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Human Immunodeficiency Virus Type 1 Nucleocapsid Protein Promotes Efficient Strand Transfer and Specific Viral DNA Synthesis by Inhibiting TAR-Dependent Self-Priming from Minus-Strand Strong-Stop DNA

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During the first strand transfer in reverse transcription, minus-strand strong-stop DNA [(−) SSDNA] is annealed to the 3′ end of the acceptor RNA in a reaction mediated by base-pairing between terminal repeat sequences in the RNA and their complement in the DNA. The large stem-loop structure in the repeat region known as TAR could interfere with this annealing reaction. We have developed an in vitro human immunodeficiency virus type 1 (HIV-1) system to investigate the effect of TAR on strand transfer. Mutational analysis demonstrates that the presence of TAR in the donor and acceptor templates inhibits strand transfer and is correlated with extensive synthesis of heterogeneous DNAs formed by self-priming from (−) SSDNA. These DNAs are not precursors to the transfer product. Interestingly, products of self-priming are not detected in HIV-1 endogenous reactions; this suggests that virions contain a component which prevents self-priming. Our results show that the viral nucleocapsid protein (NC), which can destabilize secondary structures, drastically reduces self-priming and dramatically increases the efficiency of strand transfer. In addition, the data suggest that the ability to eliminate self-priming is a general property of NC which is manifested during reverse transcriptase pausing at sites of secondary structure in the template. We conclude that this activity of NC is critical for achieving highly efficient and specific viral DNA synthesis. Our findings raise the possibility that inactivation of NC could provide a new approach for targeting reverse transcription in anti-HIV therapy.

All retroviruses synthesize a linear double-stranded DNA (dsDNA) copy of the viral RNA genome in a complex series of steps catalyzed by the virion-associated enzyme reverse transcriptase (RT) (29; for reviews, see references 5, 68, and 69). Two strand transfer reactions, namely, transfer of minus- and plus-strand DNAs, are required for this process (reviewed in reference 65).

Minus-strand transfer results from the following sequence of events. During the first step in reverse transcription, minus-strand DNA synthesis is initiated from a cellular tRNA primer (48, 70) which is bound to the primer binding site near the 5′ terminus of viral RNA (68). The product of this reaction, minus-strand strong-stop DNA [(−) SSDNA)], is translocated to the 3′ end of either the template RNA (intramolecular transfer) or the other copy of the viral genome present in the virus particle (intermolecular transfer) (40, 52) to allow further elongation of minus-strand DNA. Transfer can take place only if two conditions are met: (i) as the tRNA primer is extended, 5′ donor RNA sequences must be degraded by RNase H (14) with removal of the terminal 14 to 18 bases by the 3′-OH-independent (i.e., polymerase-independent) (28, 30, 55) mode of RNase H cleavage (6, 13, 25, 54), and (ii) (−) SSDNA must be annealed to acceptor RNA in a reaction mediated by base-pairing of sequences in the repeat (R) region of the RNA and complementary R sequences in the DNA (40, 52, 73). About two-thirds of the R sequence in human immunodeficiency virus type 1 (HIV-1) forms a large stem-loop structure known as TAR (50). The presence of TAR in the RNA and a TAR-related complementary structure in (−) SSDNA has been shown to interfere with annealing in vitro (73). Since the annealing reaction is critical for strand transfer, it is reasonable to assume that such a large secondary structure could interfere with minus-strand transfer during virus replication (73).

If this were true, it would pose a problem for the virus. An intact TAR structure is required at a late stage in virus replication for transcriptional transactivation by the viral Tat protein (50). The question then arises: how does HIV-1 overcome the potential inhibitory effect of TAR on viral DNA synthesis while maintaining the TAR structure for its role in viral RNA transcription? Presumably, there is a *trans*-acting factor(s) supplied by the virus and/or host which can modulate these apparently conflicting requirements.

One likely candidate which could fulfill this function is the viral nucleocapsid protein (NC). NC is a small basic protein, present in the virion core in association with genomic RNA, RT, primer tRNA, and integrase (12, 15, 24, 45). It binds to single-stranded nucleic acids (42, 62) and facilitates conformational changes which result in the most thermodynamically stable structure (8, 21, 23, 39, 51, 66, 67, 73). On the basis of these properties, it has been proposed that NC is a nucleic acid chaperone (38).

HIV-1 NC has two copies of an invariant zinc finger motif (37; reference 32 and references therein) which are both required for infectivity and encapsidation of genomic RNA (2, 22, 32, 34). In addition to its role in viral RNA packaging (33; reference 31 and references therein), NC, functioning as a nucleic acid chaperone, is also implicated in a number of other

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steps in the replicative cycle (reviewed in references 16 and 58). These include maturation of the genomic RNA dimer (23, 26, 27), reverse transcription (3, 18, 41, 45, 47, 53, 60, 63, 64, 71, 73, 74), and possibly integration (45).

In this report, we present evidence for a new role for the chaperone activity of NC which leads to more-efficient and more-specific viral DNA synthesis. Using an in vitro HIV-1 strand transfer system with templates containing the entire R region, we show that the efficiency of strand transfer is only 1 to 3% in the absence of HIV-1 NC. Mutational analysis indicates that this low level of strand transfer is correlated with the presence of the TAR structure in the donor and acceptor RNAs and with synthesis of a heterogeneous group of self-priming products (SP products) from (−) SSDNA. NC drastically reduces self-priming, presumably by destabilizing the TAR secondary structure. This in turn leads to a dramatic increase in the rate, extent, and efficiency of strand transfer. The ability of NC to inhibit self-priming is consistent with the observed absence of SP products in endogenous RT reactions. Our results also suggest that self-priming and its elimination by NC represent a more general phenomenon which is manifested during RT pausing at sites of secondary structure in the template.

