In human immunodeficiency virus type 1 (HIV-1), the tRNA⁄Lys primer and viral RNA template can form a specific complex that is characterized by extensive inter- and intramolecular interactions. Initiation of reverse transcription from this complex has been shown to be distinguished from subsequent elongation by early pausing events, such as at the +1 and +3 nucleotide positions. One major concern regarding the biological relevance of these results is that most kinetic studies of HIV-1 reverse transcription have been performed using tRNA⁄vRNA complexes that were formed by heat annealing. In contrast, tRNA⁄vRNA in viruses is placed onto the primer binding site by nucleocapsid (NC) sequences of the Gag protein. In this study, we have further characterized the initiation features of reverse transcription in the presence of HIV-1 NC protein. In contrast to results obtained with a heat-annealed tRNA⁄vRNA complex, we found that polymerization reactions catalyzed by HIV-1 reverse transcriptase did not commonly pause at the +1 nucleotide position when a NC-annealed RNA complex was used, and that this was true regardless whether NC was actually still present during reverse transcription. This activity of NC required both zinc finger motifs, as demonstrated by experiments that employed zinc finger-mutated forms of NC protein (H23C NC and ddNC), supporting the involvement of the zinc fingers in the RNA chaperone activity of NC. However, NC was not able to help reverse transcriptase to escape the +3 pausing event. Mutagenesis of a stem structure within the tRNA⁄vRNA complex led to disappearance of the +3 pausing event as well as to significantly reduced rates of reverse transcription. Thus, this stem structure is essential for optimal reverse transcription, despite its role in promotion of the +3 pausing event.

* This work was supported by the Canadian Institutes for Health Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† This work was performed in partial fulfillment of the requirements for a Ph.D. degree, Faculty of Graduate Studies and Research, McGill University, Montreal, Canada.

‡‡ To whom correspondence should be addressed: Mcgill AIDS Centre, Lady Davis Institute-Jewish General Hospital, 3755 Cote Ste-Catherine Rd., Montreal, Quebec H3T 1E2, Canada. Tel.: 514-340-8260; Fax: 514-340-7537; E-mail: mark.wainberg@mcgill.ca

HIV-1 reverse transcription is initiated as its cognate primer tRNA⁄Lys is annealed onto the primer binding site (PBS) of viral RNA template (vRNA). In addition to base pairing between the PBS sequence and the 3′-terminal region of tRNA⁄Lys, the resulting tRNA⁄vRNA binary complex is also characterized by other extensive but specific inter- and intramolecular interactions (1–3). The initiation of (−) strand DNA synthesis from this complex represents a specific stage, during which reverse transcriptase (RT) shows distinct binding and kinetic properties from those of the subsequent elongation stage (4). Using an in vitro reaction system, initiation of reverse transcription can be detected by the formation of short intermediate cDNA products after the tRNA primer is extended by 1, 3, or 5 nt (4–7). However, in these studies, reverse transcription had been performed with tRNA⁄vRNA complexes that were formed by heat annealing. In the virus, however, the tRNA primer is placed onto the viral RNA template by nucleocapsid (NC) sequences of the Gag protein. Therefore, it is important to compare the kinetic features of different RNA complexes that have been formed by either heat or NC annealing.

The HIV-1 NC is a small basic protein that can bind to single-stranded nucleic acid. It has two zinc finger motifs, each of which contains zinc ion binding residues, i.e. CCHC, and forms a rigid loop within the protein (8). NC protein exhibits nucleic acid chaperone activity in vitro; it catalyzes the rearrangement of nucleic acid molecules into a thermodynamically stable conformation (9, 10). This activity enables NC to participate in reverse transcription at many steps and ensures that highly specific and efficient viral cDNA synthesis will occur. For example, the overall efficiency of negative strand strong-stop DNA (−ssDNA) synthesis can be increased in the presence of NC. NC was found to function in this process in two manners, i.e. by facilitating the formation of an active form of tRNA⁄vRNA complex (11), or by transiently eliminating template secondary structures, at which the RT enzyme stalls (12–15).

To assess the roles of NC protein in initiation of reverse transcription, we employed NC to perform primer placement, and then removed it from the system by proteinase K digestion and phenol:chloroform treatment. This system allows reverse

The abbreviations used are: HIV, human immunodeficiency virus; PBS, phosphate-buffered saline; nt, nucleotide(s); RT, reverse transcriptase; vRNA, viral RNA; ssDNA, strong-stop DNA; NC, nucleocapsid; HUA, HIV-1 upstream A-rich sequence.
transcription to proceed from a NC-derived tRNA<sup>Lys<sub>3</sub></sup>-vRNA complex, while avoiding the direct involvement of NC in the reactions at the same time. These results showed that the tRNA<sup>Lys<sub>3</sub></sup>-vRNA complex that is formed by NC is already active, and can overcome +1 nt pausing during subsequent polymerization without the further involvement of NC. Therefore, NC, acting as an RNA chaperone, assists in the formation of a more functional RNA complex rather than a more thermostable one, as is obtained by heat annealing of tRNA primer onto the PBS. The zinc finger motifs of NC were found to be important in this regard, as indicated by experiments using H23C NC, which includes a point mutation within the first zinc finger structure, and ddNC, in which both zinc fingers are replaced by Gly-Gly linkages.

We further studied the causes of the +3 pausing event either with or without NC protein in the reactions. To pursue this subject, systematic mutagenesis studies were performed to disrupt a stem structure that is formed by RNA template sequences within the tRNA<sup>Lys<sub>3</sub></sup>-vRNA complex. Finally, we have compared early pausing patterns of reverse transcription from tRNA<sup>Lys<sub>3</sub></sup>-vRNA complexes derived by heat annealing, NC-mediated annealing, and within virions. Our results show that, in contrast to the tRNA<sup>Lys<sub>3</sub></sup>-vRNA complexes formed by heat annealing, the RNA complexes that are either derived from virions or formed by NC annealing barely pause at the +1 stage during initiation of reverse transcription.

