Molecular Analysis of the Second Template Switch During Reverse Transcription of the HIV RNA Template†

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Received February 22, 1996; Revised Manuscript Received June 4, 1996⊗

ABSTRACT: The molecular events leading to the second template switch during reverse transcription of the HIV genome were studied in a defined *in-vitro* system. In order to investigate displacement of the $tRNA^{lys}$ primer from the primer binding site (PBS) of the viral genomic RNA, following DNA synthesis, we produced an HIV RNA/DNA substrate that resembles the intermediate reverse transcription complex formed prior to the second template switch. Partial $tRNA^{lys}$ primer displacement was observed during plus (+) strand DNA synthesis and during minus (−) strand DNA elongation. We found two determinants that may serve as a stop signal for (+) DNA strong stop synthesis, the A^m at position 19 of the natural $tRNA^{lys}$ and the secondary structure at the PBS sequence. The later signal appears to constitute a stronger terminator *in-vitro*. The 3′ end of the nascent (−) DNA strand prior to the second template switch was also determined. It was mapped to the U5−PBS junction at the site for the first endonucleolytic cut introduced by the RNase H activity of the HIV reverse transcriptase (RT). Thus, different signals dictate the arrest of (−) and (+) nascent DNA synthesis. These stop signals appear to be required for the subsequent second template switch. However, an excess of (−) DNA "acceptor" molecules, having a 18-base sequence complementary to the (+) DNA "donor" template, was required to demonstrate the actual template switch in the *in-vitro* system. Taken together these results indicate that the reverse transcriptase can catalyze all the steps leading to the second template switch and auxiliary viral proteins may act to enhance the efficiency of this step during the reverse transcription process.

During the reverse transcription process, minus (−) strand DNA synthesis is initiated from a unique tRNA primer, bound to the primer binding site (PBS) sequence of the viral genomic RNA (Barat et al., 1989, 1991, 1993; Telesnitsky & Goff, 1993; Varmus & Brown, 1989). When (−) DNA synthesis reaches the 5′ end of the genomic RNA template, the reverse transcriptase (RT)[1] performs the first template switch which is facilitated by the R region homology found at the 3′ and 5′ ends of the retroviral RNA genome (Ben-Artzi et al., 1993). This template switch could be from the 5′ end to the 3′ end of the same template RNA molecule (intramolecular switch) or between two RNA templates (intermolecular switch) (Weiss et al., 1985; Varmus & Brown, 1989; Telesnitsky & Goff, 1993). Concomitant with (−) DNA synthesis, the RNase H activity of RT degrades the genomic RNA leaving a 15-base RNA/DNA hybrid at the polypurine track (PPT) region which serves as the primer for (+) strand DNA synthesis. Thus the nascent (−) and (+) DNAs reach the PBS from opposite directions. In order to create the long terminal repeats (LTRs), the RT must change template twice. The second template switch was postulated to occur at the PBS sequence. During this step, the 3′ end sequences of the (+) DNA should hybridize to complementary sequences at the 3′ end of the (−) DNA and synthesis of both strands resumes with each strand using the other as a template.

If indeed the second template switch occurs through (−) and (+) DNA annealing at the PBS sequence, the tRNA has to be displaced to facilitate copying of this region by both nascent DNA strands. Simultaneously, the 3′ end of the tRNA primer is degraded by the RT-associated RNase H (Weiss et al., 1985; Varmus & Brown, 1989; Champoux, 1993). Although this model is widely accepted, the molecular mechanism of the second template switch has not been elucidated yet. It has been demonstrated that RT can catalyze strand displacement synthesis through DNA/DNA duplexes but not through RNA/RNA duplexes (Boone & Skalka, 1981; Collet et al., 1978; Cho et al., 1993; Hottiger et al., 1994; Whiting & Champoux, 1994). The ability of the RT to displace the stable tRNA/RNA duplex as well as the accurate determination of the 3′ end of the (+) DNA strand prior to the second template switch has not been demonstrated. To study these putative events in the reverse transcription process, we have constructed an *in-vitro* system composed of a 5′ end of an HIV-1 genomic RNA annealed to $tRNA^{lys}$. Using this template/primer complex and various reverse transcription products derived from it, we addressed three main questions: (i) Does HIV RT displace the $tRNA^{lys}$

† This work was supported in part by National Institutes of Health Grant A1 33353A.

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⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1996.

[1] Abbreviations: HIV, human immunodeficiency virus; RT, reverse transcriptase; RNase H, ribonuclease H; PBS, primer binding site; Act D, actinomycin D; $tRNA^{lys(-m)}$, non-modified $tRNA^{lysine}$; $tRNA^{lys(+m)}$, modified $tRNA^{lysine}$.

S0006-2960(96)00439-4 CCC: $12.00

annealed at the PBS site of the HIV RNA during synthesis of the (−) or (+) DNA strands? (ii) What is the stop site for (+) and (−) DNA synthesis prior to the second template switch? (iii) Would the HIV RT perform the second template switch in a reconstructed system?

MATERIALS AND METHODS

Enzymes. Restriction enzymes and polynucleotide kinase were purchased from BioLabs Corp. SP6 and T7 RNA polymerase were from Promega Corp. Recombinant HIV RT p66/p51 heterodimer (specific activity 10000 units/mg) was purified to apparent homogeneity as previously described (Fischer et al., 1992).

Oligonucleotides. RNA primer (18 bases) (−PBS) 5′GUCCCUGUUCGGGCGCCA3′, consisted of the 3′ end of $tRNA^{lys}$ primer. Primer B (17 bases) for (−) DNA synthesis, having the sequence 5′GCCGAGTCCTGCGTCGA3′, is complementary to bases 231−247 of the HIV RNA template (Ratner et al., 1985). Primer A (18 bases) for (+) DNA synthesis is complementary to bases 22−39 of the strong stop (−) DNA, having the sequence 5′CCTCAGACCCTTTTAGTC3′. Primer C (10 bases) for (−) DNA synthesis is complementary to the 3′U5 sequence (5′CTGCTAGAGA3′).

The 48-base (−) DNA "acceptor" used to demonstrate the second template switch consisted of the entire PBS sequence and downstream sequences (nucleotides 183−231); 5′AGAGAGCTCTGGTTTCCCTTTCGCTTTCAAGTCCCTGTTCGGGCGCAA3′ (Ratner et al., 1985).

The noncomplementary 48-base control (−) DNA "acceptor" was as follows: 5′ACTTGTCATCGTCATCGTGGTGGTGGTGGTGGTGGTGCATATGAG3′.

All RNA and DNA oligonucleotides were synthesized by a nucleic acid synthesizer (Pharmacia) and were gel purified before usage.

$tRNA^{lys}$. Modified human $tRNA^{lys(+m)}$ was purified as described by Jiang et al. (1992). Synthetic unmodified $tRNA^{lys}$ was derived by *in-vitro* transcription of the appropriate plasmid (see below).