MATERIALS AND METHODS

Materials. Wild-type HIV-1 RT was purchased from Worthington; RNase H-minus HIV-1 RT, having a point mutation which changes residue E478 to Q (61), was kindly supplied by Stuart Le Grice (Center for AIDS Research, Case Western Reserve University, Cleveland, Ohio). Restriction enzymes, T4 ligase, and *Taq* polymerase were purchased from Boehringer Mannheim. T4 polynucleotide kinase and RNase ONE, a 27-kDa periplasmic *Escherichia coli* RNase which degrades RNA without base specificity, were obtained from Promega Biotech. An Ambion MEGAscript kit was used to make T7 transcripts. [γ-^{32}P]ATP (3,000 Ci/mmol), [α-^{32}P]dCTP (3,000 Ci/mmol), and [α-^{35}S]dATPαS were purchased from Amersham Life Science Inc. pUC19 was obtained from GIBCO-BRL (Gaithersburg, Md.). DNA oligonucleotides were purchased from Lofstrand (Gaithersburg, Md.). A synthetic 131-nucleotide (131-nt) (−) SSDNA oligonucleotide was obtained from Oligos Etc., Inc. (Wilsonville, Oreg.). DNA sequencing was performed by using a Sequenase kit obtained from U.S. Biochemical Corp.

Construction of plasmids. The pJD plasmid, which was designed for synthesis of a 131-nt donor RNA template, was cloned from the pDR5′ plasmid (gift from Robert Gorelick, SAIC-Frederick, NCI-Frederick Cancer Research and Development Center, Frederick, Md.), which contains the 5′ half of the HIV-1 NL4.3 proviral clone including all of U3, up to the *Eco*RI site at nt 5743 (1). A 168-nt PCR fragment was generated with (i) a forward primer (JL206) having an *Eco*RI site, T7 promoter, and bases 454 to 480 in the R region of NL4.3 (1), 5′-CG GAA TTC TAA TAC GAC TCA CTA TAG GGT CTC TCT GGT TAG ACC AGA TCT GA-3′; and (ii) a reverse primer (JL207) having *Bam*HI and *Msc*I sites and bases 560 to 584 from the U5 region of NL4.3 (1), 5′-CGC GGA TCC TGG CCA GAG TCA CAC AAC AGA CGG GCA C-3′. The 168-nt PCR fragment was digested with *Eco*RI and *Bam*HI and inserted into pUC19, which was also restricted with these enzymes.

The pJA plasmid, which was used for synthesis of a 148-nt acceptor RNA template, was cloned from pDR3′ (gift from Robert Gorelick), which contains the 3′ half of the HIV-1 NL4.3 proviral clone starting from the *Eco*RI site at nt 5743 (1) and including all of U5. The strategy used to generate a 185-nt PCR fragment was similar to that described above. The forward primer (JL204) contained an *Eco*RI site, T7 promoter, and bases 9475 to 9496 in the U3 region of NL4.3 (1): 5′-CG GAA TTC TAA TAC GAC TCA CTA TAG GGA GTG GCG AGC CCT CAG ATG-3′. The reverse primer (JL205) contained *Bam*HI and *Fsp*I sites and bases 9598 to 9622 in the R region of NL4.3 (1): 5′-CGC GGA TCC TGC GCA CTC AAG GCA AGC TTT ATT GAG G-3′. The 185-nt PCR fragment was inserted into pUC19 after double digestion of each DNA with *Eco*RI and *Bam*HI.

The pJD.S36-55 plasmid was constructed to create a substitution mutation (S36-55) which would change nucleotides 36 to 55 in the wild-type donor RNA template to the complementary sequence and disrupt the stem portion of TAR (7, 50). A DNA fragment between *Bgl*II and *Afl*II in pJD was replaced with a synthetic dsDNA made by annealing oligonucleotides JL 223 (5′-GAT CTG AGC CTG GGA CGA GAG ACC GAT TGG TCT CTA CCC ACT GC-3′) and JL 224 (5′-TTA AGC AGT GGG TAG AGA CCA ATC GGT CTC TCG TCC CAG GCT CA-3′). The same fragment was replaced in the pJA plasmid to generate a plasmid, pJA.S36-55, which was used for synthesis of acceptor RNA with the substitution mutation.

The pJD.Δ4-19 and pJA.Δ4-19 plasmids were constructed by replacing the *Eco*RI to *Bgl*II fragments in pJD and pJA, respectively, with a synthetic dsDNA made by annealing oligonucleotides JL 225 (5′-AAT TCT AAT ACG ACT CAC TAT AGG GA-3′) and JL 226 (5′-GAT CTC CCT ATA GTG AGT CGT ATT AG-3′). These two plasmids were used to create a deletion of bases 4 to 19 in the donor RNA template (Δ4-19) and in the corresponding nucleotides in the acceptor template, respectively; this deletion eliminates almost all of the bases in the left side of the TAR stem (7, 50). Donor and acceptor RNA templates carrying this mutation should be 115 and 132 nt, respectively.

For each of the clones, the sequence of the viral insert was verified by DNA sequencing.

Preparation of donor and acceptor RNA templates. Plasmids pJD and pJA, containing donor and acceptor sequences nt 454 to 584 and 9475 to 9622 (1), respectively, were linearized with *Fsp*I and *Msc*I, respectively, and then transcribed with T7 RNA polymerase according to the protocol supplied with the Ambion kit. RNA transcripts were purified by electrophoresis on a 6% denaturing polyacrylamide gel at 1,600 V for approximately 1 h, followed by excision of the full-length RNA band from the gel and elution with an RNaid kit (Bio 101), according to the manufacturer's instructions. The purified RNA was quantified by measuring the A_{260} with a UV spectrophotometer.

