Recombinant HIV-1 Nucleocapsid Protein Accelerates HIV-1 Reverse Transcriptase Catalyzed DNA Strand Transfer Reactions and Modulates RNase H Activity†

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ABSTRACT: The effect of recombinant nucleocapsid protein (NCp7) from human immunodeficiency virus type 1 (HIV-1) on HIV-1 reverse transcriptase (HIV-1 RT) catalyzed DNA strand transfer reactions has been studied using kinetic methods with a defined template−primer model system. NCp7 is shown to modulate both the rate and the efficiency of DNA strand transfer synthesis. Evidence is presented that supports the role of NCp7 in catalyzing the annealing of a nascent DNA intermediate and RNA acceptor template during strand transfer. NCp7 was also found to enhance the ribonuclease H activity of HIV-1 RT and change the specificity of RNA hydrolysis, suggesting a direct role of NCp7 in HIV-1 RT catalyzed strand transfer. The implications of these findings for retroviral reverse transcription are addressed.

The core structure of a retrovirus virion contains components required for the process of reverse transcription and host integration including the retroviral integrase and several copies of the replicative enzyme reverse transcriptase (Varmus & Brown, 1989; Whitcomb & Hughes, 1992; Skalka & Goff, 1993). The diploid viral genomic RNA is part of a ribonucleoprotein complex where the RNA is in tight association with the nucleocapsid protein NCp7. NCp7 is processed from a *gag* gene polyprotein precursor (Varmus & Brown, 1989; Darlix et al., 1990). The role of viral NCp7 in the process of viral replication is not as yet well defined, but several recent reports suggest that it is required in genomic RNA dimerization necessary for correct encapsidation (Dickson et al., 1985; Prats, et al., 1988; Bieth et al., 1990; Cornille et al., 1990; Prats et al., 1991) and in the annealing of the tRNA primer to its complementary binding site (PBS) on the genomic RNA (Prats et al., 1988; Bieth et al., 1990; Dupraz et al., 1990; Barat et al., 1991; Khan & Giedroc, 1992).

NCp7 from HIV-1 contains two conserved zinc finger domains of the form Cys-Xaa$_2$-Cys-Xaa$_4$-His-Xaa$_4$-Cys (Henderson et al., 1981; Berg, 1986; Sakaguchi et al., 1993), which can be reconstituted *in vitro* with Zn(II), Cd(II), or Co(II) (Roberts et al., 1989; South et al., 1989; Green & Berg, 1990; Fitzgerald & Coleman, 1991). High-resolution NMR studies show that while the two zinc domains fold into well-defined homologous globular structures, the remainder of the molecule, including the N-terminal and interfinger connecting polypeptide chains, is generally devoid of stable secondary structure. The mechanistic importance of the zinc-binding domains in nucleocapsid protein (NC) function and viral replication is not well understood. Mutation of the Zn(II)-coordinating ligands or the surrounding amino acids, however, results in a dramatic reduction in viral infectivity, implying that NCp7 plays an important role in RNA recognition (Meric & Sphar, 1986; Fu et al., 1988; Gorelick et al., 1988; Meric & Goff, 1989; Dupraz et al., 1990; Gorelick et al., 1990). NCp7 may function in the formation of a reverse transcriptionally active ribonucleoprotein complex. Recent data which suggests that the zinc fingers play a generalized role in stabilization and maturation of the ribonucleoprotein complex, rather than in specific RNA recognition, is consistent with this. NCp7 binds tightly and with moderate cooperativity to single-stranded RNA and DNA (Khan & Giedroc, 1992). Recent studies suggest the formation of two distinct types of binding complexes between NCp7 and RNA (Dib-Hajj et al., 1993). There is a transition[1] from a high-site-size (n = 14 nucleotides/NCp7 molecule) to a low-site-size ribonucleoprotein complex (n = 8 nucleotides/NCp7 molecule) as the ratio of NCp7 protein to RNA increases.

Consistent with their participation in tRNA primer annealing, NCp7 proteins increase the observed second-order rate constant for the renaturation of two complementary RNA strands by more than 4 orders of magnitude (Dib-Hajj et al., 1993). Also, this rate acceleration is realized only with the low-site-size ribonucleoprotein binding configuration. While zinc finger domains in other nucleic acid binding proteins are thought to be directly involved in DNA binding and recognition, this may not be the case with NCp7 since removal of such structures has no effect on renaturation catalysis (Prats et al., 1991; De Rocqui et al., 1992). Therefore, the role of the zinc finger structure in NCp7 function is as yet unknown.

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[1] Recent experiments suggest that another form of HIV-1 NC, corresponding to the N-terminal 57 amino acids of the 71 amino acid protein [cf. Summers et al. (1992)], forms only a low-site-size complex ($n = 7 \pm 1$) and exhibits an extent of DNA strand renaturation activity similar to that of the 71 amino acid protein (R. Khan and D. P. Giedroc., manuscript in preparation).

