Mechanistic Studies of Early Pausing Events during Initiation of HIV-1 Reverse Transcription

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Chen Liang‡§, Liwei Rong‡§, Matthias Götte‡, Xuguang Li‡, Yudong Quan‡,
Lawrence Kleinman¶§ and Mark A. Wainberg‡¶**

From the ‡McGill University AIDS Centre, Lady Davis Institute-Jewish General Hospital, Montreal, Quebec H3T 1E2, Canada and the Department of Medicine and ¶Microbiology, McGill University, Montreal, Quebec H3A 2B4, Canada

We have investigated the role of sequences that surround the primer binding site (PBS) in the reverse transcriptase-mediated initiation of (−) strand DNA synthesis in human immunodeficiency virus type 1. In comparisons of reverse transcription initiated from either the cognate primer tRNALys.3 or a DNA primer D-Lys.3, bound to PBS sequences, we observed that a +3 pausing site occurred in both circumstances. However, the initiation reaction with tRNALys.3 was also characterized by a pausing event after incorporation of the first nucleotide. Alteration of sequences at the 5'-end instead of the 3'-end of the PBS resulted in elimination of the +3 pausing site, suggesting that this site was template sequence-dependent. In contrast, the pausing event at the +1 nucleotide position was still present in experiments that employed either of these mutated RNA templates. The mutations at the 5'-end of the PBS also caused a severely diminished rate of initiation and the strong arrest of reactions at the +1 stage when tRNALys.3 was used as primer. Therefore, we propose that the +1 pausing event is an initiation-specific event in regard to reactions primed by tRNALys.3 and that sequences at the 5'-end of the PBS may facilitate the release of reverse transcription from initiation to elongation.

Retroviruses employ specific tRNA molecules as primers to initiate the synthesis of (−) strand DNA (1–5). These primer tRNAs bind to an 18-nt segment of viral genomic RNA termed the primer binding site (PBS). Although such binding is principally mediated by a stretch of 18 nt close to the 3'-end of tRNA, other sequences within viral genomic RNA and the tRNA primer are also involved and can modulate the initiation of (−) strand DNA synthesis, as shown in both retroviral (6–10) and other (11–16) systems. In HIV-1, this includes a stretch of four nucleotides, i.e. 625AAA628 (the A-rich loop) that interacts directly with positions 33USUU36 of the anticodon loop of primer tRNALys.3. This A-rich loop is important for initiation of (−) strand DNA synthesis and generation of progeny virus (17).

In HIV-1, the initiation stage of synthesis of (−) strand DNA, primed by tRNALys.3, can be distinguished from subsequent strand elongation in regard to both the binding and kinetic properties of reverse transcriptase (RT) (18–20). These reports showed that initiation was characterized by both early short (−) strand DNA products, resulting from pausing at the +3 or +5 nt positions, and rapid dissociation of RT from the initiation complex. The secondary structure formed between tRNALys.3 and the viral RNA template may play an important role in initiation of reverse transcription.

To pursue this subject, we developed an in vitro reverse transcription system in which low concentrations of dNTPs (i.e. 160 nm) were used to enhance our ability to detect very early pause sites, e.g., +1 and +3, in reactions primed with the tRNALys.3 cognate primer. To study the roles of sequences flanking the PBS, we generated a series of mutated HIV-1 RNA templates that contained mutations at both the 5'- and 3'-ends of the PBS that may potentially disrupt the secondary structure of complexes of tRNALys.3 and viral RNA template. Our data provide the first evidence for a +1 pausing event in reverse transcription initiated from primer tRNALys.3. We have also demonstrated on the basis of mutagenesis studies that the +3 pausing site depends on the nature of sequences at the 5'-end of the PBS and that deletion of an A-rich loop in this region or of substitutions at the 5'-end of the PBS may make it difficult for primer tRNALys.3 to be extended beyond the +1 pausing site.

MATERIALS AND METHODS

Plasmid Construction—DNA sequences at nt positions 473–1417 of HIV-1 (HxB2D) were cloned into the RNA expression vector pSP72 (Promega, Madison, WI) through use of BglII (473) and PstI (1412). The deletion of the A-rich loop (HIV/del-A) and the construction of mutation HIV/2A have been described previously (17, 21). We also engineered a substitutional mutation (HIV/HUA) by replacing the sequence 625A-ACTCTAGGCAG637 with 5'-GAACACCCAACTT-3'; this was done by PCR using an antisense primer 5'-CCCTGTCGGGGCGCAATGTGTTGGTTGTTGCTTCCCACTGACTAAGG3'-3'- and 3'- and containing the above substitutions (underlined) and a Narl restriction site (GGCGGCC), together with a sense primer, 5'-AGACACAGATGAG-ATGG-3'; this contained a BglII restriction site (AGATCT) (see Fig. 3A) (22). Partial deletions of sequences downstream of the PBS, i.e. HIV/LD1, HIV/LD2, and HIV/LD3, were generated as described (23).