In vitro strand transfer assay. A 20-nt DNA primer (JL208; 5′-CCA GAG TCA CAC AAC AGA CG-3′, complementary to nt 565 to 584 in the U5 region of HIV-1 NL4.3 [1]) was labeled at its 5′ end with ^{32}P as described previously (35). A 0.2-pmol sample of donor RNA was annealed to 0.4 pmol of 5′-end-labeled DNA primer (1×10^6 to 2×10^6 cpm) in 2 μl of buffer containing 50 mM Tris-HCl (pH 8.0) and 75 mM KCl at 65°C for 5 min and then gradually cooled to 37°C (~30 min). Reaction buffer (50 mM Tris-HCl [pH 8.0], 75 mM KCl, and 1 mM dithiothreitol), 0.2 pmol of acceptor RNA, and HIV-1 NC, where indicated, were added to the annealed donor-primer hybrid, and the mixture was incubated at 37°C for 5 min. A 0.2-pmol amount of HIV-1 RT was then added, and incubation was continued at 37°C for another 5 min. Reactions (final volume, 20 μl) were initiated by addition of $MgCl_2$ (final concentration, 7 mM) and the four deoxynucleoside triphosphates (each at a final concentration of 100 μM) and were incubated at 37°C for the times indicated below. The final concentrations of RT and the donor and acceptor RNAs were each 10 nM; the final concentration of the DNA primer was 20 nM. In some reaction mixtures, donor RNA and primer were replaced by ^{32}P-labeled synthetic 131-nt (−) SSDNA (1×10^6 to 2×10^6 cpm) at a final concentration of 20 nM. Reactions were terminated by addition of EDTA (final concentration, 50 mM) followed by addition of proteinase K to a final concentration of 0.5 μg/μl, incubation at 65°C for 30 min, and extraction with phenol-chloroform (1:1, vol/vol). Ten microliters of the aqueous phase was mixed with 4 μl of STOP solution from the Sequenase kit and was heated at 90°C for 5 min; a 2.5-μl aliquot was loaded on a 6% sequencing gel.

Radioactivity in DNA products was quantified by using a PhosphorImager (Molecular Dynamics) and ImageQuant software. In this program, the amount of radioactivity is represented by a value for "volume." To calculate the concentrations of transfer product and SP DNAs, the relative amounts of each product (fraction of total volume) were multiplied by 20 nM (concentration of primer). The data were plotted with the Excel program. To estimate transfer efficiency, the transfer product volume was divided by the sum of the volumes for transfer product, SP DNAs, and (−) SSDNA and then multiplied by 100.

HIV-1 endogenous RT assay. HIV-1 (MN) was produced from chronically infected H9 cells and was purified by a single ultracentrifugation in a sucrose density gradient, as detailed by Bess et al. (9). The assay conditions were essentially as described by Quan et al. (57). The reaction mixtures contained the following: 50 mM Tris-HCl (pH 8.0); 5 mM $MgCl_2$; 60 mM KCl; 10 mM dithiothreitol; 10 mM NaCl; 1 mM EGTA; 0.1% Nonidet P-40; 0.4 mM each dATP, dGTP, and dTTP; 10 μM dCTP; 10 μCi of [α-^{32}P]dCTP; and an amount of purified HIV-1 containing 300 ng of p24 (determined by radioimmunoassay [9]), in a final volume of 30 μl. After incubation at 39°C for the indicated times, the reactions were terminated by adding an equal volume of buffer containing 1% sodium dodecyl sulfate, 50 mM EDTA, and 0.2 M NaCl and were then incubated with 20 μg of proteinase K at 65°C for 30 min. Following extraction with phenol-chloroform, the protein-free DNA products were precipitated with ethanol; the pellet was dissolved in 8 μl of H_2O. A 4-μl portion was mixed with 0.6 μl of 10× RNase ONE buffer and 0.5 μl of RNase ONE and was incubated for 20 min at 37°C; 2 μl of STOP solution from the Sequenase kit was then added to the mixture. Another 4-μl portion was mixed with 1 μl of H_2O and 2 μl of STOP solution. Two μl each of the RNase ONE-treated and -untreated samples was heated at 90°C for 5 min and then applied to a 6% sequencing gel.

Preparation of recombinant NC. The procedures for cloning, expression, and purification of recombinant wild-type NC were performed as described by Wu et al. (71). A more detailed description of these procedures is given in reference 10.

RESULTS

In vitro strand transfer system. To determine whether the large secondary structure known as TAR (50), present in the R region at the 5′ and 3′ ends of the viral RNA genome, interferes with minus-strand DNA transfer, we designed an in vitro