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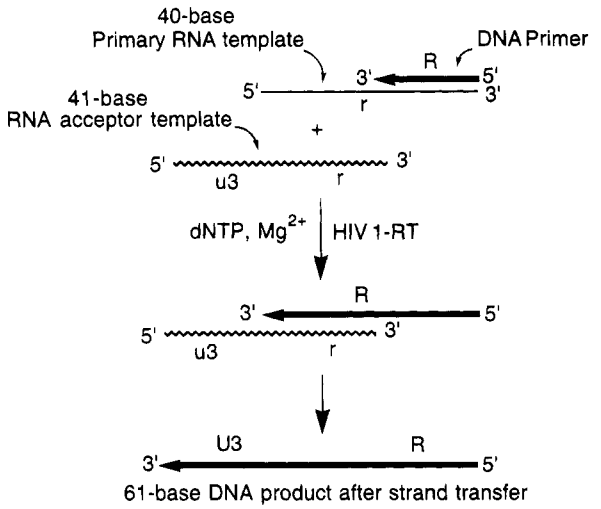

FIGURE 1: DNA strand transfer assay system. DNA is represented by thick lines; RNA is illustrated by thin lines. The 40-base primary RNA template derived from the repeat sequence (r) located at the 5′-end of the HIV-1 genome was primed with a complementary 20- or 24-base DNA oligonucleotide.[2] The acceptor RNA template was homologous to the last 20 bases (5′-end) of the primary RNA template (r) and included an additional 21 bases derived from the U3 genomic sequence. The sequences are given in Materials and Methods. In the standard assay, 5′-end-labeled [^{32}P]DNA is used, and after reaction initiation and termination (EDTA quench), the products were analyzed by polyacrylamide gel electrophoresis (PAGE) under denaturing conditions. The occurrence of strand-transfer DNA synthesis is indicated by the appearance of 61-base DNA product.

In addition to facilitating tRNA annealing to the viral genomic RNA, NCp7 likely cooperates in HIV-1 RT catalyzed strand-transfer reactions. During DNA strand transfer reactions, a nascent DNA strand synthesized by HIV-1 RT using a RNA template (primary template) is transferred onto a second RNA template (acceptor template) (Figure 1) (Luo & Taylor, 1990; Garces & Wittek, 1991; DeStefano et al., 1992; Peliska & Benkovic, 1992; Allain et al., 1994). The transfer is facilitated by both the complementarity of the RNA acceptor template with the nascent DNA strand and the RT-associated RNase H mediated removal of the primary RNA template. Since such a strand transfer involves annealing of complementary strands, NCp7 might function in the transfer event. Recently, the mechanism of strand transfer catalyzed by HIV-1 RT has been examined *in vitro* using a well-defined template−primer system (Figure 1) (Peliska & Benkovic, 1992). We have investigated the effect of NCp7 on the kinetics of this DNA strand transfer model reaction and have found that NCp7 modulates the rate of DNA strand transfer. Under conditions favoring the formation of a low-site-size complex of NCp7 and DNA/RNA substrate, a significant increase in the rate of DNA strand transfer is observed. This rate enhancement is dependent on the concentration of NCp7. In addition, NCp7 was found to change the overall efficiency of the strand-transfer reaction, giving rise to an overall lower yield of strand-transfer product in reaction mixtures containing NCp7 verses control reactions lacking the nucleocapsid protein. The kinetic factors for strand transfer influenced by NCp7 and the reason for the observed change in overall strand-transfer efficiency are discussed.

[2] Kinetics data using the 24-base DNA primer [in place of the 20-base primer used previously (Peliska & Benkovic, 1992)] give comparable results. The 24-base DNA primer gives slightly improved efficiency of DNA polymerization with HIV-1 RT, as compared to the 20-base DNA, which exhibited a pause site (data not shown).

MATERIALS AND METHODS

Materials. HIV-1 RT and HIV-1 NCp7 (denoted in previous work as NC71) were purified as described previously. Recombinant HIV-1 NCp7 is a 71 amino acid protein on the basis of the deduced amino acid sequence from Met^{377} to Phe^{447} of the Pr55 gag polyprotein precursor of the BH10-R3 HIV-1 proviral clone. Henderson et al. suggest that NCp7 is an incompletely processed product comprising mature p7 (Met^{377}−Asn^{431}), a 55 amino acid protein, and the p1 (Phe^{432}−Phe^{447}) polypeptide, with cleavage by the viral protease apparently quantitative following Asn^{431}.

The sequences of the oligonucleotides used in the kinetic studies are as follows: 24-base DNA primer, 5′-AGAGCTCCCAGGCTCAGATCTGGT-3′; 40-base RNA template, 3′-UCUCGAGGGUCCGAGUCUAGACCAGAUUGGUCUCUCUGGG-5′; 41-base RNA acceptor template, 3′-ACCAGAUUGGUCUCUCUGGGUCAUGUCCGUUUUUCGUCGAG-5′. The 20-base DNA primer is identical to the 5′ 20 bases of the 24-base DNA primer. RNA synthesis was performed by T7 RNA polymerase runoff transcription using synthetic DNA templates containing the T7 promoter. A trace amount of [α-^{32}P]UTP was incorporated into the transcription reaction to aid in purification and quantitation. The RNA was purified by electrophoresis through 20% acrylamide/8 M urea/TBE gels, eluted from excised gel slices with 0.5 M ammonium acetate at 37 °C, and precipitated with ethanol.

The RNA substrates were 5′-end-labeled by their reaction with polynucleotide kinase and [γ-^{32}P]ATP after dephosphorylation with alkaline phosphatase. 5′-End-labeled RNA was gel purified as described above.