Synthesis of HIV Template and Primer RNAs. Plasmid pSP-HIV (Ben-Artzi et al., 1992) was linearized by *Bss*HII endonuclease and transcribed using the SP6 RNA polymerase, to produce a truncated HIV RNA of 258 bases. This 5′ derived HIV RNA included the entire R, U5, PBS, and downstream sequences (nucleotides 1−258) (Ratner et al., 1985). Plasmid ptRNA^{lys} was linearized by *Bsp*MI endonuclease and transcribed by the T7 RNA polymerase to produce the 76-base unmodified $tRNA^{lys(-m)}$ (Weiss et al., 1985). Uniformly labeled $tRNA^{lys(-m)}$ (3×10^5 cpm/pmol) was synthesized by the addition of 40 μCi of [α^{32}P]CTP (800 Ci/mmol) to the *in-vitro* transcription reaction. Following RNA synthesis, the reaction mixture was treated with DNase I, phenol/chloroform extracted, and ethanol precipitated.

5′ End Labeling. [γ^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase were used in a standard procedure for 5′ end labeling of DNA primers A and B and the (−) DNA "acceptor" molecule (Sambrook et al., 1989).

Hybrid Preparation. HIV RNA and $tRNA^{lys}$ were annealed in a molar ratio of 1:5. Hybridization reaction was initiated by preincubating the reaction mixture at 65 °C for 10 min in a buffer containing 80% formamide, 40 mM Tris-HCl (pH 7.4), and 1 mM EDTA. This was followed by the addition of NaCl to 0.3 M and further incubation for 2 h at 42 °C. These stringent conditions were required to facilitate the unwinding of the 3′ end of $tRNA^{lys}$ for binding to the HIV template. Short RNA and DNA oligomers were annealed to the HIV RNA template at a molar ratio of 5:1 in a reaction mixture containing 40 mM Tris-HCl (pH 8), 100 mM KCl, and 8 mM $MgCl_2$. The mixture was heated to 65 °C for 5 min, cooled for 10 min at room temperature, and left for 10 min on ice. Using a 5-fold molar excess of primer to template, in the annealing mixture, virtually all template RNA molecules were occupied by the primer, as indicated by the slow migration of the RNA complexes on 7% polyacrylamide native gel (data not shown).

Reverse Transcriptase Reaction. Reaction mixtures (10 μL) with the appropriate RNA complex (0.2 pmol) and HIV RT (1 unit), contained 50 mM Tris-HCl (pH 8), 100 mM KCl, 2 mM DTT, 8 mM $MgCl_2$, and 1 mM dNTPs. In most experiments, the template/primer complexes used were 18-base RNA/HIV[^{32}P]RNA or $tRNA^{lys}$/HIV[^{32}P]RNA. For strong stop (−) DNA synthesis, the reaction mixture was incubated for 30 min at 37 °C. In order to demonstrate nascent (+) and (−) DNA synthesis, the corresponding 5′-[^{32}P] DNA primers A and B were added in molar excess (×3) subsequent to strong stop (−) DNA synthesis (30 min after initiation of the RT reaction). Unlabeled DNA primer C was also added to the RT reaction without preannealing. Since the RNA template is digested to small oligoribonucleotides by the RNase H activity of HIV RT, it dissociates readily to facilitate annealing of the DNA primers. The reactions demonstrating second template switch were performed in three subsequent steps (see legend to Figure 5). Reactions demonstrating strand displacement, or second template switch, were treated with 0.3 M NaOH (10 min at 95 °C) or 20 ng of RNase A (30 min at 37 °C, in the presence of 100 mM KCl) to remove the RNA (Cho et al., 1993; Sambrook et al., 1989). The reactions (10 μL) were quenched by addition of 50 mM EDTA and sample buffer (80% formamide, Tris-borate-EDTA buffer, 0.1% xylene cyanol, and 0.1% bromophenol blue) and analyzed on a 7 M urea−6% polyacrylamide sequencing gel. All samples were heated to 90 °C for 3 min in sample buffer before loading on the gel. The gels were exposed at −70 °C for 24 h to X-ray film with enhancing screens. Size of the DNA products (in bases) was determined according to the 5′[^{32}P]-labeled DNA markers: pBR322 digested by *Msp*I endonuclease and 5′[^{32}P]-labeled DNA synthetic oligomers of 39, 48, 56, 58, 63, and 87 bases.

[^{32}P]DNA products resolved by electrophoresis on a denaturing gel were quantified with a PhosphorImager (Molecular Dynamics Corp.). Efficiency of strand displacement was determined by comparing the intensity of (+) DNA products obtained using an intact and denatured primer/template complexes (Figure 2A).

RESULTS

The second template switch is postulated to take place at the PBS. During this process, the (+) DNA strand switches from hybridization with the 3′ end of the tRNA primer to hybridization with the (−) strand DNA sequence. To study the molecular events leading to the second template switch, we have constructed the HIV reverse transcription initiation complex that is utilized for strong stop (−) DNA synthesis (see Figure 1A). Two different RNA primers were annealed