MATERIALS AND METHODS

Construction of Plasmids—The primers used in our mutagenesis studies are listed in Table I. The mutated HIV-1 RNA templates designated N1-N10 were generated by PCR using primer pairs Bgl-S/Nar-A1 to Nar-A10. After digestion with BglII and NarI, the PCR products were inserted into an RNA transcription vector PBS/wild-type HIV-1, which includes a point mutation within the first zinc finger structure, and ddNC, in which both zinc fingers are replaced by Gly-Gly linkages.

Western Blot—Various concentrations of NC were used in combination with 1 pmol of template RNA and 1 pmol of primer tRNA<sup>Lys<sub>3</sub></sup> at 37°C for 1 h to promote annealing. Regardless of whether samples were subjected to proteinase K and phenol:chloroform treatment, they were fractionated on 8% denaturing polyacrylamide gels containing 7 M urea. The RTs used in this study were prepared as described (20) and included wild-type HIV-1 enzyme (p66/51), mutated HIV-1 RT containing a mutation at codon 89 (i.e. E89G), or mutated HIV-1 RT containing a mutation at codon 184 (i.e. M184V). An amount of 45 ng of wild-type RT or E89G RT was used in these reactions unless specified. Because M184V RT displays reduced processivity during reverse transcription (21, 22), a higher amount, i.e. 250 ng of M184V RT, was employed to achieve levels of cDNA synthesis similar to those attained with wild-type enzyme (data not shown).

RESULTS

A Role of NC Protein during tRNA<sup>Lys<sub>3</sub></sup>-vRNA Complex Formation—1 pmol of tRNA<sup>Lys<sub>3</sub></sup> that was prepared from human placenta (18) was annealed onto 1 pmol of RNA template by incubation at 37°C for 1 h with a calculated saturating level of NC (i.e. 30 pmol, such that the molar ratio of NC and total number of nucleotides in the template was about 1:8, based on a template of 251 nt in length) in a 10-μl reaction mixture containing 50 mM Tris-HCl (pH 7.2), 50 mM KC1, 5 mM MgCl<sub>2</sub>. Alternatively, the placement of tRNA<sup>Lys<sub>3</sub></sup> onto template RNA was performed using the same buffer conditions by denaturation of RNA molecules at 85°C for 5 min and annealing at 55°C for 10 min. To determine whether NC protein functioned during initiation of reverse transcription at the primer placement or at the primer extension phase, proteinase K digestion and phenol:chloroform extraction were performed in some cases (11). In the case of RNA isolated from virions, tRNA<sup>Lys<sub>3</sub></sup> is already naturally annealed onto the viral RNA template (19); therefore, these tRNA<sup>Lys<sub>3</sub></sup>-vRNA complexes were directly subjected to the following in vitro reverse transcription reactions.

In Vitro Reverse Transcription—1 pmol of tRNA<sup>Lys<sub>3</sub></sup> that was prepared from human placenta (18) was annealed onto 1 pmol of RNA template by incubation at 37°C for 1 h with a calculated saturating level of NC (i.e. 30 pmol, such that the molar ratio of NC and total number of nucleotides in the template was about 1:8, based on a template of 251 nt in length) in a 10-μl reaction mixture containing 50 mM Tris-HCl (pH 7.2), 50 mM KC1, 5 mM MgCl<sub>2</sub>. Alternatively, the placement of tRNA<sup>Lys<sub>3</sub></sup> onto template RNA was performed using the same buffer conditions by denaturation of RNA molecules at 85°C for 5 min and annealing at 55°C for 10 min. To determine whether NC protein functioned during initiation of reverse transcription at the primer placement or at the primer extension phase, proteinase K digestion and phenol:chloroform extraction were performed in some cases (11). In the case of RNA isolated from virions, tRNA<sup>Lys<sub>3</sub></sup> is already naturally annealed onto the viral RNA template (19); therefore, these tRNA<sup>Lys<sub>3</sub></sup>-vRNA complexes were directly subjected to the following in vitro reverse transcription reactions.

Primer tRNA<sup>Lys<sub>3</sub></sup> was extended by reverse transcriptase in a volume of 20 μl containing 50 mM Tris-HCl (pH 7.2), 50 mM KC1, 5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 10 units of RNA-guard (Amersham Pharmacia Biotech, Montreal, Quebec, Canada), and 160 nM dNTPs at 37°C for 15 min, unless otherwise specified; thereafter, reverse transcription reactions were terminated by adding EDTA (pH 8.0) to a final concentration of 50 mM. The DNA products were fractionated on 8% denaturing polyacrylamide gels containing 7 M urea. The RTs used in this study were prepared as described (20) and included wild-type HIV-1 enzyme (p66/51), mutated HIV-1 RT containing a mutation at codon 89 (i.e. E89G), or mutated HIV-1 RT containing a mutation at codon 184 (i.e. M184V). An amount of 45 ng of wild-type RT or E89G RT was used in these reactions unless specified. Because M184V RT displays reduced processivity during reverse transcription (21, 22), a higher amount, i.e. 250 ng of M184V RT, was employed to achieve levels of cDNA synthesis similar to those attained with wild-type enzyme (data not shown).
Bands are labeled in respect to numbers of nucleotides extended from transcription reactions in these cases were initiated in the absence of NC. The results show that neither H23C NC nor ddNC was able to promote the formation of an active tRNA^{Lys,3}–vRNA complex, which was achieved by heat annealing (lanes 1–5) or by incubation with synthetic HIV-1 nucleocapsid protein (lanes 11–20), as described under “Materials and Methods.” As control, same amount of tRNA^{Lys,3} and RNA template were also incubated in the absence of NC at 37 °C for 1 h (lanes 6–10). Reverse transcription reactions were performed with 45 ng of HIV-1 RT (p66/51) and 160 nm [α-32P]dCTP, dTTP, and dGTP, and were terminated at 1 min (lanes 1, 6, 11, and 16), 4 min (lanes 2, 7, 12, and 17), 16 min (lanes 3, 8, 13, and 18), 32 min (lanes 4, 9, 14, and 19), and 64 min (lanes 5, 10, 15, and 20), respectively. Lanes 16–20, NC proteins were removed after primer placement; therefore, reverse transcription reactions in these cases were initiated in the absence of NC. Bands are labeled in respect to numbers of nucleotides extended from the primer. This experiment was repeated at least three times with similar results, and a representative experiment is shown in the figure.

