In Vitro Studies on tRNA Annealing and Reverse Transcription with Mutant HIV-1 RNA Templates*

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The human immunodeficiency virus type 1 (HIV-1) RNA genome encodes a semistable stem-loop structure, the U5-PBS hairpin, which occludes part of the tRNA primer binding site (PBS). In previous studies, we demonstrated that mutations that alter the stability of the U5-PBS hairpin inhibit virus replication. A reverse transcription defect was measured in assays with the virion-extracted RNA-tRNA complexes. We now extend these studies with in vitro synthesized wild-type and mutant RNA templates that were tested in primer annealing and reverse transcription assays. The effect of annealing temperature and the presence of the viral nucleocapsid protein on reverse transcription was analyzed for the templates with a stabilized or destabilized U5-PBS hairpin, and in reactions initiated by tRNA or DNA primers. The results of this in vitro assay are consistent with the in vivo findings, in that both tRNA annealing and initiation of reverse transcription are sensitive to stable template RNA structure. Reverse transcription initiated by a DNA primer is less hindered by secondary structure in the RNA template than tRNA primed reactions. The inhibitory effect of template structure on tRNA-primed reverse transcription is more pronounced in this in vitro assay compared with the in vivo material, indicating that the heat-annealed RNA-tRNA complex differs from the virion-extracted viral RNA-tRNA complex.

The replication cycle of the human immunodeficiency virus type 1 (HIV-1)1 and other retroviruses is characterized by reverse transcription of the viral RNA genome into a double-stranded DNA, which subsequently becomes integrated into the host cell genome (1). This process is mediated by the virion-associated enzyme reverse transcriptase (RT), and the cellular tRNA\textsubscript{3\Lys} molecule is used as a primer by HIV-1 RT (2). The tRNA primer binds with its 3'-terminal 18 nucleotides to a complementary sequence in the viral genome, the primer-binding site (PBS), which is located in the untranslated leader region of the viral genome (Fig. 1A). The leader region of HIV-1 is highly structured with distinct hairpin motifs (3–9). Besides secondary structure, the HIV-1 leader RNA was recently demonstrated to adopt a compactly folded higher order structure in vitro (10). The combined results of replication studies with mutant viruses and spontaneous revertants thereof, phylogenetic analyses, RNA structure probing, and computer-assisted RNA folding suggest that part of the PBS is occluded in a hairpin structure (4, 11–13). Four nucleotides of the PBS are involved in base pairing to fold a small upstream stem-loop structure, the U5-PBS hairpin (Fig. 1A). RNA structure is not only present in the viral RNA template but also in the tRNA\textsubscript{3\Lys} primer that is known to have a stable tertiary structure. Therefore, partial unfolding of both the tRNA primer and the viral RNA template is necessary for hybridization of these molecules and to initiate reverse transcription. Although the RT enzyme itself may be able to disrupt the secondary structure of the viral RNA and the tRNA primer (14), the viral nucleocapsid (NC) protein has been proposed to be specifically involved in this process (reviewed in Ref. 15). The NC protein binds preferentially to single-stranded nucleic acids and unwinds tRNA in vitro (16–18), thereby stimulating the annealing of the tRNA primer onto the template and the synthesis of minus-strand DNA (19, 20).

In a previous study, we reported the importance of the U5-PBS hairpin for virus replication and its effect on reverse transcription (11). Mutations that alter the stability of the U5-PBS hairpin inhibit virus replication. In particular, we measured a reverse transcription defect in assays with the virion-extracted RNA-tRNA complexes as template. Stabilization of the hairpin was found to inhibit reverse transcription because of reduced tRNA primer annealing. Destabilization of the hairpin did not affect tRNA binding, and initiation of reverse transcription was in fact slightly activated. However, the interaction between the tRNA primer and this mutant genome appeared less stable than the corresponding wild-type complex, which may explain the replication defect of this mutant virus. Additional base pairing interactions between retroviral RNA sequences in the U5 region and the tRNA molecule have been suggested to stimulate primer annealing onto the PBS (21, 22). For HIV-1, a specific interaction has been proposed for the “U-rich” anticodon of tRNA\textsubscript{3\Lys} and the “A-rich” loop of the U5-PBS hairpin (23), and this interaction may be affected by mutation of the U5-PBS hairpin. These combined results suggest that the U5-PBS hairpin is involved in both the proper annealing of the tRNA primer onto the viral RNA genome and the initiation of reverse transcription. However, a more detailed analysis is difficult with the virion-derived template-primer material. For instance, this assay system does not allow one to vary the experimental conditions of the tRNA annealing step. We therefore set up reverse transcription assays with in vitro synthesized RNA templates containing the stabilized and destabilized U5-PBS hairpin. Reverse transcription was studied with the natural tRNA\textsubscript{3\Lys} and DNA primers that were annealed at different temperatures and in the presence or

