JOURNAL OF VIROLOGY, Aug. 1996, p. 4996–5004 Vol. 70, No. 8
0022-538X/96/$04.00+0

Human Immunodeficiency Virus Type 1 Nucleocapsid Protein (NCp7) Directs Specific Initiation of Minus-Strand DNA Synthesis Primed by Human $tRNA_3^{Lys}$ In Vitro: Studies of Viral RNA Molecules Mutated in Regions That Flank the Primer Binding Site

XUGUANG LI,[1] YUDONG QUAN,[1] ERIC J. ARTS,[1] ZHUO LI,[1] BRADLEY D. PRESTON,[2] HUGUES DE ROCQUIGNY,[3] BERNARD P. ROQUES,[3] JEAN-LUC DARLIX,[4] LAWRENCE KLEIMAN,[1] MICHAEL A. PARNIAK,[1] AND MARK A. WAINBERG[1]*

McGill University AIDS Center, Lady Davis Institute-Jewish General Hospital, and Departments of Medicine and of Microbiology, McGill University, Montreal, Quebec, Canada[1]; Department of Biochemistry and Eccles Institute of Human Genetics, University of Utah, Salt Lake City, Utah 84112[2]; and Unité de Pharmacochimie Moléculaire, Université René Descartes, Paris,[3] and Unité de Virologie Humaine, Institut National de la Santé et de la Recherche Médicale-U412, École Normale Supérieure, Lyon,[4] France

Received 17 January 1996/Accepted 17 April 1996

Retroviral reverse transcription starts near the 5′ end of unspliced viral RNA at a sequence called the primer binding site (PBS), where the tRNA primer anneals to the RNA template for initiation of DNA synthesis. We have investigated the roles of NCp7 in annealing of primer $tRNA_3^{Lys}$ to the PBS and in reverse transcriptase (RT) activity, using a cell-free reverse transcription reaction mixture consisting of various 5′ viral RNA templates, natural primer $tRNA_3^{Lys}$ or synthetic primer, human immunodeficiency virus type 1 (HIV-1) nucleocapsid protein (NCp7), and HIV-1 RT. In the presence of $tRNA_3^{Lys}$, NCp7 was found to stimulate synthesis of minus-strand strong-stop DNA [(−)ssDNA], consistent with previous reports. However, specific DNA synthesis was observed only at a NCp7/RNA ratio similar to that predicted to be present in virions. Moreover, at these concentrations, NCp7 inhibited the synthesis of nonspecific reverse-transcribed DNA products, which are initiated because of self-priming by RNA templates. In contrast to results obtained with $tRNA_3^{Lys}$ as primer, NCp7 inhibited the synthesis of (−)ssDNA products primed by an 18-nucleotide (nt) ribonucleotide (rPR), complementary to the PBS, even though rPR can initiate synthesis of such material in the absence of preannealing with NCp7. Primer placement band shift assays showed that NCp7 was necessary for efficient formation of the tRNA-RNA complex. In contrast, NCp7 was found to prevent formation of the rPR-RNA complex. Since NCp7 appears to exert opposite effects (annealing versus dissociation) on $tRNA_3^{Lys}$ and rPR substrates, the non-PBS binding regions of the $tRNA_3^{Lys}$ molecule may play a role in the annealing of tRNA to the template. We also investigated the roles of an A-rich loop upstream of the PBS, a 7-nt region immediately downstream of the PBS, and a 54-nt deletion further downstream of the PBS in interactions with $tRNA_3^{Lys}$. We found that deletions in the 54-nt region that may prevent formation of the U5-leader stem prevented $tRNA_3^{Lys}$ placement and priming, while deletions in the A-rich loop or the 7-nt sequence had relatively minor effects in this regard.

The 5′ ends of retroviral RNA and tRNA are highly structured, folding into several stem-loops. Therefore, partial unfolding of these molecules is prerequisite for annealing between them. Denaturation of tRNA by viral nucleocapsid protein (NCp) has been observed by circular dichroism spectroscopy (32). Although NCp has been shown to stimulate synthesis of initial DNA products, the mechanism of annealing between tRNA primer and its viral RNA template is not well understood. Therefore, it is important to use modified templates and primers to further elucidate the nature of these interactions and to understand their specificity.

Reverse transcription involves the conversion of retroviral single-stranded RNA into double-stranded DNA and is carried out by the virion-encoded enzyme reverse transcriptase (RT). All retroviral RTs utilize host cell-derived tRNAs as an initiation primer, but the specific type of tRNA employed may vary, depending on the virus (38). In the case of human immunodeficiency virus type 1 (HIV-1), this role is played by $tRNA_3^{Lys}$ (29, 43).

The binding site for $tRNA_3^{Lys}$ is positioned about 180 nucleotides (nt) from the 5′ end of unspliced viral RNA, i.e., a sequence termed the primer binding site (PBS) (43, 44). The PBS is 18 nt long and is complementary to the 3′ end of $tRNA_3^{Lys}$. The results of computer modeling, in conjunction with those of chemical and enzymatic studies, suggest that the 5′ region of viral RNA is highly structured and can fold into several stem-loops (7, 9, 13, 23). If left unfolded, these structures could hinder annealing of primer tRNA to the viral RNA template. Likewise, although tRNA is single chained, a majority of bases are hydrogen bonded to each other. Hairpin folds bring bases on the same chain into a double-helical arrangement whereby short stretches of nucleotides are complemen-

* Corresponding author. Mailing address: McGill AIDS Centre, Jewish General Hospital, 3755 Cote Ste-Catherine Rd., Montreal, Quebec, Canada H3T 1E2. Phone: (514) 340-8260. Fax: (514) 340-7537. Electronic mail address: mdwa@musica.mcgill.ca.

tary to each other. Only 4 of the 18 nt in $tRNA_3^{Lys}$, complementary to the PBS, are not base paired (38). Thus, appropriate unwinding of both tRNA and the RNA template must be prerequisite for correct annealing between these structures and in order for efficient reverse transcription to occur.

It is unclear how this unwinding takes place, although RT may itself be able to disrupt secondary structure of the 5′ viral RNA and the tRNA primer. Another viral protein of interest is the viral nucleocapsid (NCp), which bears strong similarity to other nucleic acid-binding proteins; notably it is highly basic and contains one or two copies of a conserved sequence, i.e., a so-called Cys-His box (8, 10, 22, 46, 47). In the case of HIV-1, NCp contains two copies of this Cys-His motif (43). The results of physicochemical studies suggest that NCp binds preferentially to single-stranded nucleic acids (31, 47) and unwinds primer tRNA in vitro (32). NCp also facilitates transition between single-stranded and double-stranded nucleic acids and renaturation of nucleic acids (16, 32, 50, 55), properties which could be responsible for stimulatory effects on strand transfer during DNA polymerization (3, 18, 40, 45, 55).

The addition of viral nucleocapsid protein (NCp) to reverse transcription reaction mixtures greatly stimulated synthesis of minus-strand DNA [(−)DNA], probably through enhancement of annealing between the tRNA primer and the viral RNA template (5, 42). Interactions between the tRNA primer and the viral RNA template may not be limited to the 18-nt complementary regions between them, since sequences outside the PBS of the viral RNA template are thought to be necessary for efficient initiation of retroviral reverse transcription (1, 2, 12, 13, 26, 27, 34, 36). For example, chemical and enzymatic footprint analysis showed that a conservative A-rich loop, located 10 nt upstream of the HIV-1 PBS, may be able to base pair with the anticodon loop of $tRNA_3^{Lys}$ (27); however, direct analysis of RT activity was not performed in this study. In separate experiments, six bases located downstream of the PBS were shown to be involved in specifying tRNA utilization (34), but the templates used were nonphysiological and lacked significant secondary structure. Neither of these studies examined the role of NCp.