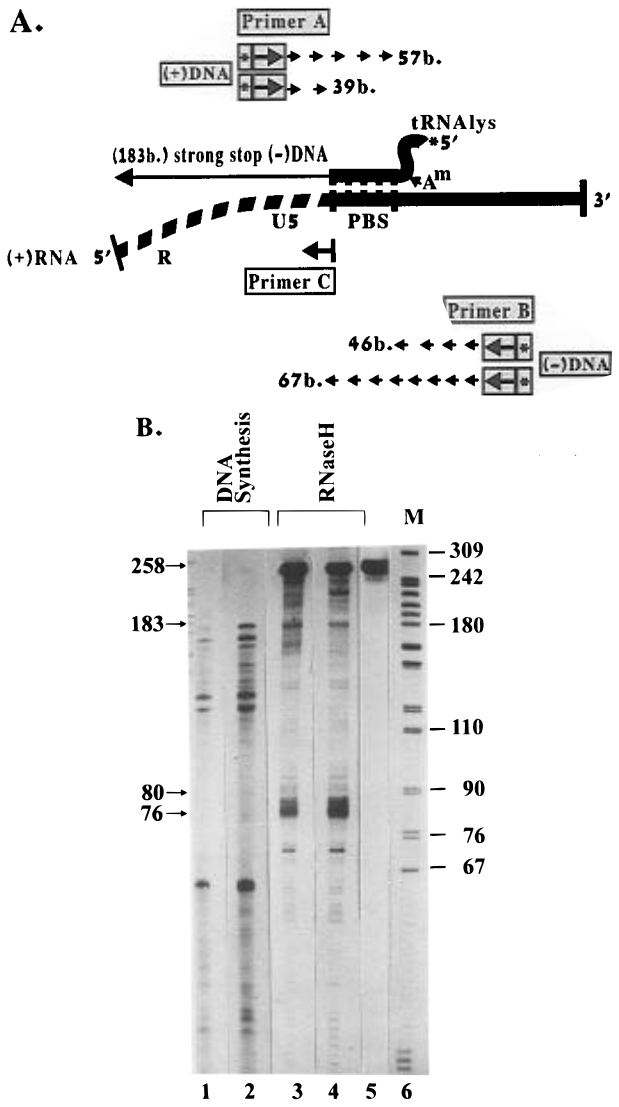

FIGURE 1: A. Diagram of the primer/template complex used to study strand displacement. (▬) $tRNA^{lys}$ (76b) primer for strong stop (−) DNA synthesis; (▬) HIV RNA (258 bases), template; stripped line designates degradation of the RNA template following strong stop (−) DNA synthesis by the RT−RNase H activity; (→) Strong stop (−) DNA (183 bases). Primer A, 18-base DNA, primer for (+) DNA synthesis. Primer B, 17-base DNA primer for (−) DNA synthesis. Primer C, 10-base DNA primer for strong stop (−) DNA synthesis. (+)DNA 39 b., the length of (+) DNA product extended to the 3′ end of $tRNA^{lys}$ primer annealed at the PBS; (+)-DNA 57 b., the length of (+) DNA product transcribing the $tRNA^{lys}$ to the A^m, or the entire 18-base RNA synthetic primer; (−)DNA 46b., the length of (−) DNA synthesized to the 5′ end of the PBS; (−)DNA 67 b., the length of (−) DNA transcribed through the 18-base PBS region and into the U5 region. Asterisks designate 5′[^{32}P] labeling of the different primers. B. Synthesis of (−) DNA and concomitant RNA template degradation using the 18-base RNA/[^{32}P]RNA (lanes 1 and 3) and $tRNA^{lys(-m)}$[^{32}P]RNA (lanes 2 and 4) as primer/template complexes. Lanes 1 and 2, strong stop (−) DNA synthesis. [^{32}P]dCTP, 5 μCi, (3000 Ci/mmol) was included in the standard RT reaction mixture. RNA was degraded by 0.3 M NaOH treatment before subjecting the [^{32}P] (−) DNA products onto the denaturing gel. Lanes 3 and 4, [^{32}P]RNA template degradation during strong stop (−) DNA synthesis. (For details see Materials and Methods). Lane 5, undigested [^{32}P]RNA template. Lane 6, 5′-[^{32}P] end-labeled DNA size marker, pBR322 digested by *Msp*I endonuclease. The amounts of the 18-base RNA/HIV[^{32}P]RNA and the $tRNA^{lys(-m)}$/HIV[^{32}P]RNA were 0.2 pmol (10^5 cpm/pmol). The numbers to the right designate the lengths of the RNA molecules, determined according to the DNA size marker and corrected since RNA mobility is 4% slower than that of DNA of the same size (Ben-Artzi et al., 1993).

to the HIV RNA template; a synthetic RNA of 18 bases complementary to the PBS sequence and the 76-base $tRNA^{lys(-m)}$. The synthesis of strong stop (−) DNA was compared with equivalent amounts of the two RNA complexes (the extent of (−) DNA synthesis was determined by calculating the amount of incorporated [α^{32}P]dCTP). Synthesis of (−) DNA was slightly more efficient with the $tRNA^{lys(-m)}$ primer than with the 18-base RNA primer. This was consistently indicated by the relative amount of the 183-base DNA product (strong stop (−) DNA) accumulated (compare lanes 1 and 2, Figure 1B). The difference in extension efficiency was not surprising since it was previously demonstrated that the HIV RNA, the HIV RT, and the $tRNA^{lys}$ primer form a specific initiation complex (Barat et al., 1991; Isel et al., 1993, 1995; Baudin et al., 1993). In this respect, Isel et al. (1996) demonstrated recently that relative priming efficiency of different primers annealed at the PBS is dependent on the molar ratio of the RT to the template/primer complex. At an excess of RT, there was a small difference in priming efficiency when comparing short RNA primer to the $tRNA^{lys(-m)}$. In our experimental system we used a high excess of HIV RT (×6.5) over template/primer complex and consistently observed slightly higher accumulation of strong stop (−) DNA using the $tRNA^{lys}$ primer compared to the short 18-base RNA primer. Premature terminated DNA products smaller than the 183-base strong stop (−) DNA were also evident in these reactions, in agreement with previous *in-vitro* studies of reverse transcription (Huber et al., 1989). It should be noted that in the absence of a preannealed primer, no (−) DNA was synthesized using the HIV RNA template (data not shown).

In order to follow RNA template degradation, which occurs concomitantly with strong stop (−) DNA synthesis, we prepared a uniformly [^{32}P]-labeled HIV RNA template of 258 bases. During strong stop (−) DNA synthesis, a portion of the [^{32}P]RNA template was degraded into distinct RNA fragments (Figure 1B, compare lanes 3 and 4 to lane 5). The 76−80-base RNA fragments derived from the 3′ end of the RNA template, including the PBS sequence and five nucleotides from the 3′ U5 region. Mapping of these RNA fragments to the 3′ end of the RNA template was further confirmed by direct sequencing of these products and by 3′ end labeling of the template RNA with [^{32}P]Cp and RNA ligase (Ben-Artzi et al., 1996). The array of larger RNAs (80−258 bases) represents cleavage products derived from the 5′ end of the RNA template, encompassing the U5 and R regions. The origin of these RNA products was confirmed by 5′[^{32}P] end labeling of the template RNA (data not shown). It is possible that these intermediate RNA products accumulated due to the non-processive mode of action of RT (Huber et al., 1989). It should be noted that the amount of the RNA degradation products accumulated was higher using $tRNA^{lys(-m)}$ primer compared to the short 18-base RNA primer (compare lanes 3 and 4). This difference correlated with the relatively efficient utilization of $tRNA^{lys(-m)}$ primer as compared to the 18-base RNA for (−) DNA synthesis (lanes 1 and 2). The RT-RNase H possesses an endonucleolytic as well as a 3′−5′ exonucleolytic activity, thus the 5′ end derived RNA products represent a slow exonucleolytic activity (lanes 3 and 4). As only a portion of the preformed template/primer is utilized for (−) DNA synthesis (lanes 1 and 2), some of the [^{32}P]-RNA template (258 bases) remained intact after the RT