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‡ The abbreviations used are: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; PBS, phosphate-buffered saline; NC, nucleocapsid; PCR, polymerase chain reaction; nt, nucleotide; LTR, long terminal repeat.
absence of NC protein. We demonstrate that both RNA annealing and initiation of reverse transcription are sensitive to stable RNA structure in the template. However, initiation of reverse transcription was hindered more dramatically by template structure with the in vivo annealed tRNA primer than with its 3' terminal. The mutations introduced in vivo differ from the duplex that is formed within the viral RNA structure. Apparently, the heat-annealed RNA-tRNA complex differs from the duplex that is formed within virion particles.

EXPERIMENTAL PROCEDURES

**DNA Constructs**—The U5-PBS hairpin of the construct Blue-5'-LTR (24) was mutated by oligonucleotide-directed in vitro mutagenesis with a Muta-Gene phagemid in vitro mutagenesis kit (Bio-Rad) as described previously (11). Oligonucleotides used are as follows: Ts, 5'-AGACCTTTTAGCACTGCTGGAAAAATCTTGAGC-3'; Td, 5'-CCTGACCCCTTTTACAAGTGGAGAAAATCTTGAGC-3' (mutagenic positions underlined). The control Blue-5'-LTR contains the XhoI-ClaI fragment of HIV-1, encompassing the 5'-LTR, PBS, and the 5'-end of the gag gene (positions 454 to +376), cloned into pBluescript (Stratagene). Nucleotide numbers refer to positions on the wild-type genomic RNA transcript, with +1 being the capped G residue. The mutations introduced were verified by sequence analysis. Sequencing was performed with the M13 universal primer AD-SD (positions 269 to +290) using the Thermo Sequenase™ dye terminator cycle sequencing kit (Amersham Pharma- macia Biotech) and an Applied Biosystems 373 DNA sequencer. The control construct PBS carries an 18-nucleotide deletion over the PBS sequence (25).

**Synthesis of RNA Templates**—The wild-type and mutant pBlue-5'-LRR plasmids were used as template for PCR amplification and subsequent in vitro transcription. The 5'-LTR region of HIV-1 was PCR-amplified with the sense primer T7-1 (positions 1 to +20) with 5'-flanking T7 RNA polymerase promoter sequence and the antisense primer AUG (position +348 to +368, with 6 additional nucleotides at its 5'-end). The PCR fragments were phenol-extracted, precipitated, and dissolved in water. The in vitro transcription reaction was performed in 10 μl of transcription buffer (40 mM Tris-HCl, pH 7.5, 2 mM spermidine, 10 mM dithiothreitol and 12 mM MgCl2) containing 0.5 μg of DNA template, 0.06 μm of ATP, GTP, CTP, and UTP; 10 units of T7 RNA polymerase (Roche Molecular Biochemicals); and 20 units of RNase inhibitor (Roche Molecular Biochemicals), and incubated for 4 h at 37 °C. Upon DNase treatment and phenol extraction, the unincorporated free nucleotides were removed by passage through a Sephadex G-50 column. Subsequently, the RNA was ethanol-precipitated and dissolved in renaturation buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl). The RNA was renatured by incubation at 85 °C for 2 min, followed by slow cooling to room temperature, and stored at −20 °C.