Fig. 1. Initiation of reverse transcription from NC-annealed tRNA^{Lys,3} primer. tRNA^{Lys,3}–wild-type template RNA complexes were achieved either by heat annealing (lanes 1–5) or by incubation with synthetic HIV-1 nucleocapsid protein (lanes 11–20), as described under “Materials and Methods.” As control, same amount of tRNA^{Lys,3} and RNA template were also incubated in the absence of NC at 37 °C for 1 h (lanes 6–10). Reverse transcription reactions were performed with 45 ng of HIV-1 RT (p66/51) and 160 nm [α-32P]dCTP, dTTP, and dGTP, and were terminated at 1 min (lanes 1, 6, 11, and 16), 4 min (lanes 2, 7, 12, and 17), 16 min (lanes 3, 8, 13, and 18), 32 min (lanes 4, 9, 14, and 19), and 64 min (lanes 5, 10, 15, and 20), respectively. Lanes 16–20, NC proteins were removed after primer placement; therefore, reverse transcription reactions in these cases were initiated in the absence of NC. Bands are labeled in respect to numbers of nucleotides extended from the primer. This experiment was repeated at least three times with similar results, and a representative experiment is shown in the figure.

The presence of NC cannot help RT to overcome the +3 pause event. Quantification of Fig. 1A shows that fewer +1 and more +5 nt products were produced when RT reactions were incubated for longer periods, whereas the proportion of +3 nt products remained relatively constant during the time course (Fig. 1B). The presence of NC during the reverse transcription reactions was able to decrease the average percentage of the +3 nt products from 60% to 50%; however, NC did not facilitate escape from the +3 nt pause event as it did for pausing at the +1 nt position (Fig. 1B). This observation suggests that the +3 nt pause during initiation of reverse transcription is biologically relevant.

To confirm that NC protein had indeed been eliminated from our reverse transcription reactions as described above, we used anti-HIV-NC monoclonal antibody to analyze NC proteins after incubation with tRNA and RNA template and subsequent proteinase K and phenol:chloroform treatment. As shown in Fig. 2, no NC protein was detected in Western blots after Proteinase K digestion and extraction. The sensitivity of the blotting is shown by a control experiment in which 3 pmol of NC protein were loaded directly onto the gel. Previous results had shown that this concentration of NC protein did not affect reverse transcription (11).

The Role of NC Protein in Initiation of Reverse Transcription Is Dependent on Intact Zinc Finger Motifs—To assess the potential roles of zinc finger motifs in the formation of active tRNA^{Lys,3}–vRNA complex, we next examined two mutated NC proteins in reactions designed to study the initiation of reverse transcription. One is H23C NC, in which a His at the 23 position has been substituted by a Cys; the other is ddNC, in which both of zinc fingers have been replaced by Gly-Gly linkages. Primer tRNA was placed onto viral RNA template through use of various concentrations of NC protein, i.e. 5, 15, 30, and 45 pmol. Thereafter, three of four dNTPs, i.e. dCTP, dTTP, and dGTP, were added to initiate reverse transcription. The results show that neither H23C NC nor ddNC was able to achieve release from the +1 pause site in these reactions (Fig. 3A, lanes 5–12), and the average ratio of the amount of +1 pausing products to total paused products was 50% in both of these cases, which is higher than the 20% average achieved through use of wild-type NC protein (Fig. 3B). It should be noted that the reactions performed with ddNC yielded a greater number of initiation intermediate products than did reactions performed with wild-type NC. Different types of preparation of NC proteins, including both wild-type and mutated varieties, were used in these experiments and gave rise to similar results each time. Therefore, wild-type zinc fingers are important in order for NC to promote the formation of an active tRNA^{Lys,3}–vRNA initiation complex.

The +3 nt Pausing Event Is Caused by a Viral RNA Template Stem Structure Located Upstream of the PBS within the tRNA^{Lys,3}–vRNA Complex—As stated above, the +3 nt pausing event...
FIG. 3. Initiation of reverse transcription from tRNA\(^{\text{Lys}}\)\(^{\text{3}}\)-vRNA complexes formed by zinc finger mutated NC proteins. Primer-template complexes were achieved by wild-type NC (lanes 1–4), H23C NC (lanes 5–8), and ddNC (lanes 9–12), as described under “Materials and Methods.” The quantities of NC proteins involved in each reaction were 5 pmol (lanes 1, 5, and 9), 15 pmol (lanes 2, 6, and 10), 30 pmol (lanes 3, 7, and 11), and 45 pmol (lanes 4, 8, and 12), respectively. The heat annealing method was used as a control, and these results are presented in the left lane, which is labeled as H. The initiation complexes were then incubated with 160 nM [\(^{\text{32}}\)P]dCTP, dTTP, and dGTP and 45 ng of HIV-1 RT (p66/51) at 37 °C for 15 min. Intermediate cDNA products were fractionated on gels, and the bands are labeled in respect to numbers of nucleotides extended from tRNALys.3. This experiment was repeated at least three times with similar results, and a representative experiment is shown in the figure.