In Vitro Reverse Transcription—These reactions were performed as described using RNA template that was generated through use of an Adenovirus M13-mp8 Plasmid Construction—DNA sequences at nt positions 473–1417 of HIV-1 (HxB2D) were cloned into the RNA expression vector pSP72 (Promega, Madison, WI) through use of BglII (473) and PstI (1412). The deletion of the A-rich loop (HIV/del-A) and the construction of mutation HIV/2A have been described previously (17, 21). We also engineered a substitutional mutation (HIV/HUA) by replacing the sequence 625A-ACTCTAGGCAG637 with 5'-GAACACCCAACTT-3'; this was done by PCR using an antisense primer 5'-CCCTGTCGGGGCGCAATGTGTTGGTTGTTGCTTCCCACTGACTAAGG3'-3'- and 3'- and containing the above substitutions (underlined) and a Narl restriction site (GGCGGCC), together with a sense primer, 5'-AGACACAGATGAG-ATGG-3'; this contained a BglII restriction site (AGATCT) (see Fig. 3A) (22). Partial deletions of sequences downstream of the PBS, i.e. HIV/LD1, HIV/LD2, and HIV/LD3, were generated as described (23).

In Vitro Reverse Transcription—These reactions were performed as described using RNA template that was generated through use of an Adenovirus M13-mp8
containing 83 mM Tris-HCl (pH 7.5) and 125 mM KCl. Reverse transcription reactions were performed in a volume of 20 µl containing 1 pmol of primer:RNA template complex, 50 mM Tris-HCl (pH 7.5), 75 mM KCl, 5 mM MgCl₂, 10 mM DTT, dNTPs, 20 units of RNA guard ribonuclease inhibitor (Amersham Pharmacia Biotech) and reverse transcriptase at 37 °C for various times, after which reactions were terminated by adding EDTA to a final concentration of 50 mM. In some experiments, reverse transcription reactions were performed in the presence of HIV-1 nucleocapsid (NC) protein (30 pmol for each reaction) as described (21). The cDNA products were fractionated on 8% denaturing polyacrylamide gels containing 7 M urea. The RT preparations used included wild-type HIV-1 enzyme (p66/51) prepared as described (25) and mutated HIV-1 RT containing a mutation at codon 478 (i.e. E478Q), responsible for defective RNase H activity (26).

RESULTS
The Initiation of HIV-1 (−) Strand DNA Synthesis from tRNALys.3 is Characterized by Rate-limiting Pause Sites at nt Positions +1 and +3—Initiation of HIV-1 reverse transcription is a distinct step from that of elongation (18–20). To further investigate this subject, we developed an in vitro reaction system that employed a PBS that contained only G, A, and C among the 5 nt at its 5′-end (Fig. 1A). Therefore, when dCTP, dTTP, and dGTP were included in these reactions, only early products of reverse transcription were generated. Reactions were also performed with only dCTP (Fig. 1A, lane 1) or both dCTP and dTTP (Fig. 1A, lane 2) to provide information on the positions of the first and second bands seen on gels. The results of Fig. 1A show that reaction products were observed at both the +1 and +3 positions in addition to a final expected product at the +5 site (lane 3). These reactions did not pause at either the +2 or +4 positions, indicating that they were rate-limiting only after the first and third nt were added. To prove that the above-mentioned pause sites (i.e. +1 and +3) were not due to an absence of dATP in the reactions, experiments performed with all four dNTPs were terminated at different times (5, 15, or 45 min), with the result that the +1 and +3 pause sites were still present (Fig. 1A, lanes 4, 5, and 6).

We next observed that the +1 and +3 pause sites were also present when increased concentrations of dNTPs, i.e. 80 nM, 160 nM, 320 nM, 640 nM, 1.28 µM, and 2.56 µM were employed in reactions that yielded higher levels of (−) strand DNA products (Fig. 1B). However, the +1 nt pausing site became increasingly faint with addition of higher concentrations of dNTP, whereas the +3 nt pause site did not diminish in intensity; this suggests that both pause sites are integral features of HIV-1 reverse transcription reactions and are seen most clearly in reactions performed at dNTP concentrations of 160 nM (Fig. 1B, lane 2).