**DNA and tRNA Primer Extension Assays**—In the DNA- and tRNA-primerized reverse transcription assays, 10 ng of in vitro synthesized RNA template was incubated with 1.5 μg of calf liver tRNA (6 pmol total RNA, of which approximately 1.2 pmol is tRNA Lys; Roche Molecular Biochemicals) or 20 ng of DNA primer in the presence or absence of 80 ng of NC protein in 12 μl of annealing buffer (83 mM Tris-HCl, pH 7.5, 125 mM KCl) at 85 or 60 °C for 10 min, followed by cooling to room temperature over a 1-h period or at 37 °C for 30 min. We tested several alternative annealing buffers, buffer B (50 mM Tris-HCl, pH 7.5, 60 mM NaCl, 5 mM MgCl2, 5 mM dithiothreitol) and buffer K (25 mM Tris-HCl, pH 7.5, 30 mM NaCl, 0.8 mM MgCl2, 5 mM dithiothreitol), and a range of NC concentrations (80, 400, and 2000 ng). The primer was extended by the addition of 6 μl of RT buffer (9 mM MgCl2; 30 mM dithiothreitol; 150 μg/ml actinomycin D; 30 μM dATP, dGTP, and dTTP; and 1.5 μM dCTP), 0.5 μl of [α-32P]dCTP, and 0.5 units of HIV-1 RT (U.S. Biochemical Corp.), and reverse transcription was performed for 30 min at 37 °C. The cDNA product was precipitated in 0.3 M sodium acetate, pH 5.2, and 70% ethanol at −20 °C, dissolved in formamide loading buffer, and analyzed on a denaturing 6% polyacrylamide-urea sequencing gel. The antisense primers used were CN1 (positions +123 to +151), Top (positions +165 to +181), and Lys21 (positions +179 to +199).

**tRNA Occupancy of the PBS**—In the PBS occupancy assay, 10 ng of in vitro synthesized RNA template was incubated with 1.5 μg of calf liver tRNA in 12 μl of annealing buffer at 85 °C for 10 min, followed by gradual cooling to room temperature over a 1-h period. Subsequently, 20 ng of the DNA primer AUG (positions +348 to +368, with 6 additional nucleotides at its 5'-end) was added, and the mixture was again incubated for 10 min at 85 °C, followed by cooling to room temperature over a 1-h period. Reverse transcription and analysis of the cDNA products was performed as described above.
in a 257-nt full-length cDNA product. Most shorter cDNAs represent RT pauses. The product marked on the right is primed by a half-tRNAlys molecule present in the calf liver tRNA preparation. A template that contains a PBS deletion is used as a control (PBS-).

FIG. 2. tRNAlys+-mediated reverse transcription of wild-type (set) and U5-PBS mutant templates. The amount of input RNA template was quantified by DNA-primer extension, with the DNA primer CN1 (lanes 1–4). The tRNA primer was annealed at different temperatures in the presence or absence of NC protein and extended by the addition of HIV-1 RT enzyme and dNTPs (lanes 5–28). Extension of the tRNA primer results in a 257-nt full-length cDNA product. Most shorter cDNAs represent RT pauses. The product marked on the right is primed by a half-tRNAlys molecule present in the calf liver tRNA preparation. A template that contains a PBS deletion is used as a control (PBS-).

RESULTS

tRNAlys+-primed Reverse Transcription on Templates with a Mutated U5-PBS Hairpin—We reported previously the construction and initial characterization of two HIV-1 mutants, designated Ts and Td, that stabilize and destabilize the U5-PBS hairpin structure, respectively. Mutant Ts is stabilized by two additional C-G base pairs compared with the wild-type hairpin. This was done by substitution of the unpaired G162 by C and by insertion of an additional C at position 165 (Fig. 1B, introduced mutations are marked by a box). This results in an increase in the thermodynamic stability of the hairpin from $\Delta G = -5.4$ kcal/mol for wild-type to $\Delta G = -18.2$ kcal/mol for mutant Ts. Mutant Td contains three nucleotide substitutions at positions 158–160. As a result, base pairing in the lower part of the stem is lost, and a relative short and instable hairpin structure is left ($\Delta G = -1.6$ kcal/mol). RNA structure probing and computer modeling of a larger region of the HIV-1 leader RNA demonstrated that these mutations do not trigger an overall structural rearrangement of the HIV-1 leader (11).