We also generated N7 and N8, in which the distance between the stem structure and the PBS was increased by the insertion of CAGs. The structures and sequences of these mutations are shown in Fig. 4 (A and B), respectively.

In the control experiment, wild-type RNA templates were annealed with tRNA\(^{\text{Lys}}\)\(^{\text{3}}\) by heat annealing, following which one (i.e. dCTP), two (i.e. dCTP, dTTP), three (i.e. dCTP, dTTP, dGTP), or all four of the dNTPs were added. As shown in Fig. 5 (A (lanes 1–4), B, E, and F), the incorporation of one or two dNTPs yielded products at the +1 nt or both the +1 and +2 nt positions. In the presence of each of dCTP, dTTP, and dGTP, pausing at both the +1 and +3 nt positions was seen in addition to the expected five-base extended product. The presence of all three intermediate products, seen with the addition of all four dNTPs, indicates that their presence in lane 3 was not caused by the absence of dATP. Longer cDNA products in lane 4 were labeled at positions +27, +28, and +40 nt. Because low concentrations of dNTPs (i.e. 160 nm) were used in these reactions, it was difficult for longer reverse transcription products, i.e. > +40 nt, to be seen on the gel.

In the case of N1, the pausing site at the +3 nt position disappeared (Fig. 5A, lanes 5 and 6), whereas pausing at the +5 nt site was diminished but still evident; at the same time, a greater number of longer cDNA products (e.g. +27, +28, and +40 nt) were produced as compared with reactions performed with wild-type template. Even stronger perturbation of the stem structure by the N2 mutation caused both a significantly decreased efficiency of initiation as well as a transition from an early to a late phase of initiation, i.e. only a one-base extension product is seen in lanes 7 and 8. To investigate the effects of N2 on the +3 pausing event, higher quantities of RT, i.e. 405 ng, were used to extend reactions beyond the +1 stage. Under this circumstance, we only detected an extended product at the +5 site (lane 9) or at higher positions (lane 10) as expected; no pausing was observed at the +3 site.
Fig. 5. A, initiation of reverse transcription with mutated N1 and N2 RNA templates. Lanes 1–4 represent experiments performed with wild-type RNA template; lanes 5 and 6 are reactions using N1 template; lanes 7–10 are reactions using N2 mutated template. The heat-annealed tRNA^Lys^3-vRNA complexes were incubated with 45 ng, (lanes 1–8) or 405 ng (lanes 9 and 10) of HIV-1 RT (p68/51) at 37 °C for 15 min, in the presence of [α-32P]dCTP only (lane 1); both [α-32P]dCTP and dTTP (lane 2); [α-32P]dCTP, dTTP, and dGTP (lanes 3, 5, 7); and all four dNTPs (lanes 4, 6, and 8). The final concentration of each of the dNTPs was 160 nM. B, initiation of reverse transcription with mutated N3 and N4 RNA templates. The order of lanes 1–8 is the same as that of A, except that lanes 5 and 6 are reactions that employed N3 template; lanes 7 and 8 are reactions with N4 mutated template. C, initiation of reverse transcription of N4 with E89G mutated RT. The RNA template used in this experiment is N4, and the RTs utilized include 45 ng of wild-type RT (p51/p66) (lanes 1–4) and 45 ng of E89G RT (lanes 5–8). D, initiation of reverse transcription of N4 with M184V mutated RT. The RNA template used in this experiment is N4, and the RTs utilized include 45 ng of wild-type RT (p51/p66) (lanes 1–4) and 250 ng of M184V RT (lanes 5–8). E, initiation of reverse transcription with mutated N5 and N6 RNA templates. The order of lanes 5–8 is the same as that of A, except that lanes 5 and 6 are reactions that used N5 template; lanes 7 and 8 are reactions that used N6 mutated template. F, initiation of reverse transcription with mutated N7 and N8 RNA templates. The order of lanes 1–10 is the same as that of A, except that lanes 5 and 6 are reactions with N7 template; lanes 7–10 are reactions with N8 template.

The N3 and N4 mutations each gave rise to a relatively complicated pausing pattern in initiation reactions. When all four dNTPs were present in these reactions, neither the +3 nt nor the +5 nt products were seen on the gel (Fig. 5B, lanes 6 and 8). When only three dNTPs (i.e. dCTP, dGTP, dTTP) were added, pausing at the +3 nt site disappeared as well. However, cDNA products longer than +16 nt were observed alongside the expected +5 and +7 nt products (Fig. 5B, lanes 5 and 7). Therefore, nucleotide misincorporation and elongation from the misincorporated nucleotide must have occurred at template sequences wherever a U was met. This result was confirmed by use of two mutated forms of RT associated with higher than average base incorporation fidelity, i.e. E89G and M184V (33–35). During reverse transcription of the N4 RNA template, nucleotide misincorporation was either diminished or eliminated in reactions performed with either the E89G or M184V RT (Fig. 5, C and D).

Fig. 5E shows the experiment performed with the N5 and N6 group. Because the stem structure was preserved, the specific intermediate initiation product at the +3 nt position was not affected (lanes 5–8). The efficiency of initiation in reactions performed with these mutated RNA templates was also near wild-type levels. However, this group of mutations significantly diminished the efficiency of the switch from the +3 to the +5 stage; cDNA products beyond +3 nt can hardly be seen on the gel.