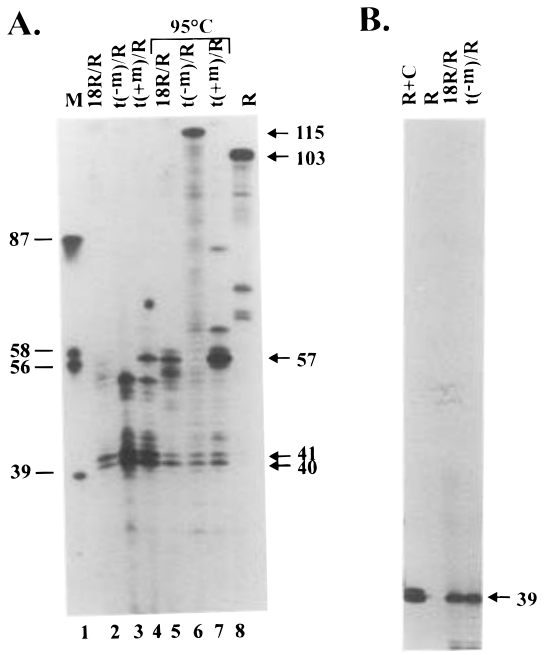

FIGURE 2: Synthesis of (+) DNA initiated from primer A using the three primer/template complexes. A. Synthesis of (+) DNA (30 min) initiated from [^{32}P] primer A, subsequent to strong stop (−) DNA synthesis (30 min). The [^{32}P] (+) DNA products were resolved by electrophoresis on 6% polyacrylamide denaturing gel. Lane 1, 5′[^{32}P]DNA markers of 39, 56, 58, and 87 bases. Lanes 2−4, (+) DNA products initiated with [^{32}P] primer A and the 18-base RNA/RNA, tRNA$^{lys(-m)}$/HIV RNA, and tRNA$^{lys(+m)}$/RNA complexes, respectively. Lanes 5−7, same as lanes 2−4, except that [^{32}P] primer A was added subsequent to strong stop (−) DNA synthesis and heat denaturation of the complexes (95 °C for 5 min). Fresh HIV RT (1 unit) was added to the RT reaction mixture subsequent to heat denaturation, and the reaction proceeded for another 30 min. Lane 8, (+) DNA synthesis initiated by (+) [^{32}P] primer A subsequent to (−) DNA synthesis primed by unlabeled DNA primer B annealed to the HIV RNA template. B. Control reactions. Lane 1, (+) DNA synthesis initiated from [^{32}P]DNA primer A, subsequent to strong stop (−) DNA synthesis. The (−) DNA synthesis was primed by the 10-base primer C that was preannealed to the HIV RNA template. Lane 2, (+) DNA synthesis in the presence of [^{32}P]DNA primer A. Lanes 3 and 4, (+) DNA synthesis primed by [^{32}P] primer A subsequent to strong stop (−) DNA synthesis, analyzed using the 18-base RNA/RNA and tRNA$^{lys(-m)}$/RNA, respectively. RNA template was removed subsequent to strong stop (−) DNA synthesis using 0.3 M NaOH treatment (see Materials and Methods). 18-base RNA/HIV[^{32}P]-RNA, tRNA$^{lys(-m)}$/HIV[^{32}P]RNA, and tRNA$^{lys(+m)}$/HIV[^{32}P]RNA were 0.2 pmol (10^5 cpm/pmol). The amount of primer [^{32}P]DNA A was 1 pmol (5×10^5 cpm/pmol), and the amount of (−) DNA primer C was 1 pmol. RNA was degraded prior to gel analysis or as indicated, by treatment with 0.3 M NaOH. The numbers to the right designate the lengths (in bases) of the DNA molecules synthesized.

reaction (compare lane 5 to lanes 3 and 4). The preliminary experiment described above demonstrates the applicability of the reconstructed system to study displacement of RNA primers from the template RNA following (+) DNA synthesis. In this experiment we added the 5′[^{32}P] primer A to the RT reaction mixture subsequent to synthesis of strong stop (−) DNA, which serves as the template for (+) DNA (Figure 2A). Consequently, a [^{32}P]DNA product of 39 bases would indicate termination of (+) DNA synthesis just before the PBS while [^{32}P]DNA products of 57 and 115 bases would indicate termination at the end of the PBS and at the end of the tRNA^{lys}, respectively (see Figure 1A). In fact, several distinct [^{32}P](+) DNA products were obtained when 18-base RNA/HIV RNA (lane 2), tRNA$^{lys(-m)}$/HIV RNA (lane 3), or tRNA$^{lys(+m)}$/HIV RNA (lane 4) was used as primers/templates. The (+) DNA products of 40−41 base suggest a major termination site for the RT at the first and second base within the PBS sequence. The (+) DNA products of 54−57 bases correspond to termination of (+) DNA synthesis three bases before and just at the end of the 18-base RNA primer that was hybridized to the HIV RNA. Taken together, the accumulation of (+) DNA products in the range of 40−57 bases indicated partial displacement of the RNA primer from the PBS by RT during (+) DNA synthesis.

Since tRNA$^{lys(-m)}$ primer was synthesized by *in-vitro* transcription of the appropriate plasmid, it lacks all nucleotide modifications that are present in cell-derived tRNA^{lys}. It was previously proposed that the first modified base A^m at position 19 from the 3′ end of tRNA^{lys} may serve as a stop signal for (+) DNA synthesis during the reverse transcription process (Roth et al., 1989). To directly analyze the role of this modified base (A^m) during (+) DNA synthesis, we compared a complex primed with tRNA$^{lys(+m)}$ purified from human tRNA (Jiang et al., 1992), to a complex primed with the synthetic tRNA$^{lys(-m)}$. Each of these complexes directed synthesis of a major (+) DNA product of 54 bases, corresponding to termination within the PBS, three bases before the modified A in tRNA$^{lys(+m)}$ (Figure 2A, lanes 3 and 4). However, the human tRNA$^{lys(+m)}$/HIV RNA complex directed synthesis of an additional (+)[^{32}P]DNA product of 57 bases, indicating arrest of (+) DNA synthesis precisely at the A^m position (Figure 2A, lane 4). In a control experiment we validated that the primer/template RNA complexes indeed remained intact following synthesis of strong stop (−) DNA and during the addition of primer A for (+) DNA initiation (Figure 2A, lanes 5−7). This was done by heat denaturing the three RNA complexes subsequent to the synthesis of strong stop (−) DNA and prior to the addition of primer A. When fresh RT and primer A were added to the denatured products obtained with the 18-base RNA/HIV RNA complex, the major (+) DNA products were of 55 and 57 bases rather than of 40−41 bases (compare lanes 2 and 5), indicating that the majority of the 40−41-base DNA products resulted from partial displacement of the 18-base RNA from the PBS during (+) DNA synthesis. Heat denaturation of the tRNA$^{lys(-m)}$/HIV RNA complex, subsequent to (−) strong stop DNA synthesis and before initiation of (+) DNA synthesis, resulted in the accumulation of a major (+) DNA product of 115 bases rather than 40−41 and 54 bases (compare lanes 3 and 6). However, when the tRNA$^{lys(+m)}$/RNA complex was heat denatured, the major (+) DNA product was of 57 bases and not of 115 bases (compare lanes 4 and 7). Two additional (+) DNA products of 63 and 82 bases were evident in this control experiment (lane 7), suggesting that the A^m modification is not an absolute stop signal for the RT. It should be noted that heat denaturation did not eliminate the premature termination of (+) DNA synthesis within the PBS (40−41 base, see lanes 5−7). This could be due to sequence dependent RT pausing. To analyze this possibility we performed (+) DNA synthesis, subsequent to (−) DNA synthesis initiated from primer B rather than from the tRNA^{lys} primer (see Figure 1A). As indicated in Figure 2A, lane 8, the 103-base expected (+) DNA product rather than the 40−41-base (+) DNA products was observed. This result suggests that the 40−41-base (+)

DNA products are not a result of sequence dependent pausing. Thus this premature termination can be explained either by a unique secondary structure or through cleavage by RNase H following (+) DNA synthesis. It should be noted that when a 18-base DNA primer complementary to the PBS was used to generate the template for (+) DNA synthesis, the 40−41-base DNA products were not detected (data not shown). In this respect, it was previously shown that tRNA primer of HIV-1 is cleaved adjacent to the first rA within the PBS (Pullen et al., 1992; Smith & Roth, 1992). Such cleavage is expected to generate some (+) DNA termination products of 40−41 bases.

