparison with NC.

These findings were also validated in experiments performed with the Tp-2 RNA template that permits strand transfer to occur. Again, a dramatic decrease in levels of self-primed cDNA products was seen in studies performed with NC, alongside increased levels of strand transfer products (Fig. 4). The addition of Tat72 also suppressed the self-priming reactions and stimulated strand transfer.

An intact TAR structure is not needed for Tat-mediated strand transfer

It is known that Tat needs to bind to TAR RNA to exert its transactivation effects on viral gene transcription. We wanted to know whether this RNA-binding event was also
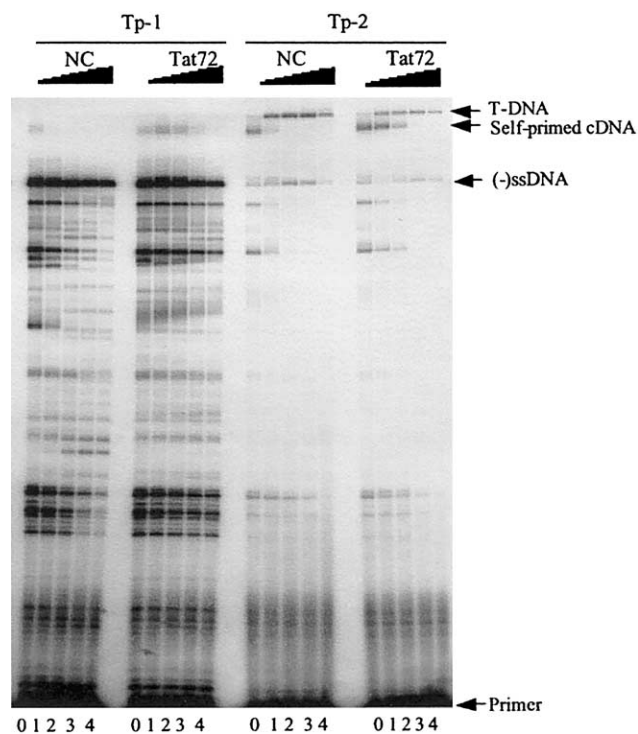

Fig. 4. Inhibition of self-priming from ($-$)ssDNA by either NC or Tat72 protein. Reverse transcription was performed with 200 ng of HIV-1 RT at 37°C for 60 min with either the Tp-1 or the Tp-2 RNA template. Lane 0, a control reaction performed with HIV-1 RT alone. Four protein concentrations of either NC or Tat72, that is, 15, 30, 60, and 90 pmol (lanes 1–4), were studied.

required for the positive role of Tat in strand transfer. Accordingly, we generated a construct termed Tp-3(dU) that contains a point mutation that changes the TAR bulge sequence 5′-UCU-3′ (nt 22–24) to 5′-CCU-3′ (Fig. 5A); this mutation has been shown to reduce the affinity of Tat for TAR (Weeks et al., 1990). The results of our reverse transcription experiments show that both NC and Tat72 were able to promote strand transfer from this mutated RNA template at levels comparable to those achieved with the wild-type RNA template (Fig. 5B).

To further assess the possible involvement of TAR in this strand transfer activity, a 13-nt segment within TAR (nt 16–28) was removed to generate the mutated RNA template Tp-3(16–28). The results of Fig. 5B show that both NC and Tat72 were able to effectively stimulate strand transfer from this mutant RNA template, although Tat72 was moderately less efficient than NC in this regard. Therefore, the activity of both NC and Tat72 in stimulation of strand transfer is largely TAR-independent.

The Cys-rich domain of Tat72 is essential for its activity in strand transfer

Tat contains a highly basic Arg-rich motif (ARM) that is essential for its binding to TAR. In addition, Tat includes a

Cys-rich region and a core domain that are both needed for transactivation of the HIV-1 LTR promoter (Gatignol and Jeang, 2000). To determine which of these regions of Tat might be involved in strand transfer, we studied a variety of mutated forms of Tat in which each of these three regions was selectively deleted. The mutant Tat proteins thus produced were termed ΔARM, ΔCore, and ΔCys-rich (Fig. 6A). The results of Fig. 6B show that neither the ΔCore nor the ΔCys-rich versions of Tat were able to inhibit reverse transcription at concentrations as high as 60 pmol, similar to results obtained with Tat72. In contrast, the ΔARM form of Tat modestly inhibited reverse transcription at 60 pmol (Fig. 6B). When the efficiency of the first-strand transfer was measured, the ΔCore and ΔARM proteins displayed the same stimulatory effect as Tat72 did (Fig. 6B); in contrast, the ΔCys-rich Tat had little or no effect (Fig. 6B). Therefore, the Cys-rich region of Tat is essential for this strand transfer activity and the ARM and Core regions play minor roles in this regard.