To study the role of the U5-PBS hairpin in the process of reverse transcription in more detail, we performed in vitro reverse transcription reactions. In these assays, we used in vitro transcribed RNA templates encompassing the complete untranslated leader region of HIV-1 (positions +1 to +368) and calf liver tRNA as a source of tRNAlys primer. The tRNA primer was annealed onto the wild-type and mutant RNA templates at different temperatures with or without NC protein, and reverse transcription was subsequently initiated by the addition of HIV-1 RT enzyme and dNTPs, including [α-32P]dCTP. We will show representative experiments that were used to calculate the reverse transcription efficiency. Similar results were obtained in three to four independent experiments, with less than 10% variation in the relative reverse transcription efficiency calculated for the different templates.

Extension of the tRNA primer on the wild-type and mutant templates produced a full-length 257-nt tRNA-cDNA product as well as shorter cDNA products (Fig. 2, lanes 5–28). No cDNA product was synthesized on the PBS-control template that carries an 18-nt deletion over the PBS, demonstrating that all products in this assay represent specific, tRNAlys+-primed cDNA molecules (26). Most shorter cDNAs represent RT pauses, due to stable RNA secondary structure in the HIV-1 leader template (27). However, one shorter cDNA (marked in Fig. 2) results from the extension of a half-tRNAlys molecule present in the calf liver tRNA preparation (results not shown; see also Ref. 28). Because the pattern of full-length and shorter cDNA products did not differ for the different RNA templates and annealing conditions, we quantified the full-length tRNA-cDNA products and corrected them for the amount of input viral RNA template as determined by primer extension with the upstream DNA primer CN1. See Fig. 2 for reverse transcription assays and Fig. 3A for a schematic of the different primers positioned on the HIV-1 RNA template. The results of the reverse transcription assay are summarized in Table I.

In the absence of NC protein (Fig. 2, lanes 5–8, 13–16, and 21–24), reverse transcription on the wild-type template is reduced about 2-fold by annealing at 60 °C compared with 85 °C. No full-length tRNA-cDNA product was obtained after annealing of the tRNA primer at 37 °C. However, we did observe the shorter cDNA product that is initiated from the half-tRNAlys molecule. This result indicates that the highly structured tRNA molecule cannot bind the PBS at 37 °C, whereas the relatively unstructured 3′-half tRNA molecule can bind. Stabilization of the U5-PBS hairpin in mutant Ts severely reduced reverse transcription at all annealing temperatures (Fig. 2, lanes 6, 14, and 22). At 85 °C, we measured 2% of the reverse transcription efficiency observed on the wild-type template, and no cDNA product was detected after annealing at lower temperatures (Table I). The 3′-half tRNAlys molecule was also unable to prime on the Ts template. Destabilization of the U5-PBS hairpin in mutant Td was found to increase reverse transcription approximately 1.5-fold compared with the wild-type level (Fig. 2, lanes 7, 15, and 23, and Table I). Thus, stabilization of the U5-PBS hairpin inhibits tRNA-primed reverse transcription, whereas destabilization of the hairpin has a modest stimulatory effect.

We also annealed the tRNA primer onto the wild-type and mutant RNA templates in the presence of NC protein at 85, 60, or 37 °C and performed reverse transcription reactions (Fig. 2, lanes 9–12, 17–20, and 25–28). On the wild-type template, a modest 2-fold stimulatory NC effect was measured in the annealing reactions at 85 and 60 °C. However, we were unable to

### Table I

| Temperature | WT Annealing | Td Annealing | PBS Annealing |
|-------------|--------------|--------------|---------------|
| 85°C        | 100%         | 10%          | 5%            |
| 60°C        | 85%          | 5%           | 3%            |
| 37°C        | 7%           | 1%           | 0.5%          |
synthesize full-length tRNA-cDNA products with NC protein at 37 °C. Because NC has been reported to stimulate tRNA annealing at physiological temperature, we repeated this experiment in several buffers and at various NC concentrations ranging from one NC molecule per 2-200 nucleotides, but we failed to measure tRNA-primed reverse transcription (results not shown). We also measured an approximately 2-fold stimulatory effect of NC on reverse transcription on the destabilized Td mutant template at 85 and 60 °C. Interestingly, reverse transcription on the structured Ts template was increased 8-fold by the addition of NC at 85 °C. A significant NC effect was also observed at 60 °C, but the fold induction could not be calculated because no cDNA product was observed in the absence of NC. The NC protein has been suggested to stimulate reverse transcription by unwinding of the structured RNA template and/or the tRNA molecule. The finding that the stabilized Ts template benefits more from the addition of NC is consistent with the idea that this protein unfolds the inhibitory structure in the template. On the other hand, the finding that NC can also stimulate reverse transcription on the destabilized Td template suggests that part of the NC effect is due to melting of the tRNA primer. Under “Discussion,” we will discuss the relative reverse transcription activities of the wild-type and mutant HIV-1 RNA templates in relation to the results that were obtained previously in the in vitro assays with virion-extracted viral RNA-tRNA complexes.