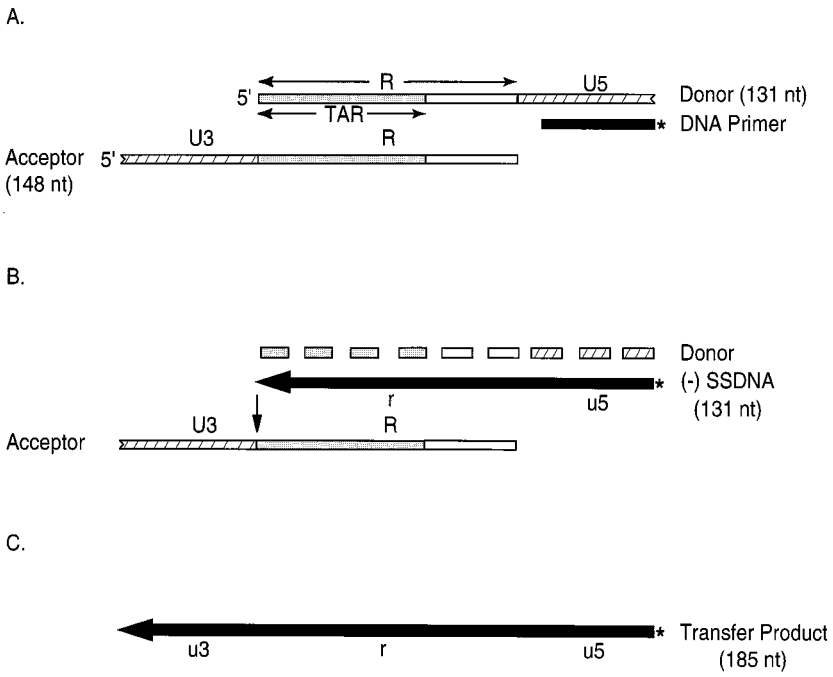

FIG. 1. Schematic diagram illustrating the HIV-1 model system for assay of the first strand transfer. (A) Templates and primer. The donor RNA template (131 nt) is composed of the entire R sequence (97 nt) and sequences from the 5′ end of U5 (34 nt) (1). The primer is a 5′-^{32}P-labeled DNA oligonucleotide complementary to the 20 nt present at the 3′ end of the donor; the radioactive label is indicated (star). The acceptor RNA template (148 nt) contains sequences from the 3′ end of U3 (54 nt) and 94 nt of R. (The 3′-terminal 4 nt of R are deleted in this template; note that the R region at the 3′ end of NL4.3 is 98 nt [1].) (B) Synthesis of (−) SSDNA, RNase H cleavage of donor RNA template, and the transfer of (−) SSDNA to acceptor RNA template. (C) Completion of minus-strand DNA synthesis and formation of the 185-nt transfer product. The TAR sequence (gray shaded bar) and the remainder of R (open rectangle), U5 and U3 sequences (hatched rectangles), and DNA molecules (solid rectangles) are indicated. The figure is not drawn to scale.

HIV-1 strand transfer system. As shown in Fig. 1A, the donor template (131 nt) contains the entire R region and the first 34 nt of U5; the acceptor template (148 nt) contains 54 nt of U3 and 94 nt of R (the last 4 nt at the 3′ end of R were deleted). The entire TAR sequence was present in the donor and acceptor RNAs; TAR comprises ~63% of the R region. A ^{32}P-labeled 20-nt DNA oligonucleotide complementary to sequences at the 3′ end of the donor was used as a primer. In this system, the (−) SSDNA is 131 nt (Fig. 1B). As synthesis of (−) SSDNA proceeds, donor RNA sequences are degraded by the RNase H activity of RT (Fig. 1B). Transfer of (−) SSDNA to the acceptor RNA results in synthesis of a 185-nt DNA product (Fig. 1C). It should be noted that to more closely reproduce the conditions of strand transfer during virus infection, equivalent amounts of donor and acceptor RNA templates were used.

DNA products generated by self-priming from (−) SSDNA. In accord with other studies (4, 13, 20, 44, 49), it was previously reported that a secondary structure in the RNA template can lead to RT pausing and a decrease in the efficiency of full-length DNA synthesis during elongation of minus-strand DNA (71). Thus, it was initially of interest to see whether the TAR secondary structure in the donor RNA (Fig. 2A) has any effect on (−) SSDNA synthesis in the absence of acceptor. Figure 3A shows a typical time course from 2 to 60 min (lanes 1 to 6). By 5 min (lane 2), (−) SSDNA was the major DNA synthesized, although smaller products were also detectable. Unexpectedly, we observed that at 20 min of incubation (Fig. 3A, lane 4), products migrating more slowly than (−) SSDNA were prominent; by 60 min (lane 6), these DNAs (labeled SP) represented the major species in the reaction mixtures. These results suggested that SP products were generated from (−) SSDNA.

To explain the appearance of the heterogeneous SP products, we considered the possibility that these DNAs were generated by self-priming from (−) SSDNA (Fig. 3D). On the basis of computer analysis, You and McHenry (73) proposed that when the TAR sequence in the donor template is copied by RT, a similar structure will be formed by the complementary sequences in (−) SSDNA. We reasoned that a TAR-related secondary structure in (−) SSDNA could induce self-annealing at the 3′ end of (−) SSDNA, thereby allowing (−) SSDNA to act as both primer and template for synthesis of plus-strand DNA sequences. In accord with this hypothesis, synthesis of SP DNAs from (−) SSDNA could be demonstrated directly by incubating a 5′-end-labeled synthetic version of the 131-nt (−) SSDNA with HIV-1 RT from 2 to 60 min in the absence of donor and primer (Fig. 3A, lanes 7 to 12). As the time of incubation increased, there was a concomitant increase in the amount of SP DNAs and a decrease in (−) SSDNA.

Synthesis of plus-strand DNA sequences in the self-priming reaction would be catalyzed by the DNA-dependent DNA polymerase activity of RT and should therefore be sensitive to the inhibitor actinomycin D. As shown in Fig. 3B (lanes 13 to 15), as little as 25 μg of this drug per ml abolished the synthesis of SP products. In addition, self-annealing of (−) SSDNA should require degradation of donor RNA sequences by the RNase H activity of RT. Not surprisingly, with an RNase H-minus version of RT (61), (−) SSDNA continued to be synthesized, whereas SP DNAs were not detectable (Fig. 3C, lanes 16 and 17). However, when *E. coli* RNase H was included in the RNase H-minus RT reaction mixture, SP products could be detected (data not shown). This indicates that for self-