Discussion

This study shows that the Tat protein is able to promote the first-strand transfer during reverse transcription. This activity was more clearly manifested with the one-exon form of Tat (Tat72) than with the two-exon form (Tat86), because the latter can simultaneously exert inhibitory effects on reverse transcription (Kameoka et al., 2001).

It has long been recognized that Tat is a strong transactivator of viral gene transcription. The Tat protein consists of special structural elements that include a basic domain, a core region, and a Cys-rich motif. The basic domain is required for the specific binding of Tat to TAR RNA, while the other two regions form part of the transactivation motif and interact with cellular factors including CDK9 and CynT1 (Wei et al., 1998; Gatignol and Jeang, 2000). Conceivably, however, these peptide motifs may also allow Tat to perform other functions than gene transcription, for example, its ability to stimulate strand transfer in reverse transcription in a manner similar to the role played by the NC protein. This functional homology between Tat and NC may be due to structural similarities between the two proteins, including the presence of Cys-rich and basic domains. However, the ability of Tat to stimulate reverse transcription might also represent a disadvantage for the virus, since any extensive premature synthesis of cDNA within cells before virus assembly could lead to degradation of the viral RNA genome by the RT-associated RNase H activity and thereby hinder viral RNA packaging. The ability of full-length Tat, that is, Tat 86, to inhibit reverse transcription (Kameoka et al., 2001) suggests that the virus may have solved this problem by including an additional amino acid sequence at its carboxyl terminus, that is, the second-exon, that helps to restrict the strand transfer activity. Indeed, the second Tat exon is nonessential for Tat to transactivate viral gene expression (Jeang et al., 2001; Jones et al., 1994). The role of this exon in restriction of reverse transcription is apparently related to its ability to inhibit strand transfer.

In general, strand transfer is mediated by annealing between homologous nucleic acid sequences. NC possesses nucleic acid charperone activity (Rein et al., 1998) and is able to stimulate strand transfer by promoting denaturation and subsequent binding between complementary stretches of nucleic acid. In addition, NC contributes to strand transfer by positive regulation of the RNase H activity of RT (Allain et al., 1994; Peliska et al., 1994; You and McHenry, 1994; Cameron et al., 1997), stabilization of RNA–DNA heteroduplexes (Driscoll et al., 2001), and inhibition of self-priming from (−)ssDNA (Guo et al., 1997; Jeang et al., 1999; Driscoll and Hughes, 2000; Lapadat-Tapolsky et al., 1997; Li et al., 1996). Tat may be able to promote strand transfer through similar mechanisms. Our results show that Tat was also able to inhibit self-priming (Fig. 4) and that the Cys-rich region as well as the basic domain of Tat were required for the strand transfer activity (Fig. 6). In addition, Tat can promote annealing between complementary nucleotide sequences (unpublished data).

Although many proteins have nucleic-acid-binding properties, and some can even promote RNA or DNA annealing, very few have been shown to function in each of primer tRNA[Lys.3] annealing, RNA dimerization, and strand transfers. Neither viral RTs nor cellular proteins such as E. coli RecA and human p53 proteins were able to promote HIV-1 RNA dimerization and primer tRNA annealing (Barat et al., 1989; De Rocquigny et al., 1992; Lapadat-Tapolsky et al., 1995). Similarly, these experiments show that a SSBP was not able to assist in strand transfer (Fig. 3) (Kim et al., 1997). The specificity and efficacy of the HIV-1 NC protein in these reactions have been attributed to both basic amino acid residues as well as to zinc finger motifs that have also been shown to be essential for the first-strand transfer (Guo et al., 2000). Prions are the only cellular proteins previously identified that can perform several of the activities characteristic of NC (Gabus et al., 2001a, 2001b), and the biologic relevance of prions in viral replication is uncertain, although it is possible that prions may become incorporated into virions and thereby complement NC in its various roles.