In the case of N7, only one CAG repeat was inserted, and the pausing event at the +3 nt position, associated with wild-type template, moved to a higher position at +6 nt (Fig. 5F, lanes 5 and 6). When more insertions were introduced into the template, e.g. N8, the reaction became defective at the initiation stage. As shown in Fig. 5F (lanes 7 and 8), the reactions were arrested after the incorporation of the first nucleotide. We next used higher concentrations of RT (i.e. 405 ng) in reactions performed with N8. Pausing at the +5, +9, and +11 nt positions can be observed when three dNTPs were added into the reactions (lane 9); these represent a common pause position (i.e. +5), the nt position just prior to the stem structure (i.e. +9), and the longest extended position that can be achieved with all three dNTPs (i.e. +11), respectively. When all four dNTPs were present in these reactions, reverse transcription was able to proceed beyond the +11 nt position (lane 10).

Because N3 and N4 included mutations in the right half of the stem, the specific pausing patterns observed may conceivably have been the result of either the altered secondary structures established or, alternatively, of modified sequences. To sort out these possibilities, two additional mutations, i.e. N9 and N10, were constructed to restore the stem base pairing that had been disrupted by N3 and N4 by introducing second site mutations into the left half of the stem of the N3 and N4 constructs. Thus, N9 and N10 keep modified sequences in the right half of the stem, similar to those of N3 and N4, while also maintaining an intact stem structure (Fig. 6, A and B). Reactions employing these two new mutations are shown in Fig. 6C. Strong pausing at the +3 nt site that was eliminated in experiments performed with the N3 and N4 constructs was reestablished when the N9 and N10 templates were used. Thus, the initiation of reverse transcription involves pausing at the +3 nt site when the RNA template used contains an intact stem structure at the 4th nt upstream of the PBS, regardless of whether the stem was formed by wild-type or exogenous sequences.

Because NC protein does not release the pause event at the +3 nt position, we mainly used heat annealing in the above in vitro reverse transcription studies. As a control, we also employed NC protein to place tRNA^Lys^3 primer onto the N1–N8 templates, and then checked for the initiation of reverse transcription from these binary RNA complexes. As shown in Fig. 7, all reactions exhibited similar pausing patterns as those performed by the heat annealing method, except that pausing bands at the +1 site were markedly reduced.

Initiation of Reverse Transcription from the Virion-derived tRNA^Lys^3-vRNA Complex—The N1, N3, N4, N5, and N7 mutations were inserted into BH10 infectious HIV-1 cDNA to assess their effects on initiation of reverse transcription of the tRNA^Lys^3-vRNA complex in the virus. COS-7 cells were trans-
HIV-1 Nucleocapsid Protein and tRNA<sup>Lys.3</sup>-vRNA Complex

Initiation of reverse transcription from virion-derived tRNA<sup>Lys.3</sup>-vRNA complexes. Viral RNAs were isolated as described under “Materials and Methods” from viral pellets containing 200 ng of CA-p24 Ag. As controls, 1 pmol of tRNA<sup>Lys.3</sup> was annealed with 1 pmol of wild-type synthetic vRNA by either the heat annealing method (H1, H2, H3) or by 30 pmol of NC (NC). The primer tRNA was then extended using 45 ng of RT (p51/p66) in the presence of 160 nM [α-<sup>32</sup>P]dCTP and dTTP, or [α-<sup>32</sup>P]dCTP, dTTP, and dGTP (H3, NC, N1, N3, N4, N5, N7), respectively. This experiment was repeated at least three times with similar results, and a representative experiment is shown here.

RESULTS

FIG. 8. Initiation of reverse transcription from virion-derived tRNA<sup>Lys.3</sup>-vRNA complexes. Viral RNAs were isolated as described under “Materials and Methods” from viral pellets containing 200 ng of CA-p24 Ag. As controls, 1 pmol of tRNA<sup>Lys.3</sup> was annealed with 1 pmol of wild-type synthetic vRNA by either the heat annealing method (H1, H2, H3) or by 30 pmol of NC (NC). The primer tRNA was then extended using 45 ng of RT (p51/p66) in the presence of 160 nM [α-<sup>32</sup>P]dCTP and dTTP, or [α-<sup>32</sup>P]dCTP, dTTP, and dGTP (H3, NC, N1, N3, N4, N5, N7), respectively. This experiment was repeated at least three times with similar results, and a representative experiment is shown here.

or N7 mutated viruses. These results further support the conclusion that the +3 pausing event during reverse transcription is caused by a stem structure present in the tRNA<sup>Lys.3</sup>-vRNA complex.

We also observed that none of the reactions that employed RNA complexes derived from virus particles paused at the +1 stage; moreover, much weaker pausing occurred at the +3 nt stage in reactions performed with wild-type virus-derived RNA complex than in those formed by either heat or NC annealing. Because all sorts of RNA complexes in this experiment were subjected to the same in vitro reverse transcription reaction conditions, the different pausing patterns obtained can only be attributed to the different conformations of the complexes used to initiate the reactions. In general, the NC-annealed RNA complex acts in a manner more similar to that of RNA complexes derived from virus particles than to those formed by heat annealing. Of course, either structural or functional differences may still remain between the tRNA<sup>Lys.3</sup>-vRNA complexes that are derived from virions or formed by NC annealing in cell-free assays.

DISCUSSION

HIV-1 NC is a small basic protein, containing two zinc finger motifs of the CX<sub>2</sub>CX<sub>2</sub>H<sub>2</sub>C<sub>2</sub> form. NC has been demonstrated to function as an RNA-chaperone protein in a wide variety of cell-free systems. It promotes annealing of complementary nucleic acid sequences (36–39). It enhances nucleic acid strand transfer from a less stable to a more stable hybrid (37, 39), it induces maturation of dimeric retroviral RNA (40), and it stimulates hammerhead ribozyme catalysis (41–44). During the viral life cycle, NC has been shown to function at multiple steps, including viral RNA packaging and dimerization, virion assembly, reverse transcription, integration, and transcription (8, 10, 45, 46). Because of these features, it has become an attractive target for the design of potential anti-HIV compounds.