The +3 Pause Site Is Dependent on Sequences at the 5′-End but not the 3′-End of the PBS—To study the role of sequences at the 5′-end of the PBS, we generated a mutated RNA template termed HIV/del-A that contained a deletion of the A-rich loop (462AAA A625) and a mutated RNA template HIV/HUA described above (Fig. 2A). The latter construct contained only T, A, and C within the 15 nt at the 5′-end of the PBS; hence, when only dATP, dTTP, and dGTP are included in reactions with the HIV/HUA template, extension should only proceed to the +13 stage. In reactions performed with 45 ng of HIV-1 RT, tRNALys.3 primer, and wt RNA genome, i.e. HIV/WT, three bands at positions +1, +3, and +5 were clearly observed (Fig. 2A, lane 9). However, when either HIV/del-A or HIV/HUA served as RNA template, only one band at position +1 was seen, even when three different dNTPs were included (lanes 1–6); furthermore, this band was weaker than that seen at the same position with the wt RNA template HIV/WT. Considering the (−) strand DNA products at the +3 and +5 positions in the case of wt RNA template HIV/WT, deletion of the A-rich loop or substitutions within the region nt 624–635 led to both greatly diminished efficiency of initiation, as well as an arrest of extension at the +1 nt position.

To investigate the effects of HIV/del-A and HIV/HUA on the +3 pausing event, higher quantities of RT, i.e. 405 ng, were used to extend reactions beyond the +1 stage. In this circumstance, extended products (both +3 and +5) were observed in reactions performed with the HIV/del-A mutated RNA template (Fig. 2B, lanes 1–3). However, when HIV/HUA was used, we detected extended products at the +5 and +13 sites and no

![Fig. 1. Synthesis of HIV-1 (−) strand DNA from primer tRNALys.3 at a dNTP concentration of 160 nt. Bands are labeled in respect to number of nucleotides extended from the 3′-end of the tRNALys.3 primer. A, reactions performed with wild-type HIV-1 RNA template (HIV/WT). Lanes 1–3, reactions performed with 405 ng of HIV-1 RT (p68/51) at 37 °C for 15 min with dCTP (α-32P) only (lane 1); both dCTP (α-32P) and dTTP (lane 2); and dCTP (α-32P), dTTP, and dGTP (lane 3). Lanes 4–6, reactions performed with 45 ng of HIV-1 RT (p68/51) and all four dNTPs (i.e. dCTP (α-32P), dTTP, dCTP, and dATP) for 5 min (lane 4), 15 min (lane 5), and 45 min (lane 6). B, reactions performed with 45 ng of HIV-1 RT and different concentrations of dNTPs (including dCTP (α-32P), dTTP, and dGTP) at 37 °C for 15 min.](image-url)
pausing at the +3 site (lanes 4–6). Therefore, the presence of the A-rich loop (622–625) and maintenance of sequences at positions 624–635 are necessary for a release from the +1 nt pause site to occur; in addition, the strong pausing at the +3 position is dependent on nt sequences 624–635 at the 5'-end of the PBS.

We also investigated the 3'-end of the PBS in the initiation of (−) strand DNA synthesis through use of appropriate deleted DNA templates, i.e., HIV/LD1, HIV/LD2, and HIV/LD3, containing deletions at wt positions 654–671, 672–691, and 692–707, respectively (Fig. 3). When tRNALys.3 was used as primer, similar band patterns were observed in reactions that used either wt RNA template (HIV/WT) or the mutated RNA templates (HIV/LD1, HIV/LD2, and HIV/LD3), although reactions proceeded less efficiently with the latter constructs (Fig. 3).

Reverse Transcription Does Not Pause at the +1 Site when Initiated from a DNA Primer—As shown above, reverse transcription initiated from tRNALys.3 still paused at the +1 position, even when sequences at both the 5'- and 3'-ends of the PBS were changed. When the first nt at the 5'-end of the PBS was changed from G to T (i.e. template HIV/WT), the addition of the first dATP still represented a rate-limiting step (Fig. 2A, lanes 1–3, 4–6, 7–9, and 10–12). To study whether this was unique to RNA primers, reactions were also performed with an 18nt DNA primer, i.e. D-Lys.3, bound to the PBS.