The Placement of tRNA<sup>548 to 348</sup> onto U5-PBS Mutant Templates—The differences in the efficiency of reverse transcription on the mutant and wild-type templates is likely to be the result of differences in the amount of tRNA primer that is annealed onto the PBS. Alternatively, normal levels of tRNA may be bound, but their extension efficiency may differ on the mutant templates. To discriminate between these two possibilities, the tRNA occupancy of the PBS was determined. As shown in the scheme in Fig. 3B, the tRNA primer was annealed onto the RNA template at 85 °C without NC protein, and this complex was subsequently used for extension of the DNA primer AUG that is positioned downstream of the PBS (Fig. 3A, position +348 to +368 region, with 6 additional nucleotides at its 5′-end). When the PBS is occupied by the tRNA primer, extension of the AUG primer will stop prematurely to produce a cDNA product of approximately 175 nt, whereas free RNA templates will produce a full-length cDNA product of 374 nt. The PBS occupancy assay with the AUG primer is shown in Fig. 4 (lanes 9–12). Control reactions were performed with the CN1 DNA primer and tRNA (Fig. 4, lanes 1–4 and 5–8, respectively). The PBS− template was included as an additional control in the PBS occupancy test and yields exclusively the full-length cDNA product that is shorter than 374 nt due to the 18-nt PBS deletion (lane 12). Extension of the downstream AUG primer on the wild-type RNA-tRNA complex produced predominantly the 175-nt stop product (lane 9). Quantitation of the premature stop and the full-length products indicated that approximately 82% of the wild-type templates have an associated tRNA primer, these results are summarized in Table II. For the destabilized Td template, a similar value of 79% was calculated. This result indicates that increased reverse transcription on the Td template is not the result of increased tRNA binding but rather the result of a more efficient extension of the tRNA primer. Extension of the AUG primer on the stabilized Ts template produced primarily the full-length cDNA product, and quantitation indicated that the tRNA occupancy is reduced to 20%. Thus, stabilization of the RNA structure that occludes part of the PBS leads to a tRNA-annealing defect (20%), but a more severe reverse transcription defect was measured (2% activity), indicating that there is also a priming defect on the Ts template.

**DNA-primed Reverse Transcription on U5-PBS Mutant Tem-**
plates—The results presented above indicate that structure in the template and primer can influence reverse transcription. To distinguish between these two effects we also performed reverse transcription with the DNA primer Lys21, which is complementary to the PBS (Fig. 3A). This DNA primer has no apparent structure and allows one to focus exclusively on reverse transcription defects imposed by template RNA structure. The 199-nt-long cDNA products (Fig. 5, lanes 10–18) were quantified and corrected for the amount of input viral RNA template as determined by CN1 primer extension (Fig. 5, lanes 1–9). The results are summarized in Table II. Unlike the results with the tRNA primer, partial annealing of the DNA primer was observed at 37 °C. No difference in DNA-primed reverse transcription was measured for the wild-type and Td template; this result was obtained after annealing at 85 and 37 °C and in the presence of NC. The stabilized Ts template demonstrated only 20% activity after primer annealing at 37 °C, but this defect was largely overcome by the addition of NC or by annealing at 85 °C (Table III). cDNA synthesis was stimulated by NC on all templates; this effect was approximately 1.5-fold for the wild-type and Td template and 3.8-fold for the Ts template. Similar to the results obtained with the tRNA primer, the stabilized Ts template benefits more from the presence of NC than the wild-type and Td templates. These results indicate that DNA-primed reverse transcription is also hindered by secondary structure in the template, although not as severely as reverse transcription primed by a tRNA molecule. For instance, we measured only 2% tRNA priming on the Ts template at 85 °C, compared with 65% DNA priming efficiency.