All reagents were the highest purity available, with all solutions prepared with RNase-free reagents. Electrophoresis was performed on denaturing gels consisting of 20% acrylamide/8 M urea/TBE (90 mM Tris/64.6 mM boric acid/2.5 mM EDTA, pH 8.3).

Strand-Transfer DNA Synthesis Reactions. A reaction mixture containing assay buffer (50 mM Tris-HCl, pH 8, 75 mM KCl, 1 mM dithiothreitol, and 0.1% Triton X-100), 200 nM 5′-end-labeled 20-base [^{32}P]DNA•40-base RNA, each dNTP at 100 μM, 7 mM $MgCl_2$, 480 nM 41-base RNA, and varying amounts of NCp7 was initiated with HIV-1 RT (200 nM final concentration) and incubated at 37 °C. At the times indicated, reaction samples were withdrawn, and the reaction was terminated by addition to EDTA (110 mM final). Reaction samples were resolved by denaturing PAGE (20% acrylamide/8 M urea/TBE), and the product bands were visualized by autoradiography using a Molecular Dynamics PhosphorImager and quantitated using ImageQuant software.

Substrate and enzyme spike experiments were performed as above except that at the designated time additional HIV-1 RT, 24-base DNA•40-base RNA, or 41-base RNA was added to the reaction mixture.

RESULTS AND DISCUSSION

During retroviral reverse transcription, the viral protein reverse transcriptase catalyzes DNA strand transfer reactions wherein a nascent DNA strand is transferred from one RNA template strand to a second RNA template (Figure 1). This reaction is exemplified by the strand transfer of minus strand

strong-stop DNA and is thought to occur during copy choice and forced copy choice recombination during minus strand DNA synthesis. We and others have developed cell-free model systems designed to examine the mechanism of DNA strand transfer catalyzed by a variety of retroviral RTs, including HIV-1 RT (Luo & Taylor, 1990; Garces & Wittek, 1991; DeStefano et al., 1992; Peliska & Benkovic, 1992; Allain et al., 1994). Many of the kinetic and mechanistic features of RT-catalyzed strand transfer have been determined: (1) The RNase H activity of reverse transcriptase is required for DNA strand transfer both *in vitro* and *in vivo*; however, exceptions have been noted (Luo & Taylor, 1990). (2) Processive DNA synthesis is not the rate-limiting step in DNA strand transfer reactions. The rate-limiting step in these reactions appears to involve the slow RNase H catalyzed hydrolysis of residual primary template RNA fragments annealed to the nascent DNA strand. Removal of these fragments allows final annealing of the nascent DNA strand to the RNA acceptor template and subsequent DNA synthesis (Peliska & Benkovic, 1992). (3) Kinetic and substrate cross-linking experiments suggest that the acceptor RNA template may begin to anneal to the nascent DNA product strand before the complete removal of residual primary RNA template, suggesting that the strand-transfer event may occur in a concerted rather than a stepwise fashion (Peliska & Benkovic, 1992).

In addition to RT, the viral core contains other virally encoded proteins including nucleocapsid protein (NC). Because of its ability to catalyze the annealing rate of complementary DNA and RNA strands, it was proposed that NCp7 might be involved along with RT in DNA strand transfer reactions (Dib-Hajj et al., 1993). This possibility was tested with HIV-1 RT and HIV-1 NCp7 using the strand-transfer model system developed earlier. This system utilizes two defined RNA templates with overlapping sequence homologies as shown in Figure 1. The size of the RNA templates (primary template, 40 bases; acceptor template, 41 bases) facilitates the analysis of polymerase and RNase H activities with single-nucleotide resolution and thus allows for the determination of kinetic factors and mechanistic details that are difficult to assess with longer, genome-sized template systems. The lack of polymerase pausing during DNA synthesis by HIV-1 RT on the RNA templates used in this study (data not shown) suggests that pronounced, stable nucleic acid secondary structures are not influencing HIV-1 RT activity (Klarmann et al., 1993).

Figure 2 illustrates the effect of NCp7 on the production of the DNA strand transfer product using the model system outlined in Figure 1. Low concentrations of NCp7 have an inhibitory effect on DNA strand transfer relative to the control reaction lacking NCp7 (Figure 2A). However, at higher concentrations of NCp7, a rate enhancement is observed. When the DNA strand transfer assay is performed at increasing concentrations of NCp7, a transition from inhibition to rate acceleration is observed in a fashion that is directly related to the concentration of NCp7 (Figure 2B). The observed increase in the rate of DNA strand transfer with increasing NCp7 concentration increases until sufficient NCp7 is present to coat all nucleotide strands, after which no further enhancement is realized. Under these reaction conditions the observed rate of DNA polymerization, which is not rate limiting for DNA strand transfer, was not sensitive to NCp7 (data not shown). The shift from inhibition to acceleration of DNA strand transfer seems to correlate with