When only dCTP was included in the reaction mixture, only one band was seen at position +1 (Fig. 4A, lane 1). When both dCTP and dTTP were present, a band corresponding to nt position +2 was observed, and that at the +1 position had disappeared (lane 2). When dCTP, dTTP, and dGTP were included, both bands corresponding to the +3 and +5 nt pause sites were observed (lane 3). When all four dNTPs were present, extension occurred to beyond the +5 position, although a strong pause site at nt position +3 was still present, regardless whether reactions were run for 5, 15, or 45 min (Fig. 4A, lanes 4, 5, and 6). Thus, when a DNA oligomer was utilized as primer, reactions did not pause at the +1 site, but they did pause at position +3.

Wild-type HIV-1 RT (p66/51) possesses RNase H activity. Because the latter might interfere in assays that used a DNA primer, experiments were performed with a mutant RT (i.e. E478Q RT), defective in RNase H activity. The results of Fig. 4B show that reactions performed with this enzyme (i.e. RNase H-) did not pause at the +1 site, although pausing still occurred at the +3 position (lane 3). When all four dNTPs were included, further extension of (−) strand DNA was observed. However, reactions were still partially arrested at the +3 position, even

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Fig. 2. Reverse transcription reactions performed with mutated (HIV/del-A and HIV/HUA) or wild-type (HIV/WT) RNA templates at 37 °C in presence of 160 nM dNTPs. The positions of pause sites are labeled with respect to number of nt extended from the 3'-end of the primer. A, the mutated RNA templates (HIV/del-A and HIV/HUA) are illustrated; the deletion of the A-rich loop is indicated by the dashed line, and the substituted nt in HIV/HUA are underlined. Reactions were performed with tRNALys.3 primer using 45 ng of HIV-1 RT (p66/51) at 37 °C for 15 min at 160 nM dNTPs. The mutated RNA templates are illustrated, including HIV/LD1, deleted of sequences 654–671, HIV/LD2, deleted of sequences 672–691, and HIV/LD3, deleted of sequences 692–707. Reactions with mutated RNA templates HIV/LD1, HIV/LD2, HIV/LD3 and wild-type template HIV/WT are shown in lanes 1–3, 4–6, 7–9, and 10–12, respectively. Reactions were performed with only dCTP (α-32P), dTTP (α-32P), dATP (α-32P), or dGTP (α-32P) (lanes 1, 4, 7, and 10), both dCTP (α-32P) and dTTP (lanes 2, 5, 8, and 11), or each of dCTP (α-32P), dTTP, and dGTP (lanes 3, 6, 9, and 12).

Fig. 3. Effects of sequences at the 3'-end of the PBS on initiation of (−) strand DNA synthesis. Reactions were performed with tRNALys.3 primer and mutated RNA templates (HIV/LD1, HIV/LD2, and HIV/LD3) or wild-type (HIV/WT) template using 45 ng of HIV-1 RT (p66/51) at 37 °C for 15 min at 160 nM dNTPs. The mutated RNA templates are illustrated, including HIV/LD1, deleted of sequences 654–671, HIV/LD2, deleted of sequences 672–691, and HIV/LD3, deleted of sequences 692–707. Reactions with mutated RNA templates HIV/LD1, HIV/LD2, HIV/LD3 and wild-type template HIV/WT are shown in lanes 1–3, 4–6, 7–9, and 10–12, respectively. Reactions were performed with only dCTP (α-32P) (lanes 1, 4, 7, and 10), both dCTP (α-32P) and dTTP (lanes 2, 5, 8, and 11), or each of dCTP (α-32P), dTTP, and dGTP (lanes 3, 6, 9, and 12).
over periods as long as 45 min (lanes 4–6). Therefore, RNase H activity was not responsible for reaction release after addition of the first nt when a DNA primer was used.

As a control for our experiments with the 18 nt DNA primer, we also employed an 18 nt RNA primer complementary to the PBS. Consistent with results obtained with tRNALys.3 as primer, we found that these reactions also paused at the +1 nt position (data not shown). Hence, the +1 nt pausing event is associated with use of RNA primers during initiation of synthesis of (−) strand DNA.