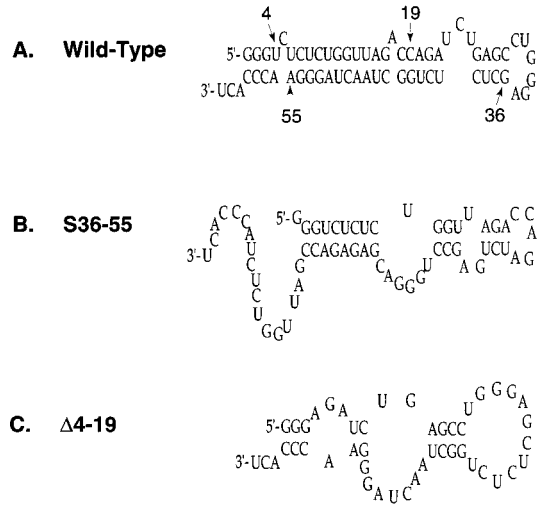

FIG. 2. Predicted secondary structures for wild-type and mutant TAR sequences. The FoldRNA (Genetics Computer Group) program was used to predict secondary structure, using the sequence from the NL4.3 HIV-1 proviral clone (1); nucleotide 1 corresponds to the 5′-terminal base in the R region. (A) Predicted TAR structure (nt 1 to 59) in the first 62 nt of wild-type donor RNA. This fold includes the first nucleotide in the genome, a G residue, in contrast to other representations of TAR which omit this G, presumably since it is methylated in authentic viral RNA (e.g., see reference 7). (B) Predicted structure of a mutant TAR sequence in which nucleotides 36 to 55 were changed to their complementary bases (S36-55). (C) Predicted structure of a mutant TAR sequence in which nucleotides 4 to 19 were deleted (Δ4-19).

priming to occur, RNase H can be added in *trans* and need not be covalently linked to the polymerase domain of RT.

SP DNAs were studied further by performing sequence analysis. Since the polyacrylamide gel data (Fig. 3) indicated that SP products are a heterogeneous class of DNAs, it was of interest to analyze the sequence of the plus-strand DNA extension at the junction with the 3′ terminus of (−) SSDNA. The material corresponding to SP DNAs was gel purified and then subjected to PCR amplification with a single primer having an *Eco*RI site at its 5′ terminus and the remaining sequence complementary to nt 521 to 538 in HIV-1 NL4.3 (1), 9 nt downstream from the 3′ end of TAR. This primer was chosen to take advantage of the fact that SP DNAs would be expected to have both plus- and minus-strand DNA sequences (Fig. 3D). The large band containing the PCR products was isolated, digested with *Eco*RI, and cloned at the *Eco*RI site in a pUC19 vector. Analysis of these clones indicated that the plus-strand sequences were diverse, i.e., in some cases, plus-strand extensions were complementary to sequences in (−) SSDNA, and in others, generation of the plus-strand sequences could not be easily explained. However, in each case, plus-strand extensions were joined to a constant sequence representing the 3′ end of (−) SSDNA (data not shown). These results suggest that self-priming did not always occur at the same nucleotide location. As expected, when (−) SSDNA was used as the template in the PCR, no product was detected with the minus-strand primer used for amplifying SP DNAs (data not shown). Independent analysis of the transfer product showed that it had the sequence predicted from the nucleotides present in the donor and acceptor RNA templates and was not derived from SP DNAs (data not shown).

Inspection of the gels in Fig. 3 also revealed that a class of smaller DNA products with the properties of SP DNAs could be formed from a minus-strand DNA pause product. For example, as may be seen from Fig. 3A (lanes 1 to 6), the amount of products ranging in size from 95 to 102 nt increased during 60 min of incubation, whereas formation of a 77-nt pause product decreased over the same interval. In addition, when the reaction mixtures were incubated with actinomycin D (Fig. 3B, lanes 14 and 15) or with RNase H-minus RT (Fig. 3C, lane 16), synthesis of the 95- to 102-nt DNA products was virtually eliminated and the amount of the 77-nt DNA was increased. The 77-nt pause product occurs at a position in the template (nt 55) where RT encounters the stem in TAR during minus-strand DNA synthesis. Computer analysis indicates that this DNA can form a folded structure which could undergo self-priming (data not shown).

Taken together, the results illustrated in Fig. 3 lead to the conclusion that SP products contain plus-strand DNA sequences which are derived by self-priming from minus-strand DNAs in a reaction requiring the RNase H and DNA-dependent DNA polymerase activities of RT. The larger class of SP products can be generated from (−) SSDNA in the absence of acceptor RNA. Since these SP DNAs are unrelated to the transfer product, we predict that they decrease strand transfer efficiency by making (−) SSDNA less available for transfer to the acceptor template (see below).

Effect of mutations in TAR on synthesis of SP DNAs and efficiency of strand transfer. The results presented above support the idea that a TAR-related secondary structure in (−) SSDNA, resulting from reverse transcription of TAR in the donor RNA, is ultimately responsible for synthesis of SP products. To provide further evidence for this conclusion, we introduced two mutational changes into the TAR sequence (Fig. 2A) of both the donor and the acceptor templates, which each disrupt base-pairing in the stem: (i) a substitution mutation (S36-55) in which nt 36 to 55 in TAR were changed to their complementary bases (Fig. 2B) and (ii) a deletion mutation (Δ4-19) in which nt 4 to 19 were deleted from the left side of the stem (Fig. 2C). These changes would be expected to reduce self-priming. In accord with this possibility, we found that in reactions without acceptor, the substitution mutation in the donor significantly reduced the synthesis of SP DNAs over a 3-h incubation period. Under the same conditions, the deletion mutation virtually eliminated SP product formation (data not shown).

To determine whether self-priming from (−) SSDNA interferes with minus-strand transfer, the activities of the wild-type and mutant donor and acceptor templates were assayed in the in vitro strand transfer system (Fig. 1). Figure 4A displays the DNA products made over a 3-h interval in reactions with the three sets of templates: wild type (lanes 1 to 6), S36-55 (lanes 7 to 12), and Δ4-19 (lanes 13 to 18). Inspection of the gel shows that the amount of transfer product was greatest in the reactions with the mutants, whereas the amount of SP DNAs was highest in the wild-type reaction. To obtain a more quantitative measure of these products, radioactivity in the gel was quantified with a PhosphorImager (see Materials and Methods).