In summary, the results presented in Figure 2A,B indicated partial $tRNA^{lys}$ displacement from the HIV RNA template during elongation of (+) DNA. In yet another control experiment we validated that the 40−41-base (+) DNA product indeed represents termination at the immediate junction of U5 and PBS. Primer C, complementary to the U5 region was annealed to HIV RNA template, in the absence of RNA primer and was extended with RT to produce strong stop (−) DNA (see Figure 1A). Subsequently, [^{32}P] primer A was added to the RT reaction and (+) DNA extension was analyzed (Figure 2B). As expected, a run off (+) DNA product of 39 bases was obtained in this reaction, representing termination just at the border of the U5 and PBS sequences (Figure 2B, lane 1). Addition of an extra base by RT, forming the 40-base DNA product, was also noticed. This was not surprising, since it was previously demonstrated that RT adds noncomplementary bases at the end of its template (Peliska & Benkovic, 1992). To demonstrate that (+) DNA extension is indeed dependent upon prior synthesis of strong stop (−) DNA, the RT reaction was carried out with HIV RNA template but in the absence of primer C. Subsequent to the addition of [^{32}P] primer A, no (+) DNA product could be visualized, as expected (Figure 2B, lane 2). To further demonstrate that the (+) DNA products larger than 39 bases resulted from utilizing the 18-base RNA or the $tRNA^{lys}$ as templates, we analyzed (+) DNA elongation using [^{32}P] primer A, following strong stop (−) DNA synthesis and the removal of all RNAs by NaOH treatment. As expected, a major (+) DNA product of 39 bases is evident, indicating termination of (+) DNA synthesis at the U5−PBS junction (Figure 2B, lanes 3 and 4).

The extent of strand displacement was quantified following separation of the [^{32}P] (+) DNA products on polyacrylamide−urea gel (Figure 2A). Using the 18-base RNA/RNA complex, 86% of (+) DNA products were arrested within the PBS forming the 40−56-base DNA products, while only 14% of the (+) DNA products copied the entire RNA primer, forming the 57-base DNA product. Using the $tRNA^{lys(-m)}$/RNA as a primer/template, 98% of the (+) DNA molecules were also arrested within the PBS, forming the 40−54-base (+) DNA products. Similar results were obtained using the $tRNA^{lys(+m)}$/RNA complex; only 10% of the (+) DNA molecules accumulated as the expected 57-base DNA product. These results indicate that strand displacement of the stable double-stranded RNA duplex situated at the PBS was relatively inefficient *in-vitro*.

We next analyzed whether the $tRNA^{lys}$ primer would also be displaced by the nascent (−) DNA initiated from [^{32}P] primer B (see Figure 1A). In this experiment [^{32}P] primer B was added to the RT reaction mixture subsequent to synthesis of strong stop (−) DNA (Figure 3). Any [^{32}P] (−) DNA product larger than 46 bases would indicate some displacement of the double-stranded RNA complex at the PBS sequence. Indeed, a major (−) DNA product of 53 bases was evident with both 18-base RNA/HIV RNA and $tRNA^{lys(-m)}$/HIV RNA complexes (Figure 3, lanes 1 and 2). The [^{32}P] (−) DNA major product of 53 bases appears to terminate seven bases within the PBS sequence. Larger [^{32}P] (−) DNA products of 60 and 65−67 bases were also detected; they appear to represent two terminations, 14 bases within the PBS and one to three bases beyond the PBS, in the U5 region. To further establish that these three major [^{32}P](−) DNA products indeed represent RT mediated displacement, and not an arrest of (−) DNA synthesis at sites of template RNA cleavages by RT-RNase H, the two complexes, 18-base RNA/RNA and $tRNA^{lys(-m)}$/RNA, were

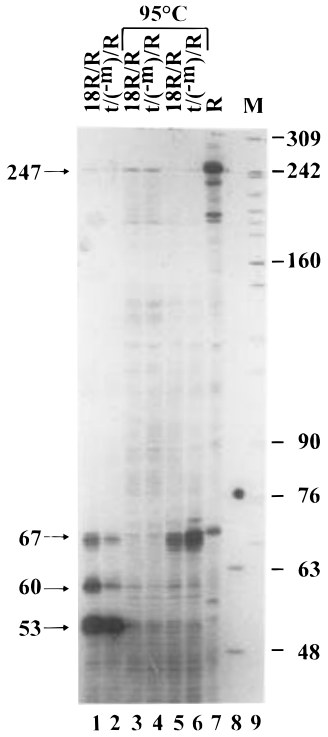

FIGURE 3: Nascent (−) DNA synthesis initiated by primer B, using the 18-base RNA/HIV RNA and the $tRNA^{lys(-m)}$/RNA complexes. Subsequent to strong stop (−) DNA synthesis (30 min), [^{32}P] primer B (1 pmol) was added to the RT reaction mixture and incubation proceeded for another 30 min. Lane 1, (−) DNA synthesis using the 18-base RNA/RNA duplex as template. Lane 2, (−) DNA synthesis using the $tRNA^{lys(-m)}$/RNA complex. Lanes 3 and 4, (−) DNA synthesis primed by [^{32}P] primer B subsequent to denaturation of the respective 18-base RNA/RNA and the $tRNA^{lys(-m)}$/RNA complexes for 5 min at 95 °C. Fresh RT (1 unit) was added to the RT reaction mixture subsequent to heat denaturation. The reaction proceeded for another 30 min. Lanes 5 and 6, (−) DNA synthesis primed by [^{32}P] primer A subsequent to denaturation of the respective 18-base RNA(−)DNA/RNA and the $tRNA^{lys(-m)}$ (−DNA)/RNA complexes formed during strong stop (−) DNA. Lane 7, (−) DNA synthesis primed by [^{32}P] primer B using the free HIV RNA template, in the absence of RNA primer. Lane 8, size markers, [^{32}P]-DNA oligonucleotides of 48 and 63 bases. Lane 9, [^{32}P] end-labeled DNA fragments of *Msp*I-digested pBR322. RT reaction conditions are described in Materials and Methods. The amounts of 18-base RNA/HIV[^{32}P]RNA (lanes 1, 3, and 5) and $tRNA^{lys(-m)}$/HIV[^{32}P]-RNA (lanes 2, 4, and 6) were 0.2 pmol (10^5 cpm/pmol). The amount of primer [^{32}P]DNA B was 1 pmol (5×10^5 cpm/pmol). RNA was degraded prior to gel analysis by treatment with 0.3 M NaOH. The numbers on the left designate the length in bases of the DNA molecules synthesized (53, 60, 67, and 247) (see Materials and Methods).