Our data demonstrate that the HIV-1 Tat protein is also able to stimulate the first-strand transfer during synthesis of minus-strand DNA. This event involves the unwinding of two complex and stable nucleic acid structures that are formed by the TAR RNA sequence and the cDNA sequence complementary to TAR. The difficulty of transforming these nucleic acid structures apparently requires both basic amino acid residues and the zinc finger motifs of NC for the first-strand transfer, in contrast to the nonrequirement for the zinc fingers in the annealing of PBS sequences during the second-strand transfer (Guo et al., 2000). The ability of Tat to promote the first-strand transfer suggests that Tat possesses nucleic acid chaperone properties similar to those of NC, and this feature of Tat may play a role in transacti-
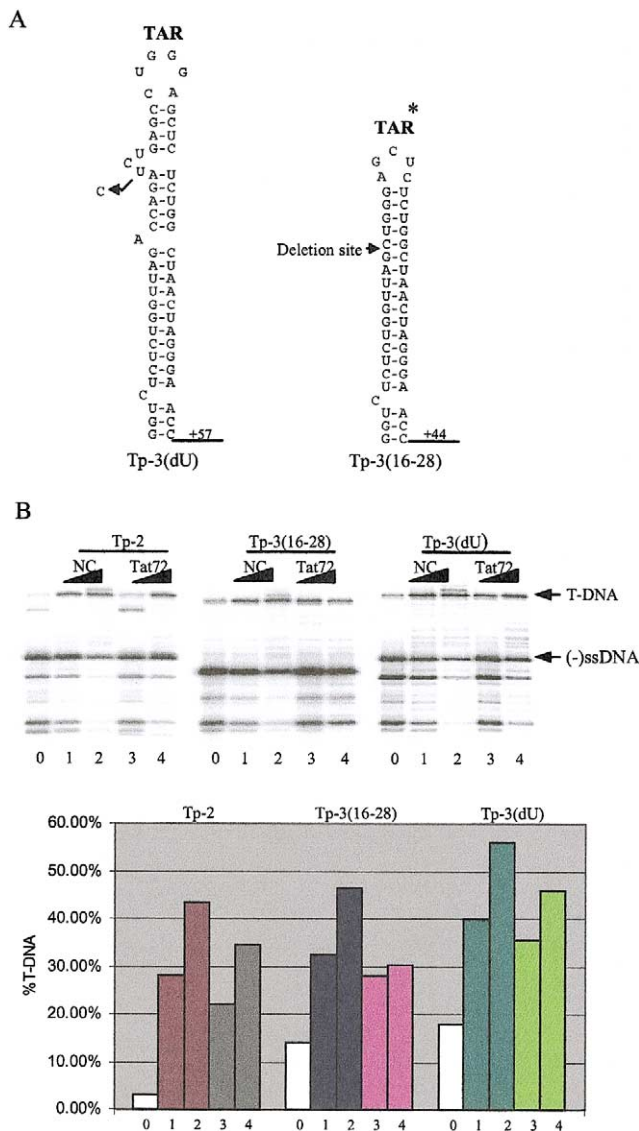
Fig. 5. (A) Illustration of mutations within the TAR region. The RNA template Tp-3(dU) contains a point mutation that changes the U22 nucleotide to a C. The mutant RNA template Tp-3(9–12) is truncated and lacks a 13-nt RNA fragment, that is, nt 16 to 28. Secondary structures of the mutant TAR were predicted on the basis of the M-Fold program (Jaeger et al., 1994; Zuker, 1989). Both the 5′ and 3′ TAR were mutated accordingly. (B) Reverse transcription was performed with 50 ng of HIV-1 RT at 37°C for 30 min. NC or Tat72 protein was used in these reactions at concentrations of either 15 or 60 pmol. The strand transfer efficiency in each reaction was determined by calculation of the percentage of T-DNA relative to the sum of both (−)ssDNA and T-DNA, and the results are plotted in the graph. The results shown are from one representative experiment of three that were performed.
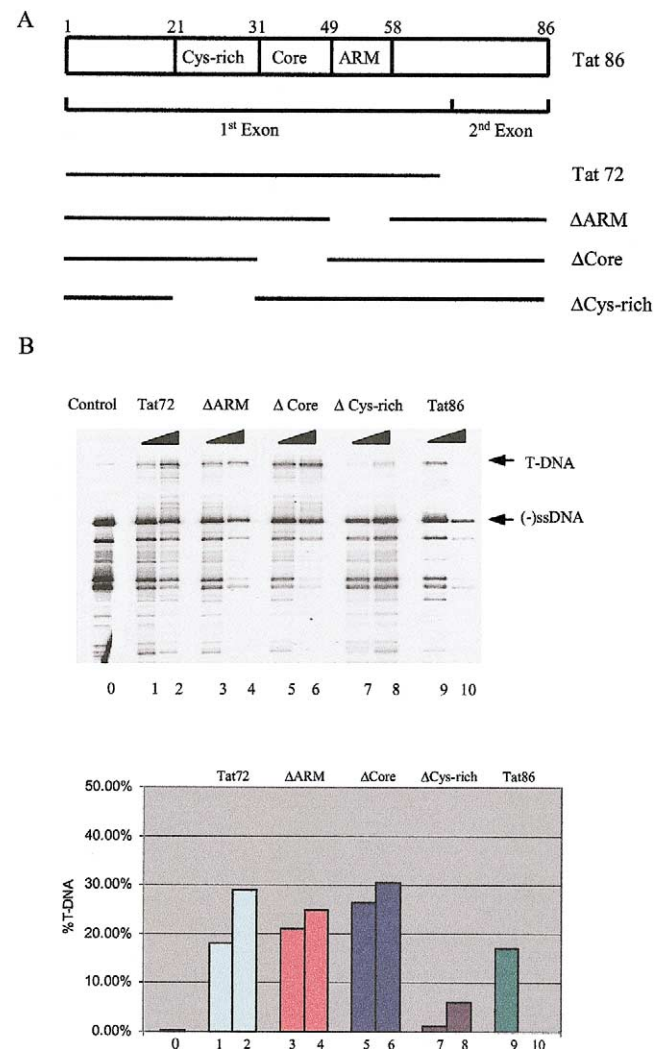