To test whether DNA priming can be inhibited more efficiently when the binding site for the primer is occluded completely by a secondary structure, we designed an additional DNA primer, termed Top (Fig. 3A). The Top primer anneals to the upper part of the U5-PBS hairpin and is perfectly complementary to the wild-type and mutant templates because its binding site does not include the nucleotides mutated in Ts or Td. The 181-nt-long cDNA products (Fig. 5, lanes 19–27) were quantified and corrected for the amount of input viral RNA template. The results are summarized in Table III. The Top primer was unable to initiate reverse transcription on the stabilized mutant Ts template, whereas reverse transcription of the wild-type and mutant Td template was initiated with equal efficiency (Fig. 5, lanes 18–27). These results indicate that annealing of a DNA primer can be precluded when the entire binding site is part of a stable RNA structure.

DISCUSSION

The HIV-1 RNA genome encodes a semistable stem-loop structure, the U5-PBS hairpin, which occludes part of the PBS. The importance of the U5-PBS hairpin for virus replication and its effect on reverse transcription was reported previously (11). These in vivo results are summarized in Fig. 6A, with the activity measured for the wild-type template set at 100%. Reverse transcription assays with these virion-extracted RNA-tRNAs complexes demonstrated that reverse transcription of the mutant Ts template was reduced to 27% of the value measured for the wild-type template. For mutant Td, a small increase in reverse transcription was consistently measured (125%). In addition, we determined the in vivo tRNA occupancy of the PBS of the wild-type and mutant genomes. We found that approximately 90% of the wild-type and mutant Td templates are associated with a tRNA primer, whereas the mutant Ts template has a PBS occupancy of only 23%. The somewhat increased reverse transcription efficiency of mutant Td results from increased initiation of reverse transcription.

The major reverse transcription defect of mutant Ts is the result of reduced tRNA binding. The analysis of virus revertants demonstrates that replication can be restored by acquisition of additional mutations that reduce the stability of the Ts hairpin from $DG = -18.2$ kcal/mol to a $DG$ value between $-15.6$ and $-5.6$ kcal/mol for the Ts revertants (11). This result suggests that reverse transcription can be initiated in vivo on
templates with an intermediate stability.

We now extended these studies on the HIV-1 U5-PBS hairpin with *in vitro* studies on primer annealing and reverse transcription. We analyzed the effect of temperature and NC protein on reverse transcription. These results are summarized in Fig. 6B, and the activity of the wild-type template was set at 100% for each experimental variation. Stabilization of the U5-PBS hairpin in mutant Ts abolishes tRNA-primed reverse transcription in this *in vitro* assay. The wild-type U5-PBS hairpin appears also somewhat inhibitory because destabilization of this structure in mutant Td has a positive effect on reverse transcription. These results are consistent with the *in vivo* findings, but the inhibitory effect of secondary structure on reverse transcription appears more pronounced *in vitro*. First, stabilization of the U5-PBS hairpin severely inhibits reverse transcription *in vitro* (Fig. 6B, 2–8%), even after annealing at high temperature or in the presence of NC protein. With the tRNA-viral RNA complex isolated from virions, a reverse transcription efficiency of 27% was measured (Fig. 6A). Second, destabilization of the hairpin in mutant Td increases the reverse transcription efficiency to 125% in the *in vivo* assay (Fig. 6A), whereas 140–170% efficiency was measured *in vitro* (Fig. 6B). We previously suggested that the repressive effect of the U5-PBS hairpin in the wild-type HIV-1 RNA may preclude premature tRNA annealing and reverse transcription in infected cells. This partial suboptimal activity of the wild-type template was partially overcome upon annealing at 85 °C or in the presence of NC, indicating that these factors induce unfolding of the wild-type U5-PBS hairpin. In the virion, the presence of co-factors other than NC may stimulate further unfolding of the hairpin.