heat denatured prior to the addition of [^{32}P]DNA primer B and RT (see Figure 3, lanes 3 and 4). Under these conditions [^{32}P] primer B was elongated on the HIV RNA template to give a multitude of large (−) DNA products. The major (−) DNA product in this reaction, 247 bases, resulted from copying the entire HIV RNA template to the end of the R region. It should be noted that (−) DNA products of 53 and 60 bases were not accumulated after heat denaturation of the RNA/RNA complex. This result strongly suggests that the 53- and 60-base (−) DNA products represent pausing sites within the RNA/RNA duplex rather than RT−RNase H-catalyzed cleavages of the template RNA. The 65−67-base (−) DNA products appear to represent termination beyond the PBS, within the U5 region at sites of the initial endonucleolytic cuts introduced by RNase H during strong stop (−) DNA synthesis (see Figure 1B, lanes 3 and 4). To confirm this assumption, the primer/template complexes were heat denatured, subsequent to strong stop (−) DNA synthesis. As can be seen in Figure 3, lanes 5 and 6, [^{32}P] primer B was elongated on the denatured RNA template to give a major (−) DNA product of 65−67 bases. Identical (−) DNA products of 65−67 bases, albeit at lower amounts, were also obtained in the absence of RNA/RNA complex denaturation (Figure 3, lanes 1 and 2). The finding that the distinct stop signal for nascent (−) DNA synthesis on the denatured RNA template at this position supports the notion of an initial endonucleolytic nick introduced by the RT−RNase H within the U5 region.

To demonstrate directly that the primer/template complexes utilized in Figures 2 and 3 were indeed stable and that the apparent strand displacement was not a result of spontaneous denaturation during the RT reaction, we analyzed the 18-base RNA/[^{32}P]RNA and the tRNA$^{lys(-m)}$/[^{32}P]-RNA complexes in a native gel before and after incubation at 37 °C for 30 min in the presence of HIV RT. The 18-base RNA/RNA and the tRNA$^{lys(-m)}$/RNA complexes remained intact following the incubation, thus demonstrating that HIV RT does not unwind the RNA/RNA complexes in the absence of DNA polymerization (data not shown).

The results illustrated in Figures 2 and 3 demonstrate the crucial steps that were postulated to precede the second template switch, namely, (+) and (−) DNA elongation and strand displacement of the tRNA primer from the PBS. To analyze whether RT can catalyze the actual second template switch in an *in-vitro* system, we have synthesized an "acceptor template", consisting of the 18-base (−)PBS sequence and 30-base downstream sequences (Figure 4A). Previous work of our laboratory (unpublished data) as well as by others (Luo & Taylor, 1990; Peliska & Benkovic, 1992) has demonstrated that efficiency of the first template switch *in-vitro* was affected by the relative amounts of the "acceptor" and "donor" templates. We anticipated, therefore, that an excess of "acceptor" templates might also be required for the second template switch *in-vitro*. Therefore (−) DNA "acceptor" (Ac) was added in molar excess (×3) subsequent to strong stop (−) DNA synthesis and (+) DNA synthesis was initiated by the addition of [^{32}P] (+) DNA primer A. In this experiment, we expected that the (+) DNA would copy the 18-base RNA primer sequence and would extend beyond the PBS by switching to the (−) DNA "acceptor" template. A proper template switch should result in the accumulation of an 87-base (+) DNA product (see Figure 4A). As can be seen in Figure 4B, lane 1, in the absence of the "acceptor" molecule, [^{32}P] (+) DNA products of 40−57 bases accumulated, demonstrating partial strand displacement of the RNA/RNA duplex, as described above. However, upon addition of the "acceptor" (−) DNA, a major (+) DNA product of 87 bases accumulated (Figure 4B, lane 2). The 87-base DNA product represented elongation of the (+) DNA beyond the PBS by switching from (−) strong stop DNA to the "acceptor" template. It should be noted that in the absence of "acceptor" template, (+) DNA products larger than 57 bases were not accumulated (compare Figure 4B, lanes 1 and 2), thus suggesting that template switch is dependent on the presence of "acceptor" molecules.

However, since only part of the RNA template was utilized for strong stop (−) DNA synthesis (see Figure 1) we could not exclude the possibility that the residual HIV RNA serves as a template for (−) DNA initiated by the (−) DNA "acceptor" molecule and that primer A subsequently copied continuously this (−) DNA template. To exclude this possibility, we added the (−) DNA "acceptor" following degradation of the RNA template by RNase A (Figure 4B, lane 3). Thus, template switch in the absence of the RNA template could only have been a result of DNA dependent DNA synthesis.

Since RNase A treatment was performed in low salt concentration, we expected the removal of the PBS RNA/RNA duplex. RNase A treatment was utilized instead of NaOH treatment in order to keep the double-stranded DNA structures intact. RNase A treatment decreased but did not eliminate the second template switch, and some (+) DNA was still able to translocate to the (−) DNA "acceptor" to produce the expected 87-base product.

To further investigate the second template switch, we performed some more control experiments: we first analyzed the effect of RNA degradation (RNase A treatment) on subsequent (+) DNA synthesis and template switch before the addition of the (−) DNA "acceptor" (Figure 4B, lane 4). As expected, in the absence of the RNA/RNA duplex (RNase A treatment), the only (+) DNA product accumulated was of 40 bases, representing termination of (+) DNA synthesis at the U5−PBS junction. This would suggest that the template switch is dependent upon copying the RNA primer by the nascent (+) DNA. Since RNase A cleaves the RNA primer adjacent to pyrimidine residues, an rA residue remains attached to 5′ end of the strong stop (−) DNA. This RNA derived residue is transcribed by RT, thus forming the 40-base rather than the expected 39-base (+) DNA product. In yet another control experiment (Figure 4B, lane 5), we added primer C to a reaction mixture containing the HIV RNA template. Following (−) DNA synthesis, the RNA template was removed by RNase A treatment and [^{32}P](+) DNA primer A and the (−) DNA "acceptor" template were added to the reaction mixture simultanously. As in the previous control experiment, only the 39- and 40-base (+) DNA products accumulated, indicating again that reverse transcription of the RNA primer into a (+) DNA copy was a prerequisite for obtaining the 87-base DNA template switch product. To demonstrate that the second template switch is indeed a DNA dependent DNA synthesis process, we added actinomycin D (Act D) to the RT reaction mixture along with the (−) DNA "acceptor" (Figure 4B, lane 6). Addition of the "acceptor" molecule and actinomycin D was carried out after the following processes: (i) strong stop (−) DNA synthesis, (ii) (+) DNA