To further investigate this subject, we performed cell-free reverse transcription reactions that included various modified 5′ HIV-1 RNA templates, nucleocapsid protein (NCp7), recombinant RT, human $tRNA_3^{Lys}$ or synthetic primer, and radiolabeled substrates. Consistent with previous observations (42), NCp7 was found to stimulate the synthesis of minus-strand strong-stop DNA [(−)ssDNA] in our system. However, this effect was obtained only if the protein/nucleic acid ratio was one NCp7 molecule per 6 to 20 tRNA or RNA nt residues. Moreover, a maximal effect was obtained at a ratio of one NCp7 per 6 tRNA or RNA nt residues, i.e., close to the predicted NCp/nucleic acid ratio in virions (16, 31). Remarkably, at these concentrations, NCp7 suppressed the synthesis of nonspecific reverse-transcribed DNA products, which were probably initiated because of self-priming by RNA templates. We also found that synthesis of (−)ssDNA, initiated by a 18-nt ribooligonucleotide primer (rPR), complementary to the PBS, was inhibited by NCp7 in a concentration-dependent manner. With a primer placement assay, NCp7 was found to be necessary for efficient formation of the $tRNA_3^{Lys}$-RNA complex but prevented formation of an rPR-RNA complex. Finally, a 54-nt sequence, located downstream of the PBS, was essential for efficient formation of the $tRNA_3^{Lys}$ RNA complex and synthesis of (−)ssDNA.

(This research was performed by Xuguang Li in partial fulfillment of the requirements for a Ph.D. degree from McGill University, Montreal, Quebec, Canada, 1996.)

MATERIALS AND METHODS

Chemicals and reagents. All chemicals were purchased from Sigma Inc. (St. Louis, Mo.) unless specified. Radioisotopes were obtained from Dupont Inc. (Mississauga, Ontario, Canada). Restriction enzymes, modifying enzymes, and RNAguard (RNase inhibitor) were obtained from Pharmacia, Inc. (Montreal, Quebec, Canada). $tRNA_3^{Lys}$ was purified from human placenta as previously described (29). The 18-mer ribonucleotide (rPR), complementary to the PBS, was obtained from General Synthesis Diagnosis, Inc. (Toronto, Ontario, Canada). rPR was purified by high-pressure liquid chromatography and shown to be free of smaller ribonucleotides as monitored through use of 20% denaturing polyacrylamide gel electrophoresis. The sequence of the synthetic rPR is 5′-GUC CCUGUUCGGGCGCCA-3′. HIV-1 RT (p66-p51) was a gift of Stuart F. J. Le Grice of Case Western Reserve University, Cleveland, Ohio (54). HIV-1 nucleocapsid protein (NCp7 [72 amino acids long]) was chemically synthesized as previously described (20, 21). We have previously characterized this synthetic NCp7 with regard to structure and its nucleic acid-binding properties (20, 21, 39). Purified calf histone was purchased from ICN Inc., Montreal, Canada, and bovine serum albumin (BSA) was obtained from Sigma Inc.

Construction of RNA expression plasmids and preparation of HIV-1 RNA transcripts. Construction of PBS/WT, a plasmid for making a 483-nt 5′ HIV-1 RNA transcript comprising R, U5, PBS, and part of the Gag-encoding region has been described previously (4), as has the PBS(−) plasmid (with a complete deletion of the 18-nt PBS) (37). Deletions of either 4 nt, i.e., AAAA (10 nt upstream of the PBS, positions 169 to 172) (43), or 7 nt, immediately downstream of the PBS (positions 202 to 208 in PBS/WT) (43), were made by a modified PCR-based megaprimer mutagenesis method (41) and designated PBS/del-A and PBS/del-7, respectively. A deletion of 54 nt immediately downstream of the PBS (positions 202 to 255) (43) was generated by cutting PBS/WT with *Nar*I and *Bss*HII, following which the PBS was rebuilt by the same mutagenesis strategy described above. Templates containing this modification were designated PBS/del-LD. For RNA transcript preparations, plasmids were linearized by *Acc*I and used as templates in an Ambion Mega-scripts kit (Austin, Tex.) according to the manufacturer's instructions. The integrity of RNA transcripts was routinely checked using a denaturing gel (5% polyacrylamide–7 M urea) before their use in reverse transcription assays.

Reverse transcription. Reverse transcription reactions (unless otherwise specified) were performed in a volume of 10 μl with reaction mixtures containing 50 mM Tris-Cl (pH 7.2), 50 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 200 μM dATP, 200 μM dGTP, 200 μM dTTP, 50 μM dCTP, 1.5 μl of [α-^{32}P]dCTP (specific activity, 3,000 Ci/mmol), 5 U of RNAguard, 100 nM RNA template, 100 nM primer (either $tRNA_3^{Lys}$ or rPR), and various amounts of NCp7 depending on the individual experiment. NCp7 has been prepared by either peptide synthesis (20) or by expression of recombinant clones (28) as previously described; identical results were obtained with both preparations in these studies. Reaction mixtures were preincubated at 37°C for 30 min prior to the addition of RT (final concentration, 50 nM). The reaction mixture was then incubated at 37°C for 15 to 30 min before termination by the addition of EDTA (final concentration, 50 mM). These reactions yielded linear results for up to 60 min after initiation, most likely because of continuous denaturation and refolding of the nucleic acids involved. After phenol-chloroform extraction, the reaction products were precipitated with ethanol and boiled for 5 min in formamide denaturing buffer before being fractionated in a 5% denaturing polyacrylamide gel containing 7 M urea. Finally, the gel was dried and exposed to Kodak film at −70°C. In some cases, the reaction products were treated with NaOH to remove the RNA primer before being fractionated on a denaturing gel (11).

Placement of primers onto RNA template by NCp7. 5′-end-labeled primer (tRNA or rPR) was incubated with RNA template in the absence of deoxynucleoside triphosphates (dNTPs) but in the presence of various amounts of NCp7 under the reverse transcription conditions described above. Reaction products were treated with 200 μg of proteinase K per ml at 37°C for 30 min and then extracted with phenol-chloroform. To the resultant aqueous phase, we added an equal volume of 2× sample loading buffer, consisting of 50 mM Tris-Cl (pH 7.2), 25 mM EDTA, 25% glycerol, 2% sodium dodecyl sulfate, and 0.01% bromphenol blue (55). This step was followed by fractionation on a 1.5% agarose gel. The gel was dried and exposed to Kodak film at −70°C.

RESULTS

Effect of NCp7 on reverse transcription primed by human $tRNA_3^{Lys}$. To investigate the effect of NCp7 on (−)ssDNA synthesis, we reconstituted a reverse transcription reaction mixture consisting of HIV-1 template, human $tRNA_3^{Lys}$, RT, and NCp7. As shown in Fig. 1, the template and tRNA were preincubated with various concentrations of NCp7 prior to the addition of enzyme. The final product is 259 nt long and consists of (−)DNA (183 nt) and the attached primer $tRNA_3^{Lys}$ (76 nt) at the 5′ end. Figure 2A shows that (−)ssDNA was clearly detected when NCp7 was present at concentrations of 14, 9.3,