Fig. 6. Mutant Tat proteins and their effects on the first-strand transfer. (A) Illustration of Tat mutations. Tat72 contains the first exon; the ΔARM, ΔCore, and ΔCys-rich mutants lack the amino acids that span positions 50 to 58, 40 to 47, and 21 to 39, respectively. (B) Reverse transcription was performed with 50 ng of HIV-1 RT at 37°C for 30 min. Either 15 or 60 pmol of each type of Tat protein was used in the reactions. Lane 0, a reaction performed with HIV-1 RT alone. The percentage of T-DNA relative to (−)ssDNA and T-DNA was calculated and plotted. The results shown are from one representative experiment of three that were performed.

ent discrepancy in regard to the Tat protein simultaneously possessing the ability to both positively and negatively regulate RT activity. As stated, intracellular localization of various forms of Tat may be an important consideration in determining temporal relationships between Tat expression and the role of Tat in regulation of reverse transcription.

It has been widely observed that Tat may not be incorporated into progeny virions responsible for a new round of infection. We have suggested that this exclusion may be necessary to prevent Tat from being able to play a negative regulatory role in regard to RT during a subsequent replication event. At the same time, the negative role of Tat in regard to RT helps to explain why multiple rounds of

vation. For example, the binding of Tat to TAR, together with CynT1 and CDK9, may induce conformational rearrangements of TAR that further stabilize the RNA–protein complex.

The finding reported here that Tat72 can stimulate strand transfer in reverse transcription is consistent with earlier reports (Harrich et al., 1997) and helps to resolve an appar-

reverse transcription are not initiated within cells that have been chronically infected by HIV.