As shown in Fig. 4B, by 3 h, ~20-fold more of the transfer product was made in the Δ4-19 reaction than in the reaction with the wild-type templates. Synthesis of transfer product in the S36-55 reaction was more modest but was still considerably higher (approximately fivefold) than with the wild-type templates. Even after 3 h of incubation, very little transfer product was made in the wild-type reaction, the amount increasing only ~6-fold between 10 min and 3 h; in the same interval, the transfer products in the S36-55 and Δ4-19 reactions increased ~32- and 42-fold, respectively. In addition to these effects on overall extent of transfer, the mutations were also associated with an increase in the rate of synthesis of the transfer product. The most significant increase was seen in the reaction with the

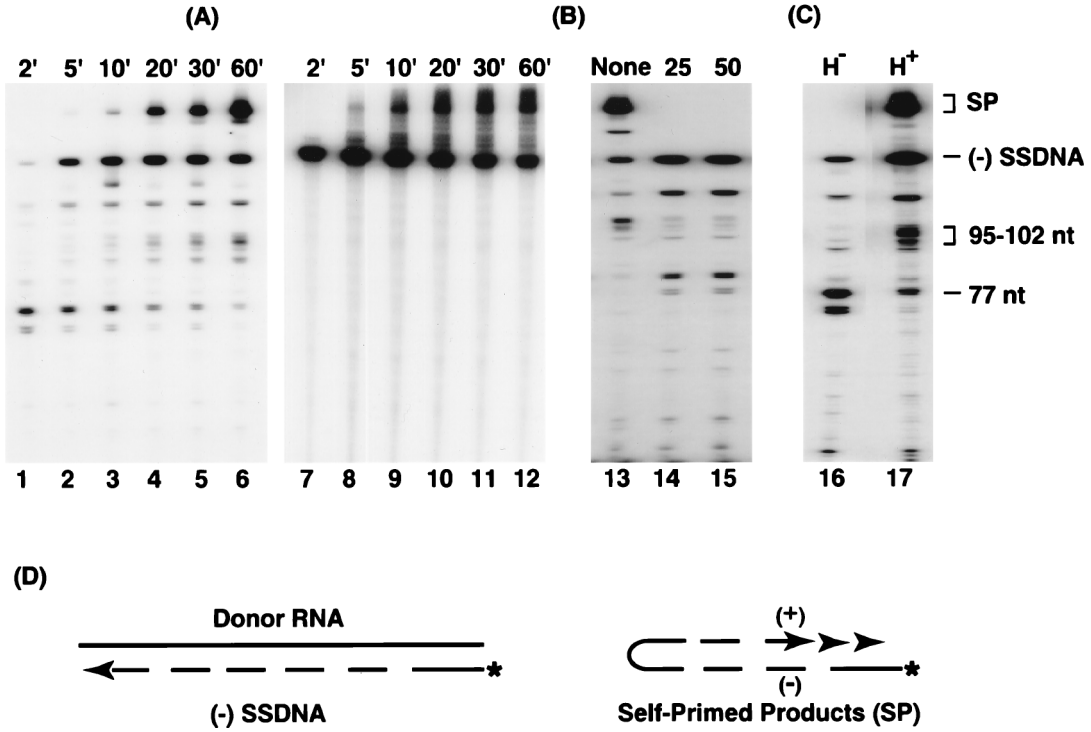

FIG. 3. Synthesis of (−) SSDNA in the absence of acceptor RNA template. Reaction conditions were the same as those described in Materials and Methods, except that the acceptor RNA template was omitted. DNA products were resolved in a 6% sequencing gel. (A) Time course of DNA synthesis. Twenty-microliter aliquots were removed at the indicated times and analyzed as described in Materials and Methods. Lanes 1 to 6, reaction with donor and ^{32}P-labeled 20-nt DNA oligonucleotide primer; lanes 7 to 12, reaction with ^{32}P-labeled 131-nt synthetic (−) SSDNA. (B) Synthesis of DNA products in the presence or absence of actinomycin D. Lanes 13 to 15, reaction mixtures containing no drug and 25 and 50 μg of actinomycin D per ml, respectively. (C) Synthesis of DNA products with HIV-1 RNase H-minus RT (H^-) or wild-type RT (H^+). The reaction mixtures in panels B and C were incubated at 37°C for 30 min. The positions of SP products, (−) SSDNA, bands ranging in size from 95 to 102 nt, and a 77-nt pause product are indicated to the right of the gel in panel C. The 95- to 102-nt bands and the 77-nt pause product are aligned in panels A and C but are in slightly higher positions in the gel shown in panel B. (D) DNA products. Schematic diagrams depict synthesis of (−) SSDNA by primer extension from the donor RNA template (on the left) and formation of SP products from (−) SSDNA (on the right). The plus-strand polarity of sequences joined to (−) SS DNA in SP products [(+)] and the minus-strand polarity of the (−) SS DNA moiety in SP products [(−)] are indicated. The ^{32}P label at the 5′ end of the primer (*) is also shown. The diagrams are not drawn to scale.

Δ4-19 templates, particularly during the first 60 min of incubation.

Analysis of SP DNAs in these reaction mixtures (Fig. 4C) showed that in contrast to the pattern observed for the transfer product, the wild-type reaction mixture contained high levels of SP products, reaching a maximum value between 60 and 90 min of incubation. The S36-55 reaction mixture contained very small amounts of the SP DNAs, and the Δ4-19 reaction mixture had virtually none.

The transfer efficiency of each of the reactions was estimated as described in Materials and Methods. At 30 min, the values for the wild-type, S36-55, and Δ4-19 reactions were 0.9, 2.8, and 3.7%, respectively. At 3 h, the transfer efficiency of the wild-type reaction was only slightly increased, to 1.3%; in contrast, the transfer efficiencies of the S36-55 and Δ4-19 reactions were increased to 16 and 31%, respectively.