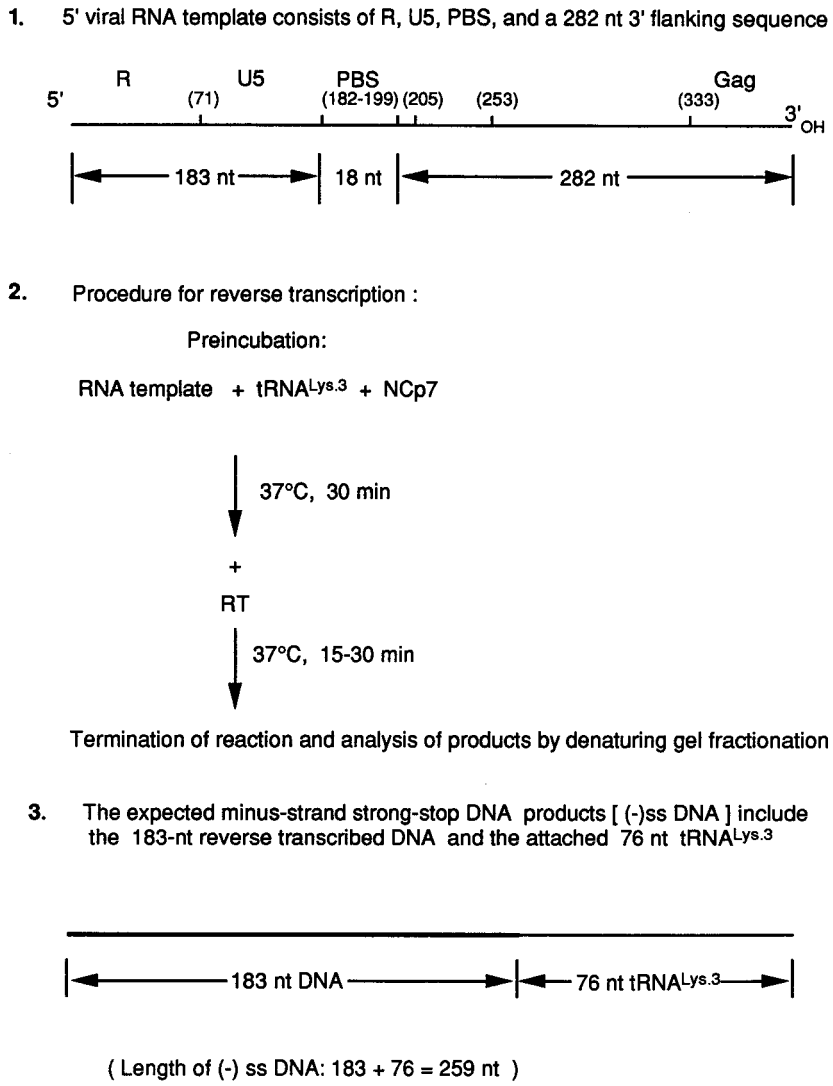

FIG. 1. Graphic description of reverse transcription reaction. (Part 1.) The RNA template consists of R, U5, PBS, and a 282-nt 3′-flanking sequence. The position and size of each region are indicated. The numbers in parentheses designate nucleotide positions within the viral RNA template. (Part 2.) RNA template and $tRNA_3^{Lys}$ were preincubated with variable amounts of NCp7 at 37°C for 30 min, RT was added (final concentration, 50 nM), and the reaction mixture was incubated at 37°C for up to 30 more min. Reactions were terminated, and products were fractionated on 5% polyacrylamide gels containing 7 M urea. (Part 3.) The expected full-length (−)ssDNA products initiated by $tRNA_3^{Lys}$ consist of a 183-nt (−)cDNA and a 76-nt (−)tRNA. The use of an 18-nt ribonucleotide (rPR) (complementary to the PBS) as the primer instead of $tRNA_3^{Lys}$ is expected to yield full-length (−)ssDNA of 183 nt and a dPR of 18 nt. $tRNA^{Lys.3}$, $tRNA_3^{Lys}$.

6.2, 4.1, and 2.7 μM. This corresponds to a ratio of one NCp7 molecule to 4, 6, 9, 14, and 21 nt residues, respectively (lanes 1, 2, 3, 4, and 5, respectively). The strongest (−)ssDNA bands were observed at a NCp/nucleotide ratio of 1:6 (lane 2). Bands smaller than 259 nt probably represent incomplete DNA products (4, 24, 32, 36). Note that no specific (−)ssDNA products were detected when a template from which the PBS was deleted [i.e., PBS(−)] was used in either the presence of NCp7 (one NCp per 4 nt, lane 9) or its absence (lane 10). Indeed, the 259-nt (−)ssDNA product was never present under these conditions, no matter how much NCp7 or $tRNA_3^{Lys}$ was added (data not shown). Thus, the 259-nt reverse-transcribed DNA product results from tRNA priming at the PBS.

Unexpectedly, we found that nonspecific reverse-transcribed DNA products (mostly >259 nt) accumulated when lower concentrations of NCp7 were employed (Fig. 2A, lanes 4, 5, 6, and 7). Indeed, maximal amounts of these DNA products were detected in the absence of NCp7 (lane 8). An amount of nonspecific DNA product equivalent to that in reaction mixtures containing $tRNA_3^{Lys}$ (lane 8) was also detected in reaction mixtures that contained PBS/WT template, RT, and dNTPs but excluded NCp7 and $tRNA_3^{Lys}$ (lane 12). This suggests that these products might be due to self-priming of the RNA template itself and that this could be inhibited by NCp7 (lane 11).

We also blocked the terminal nucleoside 3′ hydroxyl of the RNA template by treatment with excess cytidine 3′,5′-biphosphate (pCP), prior to the addition of template to the reverse transcription mixture (29). This resulted in a disappearance of nonspecific products (Fig. 2B, lanes 1 to 3). In the absence of NCp7, no nonspecific DNA products were detected and synthesis of (−)ssDNA was less efficient (lane 1) than when NCp was present (lane 3). Denaturation of the RNA template (92°C for 2 min followed by quick chilling on ice) (4, 37) before addition to the reaction mixture resulted in synthesis of more