As stated, the +13 pause site was dependent on the nature of the template but not the primer (i.e., DNA or RNA) used in these reactions. To further verify such template dependence, experiments were performed with the DNA primer d-Lys.3 and the mutated viral RNA templates HIV/del-A and HIV/HUA. The results of Fig. 5 show that extension from the DNA primer occurred normally in reactions performed with either wt (HIV/WT) or the mutated (HIV/del-A, HIV/HUA) RNA templates. However, when HIV/HUA was used, no strong pausing was observed at the +1 nt position (lane 6), consistent with results obtained with the cognate primer, tRNALys.3 (Fig. 2B).

NC Protein Helps Reverse Transcription Escape from the +1 Pausing Event—To study whether NC protein could affect the +1 and +3 pausing events in reverse transcription reactions primed with tRNALys.3, 30 pmol of NC protein was added to our 20-μl reaction system, and reactions were terminated at various times (1, 4, 16, 32, and 64 min) (Fig. 6, lanes 1–5). A similar time-course without NC protein was also performed as a control (Fig. 6, lanes 6–10). The data show that inclusion of NC resulted in significantly less pausing at the +1 nt position and did not affect the +3 nt pause site. Furthermore, more +5 nt product was generated in the presence of NC protein, suggesting that NC had contributed to more efficient elongation of reverse transcription.

![Fig. 4](https://example.com/fig4.jpg)

**Fig. 4.** Reverse transcription reactions performed with a DNA primer, d-Lys.3, and wild-type template (HIV/WT) in the presence of 160 nm dNTPs. A, reactions were run with 45 ng of HIV-1 RT at 37 °C. Lanes 1–3, reactions were performed for 15 min with only dCTP (α32P) (lane 1); both dCTP (α32P) and dTTP (lane 2); and dCTP (α32P), dTTP, and dGTP (lane 3). Lanes 4–6, reactions performed with all four dNTPs (i.e., dCTP (α32P), dTTP, dGTP, and dATP) for 5 min (lane 4), 15 min (lane 5), and 45 min (lane 6). B, reactions performed with mutated HIV-1 RT (45 ng) (RNase H−). Lanes 1–6, same order of reactions as in A.

![Fig. 5](https://example.com/fig5.jpg)

**Fig. 5.** Reverse transcription reactions (45 ng of HIV-1 RT) were primed with d-Lys.3 DNA using either mutated (HIV/del-A or HIV/HUA) or wild-type (HIV/WT) RNA as a template at 37 °C in the presence of 160 nm dNTPs and were run for 15 min. The order of lanes 1–9 is the same as that of Fig. 2A.
Early Pause Sites in HIV Reverse Transcription

DISCUSSION

Biochemical analysis has shown that the initiation of HIV-1 reverse transcription can be distinguished from subsequent elongation (18–20). The biological relevance of this observation is suggested by the fact that RT can lose its ability to discriminate against a nonself tRNA primer when the latter was extended by two nt (27). Second, in vitro labeling revealed that primer tRNALys3 was extended by two nt within virus particles that had engaged in synthesis of (−) strand DNA (28). In our system, initiation has been characterized on the basis of several early pause sites (e.g. +1 and +5) that add substantially to our understanding of early events in reverse transcription.

This is the first demonstration that pausing at the +1 nt site represents a rate-limiting step in reverse transcription reactions performed with an HIV-1 RNA template and the cognate primer tRNALys3. This is not an unexpected finding because HIV-1 RT possesses both RNA-dependent DNA polymerization and DNA-dependent DNA polymerization activities. During reverse transcription, the enzyme is bound to either RNA-DNA or DNA-DNA hybrids during RNA-dependent DNA polymerization and DNA-dependent DNA polymerization respectively, except at the initiation of synthesis of (−) strand DNA (−) ssDNA, when the enzyme is bound to a RNA-RNA hybrid and employs tRNALys3 as primer for production of cDNA. After initiation takes place, the role of primer is effectively replaced by the newly made DNA, from which further extension will occur (5). Therefore, the initiation of (−) ssDNA synthesis is a distinct stage of reverse transcription, especially in regard to incorporation of the first dNTP.