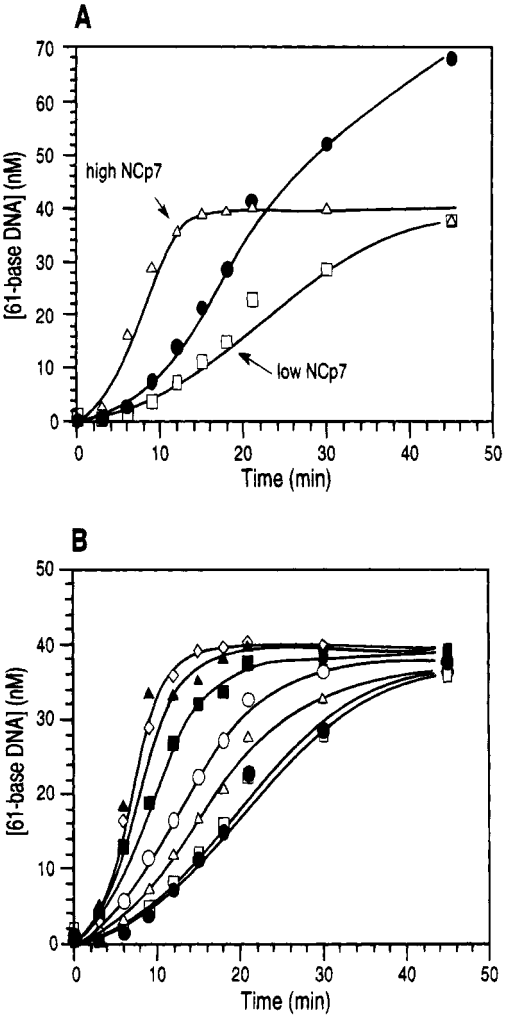

FIGURE 2: Concentration dependence of NCp7 on DNA strand transfer. DNA strand transfer was studied in the presence of varying concentrations of NCp7. (A) Effects of high and low concentrations of NCp7. A reaction mixture containing assay buffer [50 mM tris-HCl, pH 8, 75 mM KCl, 1 mM DTT (dithiothreitol), and 0.1% Triton X-100], 200-nM 5′-end-labeled 20-base [^{32}P]DNA·40-base RNA, each dNTP at 100 μM, 7 mM $MgCl_2$, 480 nM 41-base RNA, and 0 (●), 0.5 (□), or 16 μM NCp7 (△) was initiated with HIV-1 RT (200 nM final concentration) and incubated at 37 °C. At the times indicated, reaction samples were withdrawn, and the reaction was terminated by addition to EDTA (110 mM final). Reaction samples were analyzed by PAGE on 20% acrylamide/8 M urea/TBE gels. The product bands were visualized following autoradiography using a Molecular Dynamics PhosphorImager and quantitated using ImageQuant software. (B) Titration with NCp7. The reaction conditions were as for (A) except the NCp7 concentrations were 0.5 (●), 1 (□), 2 (△), 4 (○), 8 (■), 12 (▲), and 16 μM (◇).

the previously observed transition from a site size of 14 nucleotides/NCp7 molecule to one of 8 nucleotides/NCp7 molecule (Dib-Hajj et al., 1993). These earlier studies have shown that, in the presence of sufficient NCp7 to coat all nucleotide strands (site size $n = 8$), NCp7 increases the second-order rate constant for the annealing of complementary nucleotide strands. Under conditions of low NCp7 protein concentration (site size $n = 14$), the rate acceleration is not realized. The correlation between these results and the observed modulation in DNA strand transfer kinetics would be in accord with NCp7 participating in some annealing event during the DNA strand transfer process. The physical and mechanistic significance of this transition is not yet understood and is the subject of current investigations.

It was observed previously that the rate of DNA strand transfer was sensitive to the concentration of 41-base RNA

acceptor template (Peliska & Benkovic, 1992). Under similar conditions, but in the presence of NCp7, this concentration dependence is largely removed and rate enhancements approaching an order of magnitude can be achieved at low concentrations of 41-base RNA acceptor template (data not shown).

The presence of NCp7 also affects the overall efficiency of DNA strand transfer (Figure 2A). In the presence of high concentrations of NCp7, the accumulation of DNA strand transfer product terminates at a lower concentration than is observed in the absence of NCp7. This phenomenon could be the result of either the rapid consumption of acceptor template RNA by the RNase H activity of HIV-1 RT in the presence of NCp7 or the formation of a dead-end complex involving HIV-1 RT, thereby preventing further enzyme turnover. To investigate these possibilities, substrate and enzyme spike experiments were performed. In these experiments, a strand-transfer reaction was allowed to proceed until the characteristic plateau in product formation was attained, and then a second aliquot of 41-base RNA acceptor template (Figure 3A), HIV-1 RT (Figure 3B), or 24-base DNA•40-base RNA template•primer (Figure 3C) was added. When HIV-1 RT was added to the strand-transfer reaction, no subsequent synthesis of additional 61-base DNA strand transfer product was observed. Since a second addition of active HIV-1 RT does not affect the yield of strand-transfer product, it is unlikely that the observed plateau is caused by a dead-end complex involving HIV-1 RT. A similar result was obtained when 24-base DNA•40-base RNA template•primer was added. However, addition of 41-base RNA acceptor template to the strand-transfer reaction resulted in the subsequent production of additional strand-transfer product. This strongly suggests that the observed plateau in strand-transfer product results from the rapid depletion of the pool of 41-base RNA acceptor template.