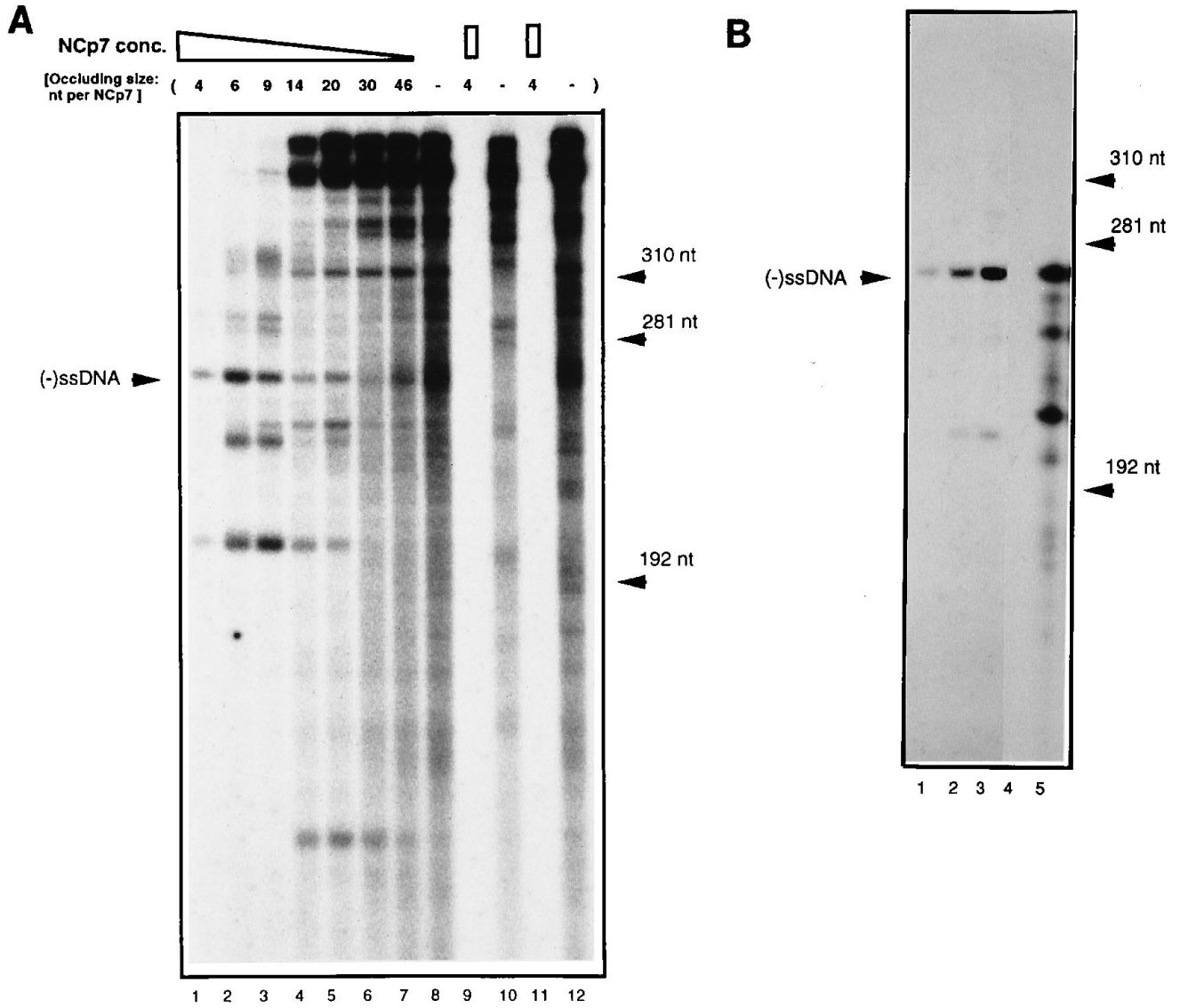

FIG. 2. (A) Effect of NCp7 concentration on DNA synthesis. Lanes 1 to 7 represent reverse transcription reactions performed with wild-type template (PBS/WT), $tRNA_3^{Lys}$, RT, and decreasing concentrations (conc.) of NCp7, i.e., 14, 9.3, 6.2, 4.1, 2.7, 1.8, and 1.2 μM (corresponding to one NCp7 molecule per 4, 6, 9, 14, 20, 30, and 46 nt residues). Lane 8 represents a reaction performed without NCp7. Lane 9 shows results from a reaction that included PBS(−) template, $tRNA_3^{Lys}$, RT, and 14 μM NCp7 (one NCp7 for 4 nt residues). Lane 10 contained only PBS(−) template and RT. Lane 11 contained PBS/WT template, RT, and 12 μM NCp7 to achieve a ratio of one NCp7 per 4 nt (in order to compare with lane 1 in which $tRNA_3^{Lys}$ was added). Lane 12 contained PBS/WT template and RT. The minus sign over lanes 8, 10, and 12 designates the absence of NCp7. No reverse-transcribed products were detected in a control experiment performed with $tRNA_3^{Lys}$, RT, and dNTP but without RNA template and NCp. (B) Reverse transcription reactions using RNA templates with modified 3′ ends. The 3′-terminal hydroxyl of the RNA template was blocked by labeling with pCp (29). The resultant RNA templates were then used in reverse transcription reactions. Lane 1, reaction performed in the absence of NCp7; lane 2, reaction performed in the absence of NCp7, but in which the modified RNA template was denatured by heating (92°C for 2 min, followed by quick chilling on ice) before being added into the reaction mixture (4, 37); lane 3, reaction performed in the presence of NCp7; lane 4, reaction with a negative control performed with a RNA template with the PBS deleted, i.e., PBS(−); lane 5, reaction with positive control involving preannealing of $tRNA_3^{Lys}$ to the modified RNA template by a heating-annealing procedure (4, 37) followed by the addition of RT and dNTPs.

(−)ssDNA (lane 2) than when NCp7 was absent (lane 1), probably because of disruption of secondary structure of the RNA template. As a control, preannealing of $tRNA_3^{Lys}$ to the RNA template by a heating-annealing process (92°C for 2 min, followed by 55°C for 10 min and 37°C for 10 min) (4, 37) also resulted in efficient synthesis of (−)ssDNA (lane 5). The incomplete DNA products in this lane reflect increased pausing during DNA polymerization due to an absence of NCp7 (28). No DNA synthesis was observed when the PBS(−) template was used under the same conditions (lane 4). These results suggest that the (−)ssDNA products generated in the presence of NCp are specific and provide additional evidence that synthesis of nonspecific reverse-transcribed products was caused by self-priming of the RNA template. The fact that other investigators did not observe nonspecific reverse-transcribed DNA products may be due to their use of 5′-end labeled tRNA (3, 42, 52), rather than our use of radiolabeled dNTPs to enhance sensitivity. Overall, we found that increased concen-

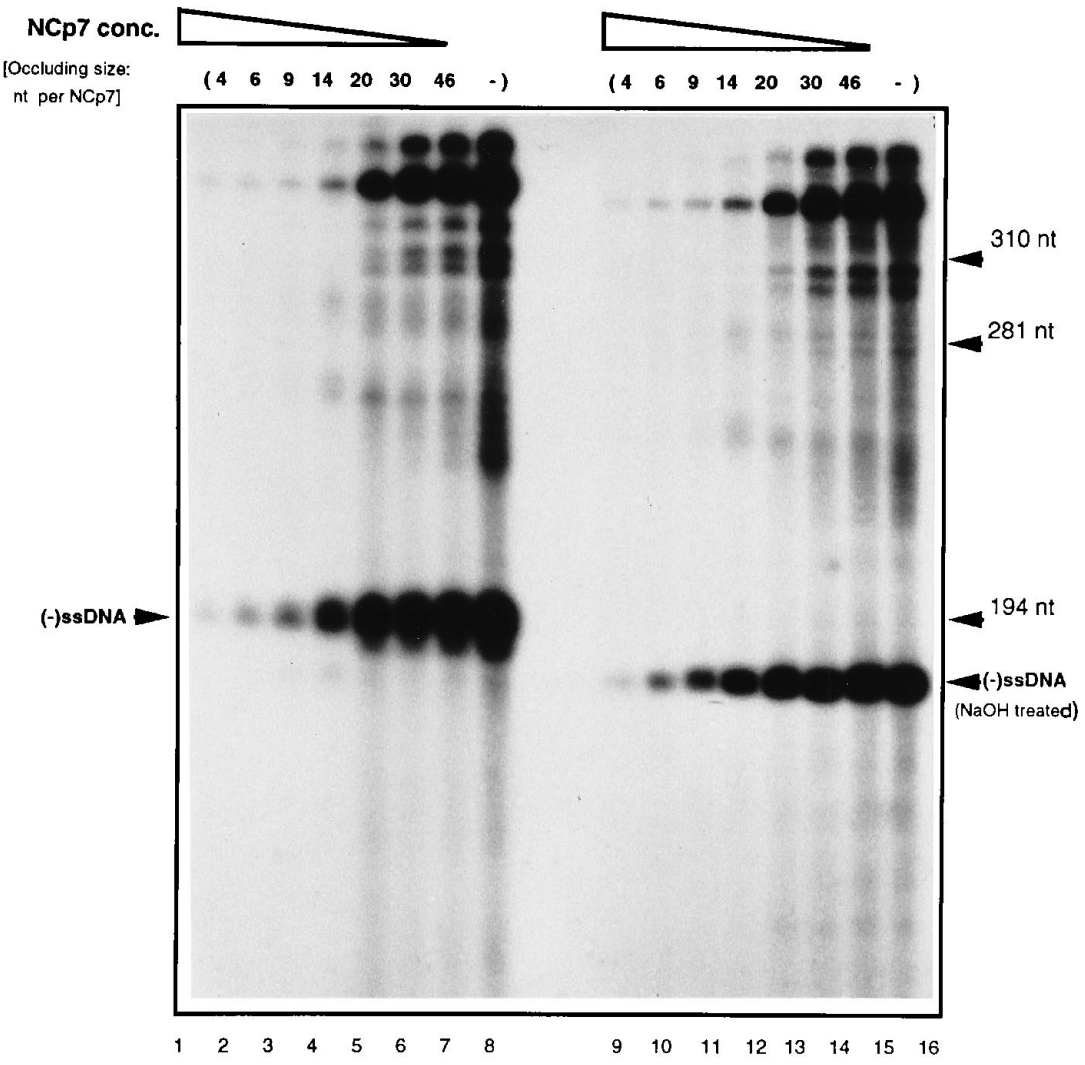

FIG. 3. Effect of NCp7 concentration on rPR-initiated reverse transcription. Lanes 1 to 8 represent experiments performed with PBS/WT template, rPR, RT and decreasing concentrations (conc.) of NCp7, i.e., 12.5, 8.3, 5.5, 3.7, 2.4, 1.6, and 1 μM, to achieve the NCp/nucleotide ratios described in the legend to Fig. 2. The experiment in lane 8 was performed without NCp7. Lanes 9 to 16 represent the same reactions as lanes 1 to 8 but with NaOH treatment of reaction products to digest the RNA portion (11). The minus sign over lanes 8 and 15 designates the absence of NCp7.

trations of NCp7 led to decreased accumulation of nonspecific reverse-transcribed DNA products and increased generation of specific (−)ssDNA (Fig. 2A, lanes 1 to 8).

Inhibition effect of NCp7 on reverse transcription primed by either viral template or by ribooligonucleotide (rPR) complementary to the PBS. We next investigated reverse transcription using a 18-mer ribooligonucleotide (rPR) complementary to the PBS, as a primer in the place of $tRNA_3^{Lys}$. In this instance, NCp7 displayed dose-dependent inhibitory rather than stimulatory effects on rPR-primed synthesis of (−)ssDNA (Fig. 3, lanes 1 to 8). Indeed, the strongest (−)ssDNA signal was obtained in the absence of NCp7 (Fig. 3, lane 8). Figure 3 also shows that NCp7 inhibited formation of nonspecific DNA products of reverse transcription (lanes 1 to 8), as described above. No specific (−)ssDNA products were detected when PBS(−) template was used in place of the PBS-containing PBS/WT template in the reverse transcription reaction (not shown). Treatment of the reaction products with NaOH to digest the RNA portion of the reaction products resulted in a smaller size of (−)ssDNA (approximately 18 nt shorter), confirming that these products were indeed initiated by rPR (Fig. 3, lanes 9 to 16). The smaller size of nonspecific DNA products after NaOH treatment also confirmed that these products were caused by self-priming of RNA templates (Fig. 3, lanes 9 to 16). Thus, NCp7 appears to inhibit formation of reverse-transcribed DNA products initiated either by rPR or by RNA template.