Our studies focused on the RNA-chaperone activity of HIV-1 NC protein during the annealing of tRNA<sup>Lys.3</sup> to viral RNA template containing the PBS. In a previous study, we found that the tRNA<sup>Lys.3</sup>-vRNA initiation complex, formed in the presence of NC, resulted in elevated efficiency of the switch from initiation to elongation in the subsequent synthesis of (−) ssDNA; the zinc finger motifs of NC were required for this activity (11). In this work, we have shown that the role of NC during initiation of reverse transcription, i.e. the release of pausing from the +1 nt position, was also the result of the activity of NC during the RNA annealing process. An intact zinc finger structure was indispensable for this specific activity.

On the basis of these observations, the following conclusions regarding the RNA-chaperone activity of HIV-1 NC can be

Acknowledgments: We thank Dr. F. May for providing us with wild-type and NC mutants of HIV-1 and Dr. K. Chinnan for providing us with CA-p24Ag. This work was supported by grants from the National Institutes of Health (AI-28835, AI-26443) to R. C.

The authors declare no conflict of interest. This work was supported by grants from the National Institutes of Health (AI-28835, AI-26443) to R. C.

Fig. 6. A, illustration of the secondary structures of the complexes formed between tRNA<sup>Lys.3</sup> and the wild-type, N9, and N10 viral RNA templates. B, lists of wild-type as well as N9 and N10 viral RNA template sequences. C, initiation of reverse transcription with the mutated N9 and N10 RNA templates. The order of lanes 5–8 is the same as that of Fig. 5A, except that lanes 5 and 6 are reactions in which N9 template was employed; lanes 7 and 8 are reactions that used N10 mutated template.

Fig. 7. Initiation of reverse transcription from NC-annealed tRNA<sup>Lys.3</sup>-vRNA complexes. tRNA<sup>Lys.3</sup> was placed onto various mutated viral RNA templates by incubation with synthetic HIV-1 NC protein. Reverse transcription was performed by addition of 45 ng of RT and 160 nM each of dCTP, dTTP, and dGTP in the presence of 45 (lanes N1 and N3–N7) or 405 ng (lanes N2 and N8) of wild-type reverse transcription (p51/p66) as described under “Materials and Methods.”

The results of Fig. 8 show that both the +3 and +5 nt products were observed with RNA from wild-type virus; in contrast, +3 pausing was hardly seen with RNA from any of the N1, N3, N4, N5,
drawn. (i) An RNA complex with the most thermostable structure, i.e. that formed by heat annealing, may not always be the most functional. (ii) The RNA-chaperone mechanism to explain the formation of the most stable RNA complexes includes a transient base pair destabilization, followed by random repair until the maximal number of base pairs is reached (9, 10, 47). However, in our system, NC was shown to assist in the formation of a functional RNA complex rather than a thermostable one. This shows that a random or nonspecific model does not fit well with our findings. (iii) Zinc finger motifs were found to be important; this provides further evidence that specific interactions between protein and RNA may involve in the proper folding of the tRNA\(^{\text{Lys.3}}\)-vRNA complex. Therefore, the tRNA\(^{\text{Lys.3}}\)-vRNA complex formed in the presence of HIV-1 NC protein is a specialized one, which may involve both nonspecific and specific interactions between the protein and the RNA.

Previous studies have shown that it is the basic residues rather than the zinc finger motifs of NC protein that are essential for its RNA-chaperone activity (8, 10, 39, 40, 44, 48). In agreement, we found that mutations of the zinc fingers did not affect the tRNA primer annealing capability of NC protein (11). However, the tRNA\(^{\text{Lys.3}}\)-vRNA binary complex that was formed in the presence of zinc finger mutated NC showed a lower efficiency of initiation of reverse transcription in this work; this is consistent with our previous data, which showed that such mutated NC was less efficient at mediating switch from initiation to elongation (11). Similar conclusions have been drawn in in vivo studies, in which sequences between the two Cys-His boxes of NC were found to be essential for packaging and placement of tRNA\(^{\text{Lys.3}}\), whereas mutations that showed the most deleterious effects in regard to initiation of reverse transcription were those that mapped to either of the two NC Cys-His boxes (49).

The zinc finger-dependent nucleic acid chaperone activity of NCp7 has also been reported in other in vitro systems. For example, although NC (12–53) can promote the efficient annealing of tRNA\(^{\text{Lys.3}}\) to the PBS, modification of the first zinc finger in this truncated form of NC led to a large reduction in its primer annealing capacity (50). Zn\(^{2+}\)-binding residues were also found to play important roles during minus and plus strand transfer during HIV-1 reverse transcription, by promoting the unfolding of highly structured RNA and DNA strand transfer intermediates (51). The helix-coil transition of single DNA molecules has been measured through use of an optical tweezers instrument; intact zinc finger structures were shown to be indispensable for two essential activities of NC, i.e. to destabilize double-stranded DNA and to decrease the cooperativity of the helix-coil transition (47). Such prolonged intermediate states between the helix and coil induced by NC may provide an good environment for the specific recognition of nucleotides by zinc finger motifs, which may lead to formation of a reverse transcription competent tRNA\(^{\text{Lys.3}}\)-vRNA complex, as shown both in this paper and in previous reports (11).

In contrast to the +1 nt pausing event, the +3 nt position cannot be bypassed by the presence of NC protein during the annealing process. Similar differences in formation of the +1 and +3 nt pause sites were also observed in our previous studies. For example, initiation of reverse transcription paused at the +3 nt site, whenever tRNA\(^{\text{Lys.3}}\) or a DNA primer was employed; however, the +1 nt intermediate product disappeared when RT reactions were primed from an oligo DNA (7). In vitro probing data have described the existence of a 8-base pair stem structure, located at the fourth nt position upstream of the PBS within the tRNA\(^{\text{Lys.3}}\)-vRNA complex (1–3). Accordingly, we reasoned that reverse transcription must have paused after the third nucleotide was incorporated to dissolve the base pairs of the stem structure. To prove this point, we introduced a series of mutations into the viral RNA template, such that the stem structure was deliberately destabilized. Our results show that reverse transcription of RNA templates containing these mutations no longer paused at the +3 nt position, regardless of whether a heat annealing or NC annealing method was employed.