These results indicate that when the TAR structure is destabilized by mutational change, strand transfer is increased and synthesis of SP products is significantly reduced (S36-55) or essentially eliminated (Δ4-19). In contrast, the presence of TAR in the wild-type donor and acceptor templates is correlated with inefficient strand transfer and synthesis of large amounts of SP DNAs.

Analysis of DNA products in endogenous HIV-1 RT assays. Since SP DNAs are formed in the in vitro strand transfer reactions, it was of interest to determine whether these products are detectable in endogenous RT reactions with HIV-1 detergent-disrupted particles. Synthesis of (−) and (+) SS DNAs was assayed over a 0.5- to 6 h-interval; at each time point, portions were taken and applied directly to the gel (Fig. 5, odd-numbered lanes) or were first treated with RNase ONE to remove RNA primers which might still be attached to the DNA products (Fig. 5, even-numbered lanes). Following RNase ONE treatment, (−) and (+) SSDNAs migrated to positions expected on the basis of their molecular sizes, i.e., 182 and 635 nt, respectively (1). [Note that the (−) SSDNA is larger than the in vitro-synthesized product (131 nt) since the donor RNA used in the in vitro assay does not contain the complete U5 sequence (see Materials and Methods and legend to Fig. 1).] In the absence of RNase ONE treatment, (−) SSDNA migrated to a position corresponding to 258 nt, as expected if the DNA were still bound to the $tRNA_3^{Lys}$ primer. The mobility of (+) SSDNA with bound RNA was only slightly less than that of (+) SSDNA itself, since the HIV-1 polypurine tract primer which initiates plus-strand DNA synthesis is just 15 nt long (11).

Both (−) and (+) SSDNAs were detected as early as 0.5 h (Fig. 5, lanes 1 and 2). With increasing time of incubation, the amount of (−) SSDNA was reduced, and after 2 h (lanes 5 and 6), (−) SSDNA was not the major product of the reaction. In contrast, the amount of (+) SSDNA increased with time of incubation and was more prominent at later times, i.e., at 4 h (lanes 7 and 8) and 6 h (lanes 9 and 10). Most importantly, at each time point, a smear of bands in the position expected for