Effect of NCp7 on placement of primers onto RNA template. To initiate synthesis of (−)ssDNA, the primer must be annealed onto the PBS of the RNA template. Since NCp7 had differential effects on $tRNA_3^{Lys}$ versus rPR-initiated DNA synthesis, it was of interest to investigate how NCp7 could influence annealing between primer and RNA template. Toward this end, 5′-end-labeled primer was incubated with RNA template and NCp7, under the same conditions as those of reverse transcription reactions (see Materials and Methods). Following incubation at 37°C for 30 min, the reaction complex was fractionated on a 1.5% agarose gel. As expected (42), increas-

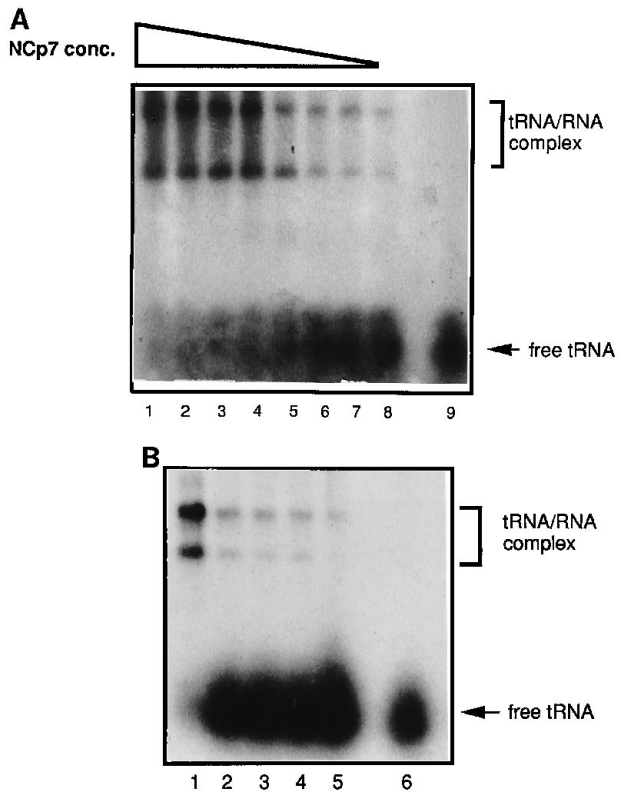

FIG. 4. (A) Effect of NCp7 concentration on formation of complexes between $tRNA_3^{Lys}$ and RNA template. Lanes 1 to 8 represent experiments performed with RNA template, 5′-end-labeled $tRNA_3^{Lys}$, and decreasing concentrations of NCp7, i.e., 14, 9.3, 6.2, 4.1, 2.7, 1.8, and 1.2 μM. Lane 8 excluded NCp7. Lane 9 contains a 5′-end-labeled $tRNA_3^{Lys}$ control. The presence of the tRNA-RNA complex is indicated. Note that the upper bands represent complexes between tRNA and the dimeric RNA template, while the lower bands represent complexes between tRNA and monomeric RNA (6, 16, 21, 42, 52). (B) Inability of histone, BSA, or RT to substitute for NCp7 in formation of complexes between $tRNA_3^{Lys}$ and the RNA template. Lane 1, reaction performed with 9.3 μM NCp7; lane 2, reaction performed with 9.3 μM histone; lane 3, reaction performed with 9.3 μM BSA; lane 4, reaction performed with 9.3 μM RT; lane 5, reaction performed in the absence of proteins; lane 6, reaction performed with 5′-end-labeled $tRNA_3^{Lys}$ only (lane 6).

ing concentrations of NCp7 gave rise to increasingly intense signals representing the $tRNA_3^{Lys}$-RNA complex (Fig. 4A). In the absence of NCp7, barely detectable annealing was observed (lane 8). The amount of NCp7 needed for optimal annealing between $tRNA_3^{Lys}$ and the RNA template, i.e., one NCp per 6 to 20 nt (Fig. 4A), corresponded reasonably well with the amount of NCp7 required for specific $tRNA_3^{Lys}$-primed synthesis of (−)ssDNA (Fig. 2A). The upper bands represent complexes between $tRNA_3^{Lys}$ and the dimeric RNA template, while the lower bands represent complexes between $tRNA_3^{Lys}$ and monomeric RNA template (6, 16, 21, 42, 52). No annealing of $tRNA^{Lys}$ onto the template was observed when the PBS(−) RNA template was used in a control reaction mixture (not shown here but shown below).

As an additional control, we asked what the effect would be on the formation of the tRNA-RNA complex if we replaced NCp7 by other proteins. In comparison with high-level complex formation obtained in the presence of NCp7 (Fig. 4B, lane 1), only minimal placement of tRNA onto RNA was obtained in the presence of a basic protein, e.g., histone (lane 2), an acidic protein, e.g., BSA (lane 3), or RT (lane 4). No proteins were present in the experiment of lane 5, while only ^{32}P-5′-end-labelled $tRNA_3^{Lys}$ was present in lane 6. This shows that the effects of NCp7 on formation of the primer-template complex are specific, consistent with previous findings (42).

In contrast to the results of the primer placement experiment with $tRNA_3^{Lys}$, different findings were obtained when rPR was used as the primer (Fig. 5). In this case, increasing concentrations of NCp7 prevented formation of a complex between rPR and the RNA template. In the absence of NCp7, the RNA template annealed less efficiently to $tRNA_3^{Lys}$ (Fig. 4A, lane 8) than to rPR (Fig. 5, lane 8). The ability of NCp7 to inhibit annealing between rPR and viral RNA correlated with its inhibitory effect on the synthesis of (−)ssDNA (Fig. 3).

Effect of NCp7 on reverse transcription involving mutant RNA template. Previous investigations have shown that HIV RNA sequences that flank the PBS may be important in interactions between tRNA and the RNA template but did not examine the potential role of NCp7 in this regard (26, 27, 34). To test how NCp7 might influence such interactions, we created RNA templates that contained deletions of the A-rich loop (PBS/del-A) (nt 169 to 172) or of 7 nt located immediately downstream of the PBS (PBS/del-7) (nt 202 to 208). In addition, a deletion of 54 nt, located immediately downstream of the PBS (PBS/del-LD) (nt 202 to 255), was constructed to disrupt the U5-leader stem of viral RNA (36). Figure 6A shows the results of reverse transcription reactions performed with these mutant RNA templates with $tRNA_3^{Lys}$ as the primer. As expected, if the template contained a deletion in the PBS, i.e., PBS(−) template, no (−)ssDNA was generated (lane 1). Furthermore, within the time frame of these experiments (15 to 30 min), del-A and del-7 had only minor effects on $tRNA_3^{Lys}$ placement and priming. Deletion of the A-rich loop (PBS/del-A) or of the 7-nt sequence, downstream of the PBS (PBS/del-7), had a minor effect on synthesis of (−)ssDNA (lanes 3 and 4) compared with that of wild-type DNA (lane 2). However, the larger deletion located downstream of the PBS (PBS/del-LD) resulted in a significant reduction in the amount of (−)ssDNA. This result was obtained in each of three separate experiments. These data were further confirmed by testing these mutant templates in time course reverse transcription assays (not shown).