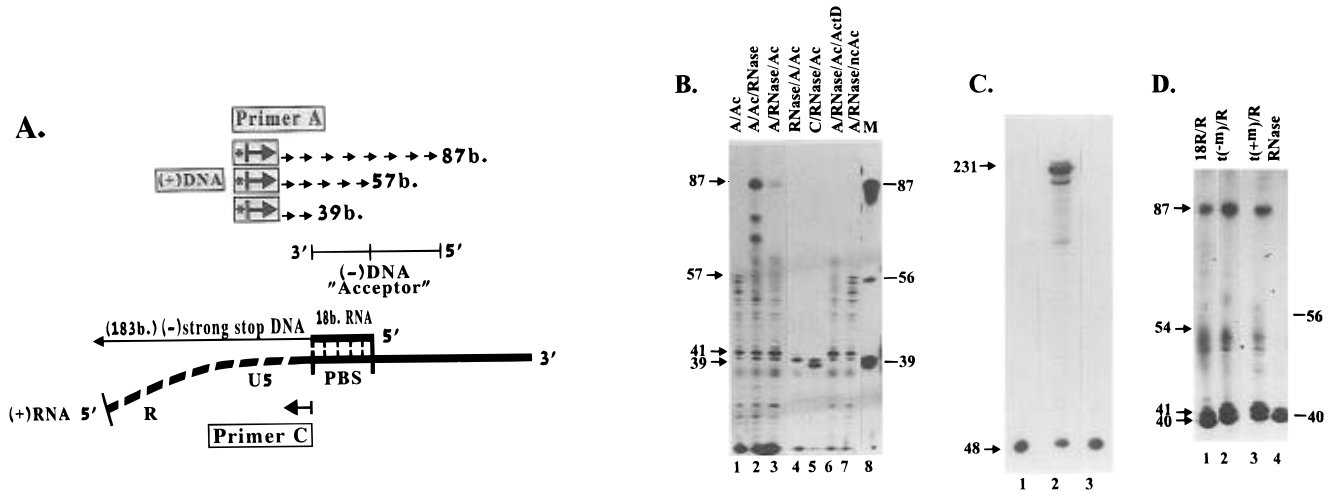

FIGURE 4: Analysis of the second template switch. A. Scheme of the experimental system used for analysis of the second template switch. (▬) The RNA primer used to initiate strong stop (−) DNA synthesis (the 18-base RNA, $tRNA^{lys(-m)}$, or $tRNA^{lys(+m)}$). (▬) HIV RNA template (258 bases). The stripped line designates degradation of the RNA template following strong stop (−) DNA synthesis by the RT−RNase H activity. (→) Strong stop (−) DNA, 183 bases. Primer C, 10-base DNA primer for strong stop (−) DNA synthesis. Primer A, 18-base DNA to initiate (+) DNA synthesis. (−)DNA "acceptor", 48 b., consisting of the 18-base PBS sequence and 30 bases of downstream sequences. (+) DNA, 39 b., the length of the nascent (+) DNA initiated with primer A to the junction of U5 and PBS. (+) DNA, 57 b., the length of the nascent (+) DNA to the end of the of the PBS. (+) DNA, 87 b., the length of (+) DNA translocating to the (−) DNA "acceptor" template to perform the second template switch. B. Second template switch using the 18-base RNA/RNA complex and the (−) DNA "acceptor" molecule. The experiment was carried out in three subsequent stages: (i) synthesis of strong stop (−) DNA (30 min), (ii) [^{32}P] (+) DNA synthesis after the addition of [^{32}P] primer A but in the absence of (−) DNA "acceptor" molecule (30 min); and (iii) [^{32}P] (+) DNA synthesis after the addition of (−) DNA "acceptor" molecule (Ac) and treatment with RNase A. The timing of RNase A treatment is indicated above each lane. Lane 1, (+) DNA synthesis subsequent to strong stop (−) DNA synthesis, in the absence of the (−) DNA "acceptor" molecule. Lane 2, (+) DNA synthesis, in the presence of (−) DNA "acceptor" template. RNA degradation was after the addition of the (−) DNA "acceptor" template. Lane 3, same as lane 2 except that RNA degradation was prior to the addition of the (−) DNA "acceptor" template. Lane 4, (+) DNA synthesis subsequent to strong stop (−) DNA synthesis and RNA degradation by RNase A. Lane 5, (+) DNA synthesis subsequent to strong stop (−) DNA synthesis initiated with primer C (10 bases) annealed to the U5−PBS junction. Lane 6, (+) DNA synthesis in the presence of (−) DNA "acceptor" molecule and actinomycin D (50 μg/mL). Actinomycin D and the (−) DNA "acceptor were added simultanously. Lane 7, (+) DNA synthesis in the presence of non-complementary (−) DNA "acceptor" molecule of 48 bases. Lane 8, size markers of [^{32}P]DNA: 39, 56, and 87 bases. The amounts of the [^{32}P] 18-base RNA/RNA and the DNA "acceptor" were 0.2 pmol (10^5 cpm/pmol) and 0.6 pmol, respectively. The [^{32}P] primer A was 1 pmol (10^5 cpm/pmol). The numbers to the left designate the length in bases of the [^{32}P] (+) DNA products. C. Control experiment. Lane 1, [^{32}P] (−) DNA "acceptor". Lane 2, RT-mediated (−) DNA synthesis initiated with [^{32}P] (−) DNA "acceptor" on the HIV RNA template. Lane 3, (−) DNA synthesis in the presence of [^{32}P] (−) DNA "acceptor", subsequent to template degradation by RNase A. D. Comparison of template switch using the three primer/template complexes, 18-base RNA/RNA, $tRNA^{lys(-m)}$/RNA, and $tRNA^{lys(+m)}$/RNA. The reaction was carried out, as described in the legend to Figure 4B. The (−) DNA "acceptor" molecule was added to the reaction subsequent to strong stop (−) and (+) DNA synthesis. Lanes 1−3, (+) DNA synthesis initiated with [^{32}P] primer A and (−) DNA "acceptor" molecule, using the 18-base RNA/RNA, the $tRNA^{lys(-m)}$/RNA, and the $tRNA^{lys(+m)}$/RNA primer/template complexes, respectively (0.2 pmol, 10^5 cpm/pmol). Lane 4, control, [^{32}P] (+) DNA synthesis subsequent to RNA degradation (RNase A treatment) of the $tRNA^{lys(-m)}$/RNA complex and in the presence of (−) DNA "acceptor". The amount of (−) DNA "acceptor" molecule was 0.6 pmol. [^{32}P] (+) primer A and [^{32}P] (−) DNA "acceptor" were used at 1 pmol (5×10^5 cpm/pmol).

synthesis, and (iii) RNase A treatment. As observed in Figure 4B, lane 6, major (+) DNA products of 40−41 bases and some larger (+) DNA products accumulated. However, the 87-base (+) DNA product, which indicates a template switch, was not detected. This control experiment further indicates that the 87-base DNA product obtained in Figure 4B, lane 3, is indeed a result of (+) DNA elongation beyond the PBS, using the (−) DNA "acceptor" as a template. Furthermore, to demonstrate that the second template switch is dependent on the annealing of the (−) and (+) complementary PBS DNA sequences, we carried out the template switch reaction in the presence of a nonspecific "acceptor" DNA molecule of 48 bases (non-complementary (nc) Ac). As demonstrated in Figure 4B, lane 7, there was no accumulation of the 87-base (+) DNA product.