To further investigate this reaction, the RNase H catalyzed degradation of 41-base RNA acceptor template during DNA strand transfer was monitored by 5′-^{32}P end labeling of RNA acceptor template (Figure 4). This experiment corroborates the conclusions of the substrate spike experiments in that it clearly shows that, in the reaction containing NCp7, 41-base RNA acceptor template is degraded approximately 8-fold faster than in the control lacking NCp7.[3] Furthermore, the RNase H catalyzed depletion of 41-base RNA acceptor template tracks with the observed termination of 61-base DNA strand transfer product formation (Figure 4B). Within 20 min all of the 41-base RNA has been consumed, and the accumulation of 61-base DNA is necessarily terminated.

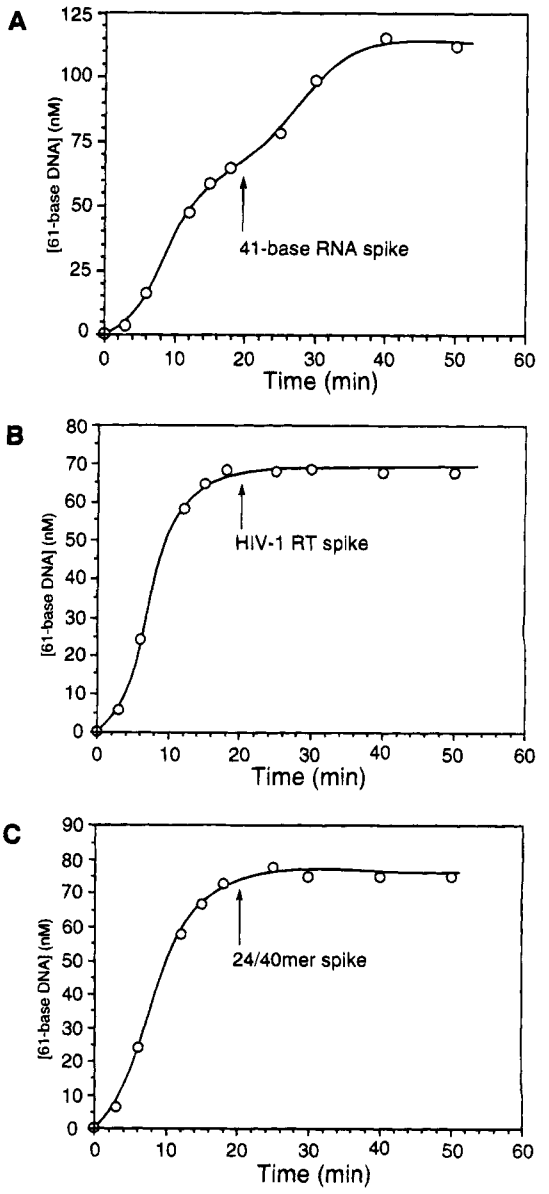

FIGURE 3: Substrate and enzyme spike experiments. DNA strand transfer reactions were allowed to proceed until a reaction product plateau was achieved, and then reactions were spiked with additional substrate or enzyme. Reaction mixtures containing assay buffer, 200 nM 5′-end-labeled 20-base [^{32}P]DNA•40-base RNA, each dNTP at 100 μM, 7 mM $MgCl_2$, 825 nM 41-base RNA, and 15 μM NCp7 were incubated at 37 °C for 5 min, and then the strand-transfer reaction was initiated with HIV-1 RT (100 nM final concentration) at 37 °C. At the indicated times, reaction samples were withdrawn, and the reaction was quenched. After a reaction time of 20 min, the reaction mixture was spiked with either (A) 41-base RNA acceptor template (1 μM additional), (B) HIV-1 RT (100 nM additional), or (C) 24-base DNA•40-base RNA (200 nM additional), and the reaction was allowed to proceed. Reaction products were analyzed as in Figure 2.

These results conclusively show that the efficiency of total 61-base DNA formation is directly dependent on the depletion of 41-base RNA acceptor template. Identical reactions carried out in the absence of NCp7 showed that the depletion of 41-base RNA acceptor template is comparatively slow (Figure 4A, left panel), and as a result the overall efficiency of DNA strand transfer product formation increases as shown in Figure 2A. Interestingly, at low concentrations of NCp7 a similar plateau is observed (Figure 2) even though strand transfer is inhibited under these conditions. The reason for this is not known, but it suggests that NCp7 may play a role

[3] Under the conditions described in Figure 4, the net rate of 41-base RNA hydrolysis in the presence of NCp7 is approximately 6-fold higher than in the absence of NCp7. The hydrolysis is the sum of both the productive (Figure 5, steps i−ii) and the nonproductive (Figure 5, steps iii−iv) DNA strand transfer reactions. The approximate rates for productive DNA strand transfer are 6 nM/min (+NCp7) and 3 nM/min (−NCp7), and 60 nM/min (+NCp7) and 8 nM/min (−NCp7) are the approximate rates for nonproductive DNA strand transfer. Taking the net rate of DNA strand transfer as the sum of the productive and nonproductive processes, NCp7 accelerates the intrinsic rate of DNA strand transfer by a factor approaching an order of magnitude. The 41-base RNA acceptor template is kinetically partitioned in favor of its own degradation rather than toward formation of 61-base DNA strand transfer product. This is presumably due to a greater region of complementary overlap between the 61-base DNA strand transfer product and the 41-base RNA acceptor template (41 bases) compared to the 40-base primary DNA product (20 bases).