To further investigate the mechanisms involved, tRNA primer placement studies were performed. As expected, no significant differences regarding formation of a tRNA-RNA template complex, promoted by NCp7, were observed among

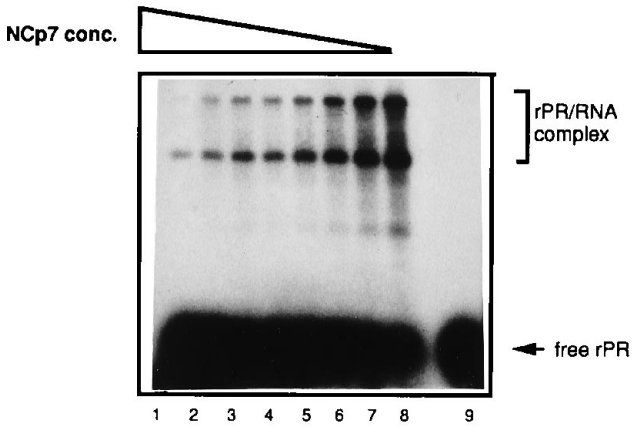

FIG. 5. Effect of NCp7 concentration on formation of complexes between rPR and the RNA template. Lanes 1 to 8 represent results from reactions including RNA template, 5′-end-labeled rPR, and decreasing concentrations (conc.) of NCp7, i.e., 12.5, 8.3, 5.5, 3.7, 2.4, 1.6, and 1 μM.

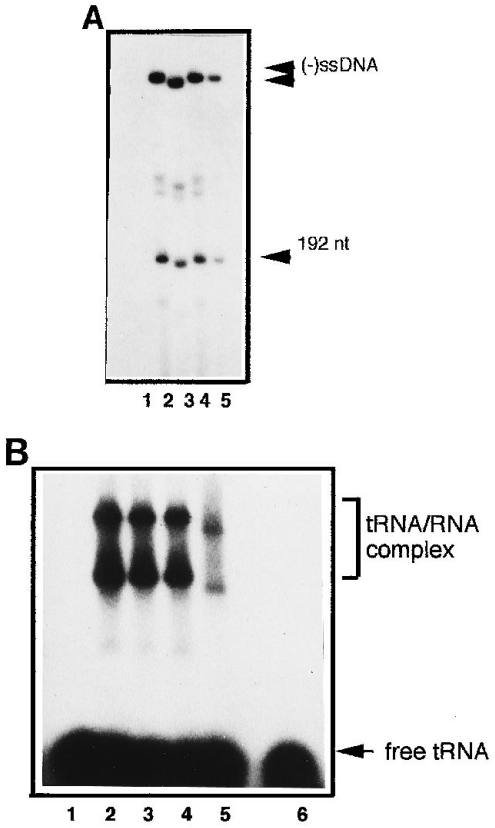

FIG. 6. (A) Effect of NCp7 on the synthesis of (−)ssDNA with various RNA templates. $tRNA_3^{Lys}$ was used as the primer. Lane 1, PBS(−); lane 2, PBS/WT; lane 3, PBS/del-A; lane 4, PBS/del-7; lane 5, PBS/del-LD. The NCp7 concentration was adjusted to one NCp7 molecule per 6 nt. Note that the (−)ssDNA generated in lane 3 (lower arrow), using PBS/del-A as the template, is smaller than other (−)ssDNA molecules (upper arrow) because of a 4-nt deletion in the template. The smaller products (normally 192 and 210 nt) are also reduced in length, as expected from paused polymerization during synthesis of (−)ssDNA (4, 25, 33, 37). (B) Effect of NCp7 on formation of complexes between $tRNA_3^{Lys}$ and various RNA templates. Lane 1, PBS(−); lane 2, PBS/WT; lane 3, PBS/del-A; lane 4, PBS/del-7; lane 5, PBS/del-LD; lane 6, 5′-end-labeled $tRNA_3^{Lys}$ as a control marker.

reactions performed with PBS/WT, PBS/del-A, and PBS/del-7 (Fig. 6B, lanes 2, 3, and 4). However, less primer-template complex was formed when PBS/del-LD was employed (lane 5), while no primer-template could be detected in the case of the PBS(−) template (lane 1). Similar results were obtained when NCp7 concentrations in these reactions corresponded to one NCp7 molecule per 4, 8, or 14 nt residues (data not shown).

DISCUSSION

Initiation of retroviral reverse transcription requires primer tRNA to be annealed to the 5′ end of the viral RNA template at a region called the PBS. As stated above, both tRNA and the PBS-containing 5′ end of the viral RNA are highly structured and fold into several stem-loops (7, 9, 17, 23). Therefore, appropriate unfolding or denaturation of these structures is necessary for annealing between the tRNA primer and the RNA template to occur. The fact that few RT-catalyzed reverse-transcribed DNA products were observed in the absence of other proteins suggests that RT alone may not suffice to destabilize secondary structures of nucleic acid sequences (3, 5, 42, 52). While it is likely that NCp7 may melt tRNA by virtue of its high affinity for single-stranded RNA (31, 32), the nature of the annealing between tRNA and the viral RNA template is not well understood.

Interactions between the viral RNA template and $tRNA_3^{Lys}$ are not limited to base pairing between the PBS and an 18-nt segment at the 3′ end of the latter molecule (1, 2, 26, 27, 34, 36). It is unclear whether NCp7 can induce melting of viral template RNA that possesses extensive secondary and tertiary structure in spite of its ability to facilitate transitions between single- and double-stranded DNA (50). The use of altered RNA templates and primers addresses these issues.

We have both confirmed and extended previous observations (3, 5, 42, 52) by using a cell-free reverse transcription assay mixture consisting of 5′ viral RNA, $tRNA_3^{Lys}$, RT, and NCp7. This system also enabled us to study primer placement. Consistent with the data of others, we found that NCp7 stimulated generation of (−)ssDNA (3, 5, 42, 52). A novel observation, however, is that NCp7 conferred exquisite specificity to reverse transcription, as shown by the suppression of nonspecific initiation from self-primed RNAs and the concomitant stimulation of specific initiation by $tRNA_3^{Lys}$. Moreover, the most efficient generation of specific (−)ssDNA occurred at NCp concentrations close to those predicted to be present in virions (16, 31, 32). The fact that previous investigators failed to observe nonspecific DNA products of RT reactions may be due to their use of less sensitive conditions, e.g., use of end-labeled tRNA primer rather than radiolabeled dNTPs as employed in this study.

Self-priming most likely resulted because the 3′ end of the RNA template folded back to initiate DNA synthesis. This may be due to a tendency of the RNA template to form secondary structure (7, 9, 17, 23). NCp7-mediated inhibition of synthesis of nonspecific DNA may be due to destabilization of the RNA helix (24, 28, 34, 50) and/or the coating of viral template RNA in a manner that renders it nonrecognizable by RT. Further work will be necessary to clarify the mechanisms involved and to determine whether NCp7 might inhibit the generation of nonspecific DNA in vivo. Virion genomic RNA contains nicks that might initiate synthesis of nonspecific reverse-transcribed products (14, 15, 16, 30, 48, 49).

In sharp contrast to $tRNA_3^{Lys}$-primed synthesis of (−)ssDNA, reverse transcription initiated by the 18-nt rPR complementary to the PBS was inhibited by NCp7 in a dose-dependent manner (Fig. 3). This result was apparently due to NCp7-mediated prevention of annealing between rPR and the viral RNA template (Fig. 5). Indeed, in the absence of NCp7, rPR was modestly annealed onto the RNA template during an incubation period of 30 min at 37°C. In contrast, little $tRNA_3^{Lys}$ was found to be annealed with the RNA template in the absence of NCp7, and the addition of NCp7 stimulated this process by more than 50-fold (molecular imaging analysis not shown) (Fig. 4A). Therefore, it appears that the remaining portion of the tRNA molecule (i.e., sequences other than the 18 nt that base pair with the PBS) might be the driving force for an equilibrium that favors formation of a stable tRNA-RNA duplex mediated by NCp7. This might occur through interaction with sequences outside the PBS.

Removal of the remaining portion of the $tRNA_3^{Lys}$ molecule (as in the case of rPR) resulted in poor hybridization between rPR and the RNA template in the presence of NCp7. Thus, NCp7 apparently confers specificity by stabilizing and/or mediating the formation of the $tRNA_3^{Lys}$-RNA complex that is recognized by RT for initiation of (−)ssDNA synthesis. Indeed, mutant retroviruses, containing a PBS replaced by sequences complementary to other tRNAs, reverted back to the wild type in culture, even though these mutant viruses can use

alternate tRNAs as replication primers during early stages of infection (19, 37, 51, 53). These results indicated that the NCp-mediated configuration of the wild-type tRNA-RNA complex is essential for recognition by RT and that NCp might cause more efficient annealing between viral template RNA and $tRNA_3^{Lys}$ than between viral RNA and other tRNA isoacceptor species (53). Protein-protein interactions involving both RT and NCp7 may also be important in this regard (16, 32, 35).