Materials and methods

Plasmid construction

HIV-1 nt sequences from the BH10 infectious cDNA clone were used in these studies. The vector pSP72 (Promega, Madison, WI) contains the T7 promoter and was employed to generate the constructs described below. Viral cDNA sequences at nt positions 454 to 920 were first amplified using primer pair P1/P2 (5′-GCA GGT CCT GGC TTA TCG AAA TTA ATA CGA CTC ACT ATA GGG TCT CTC TGG TTA GAC-3′/5′-ACG TGG TAC CAT TAA CTG CGA ATC GTT CTA GC-3′); the PCR products were digested with restriction enzymes *Hpa*I and *Kpn*I and then inserted into pSP72 to engineer construct Tp-1. Construct Tp-2 was generated by insertion of the viral cDNA fragment at nt 9516 to 9360 into construct Tp-1 between the restriction sites *Bss*HII and *Pst*I (Fig. 1A). A single nucleotide change from U22 to C22 within the TAR structure was generated by PCR through use of primer pair P2/P3 (5′-GCA CAC GCG CGC AGC AGC TGC TTT TTG CCT GTA CTG GGT CTC TCT GGT TAG ACC AGA CCT GAG CC-3′). The construct thus generated was termed Tp-3(dU) (Fig. 5A). Truncation of the nucleotide sequence from nt 16 to 28 within TAR was generated by PCR using primer pairs P2/P4 (5′-TGC AGG TTA ACC TGG CTT ATC GAA ATT AAT ACG ACT CAC TAT AGG TCT CTC TGG TTA GCT GGG AGC TCT CTG GC-3′) and P5/P6 (5′-CAC CTC AGG TAC CTT TAA GAC-3′/5′-GCT TTT TGC CTG TAC TGG GTC TCT CTG GTT AGC TGG GAG CTC TCT GGC-3′). The construct thus generated was termed Tp-3(16–28) (Fig. 5A).

Synthesis of RNA templates

RNA was transcribed using a Mega-scripts kit (Ambion, Austin, TX). An RNA template of 262 nt was synthesized from the Tp-1 construct that had been digested with restriction enzyme *Bss*HII. This RNA template contained intact R and U5 regions as well as the PBS and was used for the exclusive synthesis of (−)ssDNA. Longer RNA templates were transcribed from the Tp-2 (Fig. 1A), Tp-3(dU), or Tp-3(16–28) (Fig. 5A) DNA constructs that had been linearized by digestion with restriction enzyme *Pst*I. These RNA templates contain both the 5′R and the 3′R regions and therefore are competent to support strand transfer. The integrity of all RNA templates was verified on 5% denaturing polyacrylamide gels containing 7 M urea.

RT, Tat, and NC proteins

Recombinant HIV-1 RT (p66/51) was prepared as previously described (Wohrl et al., 1993). The wild-type form of the Tat protein (Tat86) as well as various mutated forms, including Tat72, ΔARM, ΔCore, and ΔCys-rich (Fig. 6A), were produced in *E. coli* M15 (pREP4) and purified using nickel–nitrilotriacetic acid resin (Qiagen, Mississauga, Ontario, Canada) as described (Kameoka et al., 2001). The NC protein used in this study contains 72 amino acids and was generated by stepwise solid-phase synthesis (De Rocquigny et al., 1991). The SSBP was purchased from Promega (St.-Laurent, Quebec, Canada).

Reverse transcription

An 18-nt DNA oligonucleotide (5′-GTCCCTGT-TCGGGCGCCA-3′), complementary to the PBS, was used as primer in the reactions described below. This DNA oligomer was labeled at the 5′ end with ^{32}P (ICN, Irvine, CA) and was annealed onto RNA template by heating at 85°C for 5 min and a further incubation at 55°C for 10 min (Liang et al., 1998). Reactions were performed at 37°C in a volume of 20 μl containing 1 pmol of RNA template, 1 pmol of primer, 0.2 mM each dNTPs, 100 ng of RT, 50 mM Tris–Cl (pH 7.5), 75 mM KCl, 5 mM Mg$_2$Cl, 10 mM DTT, and 10 units of ribonuclease inhibitor (Amersham Pharmacia Biotech., Baie d'Urfe, Quebec, Canada). Various concentrations of NC, Tat, or SSBP proteins were added into the reaction mixtures to test their effects on strand transfer. The reaction products were fractionated on 5% denaturing polyacrylamide gels containing 7 M urea and visualized by exposure to X-ray film (Kodak, Rochester, NY).

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