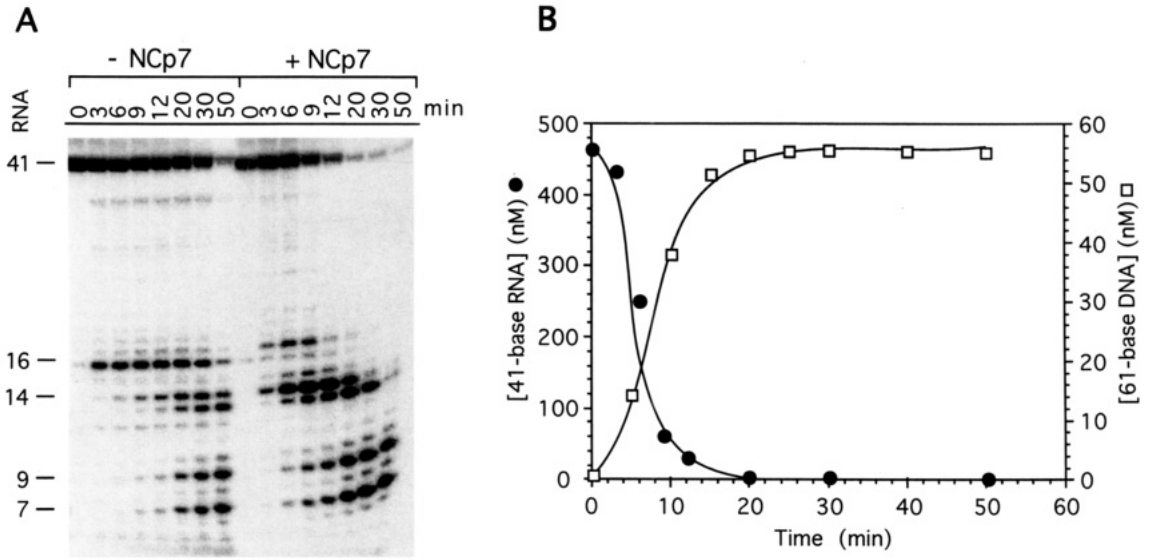

FIGURE 4: Analysis of substrate processing during DNA strand transfer in the presence of NCp7. (A) Analysis of the HIV-1 RT catalyzed degradation of 41-base [^{32}P]RNA acceptor template during strand transfer in the absence (left panel) or the presence (right panel) of NCp7. The reactions were carried out as in Figure 2 except that the degradation of the 5′-end-labeled 41-base [^{32}P]RNA acceptor RNA was monitored. Reaction mixtures contained 200 nM 24-base DNA·40-base RNA, 500 nM 41-base [^{32}P]RNA, each dNTP at 100 μM, 7 mM $MgCl_2$, 100 nM HIV-1 RT, and 0 or 15 μM NCp7 in assay buffer at 37 °C. Reaction samples were withdrawn at the indicated times and analyzed as described in Figure 2. The sizes of the RNA products are shown at the left. (B) Quantitative analysis of DNA strand transfer product formation (□) and 41-base RNA acceptor template degradation (●) during strand transfer. Except for the presence of the ^{32}P substrate label, both reactions were conducted under conditions identical with those described in (A). Note the difference in concentration scales.

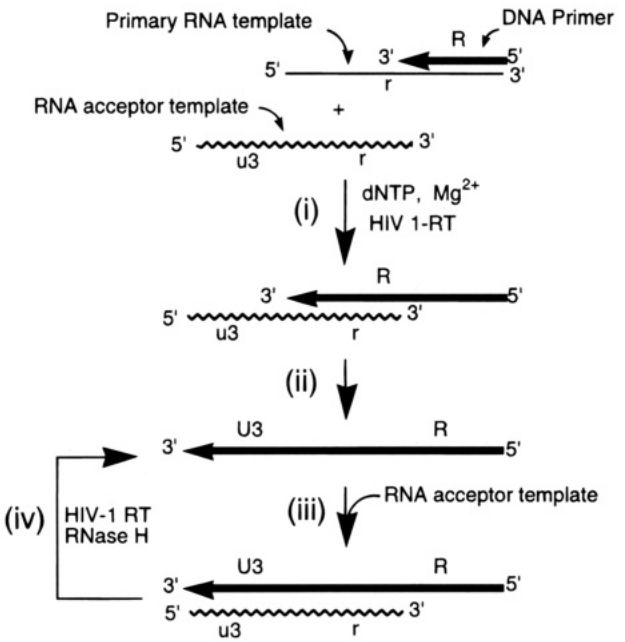

FIGURE 5: Model for nonproductive 41-base RNA hydrolysis during DNA strand transfer. The 61-base DNA formed during DNA strand transfer (steps i and ii) is capable of hybridization with 41-base RNA acceptor template (step iii) to form an intermediate that is subsequently hydrolyzed by the RNase H activity of HIV-1 RT (step iv), releasing the 61-base DNA, which can participate in further cycles, resulting in the depletion of 41-base RNA acceptor template and termination of 61-base DNA strand transfer product formation (Figure 4).

in several steps of the strand-transfer process in addition to its known strand-annealing activity.