Recent genetic and biochemical evidence suggests that interactions at multiple sites between tRNA and 5′ viral RNA (not merely complementary sequences between the PBS and the 3′ end of the tRNA primer) may be required for efficient reverse transcription (1, 2, 12, 13, 26, 27, 34, 36). In the avian retroviral system, interactions between the TΨC loop of $tRNA^{Pro}$ and sequences located upstream of the PBS can help to stabilize the tRNA-RNA complex (1, 2). In HIV-1, the A-rich loop located upstream of the PBS and a 6-nt sequence located immediately downstream of the PBS have been shown to interact with the anticodon region of $tRNA_3^{Lys}$ (27, 34). In our system, deletion of either of these regions had little effect on synthesis of (−)ssDNA. Any discrepancies in results might be attributable to the use of different viral strains and/or experimental conditions (27, 34). It is also possible that the templates used by other investigators had different secondary structures than those of templates employed here. However, we found that a larger deletion, located downstream of the PBS (PBS/del-LD), did result in decreased formation of the primer-template complex as well as synthesis of (−)ssDNA (Fig. 6). We have also tested this modification in the context of an infectious molecular clone and found that it greatly reduced infectivity, while the other two deletions tested, i.e., PBS/del-A and PBS/del-7, had little or no effect in this regard (36a). Determination of nascent reverse-transcribed DNA products from infected cells by a quantitative PCR revealed no differences among PBS/del-A, PBS/del-7, and wild-type virus, but a >10-fold reduction in DNA synthesis was observed in the case of the PBS/del-LD mutants. We are currently investigating the role of the latter region in maintaining an appropriate configuration of template and in whether specific sequences within it are necessary for direct interaction with $tRNA_3^{Lys}$.

ACKNOWLEDGMENTS

This research was supported by grants from the Medical Research Council of Canada and by Health Canada. Mark A. Wainberg is a National AIDS Scientist funded by Health Canada.

We thank Stuart F. J. Le Grice, Case Western Reserve University, Cleveland, Ohio, for a gift of HIV RT.

REFERENCES

1. **Aiyar, A., D. Cobrinik, Z. Ge, H.-J. Kung, and J. Leis.** 1992. Interaction between retroviral U5 RNA and the TΨC loop of the $tRNA^{Trp}$ primer is required for efficient initiation of reverse transcription. J. Virol. **66:**2464–2472.
2. **Aiyar, A., Z. Ge, and J. Leis.** 1994. A specific orientation of RNA secondary structure is required for initiation of reverse transcription. J. Virol. **68:**611–618.
3. **Allain, B., M. Lapadat-Tapolsky, C. Berlioz, and J.-L. Darlix.** 1994. Transactivation of the minus strand DNA transfer by nucleocapsid protein during reverse transcription of the retroviral genome. EMBO J. **13:**973–981.
4. **Arts, E. J., X. Li, Z. Gu, L. Kleiman, M. A. Parniak, and M. A. Wainberg.** 1994. Comparison of deoxyoligonucleotide and $tRNA^{Lys.3}$ as primers in an endogenous HIV-1 in vitro reverse transcription/template switching reaction. J. Biol. Chem. **269:**14672–14680.
5. **Barat, C., V. Lullien, O. Schatz, F. Gruninger-Leitch, M. Nugeyere, F. Barre-Sinoussi, S. F. J. LeGrice, and J. L. Darlix.** 1989. HIV-1 reverse transcriptase specifically interacts with the anti-codon domain of its cognate primer $tRNA^{Lys.3}$. EMBO J. **8:**3279–3285.
6. **Barat, C., O. Schatz, S. F. J. Le Grice, and J. L. Darlix.** 1993. Analysis of the interactions of HIV-1 replication primer $tRNA^{Lys.3}$ with nucleocapsid protein and reverse transcriptase. J. Mol. Biol. **231:**185–190.
7. **Baudin, F., R. Marquet, C. Isel, J.-L. Darlix, B. Ehresmann, and C. Ehresmann.** 1993. Functional sites in the 5′ region of HIV-1 RNA form defined structural domains. J. Mol. Biol. **229:**382–397.
8. **Berg, J.** 1986. Potential metal-binding domains in nucleic acid binding proteins. Science **25:**485–487.
9. **Berkhout, B., and L. Schoneveld.** 1993. Secondary structure of the HIV-2 leader RNA comprising the tRNA-primer binding site. Nucleic Acids Res. **21:**1171–1178.
10. **Bess, J. W., Jr., P. J. Powell, H. J. Issaq, L. J. Schumack, M. K. Grimes, L. E. Henderson, and L. O. Arthur.** 1992. Tightly bound zinc in human immunodeficiency virus type 1, human T-cell leukemia virus type I, and other retroviruses. J. Virol. **66:**840–847.
11. **Blain, S. W., W. A. Hendrickson, and S. P. Goff.** 1995. Reversion of a Moloney murine leukemia virus RNase H mutant at a second site restores enzyme function and infectivity. J. Virol. **69:**5113–5116.
12. **Cobrinik, D., A. Aiyar, Z. Ge, M. Katzman, H. Huang, and J. Leis.** 1991. Overlapping U5 sequence elements are required for efficient integration and initiation of reverse transcription. J. Virol. **65:**3864–3872.
13. **Cobrinik, D., L. Soskey, and J. Leis.** 1988. A retroviral RNA secondary structure required for efficient initiation of reverse transcription. J. Virol. **62:**3622–3630.
14. **Coffin, J. M.** 1979. Structural replication and recombination of retrovirus genomes: some unifying hypotheses. J. Gen. Virol. **42:**1–26.
15. **Coffin, J. M.** 1985. Genome structure, p. 17–74. *In* R. Weiss, N. Teich, H. Varmus, and J. Coffin (ed.), RNA tumor viruses, part 1, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Darlix, J.-L., M. Lapadat-Tapolsky, H. De Rocquigny, and B. P. Roques.** 1995. First glimpses at structure-function relationships of the nucleocapsid protein of retroviruses. J. Mol. Biol. **254:**523–537.
17. **Darlix, J.-L., M. Schwager, P. F. Spahr, and P. A. Bromley.** 1980. Analysis of the secondary and tertiary structure of the Rous sarcoma virus RNA. Nucleic Acids Res. **8:**3335–3354.
18. **Darlix, J.-L., A. Vincent, C. Gabus, H. DeRocquigny, and B. Roques.** 1993. Trans-activation of the 5′ to 3′ viral DNA strand transfer by nucleocapsid protein during reverse transcription of HIV-1 RNA. Life Sci. **316:**763–771.
19. **Das, A. T., B. Klaver, and B. Berkhout.** 1995. Reduced replication of human immunodeficiency virus type 1 mutants that use reverse transcription primers other than the natural $tRNA_3^{Lys}$. J. Virol. **69:**3090–3097.
20. **De Rocquigny, H., D. Ficheux, M. C. Fournie-Zaluski, J. L. Darlix, and B. P. Roques.** 1991. First large scale chemical synthesis of the 72 amino acid HIV-1 nucleocapsid protein Ncp7 in an active form. Biochem. Biophys. Res. Commun. **180:**1010–1018.
21. **De Rocquigny, H., C. Gabus, A. Vincent, M. C. Fournie-Zaluski, B. P. Roques, and J. L. Darlix.** 1992. Viral annealing activities of human immunodeficiency virus type 1 nucleocapsid protein require only peptide domains outside the zinc fingers. Proc. Natl. Acad. Sci. USA **89:**6472–6476.
22. **Fitzgerald, D., and J. Coleman.** 1991. Physicochemical properties of cloned nucleocapsid protein from HIV interactions with metal ions. Biochemistry **30:**5195–5201.
23. **Harrison, G. P., and A. M. L. Lever.** 1992. The human immunodeficiency virus type 1 packaging signal and major splice donor region have a conserved stable secondary structure. J. Virol. **66:**4144–4153.
24. **Herschlag, D., M. Khosla, Z. Tsuchihashi, and R. L. Karpel.** 1994. An chaperone activity of non-specific RNA binding proteins in hammerhead ribozyme catalysis. EMBO J. **13:**2913–2924.
25. **Huber, H. E., J. M. McCoy, J. S. Seehra, and C. C. Richardson.** 1989. Human immunodeficiency virus 1 reverse transcriptase. Template binding, processivity, strand displacement synthesis and template switching. J. Biol. Chem. **264:**4669–4678.
26. **Isel, C., C. Ehresmann, G. Keith, B. Ehresmann, and R. Marquet.** 1995. Initiation of reverse transcription of HIV-1: secondary structure of the HIV-1 RNA/$tRNA^{Lys.3}$ (template/primer) complex. J. Mol. Biol. **247:**236–250.
27. **Isel, C., R. Marquet, G. Keith, C. Ehresmann, and B. Ehresmann.** 1993. Modified nucleotides of $tRNA^{Lys.3}$ modulate primer/template loop-loop interaction in the initiation complex of HIV-1 reverse transcription. J. Biol. Chem. **269:**1388–1393.
28. **Ji, X., G. J. Klarmann, and B. D. Preston.** 1996. Effects of HIV-1 nucleocapsid protein on HIV-1 reverse transcriptase activity in vitro. Biochemistry **35:**132–143.
29. **Jiang, M., J. Mak, A. Ladha, E. Cohen, M. Klein, B. Rovinski, and L. Kleiman.** 1993. Identification of tRNAs incorporated into wild-type and mutant human immunodeficiency virus type 1. J. Virol. **67:**3246–3253.
30. **Junghans, R. P., L. R. Boone, and A. M. Skalka.** 1982. Retroviral DNA H structures: displacement-assimilation model of recombination. Cell **30:**53–62.
31. **Karpel, R. L., L. Henderson, and L. E. Oroszlan.** 1987. Interactions of retroviral structural proteins with single stranded nucleic acids. J. Biol. Chem. **262:**4961–4967.
32. **Khan, R., and D. Giedroc.** 1992. Recombinant human immunodeficiency virus type 1 nucleocapsid protein unwinds tRNA. J. Biol. Chem. **267:**6689–6695.