Since our analysis of the second template switch was dependent on the removal of RNA by RNase A treatment, we had to validate that indeed all RNAs were degraded prior to the addition of (−) DNA "acceptor" molecule. To this end, the (−) DNA "acceptor" molecule was 5′[^{32}P] labeled and incubated in the presence of HIV RNA template and the four deoxyribonucleotides triphosphates either with or without RNase A (Figure 4C, lanes 2 and 3, respectively). In the absence of RNase A, the [^{32}P] (−) DNA "acceptor" was extended, producing the expected 231-base DNA product (Figure 4C, lane 2), while in the presence of RNase A, extension of the (−) DNA "acceptor" was not detected (Figure 4C, lane 3). Thus this control experiment indicated that RNase A treatment was effective. In the last experiment we compared accumulation of the second template switch DNA products using the three primer/template complexes; 18 base RNA/HIV RNA, $tRNA^{lys(-m)}$/HIV RNA and $tRNA^{lys(+m)}$/HIV RNA (Figure 4D). These three RNA/RNA complexes together with the (−) DNA "acceptor" molecule, facilitated template switch to produce the 87-base (+) DNA product (Figure 4D, lanes 1−3). When the 18-base/RNA duplex was eliminated by RNase A treatment subsequent to strong stop (−) DNA synthesis template switch did not occur and [^{32}P] (+) DNA synthesis terminated at the U5−PBS junction, resulting in accumulation of the 39- and the 40-

base (+) DNA products (Figure 4D, lane 4). The same result was obtained using the $tRNA^{lys(-m)}$/HIV RNA and the $tRNA^{lys(+m)}$/HIV RNA complexes (data not shown). The experiment presented in Figure 4D indicates that the amount of the template switch products [(+) DNA, 87 bases] is similar using the three template/primer complexes.

DISCUSSION

According to current models of the reverse transcription process, the nascent (+) and (−) DNA strands reach the PBS sequence from opposite directions. The second template switch occurs at the PBS sequence through the annealing of the 3′ ends of the two complementary strands (Telesnitsky & Goff, 1993). To facilitate this step, the RT is postulated to displace the tRNA/RNA duplex at the PBS and subsequently to degrade the RNA moiety.

In order to study the steps leading to the second template switch in detail, we have constructed a model system consisting of the appropriate template/primer and purified HIV RT. The template/primer consisted of authentic human $tRNA^{lys(+m)}$, synthetic $tRNA^{lys(-m)}$, or 18-base RNA primers bound to the PBS region of HIV RNA template. We analyzed tRNA/RNA duplex unwinding in the presence and absence of DNA synthesis. This reaction is thought to precede the template switch. We were able to demonstrate partial displacement of $tRNA^{lys}$ from the template RNA through either copying of the $tRNA^{lys}$ sequences into (+) DNA or through extension of the nascent (−) DNA into the PBS of the HIV RNA. However, the pausing sites within the RNA/RNA duplex were different, dependent on (+) or (−) DNA synthesis. While a large fraction of nascent (−) or (+) DNA intermediates terminated along the PBS sequence, only 2%−10% of the DNA molecules copied the entire 18 bases of the $tRNA^{lys}$ annealed to the PBS. Thus, the process of strand displacement by RT appears to be relatively inefficient *in-vitro*. Furthermore, we have demonstrated that RT is incapable of unwinding RNA/RNA duplex in the absence of DNA synthesis. This later result is consistent with a previous observation of Collet et al. (1978). There are several studies on the capacity of polymerases in general and RT in particular to displace the nontemplate double-stranded structures, concomitant with DNA synthesis. It has been demonstrated by several investigators that RT catalyzes strand displacement synthesis through short as well as long DNA/DNA structures (Boone & Skalka, 1981; Schultz et al., 1995; Hottiger et al., 1994; Huber et al., 1989; Whiting & Champoux, 1994). In contrast, long RNA/RNA duplexes were resistant to displacement by RT (Cho et al., 1993). Since the ability of HIV RT to displace the stable 18-base $tRNA^{lys}$/RNA duplex in our reconstructed system is limited and copying of these sequences may be crucial for the second template switch, we suggest that auxiliary viral proteins may be involved in this step during viral infection. In this respect, it was recently suggested that nucleocapsid proteins might be involved in strand displacement (Tsuchihashi & Brown, 1994).

We have also determined the 3′ ends of both the (−) and the (+) DNA strands, prior to the second template switch. The nascent (−) DNA arrested at the site of the first RNase H endonucleolytic cuts one to three bases within the U5 region of the HIV RNA template. This cleavage site was defined by us (Ben-Artzi et al., 1996) as well as by others (Gotte et al., 1995; Schultz et al., 1995). The 3′ end of the (+) DNA strand was determined partially by the first modified base (A^m) in the $tRNA^{lys(+m)}$ primer. In this respect, genetic studies with MuLV have previously suggested that the stop signal for (+) DNA synthesis is nucleotide 19 (A^m) of the $tRNA^{pro}$ primer (Roth et al., 1989). Several lines of evidence indicate that this A^m modification of $tRNA^{lys}$ is not the sole signal for (+) DNA termination: (i) The complex of $tRNA^{lys(-m)}$/HIV RNA gave virtually no readthrough of (+) DNA synthesis beyond the PBS sequence; in contrast, denaturation of the complex resulted in extensive synthesis of the expected 115-base (+) DNA readthrough product. (ii) Comparison of (+) DNA synthesis on intact and denatured $tRNA^{lys(+m)}$/RNA complex indicated that the A^m is not an absolute termination signal for (+) DNA synthesis. Taken together, we suggest that termination of (+) DNA synthesis at the PBS sequence is also dictated by the structure of the RNA/RNA complex.

Mapping the 3′ ends of both (+) and (−) DNA strands indicated the formation of complementary PBS sequences. Therefore, hybridization of the two nascent DNAs at the PBS sequence should created a stable DNA/DNA structure to facilitate the second template switch.

Although we did demonstrate some of the steps leading to the second template switch *in-vitro*, this last reaction was not observed initially. Nonetheless, the second template switch in our *in-vitro* system required a 3-fold excess of (−) DNA "acceptor" over the "donor" (+) DNA molecules. We demonstrated the second template switch using three primer/template complexes, 18-base RNA/RNA, $tRNA^{lys(-m)}$/RNA, and $tRNA^{lys(+m)}$/RNA. The products as well as the efficiencies of the second template switch reaction using the three primer/template complexes were similar, indicating that this step can be studied with the simple complex, 18-base RNA/RNA primer/template.

Taken together, our studies on the second template switch and those of Peliska and Benkovic (1992) and Luo and Taylor (1990) regarding the first template switch suggest several common features: (i) Complementarity between the "donor" and the "acceptor" sequences; (ii) requirement for an excess of "acceptor" molecule; and (iii) relatively low efficiency of template switch in the reconstituted system. It is therefore possible that nucleocapsid proteins are required to enhance the second template switch as was demonstrated for the first template switch (Tsuchihashi & Brown, 1994; You & McHenry, 1994).

ACKNOWLEDGMENT

We thank Dr. G. Kaufmman and R. Hozarsky for helpful discussion and L. Nir for contribution to this work.

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BI960439X