As stated, the arrest of RT reactions at the +3 nt stage is caused by the existence of the stem within the tRNA\(^{\text{Lys.3}}\)-vRNA complex. In addition, the introduction of mutations that altered either the structure (N1, N2, N3, and N4) or the position (N7, N8) of the stem resulted in less efficient initiation of reverse transcription, as shown in Fig. 5 (A, B, and E); this effect was most pronounced in the case of the N2 and N8 mutated templates (Fig. 5, A and E). In contrast, the N5, N6, N9, and N10 templates represent constructs that preserved stem structure, whereas sequences within the stem were changed. The overall efficiency of initiation of RT reactions performed with these templates approached wild-type levels, although the switch from the +3 to the +5 stage was compromised. Therefore, as long as the stem structure was not disturbed, or the distance of the stem from the PBS remained unchanged, the tRNA-vRNA complex would remain functional, and an optimal initiation rate of reverse transcription would be achieved. Because of its special position within the tRNA-vRNA complex, the stem structure studied may help to promote efficient reverse transcription by stabilizing two adjacent intermolecular interactions between the primer and the template, i.e. the PBS/3’ end of tRNA and the A-rich loop/anti-codon loop of tRNA; both of these are essential for the efficient initiation of (--) ssDNA synthesis (4, 7, 27–32).

Although its structure is key, the sequence of the stem can also affect the efficiency of reverse transcription. For example, the sequence of the right half of the stem structure was found to be important in the switch from the +3 nt pause site to a even later initiation intermediate at the +5 nt position. Reverse transcription of the N5 and N6 templates, which exchanged base pair sequences without disturbance of the stem structure, barely proceeded beyond the +3 stage, although the reaction was initiated in near normal fashion (Fig. 5E). The fidelity of reverse transcription was also affected when sequences on the right half of the stem were mutated (Fig. 5B).

The sequence within/around the stem structure may also affect patterns of pausing during initiation of reverse transcription. For instance, in the experiments performed with the N7 and N8 templates, bands at the +5 and +8 nt positions were visible in addition to the expected pause sites at +6 and +9 (Fig. 5F). The question that arises is whether it is the sequence or the stem structure that determines the +3 pause site. Fig. 5 (A and E) answers this because disruption of the stem structure in the RNA templates N1 and N2 eliminated pausing at the +3 site (Fig. 5A). Restoration of stem base pairing by second-site mutations in the N5 and N6 RNA templates led to reappearance of the +3 pause site (Fig. 5E). Thus, formation of the specific pause site at the +3 nt position is a result of the existence of the stem structure rather than of specific RNA sequences.

This conclusion is further supported through experiments performed with constructs N9 and N10, in which the stem structure that had been destabilized by the N3 and N4 mutations was restored by second-site compensatory mutations (Fig. 6, A and B). The results of Fig. 6C show that the +3 pause event that was eliminated in experiments performed with the N3 and N4 templates was reestablished when the N9 and N10 RNA templates were used (Fig. 6C). Thus, the pause event at the +3 nt site is highly specific and is caused by the stem
structure located at the fourth nucleotide upstream of the PBS, even though it is also true that other template sequences proximal to the PBS can affect patterns of pausing to a limited extent.

In general, our mechanistic studies of early pausing events and the role of the NC protein in this process contribute further to an understanding of the initiation of reverse transcription. First, we have demonstrated that the +1 pausing event is a distinct rate-limiting step during initiation, because it can only be observed when RNA is used to prime reverse transcription (7), and because the addition of NC protein containing intact zinc finger motifs can help RT to escape this pause site. Second, the presence of the +3 nt pausing event is solely a result of the specific secondary structure of the tRNA\textsuperscript{Lys3,vRNA} complex. Therefore, the mechanisms responsible for the formation of these distinct intermediate products during initiation of HIV-1 reverse transcription are different.

Finally, early pause events at the +3 and +5 nt positions were observed when tRNA\textsuperscript{Lys3,vRNA} was prepared from virus particles, indicating that similar initiation events also transpire in the virus. However, comparison of relative amounts of each short cDNA product revealed that the initiation complex, isolated from the virus, more closely resembles that annealed by NC than that prepared by heat annealing (Fig. 8). This implies that NC protein plays an important role during initiation of reverse transcription both in vitro and in vivo.

Acknowledgment—We are grateful to Dr. Stuart LeGrice for helpful discussions.