33. **Klarmann, G., C. A. Schauber, and B. D. Preston.** 1993. Template-directed pausing of DNA synthesis by HIV-1 reverse transcriptase during polymerization of HIV-1 sequences in vitro. J. Biol. Chem. **268:**9793–9802.
34. **Kohlsteadt, L., and T. Steitz.** 1992. Reverse transcriptase of HIV-1 can use either human $tRNA^{Lys.3}$ or E. coli tRNAglu as primer in an in vitro utilization assay. Proc. Natl. Acad. Sci. USA **89:**4652–4656.
35. **Lapadat-Tapolsky, M., C. Pernelle, C. Borie, and J.-L. Darlix.** 1995. Analysis of the nucleic acid annealing activities of nucleocapsid protein from HIV-1. Nucleic Acids Res. **23:**2434–2441.
36. **Leis, J., A. Ashok, and D. Cobrinik.** 1993. Regulation of initiation of reverse transcription of retroviruses, p. 33–47. *In* A. M. Skalka and S. P. Goff (ed.), Reverse transcriptase. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.

36a. **Li, X., C. Liang, Y. Quan, R. Chandock, M. A. Parniak, L. Kleiman, and M. A. Wainberg.** Impact on replication of the immunodeficiency virus type 1 of mutations in sequences that flank the primer binding sequence. Submitted for publication.

37. **Li, X., J. Mak, E. J. Arts, Z. Gu, L. Kleiman, M. A. Wainberg, and M. A. Parniak.** 1994. Effects of alterations of primer binding site sequences in human immunodeficiency virus type 1 replication. J. Virol. **68:**6198–6206.
38. **Litvak, S., L. Sarih-Cottin, M. Fournier, M. Andreola, and L. Tarrago-Litvak.** 1993. Priming of HIV-1 replication by $tRNA^{Lys.3}$: role of reverse transcriptase. Trends Biochem. Sci. **19:**114–118.
39. **Moreller, N., N. Jullian, H. De Rocquigny, B. Maigret, J. L. Darlix, and B. P. Roques.** 1992. Determination of the structure of the nucleocapsid protein NCp7 from the human immunodeficiency virus type 1 by ^1H NMR. EMBO J. **11:**3059–3065.
40. **Peliska, J. A., S. Balasubramanian, D. P. Giedroc, and S. J. Benkovic.** 1994. Recombinant HIV-1 nucleocapsid protein accelerates HIV-1 reverse transcriptase catalyzed DNA strand transfer reactions and modulates RNase H activity. Biochemistry **33:**13817–13823.
41. **Picard, V., E. Ersdal-Badju, A. Lu, and S. C. Bock.** 1994. A rapid and efficient one-tube PCR-based mutagenesis technique using Pfu DNA polymerase. Nucleic Acids Res. **22:**2587–2591.
42. **Prats, A. C., L. Sarih, C. Gabus, S. Litvak, G. Keith, and J. L. Darlix.** 1988. Small finger protein of avian and murine retroviruses has nucleic acid annealing activity and positions the replication primer tRNA onto genomic RNA. EMBO J. **7:**1777–1783.
43. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal.** 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313:**277–284.
44. **Rhim, H., J. Park, and C. D. Morrow.** 1991. Deletions in the $tRNA^{Lys}$ primer binding site of human immunodeficiency virus type 1 identify essential regions for reverse transcription. J. Virol. **65:**4555–4564.
45. **Rodriguez-Rodriguez, L., Z. Tsuchihashi, G. M. Fuentes, R. A. Bambara, and P. J. Fay.** 1995. Influence of human immunodeficiency virus nucleocapsid protein on synthesis and strand transfer by the reverse transcriptase in vitro. J. Biol. Chem. **270:**15005–15011.
46. **South, T. L., P. R. Blake, R. C. Sowder III, L. A. Arthur, L. Henderson, and M. F. Summers.** 1990. The nucleocapsid protein from HIV-1 particles binds zinc and forms retroviral-type zinc fingers. Biochemistry **29:**7786–7789.
47. **Surovoy, A., J. Dannull, O. K. Moelling, and G. Jung.** 1993. Conformational and nucleic acid binding studies on the synthetic nucleic acid protein of HIV-1. J. Mol. Biol. **229:**94–104.
48. **Tanchou, V., C. Gabus, V. Rogemond, and J. L. Darlix.** 1995. Formation of stable and functional HIV-1 nucleoprotein complexes in vitro. J. Mol. Biol. **252:**563–571.
49. **Temin, H. M.** 1993. Retroviral variation and reverse transcription: abnormal transfers result in retrovirus genetic variation. Proc. Natl. Acad. Sci. USA **90:**6900–6903.
50. **Tsuchihashi, Z., and P. O. Brown.** 1994. DNA strand exchange and selective DNA annealing promoted by the human immunodeficiency virus type 1 nucleocapsid protein. J. Virol. **68:**5863–5870.
51. **Wakefield, J. K., A. G. Wolf, and C. D. Morrow.** 1995. Human immunodeficiency virus type 1 can use different tRNAs as primers for reverse transcription but selectively maintains a primer binding site complementary to $tRNA_3^{Lys}$. J. Virol. **69:**6021–6029.
52. **Weis, S., B. Konig, Y. Morikawa, and I. Jones.** 1992. Recombinant HIV-1 nucleocapsid protein p15 produced as a fusion protein with glutathione-S-transferase in E. coli mediates dimerization and enhances reverse transcription of retroviral RNA. Gene **121:**203–312.
53. **Whitcomb, J. M., B. A. Ortiz-Conde, and S. H. Hughes.** 1995. Replication of avian leukosis viruses with mutations at the primer binding site: use of alternative tRNAs as primers. J. Virol. **69:**6228–6238.
54. **Wohrl, B. M., B. Ehresmann, G. Keith, and S. F. J. Le Grice.** 1993. Nuclease footprintings of human immunodeficiency virus reverse transcriptase/$tRNA^{Lys.3}$ complexes. J. Biol. Chem. **268:**13617–13624.
55. **You, J. C., and C. S. McHenry.** 1994. HIV nucleocapsid protein accelerates strand transfer of the terminally redundant sequences involved in reverse transcription. J. Biol. Chem. **269:**31491–31495.