This correlation is best explained by the strand-transfer model shown in Figure 5, where in the presence of NCp7 there is a competition for annealing to the 41-base RNA acceptor template between 40-base DNA primary product (Figure 5, step i) and newly formed 61-base DNA (Figure 5, step iii). Annealing to the 40-base DNA gives rise to 61-base DNA strand transfer product (Figure 5, step ii), while annealing to 61-base DNA results in degradation of the 41-base RNA acceptor template by the RNase H activity of HIV-1 RT but without further observable DNA synthesis (Figure 5, step iv). This second, nonproductive cycle is significantly enhanced in the presence of NCp7 and suggests that the strand-transfer process leading to this event is itself promoted.[3] The apparent lag in both 61-base DNA strand transfer product formation and 41-base RNA degradation supports the need to accumulate 40-base DNA primary product before initiation of the nonproductive cycle.

Recent evidence shows that complete proviral DNA synthesis can occur using only one of the copackaged RNA templates (Jones et al., 1994). Also, while there are two copies of genomic RNA within each retrovirus particle, each of which could potentially generate independent copies of preintegrated proviral DNA, only a single provirus is generated per infection (Panganiban & Fiore, 1988; Hu & Temin, 1990). This could result from a sufficiently low integration frequency that results in only a single integration event per infection, or the preintegration multiprotein complex required for DNA integration could be formed on only one of the two synthesized proviral DNA molecules. Alternatively, the nonproductive consumption of one of the two RNA templates during reverse transcription, *via* a process analogous to that shown in Figure 5, would result in the production of a single copy of proviral DNA. This predicts that DNA synthesis occurs primarily or exclusively on only one of the RNA templates, with the second RNA template acting as an acceptor template for DNA strand transfer. This would eliminate the need for a "search" mechanism for transfer when RT encounters a strong replication pause site or block, and it assures that the second RNA template is always available for strand transfer.

The mechanism of inhibition by low concentrations of NCp7 is not yet understood. Under these experimental conditions, NCp7 appears to bind to single-stranded DNA

and RNA in a high-site-size conformation (14 nt/NCp7 molecule). It has been suggested (Dib-Hajj et al., 1993), but not yet definitively demonstrated, that the putative high-site-size binding mode consists of two subsites of approximately 8 nt. In the low-site-size complex, one subunit is bound to ssDNA while the other is free and available to mediate strand annealing. Possibly this high-site-size configuration dictates an RNA acceptor template configuration that cannot efficiently participate in strand transfer. Previous studies have demonstrated that while NCp7 catalyzes the renaturation of complementary nucleic acid strands from the low-site-size complex, the high-site-size complex does not appear to inhibit the annealing of complementary strands, although small changes in the non-protein-assisted rate could have been missed in these experiments (Dib-Hajj et al., 1993). The inhibition observed during HIV-1 RT catalyzed strand transfer is therefore unlikely due to an inhibition of acceptor RNA annealing at low NCp7 concentrations. This further emphasizes the possibility that NCp7 plays a role in addition to strand renaturation during the catalysis of DNA strand transfer reactions (see below).

The above data show that NCp7 enhances the rate of DNA strand transfers catalyzed by HIV-1 RT by over an order of magnitude. Presumably, NCp7 participates in stimulating annealing between the DNA substrate and the RNA acceptor template, consistent with previously observed NCp7 activities (Prats et al., 1988; Khan & Giedroc, 1992; Dib-Hajj et al., 1993). However, this activity of NCp7 is not sufficient to explain all of the kinetic data. The observed rapid depletion of 41-base RNA acceptor template (Figure 4A) requires an activation of the RNase H activity of HIV-1 RT in the presence of NCp7. Furthermore, examination of the cleavage specificity of RNase H catalyzed degradation of 41-base acceptor template (Figure 4A) shows that the locations of RNA cleavage differ in the presence of NCp7 and in its absence. In the absence of NCp7, 41-base acceptor RNA hydrolysis gives rise to the initial formation of a 16-base 5′-end RNA cleavage product. Quantitation of the reaction substrates and products shows that this initial product gives rise to smaller RNA fragments at longer reaction times (data not shown). In the initial phase of the reaction (12 min) only a fraction (20%) of the 41-base RNA substrate is converted to the 16-base cleavage product. This corresponds to processing of the available 24-base DNA·40-base RNA template·primer, with a slower processing of the excess 41-base RNA acceptor template (Figure 5).

In the presence of NCp7, there is no initial production of the 16-base RNA intermediate; only the production of smaller RNA fragments is observed. These smaller RNA fragments are presumably sufficiently small to allow their dissociation from the DNA strand under these experimental conditions, thereby facilitating the subsequent annealing of additional RNA templates for polymerase or RNase H processing. Importantly, even that portion of the 41-base RNA acceptor template in excess of the 24-base DNA·40-base RNA template·primer is rapidly degraded (~94%) within the first 12 min of the reaction. This is consistant with the mechanism for nonproductive acceptor template hydrolysis shown in Figure 5 and suggests that NCp7 can modulate the RNase H activity of HIV-1 RT.