The NC protein has been reported to stimulate tRNA annealing and the initiation of reverse transcription *in vitro*, and the annealing of the tRNA primer at physiological temperature in the presence of NC was reported (19, 20). We found that the presence of NC had a modest 2-fold stimulatory effect on reverse transcription on the wild-type and destabilized Td templates after annealing at 60 °C or even at 85 °C. This high temperature apparently does not interfere with NC function, suggesting that the NC zinc fingers, which are expected to be denatured at this temperature, are not required for NC-RNA binding. Consistent with this idea, *in vitro* RNA annealing activity was reported previously for mutant NC proteins lacking the zinc finger domains (29–31). In this study, NC did not facilitate tRNA annealing at physiological temperature. Another study also reported a very modest effect of NC on *in vitro* reverse transcription (32). The discrepancy with other studies may be caused by differences in RNA template, tRNA primer, or RT enzyme. Up to 8-fold stimulatory NC effect was measured for the stabilized Ts template. This result indicates that the stabilized Ts template benefits more from RNA unfolding by NC than the wild-type and mutant Td template. The finding that NC can activate reverse transcription on the unstructured Td template suggests that part of the NC effect is due to melting of the tRNA primer.

To discriminate between the effect of structure in the template and in the primer on reverse transcription, we also performed reverse transcription assays with a DNA primer complementary to the PBS. In contrast to the tRNA primer, this DNA primer has no apparent structure and allows one to study exclusively the effect of template RNA structure. The results are summarized in Fig. 6C, and the reverse transcription activity of the wild-type template was set at 100%. No difference was found between the wild-type and mutant Td template. This indicates that the secondary structure in the wild-type template is not inhibitory to DNA-primed reverse transcription, whereas it has a modest negative effect on tRNA-primed reverse transcription *in vivo* (Fig. 6, A and B). The DNA-primed reverse transcription on the Ts template ranged from 20 to 65% (Fig. 6C), depending on the annealing temperature and the presence of NC, whereas this template abolished tRNA-primed reverse transcription *in vitro* (Fig. 6B, 0–8%...
PBS occupancy (20%) for the Ts template, but a more severe tRNA priming defect was apparent (2%). Thus, the reverse transcription defect of mutant Ts in the *in vitro* assay results both from a reduced PBS occupancy and from a less efficient tRNA extension. This indicates that both tRNA annealing and the initiation of reverse transcription are sensitive to stable RNA structure in the template. However, initiation of reverse transcription by an *in vitro* annealed tRNA is hindered more effectively by the secondary structure than initiation by an *in vivo* placed tRNA. This suggests that there may be additional features within the virion particle that facilitate efficient reverse transcription.

We reported previously efficient reverse transcription for mutant Td in *in vivo*, although reduced PBS occupancy was measured (11). Apparently, the tRNA primer was lost in the PBS occupancy assay during the heat denaturing step to anneal the downstream primer. This result indicates that the interaction between the tRNA primer and the mutant Td genome is less stable than the complex with the wild-type template, although both templates have an identical PBS. Moreover, several additional stop products upstream and downstream of the PBS were observed for the wild-type template during extension of the downstream primer. These stops are due to tRNA annealing, because the signals are not observed with the mutant Ts template. Most importantly, these stops were also not observed for the mutant Td template, indicating that a different conformation of the viral RNA-tRNA complex is reached on the wild-type template compared with mutant Td. This result suggests that the U5-PBS hairpin is directly or indirectly involved in correct tRNA annealing onto the viral RNA genome. Several studies suggest that the A-rich loop of the U5-PBS hairpin interacts directly with the anticodon of tRNA<sub>Asp</sub> (23, 37–46). This interaction may be affected by destabilization of the hairpin in mutant Td. We measured no difference in the placement of the tRNA primer onto the wild-type or destabilized Td template, but this could be measured only upon heat annealing. These results suggest that the heat-annexed RNA-tRNA complex differs from the virion-extracted vRNA-tRNA complex, as has been suggested previously (47, 48). This may explain the more severe inhibitory effect of secondary structure on reverse transcription in *in vitro* and indicates that the complex process of HIV-1 reverse transcription cannot be faithfully studied in simplified *in vitro* reactions.

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