When the first C deoxyribonucleotide from the dNTP pool is added to the 3′-OH of the A ribonucleotide at the 3′-end of tRNALys3, displacement of a ribonucleotide-ribonucleotide pair (A-U) must occur in favor of a newly formed deoxyribonucleotide-ribonucleotide pair (dC-G) at the RT polymerization active site. Due to the absence of a 2′-OH residue in dC, the two
nucleotide pairs (A-U and dC-G) may assume different conformations. Therefore, a structural rearrangement of the polymerization active site is required for the RT enzyme to adapt to the new dC-G pair and to add the next deoxyribonucleotide (dT) to the one-base extended primer. It is generally believed that a conformational change of RT must precede the chemical step, resulting in a rate-limiting event (29–32). Our results show that this rate-limiting step results in the pause in the RT reaction without a change in conformation. Consequently, rearrangement of the first dC at the 3′-end of primer tRNALys.3 necessitates that the primer-DNA hybrid to which RT is bound will always be a DNA-RNA hybrid, thus permitting the enzyme to catalyze the extension of the new dC-G pair and to add the next deoxyribonucleotide (dT) to the nascent primer 3′ terminus. However, a distance of 19 nt instead of 18 nt was observed between these sites after incorporation of the first dC at the 3′-end of primer tRNALys.3. This suggests that the spatial relationship between the functional RNase H cleavage site and the nascent primer 3′ terminus is required for the RT enzyme to adapt to the 5′-end of the PBS, in which the three nt position may represent the actual point of transition from initiation to elongation, and the fourth nt (G) is a start site in the stem structure. Therefore, when reactions are extended to the third base, the hydrogen bonds between G-C must be disrupted before the fourth nucleotide (dC) can be incorporated. Consequently, these reactions must pause at the +3 nt position. Replacement of the sequence 5′-AAUUCUAGCCG-3′ at the 5′-end of the PBS with an irrelevant sequence, i.e., 5′-GAACACCGCAAU-3′ (i.e., HIV/HUA), results in disruption of the stem structure, eliminating the +3 nt pause site.

Our data add significantly to the notion that the initiation of HIV-1 reverse transcription represents a distinct phase in RT reactions (18–20). First, we have documented that a pause site is observed at +1 position in reactions using wild-type RNA template (HIV/WT). This result might also be attributable to a destabilization of the stem-loop caused by deletion of the A-rich loop, because mutations in this region can cause disturbances in secondary structure of complexes between tRNALys.3 and viral genomic RNA (34). Therefore, template structure may be responsible for the type of pausing seen at the +3 position during initiation of synthesis of (−) strand DNA.

Our data add significantly to the notion that the initiation of HIV-1 reverse transcription represents a distinct phase in RT reactions (18–20). First, we have documented that a pause site is observed at +1 position in reactions using wild-type RNA template (HIV/WT). This result might also be attributable to a destabilization of the stem-loop caused by deletion of the A-rich loop, because mutations in this region can cause disturbances in secondary structure of complexes between tRNALys.3 and viral genomic RNA (34). Therefore, template structure may be responsible for the type of pausing seen at the +3 position during initiation of synthesis of (−) strand DNA.

Second, the transition from initiation to elongation probably occurs at the +1 site as well as at the previously observed +3 and +5 positions. On the basis of our studies, the +3 pause site strongly depends on the secondary structure of the viral RNA template. Conceivably, the +1 position may represent the actual point of transition from initiation to elongation, and the +3 pause site may be involved in the arrest of reactions at other early stages. Finally, the A-rich loop, as well as other sequences at the 5′-end of the PBS, may be involved in both the efficiency of initiation as well as release from the +1 pause site.

The +1 and +3 pausing events in viral reverse transcription may play a regulatory role similar to that observed in the case
of E. coli RNA polymerase, for which initiation of gene transcription begins in an abortive mode and pauses near the start site, and for which a regulatory subunit, σ, is required for transcription to proceed to elongation (35, 36). In HIV-1, the NC protein may play a role analogous to that of the σ factor, because NC can help to overcome the +1 pausing event (Fig. 6). NC protein is also able to unwind the secondary structure of template RNA, facilitating the synthesis of long products of reverse transcription (37), and can stimulate the annealing of complementary RNA and/or DNA sequences to stabilize hybrids (38). We believe that NC helps to form and stabilize complexes between primer tRNALys.3 and viral genomic RNA (38). We believe that NC helps to form and stabilize complementary RNA and/or DNA sequences to stabilize hybrid RNA, facilitating the synthesis of long products of reverse transcription (37), and can stimulate the annealing of complementary RNA and/or DNA sequences to stabilize hybrids (38). We believe that NC helps to form and stabilize complexes between primer tRNALys.3 and viral genomic RNA (38).

This deficit in viral replication caused by deletion of the A-rich loop may have been partially compensated by elimination of the pause sites at positions +11 through +14.

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