REFERENCES

1. Isel, C., Marquet, R., Keith, G., Ehresmann, C., and Ehresmann, B. (1993) J. Biol. Chem. 268, 25269–25272
2. Isel, C., Ehresmann, C., Keith, G., Ehresmann, B., and Marquet, R. (1995) J. Mol. Biol. 247, 236–250
3. Skripkin, E., Isel, C., Marquet, R., Ehresmann, B., and Ehresmann, C. (1996) Nucleic Acids Res. 24, 509–514
4. Isel, C., Lanchy, J. M., Le Grice, S. F., Ehresmann, C., Ehresmann, B., and Marquet, R. (1996) EMBO J. 15, 917–924
5. Lanchy, J. M., Ehresmann, C., Le Grice, S. F., Ehresmann, B., and Marquet, R. (1996) EMBO J. 15, 7178–7187
6. Lanchy, J. M., Isel, C., Ehresmann, C., Marquet, R., and Ehresmann, B. (1996) J. Mol. Biol. 268, 297–301
7. Liang, C., Rong, L., Gotte, M., Li, X., Quan, Y., Kleiman, L., and Wainberg, M. A. (1998) J. Biol. Chem. 273, 21309–21315
8. Darlix, J.-L., Lapadat-Tapolsky, M., de Roquigny, H., and Roques, B. P. (1995) J. Mol. Biol. 254, 523–537
9. Herschlag, D. (1995) J. Biol. Chem. 270, 20871–20874
10. Rein, A., Henderson, L. E., and Levin, J. G. (1998) Trends Biochem. Sci. 23, 297–301
11. Rong, L., Liang, C., Hsu, M., Kleiman, L., Petitjean, P., de Roquigny, H., Roques, B. P., and Wainberg, M. A. (1998) J. Virol. 72, 9353–9358
12. Rodriguez-Rodriguez, L., Tsuchihashi, Z., Fuentes, G. M., Bambara, R. A., and Fay, P. J. (1995) J. Biol. Chem. 270, 15095–1511
13. Tanchou, V., Gabus, C., Rogemond, V., and Darlix, J.-L. (1995) J. Mol. Biol. 232, 563–571
14. Ji, X., Klarmann, G. J., and Preston, B. D. (1996) Biochemistry 35, 132–143
15. Wu, W., Henderson, L. E., Copeland, T. D., Gorlich, R. J., Boche, W. J., Rein, A., and Levin, J. G. (1996) J. Virol. 70, 7132–7142
16. Arts, E. J., Li, X., Gu, Z., Kleiman, L., Parniak, M. A., and Wainberg, M. A. (1994) J. Biol. Chem. 269, 14672–14679
17. de Roquigny, H., Ficheux, D., Fournier-Zaluski, M.-C., Darlix, J.-L., and Roques, B. P. (1991) Biochem. Biophys. Res. Commun. 180, 1010–1018
18. Jiang, M., Mark, J., Ladha, A., Cohen, E., Klein, M., Rovinski, B., and Darlix, J.-L. (1993) J. Biol. Chem. 268, 13596–13603
19. Huang, Y., Wang, J., Shalom, A., Li, Z., Khor childish, A., Wainberg, M. A., and Kleiman, L. (1997) J. Virol. 71, 726–728
20. Wöhrl, B. M., Ehresmann, B., Keith, G., and Le Grice, S. F. (1993) J. Biol. Chem. 268, 13617–13624
21. Back, N. K. T., Nijhuis, M., Keulen, W., Boucher, C. A. B., Oude Essink, B. B., and van Kuilenburg, A. B. P., van Gennip, A. H., and Berkhout, B. (1996) EMBO J. 15, 4040–4049
22. Boyer, P. L., and Hughes, S. H. (1995) Antimicrob. Agents Chemother. 39, 1624–1628
23. Wakefield, J. K., Kang, S. M., and Morrow, C. D. (1996) J. Virol. 70, 966–975
24. Kang, S. M., Wakefield, J. K., and Morrow, C. D. (1996) Virology 222, 401–414
25. Das, A. T., Klaver, B., and Berkhoort, B. (1997) J. Gen. Virol. 78, 837–843
26. Yu, G., and Morrow, C. D. (1999) Virology 254, 161–168
27. Das, A. T., and Berkhoort, B. (1995) Nucleic Acids Res. 23, 1319–1326
28. Arts, E. J., Steter, S. R., Li, X., Rausch, J. W., Howard, K. J., Ehresmann, B., North, T. W., Wöhrl, B. M., Goode, R. S., Wainberg, M. A., and Le Grice, S. F. (1990) Proc. Natl. Acad. Sci. U. S. A. 93, 10663–10666
29. Wakefield, J. K., and Morrow, C. D. (1996) Virology 220, 290–298
30. Liang, C., Li, X., Rong, L., Inouye, P., Gu, Z., Kleiman, L., and Wainberg, M. A. (1997) J. Virol. 71, 5759–5757
31. Zhang, Z., Kang, S. M., Li, Y., and Morrow, C. D. (1998) Nucleic Acids Res. 26, 394–406
32. Kang, S. M., and Morrow, C. D. (1999) J. Virol. 73, 1818–1827
33. Drossopoulou, W. C., and Prasad, V. R. (1996) J. Virol. 70, 4834–4838
34. Pandey, V. N., Kang, S. M., Sarafianos, S. G., Yadav, P. N. S., and Madok, M. J. (1996) Biochemistry 35, 2166–2179
35. Wainberg, M. A., Drossopoulou, W. C., Salomon, H., Hsu, M., Borkow, G., Parniak, M. A., Gu, Z., Song, Q., Mange, J., Islam, S., Castrioti, G., and Prasad, V. R. (1996) Science 271, 1282–1285
36. Bibi, F., Kahn, R., and Giedroc, D. P. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 7178–7181
37. Tsuchihashi, Z., and Brown, P. O. (1994) J. Biol. Chem. 269, 13617–13624
38. Wakefield, J. K., and Darlix, J.-L. (1992) J. Virol. 66, 6770–6779
39. Boyer, P. L., and Hughes, S. H. (1995) Antimicrob. Agents Chemother. 39, 1624–1628
HIV-1 Nucleocapsid Protein and the Secondary Structure of the Binary Complex Formed between tRNA<sub>Lys</sub> and Viral RNA Template Play Different Roles during Initiation of (−) Strand DNA Reverse Transcription

Liwei Rong, Chen Liang, Mayla Hsu, Xiaofeng Guo, Bernard P. Roques and Mark A. Wainberg

*J. Biol. Chem.* 2001, 276:47725-47732.  
doi: 10.1074/jbc.M105124200 originally published online October 15, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M105124200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 51 references, 28 of which can be accessed free at http://www.jbc.org/content/276/50/47725.full.html#ref-list-1