The data in Figure 4A suggest that NCp7 plays a direct role in dictating the specificity and activity of HIV-1 RT catalyzed RNA hydrolysis. While the DNA polymerase and RNase H activities of HIV-1 RT are spatially separated by

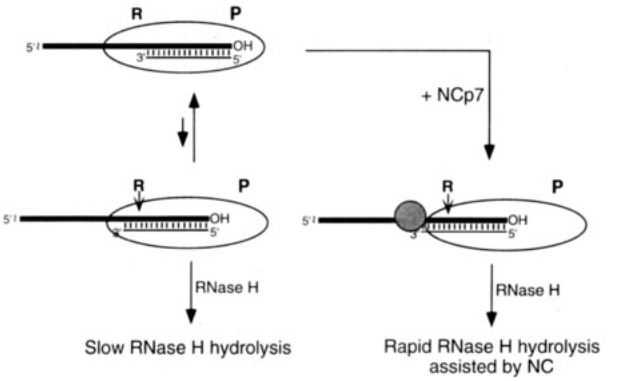

FIGURE 6: Model for NCp7-stimulated RNase H activity facilitated by substrate−protein and protein−protein interactions. During strand transfer, DNA synthesis proceeds until the end of the primary RNA template is encountered. Concomitant with DNA synthesis, the RNase H activity of HIV-1 RT hydrolyzes the RNA template except for an RNA fragment annealed to the DNA 3′-terminus. The distance between the polymerase and the RNase H active sites of HIV-1 RT is approximately 18 or 19 nt. For hydrolysis of the remaining RNA fragment, the DNA·RNA substrate must release from the polymerase active site and rebind in the RNase H active site. The slow rate of RNA hydrolysis in the absence of NCp7 (left) suggests that this repositioning may be unfavorable, with the 3′-terminus of the DNA preferentially binding in the polymerase active site. The binding of NCp7 (right) to the exposed single-stranded DNA and interactions between NCp7 and HIV-1 RT could overcome this thermodynamic barrier and drive the binding of the DNA·RNA hybrid into the RNase H site for hydrolysis.

approximately 18 or 19 nucleotides, DNA·RNA substrates bind within a cleft on the protein that spans both active sites (Kohlstaedt et al., 1992; Jabobo-Molina et al., 1993). This finding led to the proposal that both polymerase and RNase H activity can occur concomitantly during DNA synthesis. Kinetic and biochemical studies indicate that two kinetically distinct RNase H activities can be associated with HIV-1 RT. First, polymerase-dependent RNase H activity cleaves the RNA of the nascent DNA·RNA during concomitant DNA synthesis until the polymerase encounters the end of the RNA template. Since the polymerase and RNase H active sites are spatially separate, the termination of DNA synthesis generates RNA cleavage products ranging from 14 to 19 nt distant from the primer 3′ terminus (Furfine & Reardon, 1990; Schatz et al., 1990; Wohrl & Moelling, 1990; Jacobo-Molina & Arnold, 1991; Gopalakrishnan et al., 1992). These remaining RNA fragments are cleaved by a slow polymerase-independent RNase H activity. Previous studies have indicated that this polymerase-independent RNase H activity of HIV-1 RT is responsible for the rate-limiting degradation of residual RNA fragments annealed to the nascent DNA strand prior to strand transfer (Gopalakrishnan et al., 1992; Peliska & Benkovic, 1992).

This slow RNase H activity is characterized by the requirement for repositioning of the DNA·RNA duplex from binding in the polymerase active site to the RNase H site situated approximately 19 bp away (Gopalakrishnan et al., 1992; Kohlstaedt et al., 1992; Jabobo-Molina et al., 1993) (Figure 6). This repositioning of DNA·RNA hybrid appears to be thermodynamically unfavorable, with the 3′-end of the nascent DNA preferentially binding in the polymerase site of HIV-1 RT (Gopalakrishnan et al., 1992) (Figure 6). Since this slow RNase H activity appears to be at least partly rate limiting in DNA strand transfer, it is an attractive step for possible rate enhancement by NCp7. While a detailed mechanism for the NCp7-induced change in rate and RNase H specificity during HIV-1 RT catalyzed strand-transfer

reactions cannot be constructed using the current experimental data, a working model consistent with these results can be proposed where NCp7 interacts with HIV-1 RT through direct protein−protein and DNA/RNA−protein interactions (Figure 6). In this model, the NCp7 binds to the DNA strand and through interaction with HIV-1 RT facilitates the delivery of the DNA•RNA duplex into the RNase H site for cleavage, thereby altering the rate and location of RNase H cleavage (Figures 5 and 6). Consistent with this model, preliminary evidence for a direct protein−protein interaction between HIV-1 RT and NCp7 has recently been obtained (unpublished results).

Recently, a report describing the transactivation of DNA strand transfer by nucleocapsid protein and reverse transcriptase from Moloney murine leukemia virus has appeared (Allain et al., 1994). In the described system, larger RNA templates were utilized and an enhancement in DNA strand transfer was observed. However, no kinetic analysis was performed, so the mechanism of the enhancement could not be delineated. Whether the NC participated directly in DNA strand transfer, as indicated in this report, or indirectly by eliminating template secondary structure was not determined. The experiments described here utilizing shorter RNA templates, while simplified from the nature of genomic RNA, allow for a quantitative evaluation of the strand-transfer reaction. Utilization of this defined model system should aid in the determination of the mechanistic details of DNA strand transfer reactions catalyzed by HIV-1 RT and nucleocapsid protein.

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