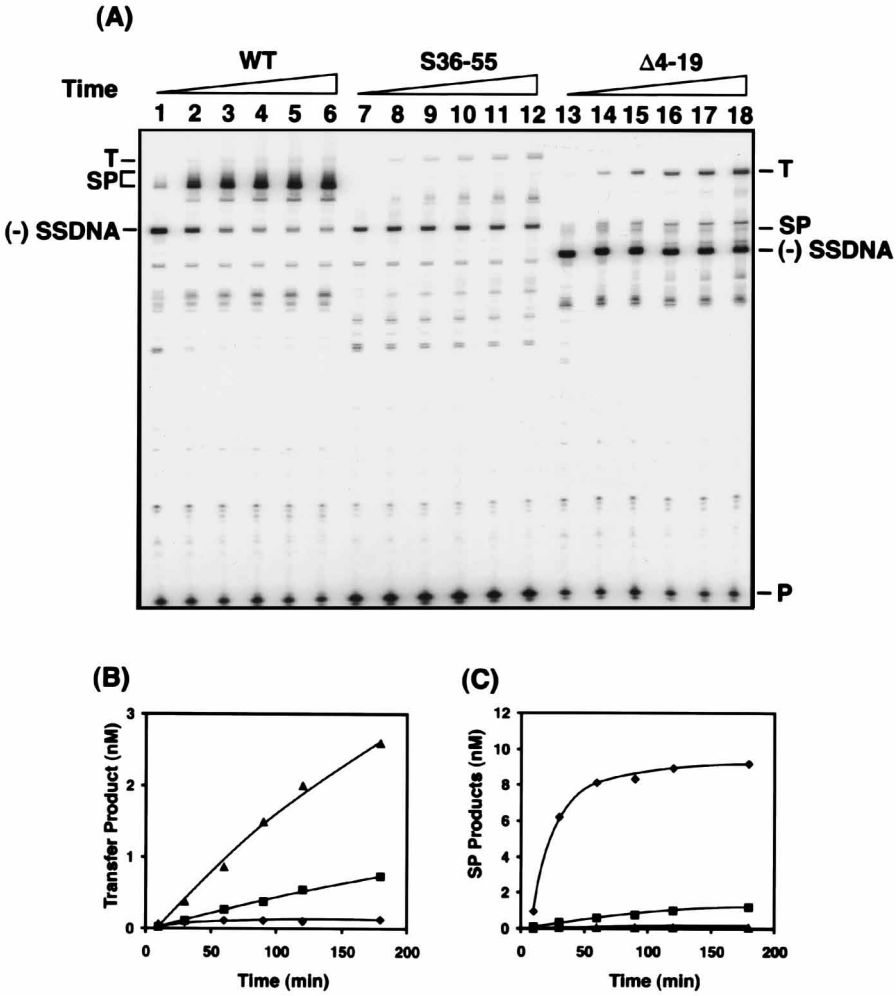

FIG. 4. Effect of the TAR-related secondary structure in (−) SSDNA on the synthesis of transfer product and SP products. Plasmids containing wild-type and mutant TAR sequences were constructed and transcribed as described in Materials and Methods. Mutations were present in both the donor and the acceptor templates. (A) Gel analysis. At the times indicated below, 20-μl aliquots were removed from each reaction mixture and were processed and analyzed as described in Materials and Methods. Reaction mixtures contained the following donor and acceptor templates: wild type (WT) (lanes 1 to 6); S36-55, containing substitution mutations in residues 36 to 55 of R (see Fig. 2B) (lanes 7 to 12); and Δ4-19, containing a deletion of residues 4 to 19 in R (see Fig. 2C) (lanes 13 to 18). The times of incubation were as follows: lanes 1, 7, and 13, 10 min; lanes 2, 8, and 14, 30 min; lanes 3, 9, and 15, 60 min; lanes 4, 10, and 16, 90 min; lanes 5, 11, and 17, 120 min; and lanes 6, 12, and 18, 180 min. The positions of the transfer product (T), SP products, and (−) SSDNA are indicated on the left for the WT and S36-55 reactions; the positions of each of these products for the Δ4-19 reaction are indicated on the right. The position of the 20-nt DNA oligonucleotide primer (P) is also indicated on the right. Note that only bands which had mobilities intermediate between the transfer product and (−) SSDNA and also appeared in the absence of acceptor (data not shown) were identified as SP products. Thus, in lanes 13 to 18, only one band was identified as an SP product since the other bands migrating between (−) SSDNA and the transfer product were present only in reaction mixtures with acceptor RNA. (B and C) Quantitative PhosphorImager analysis. The concentrations of transfer product (B) and SP products (C) were plotted against time of incubation. Symbols: diamonds, wild type; squares, S36-55; and triangles, Δ4-19.

SP DNAs was not detected in any of the samples. These results suggest that SP DNAs are not synthesized in endogenous RT reactions and also imply that a component in the virus is responsible for preventing self-priming from (−) SSDNA.

Effect of HIV-1 NC on synthesis of SP DNAs and the efficiency of strand transfer. Retroviral NC is a nucleic acid chaperone (38) which has the ability to destabilize secondary structures (38, 43, 66, 73). Thus, it is possible that one function of virion-associated NC is to promote strand transfer by preventing self-priming from (−) SSDNA. To test this possibility, we performed the strand transfer assay (Fig. 1) in the presence of HIV-1 NC (Fig. 6A and B). Inspection of the gel (Fig. 6A) shows that as the concentration of NC was increased, synthesis of the transfer product was increased, while formation of SP DNAs was decreased. PhosphorImager analysis (Fig. 6B) of the gel data (Fig. 6A) shows that at the highest concentrations of NC, synthesis of the transfer product was stimulated ~10-fold, whereas synthesis of SP DNAs was decreased ~10-fold. Similar dose responses were obtained when NC was incubated with ^{32}P-labeled synthetic 131-nt (−) SSDNA and acceptor RNA in the absence of donor RNA and primer (data not shown). In addition, in the absence of acceptor, NC stimulated synthesis of (−) SSDNA and inhibited formation of SP products from (−) SSDNA in a dose-dependent manner (data not shown).

To measure the effect of NC on the rate of strand transfer, we incubated reaction mixtures over a 30-min interval without NC and with three different concentrations of NC: 0.4, 1.6, and 3.2 μM (Fig. 6C). At the 0.4 μM concentration, the ratio of nucleotides in the two RNA templates to NC molecules (i.e., 7 nt per NC molecule) corresponds to the size of the binding site calculated from in vitro experiments (21, 42, 72) and is in

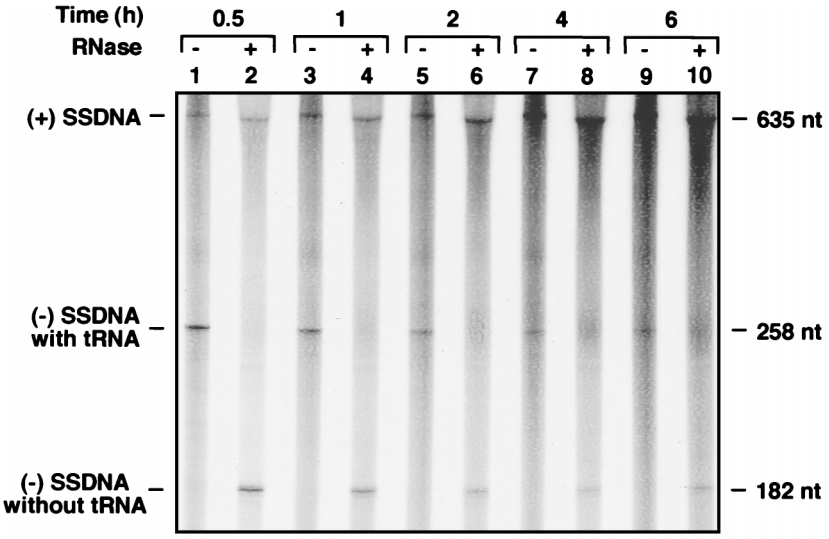

FIG. 5. Synthesis of (−) and (+) SSDNA in an HIV-1 endogenous reaction. Reaction mixtures were incubated from 0.5 to 6 h, as described in Materials and Methods. Odd-numbered lanes, untreated samples; even-numbered lanes, samples treated with RNase ONE. Lanes 1 and 2, 0.5 h; lanes 3 and 4, 1 h; lanes 5 and 6, 2 h; lanes 7 and 8, 4 h; and lanes 9 and 10, 6 h. (−) SSDNA with the tRNA primer attached or without the tRNA primer migrates to positions corresponding to 258 and 182 nt, respectively. Since the polypurine tract is only 15 nt, there is only a small change in the mobility of (+) SSDNA following RNase ONE treatment (compare odd- and even-numbered lanes for each time point). (+) SSDNA without the polypurine tract primer migrates to a position corresponding to 635 nt. The sizes of the products were determined by running a sequencing ladder generated with MP18 DNA, which is included in the Sequenase kit. Longer minus-strand DNAs were not detected in this gel.

agreement with the estimated ratio of RNA nucleotides to NC molecules in the retrovirus particle (37). At 1.6 and 3.2 μM, the ratios of nucleotides per NC molecule are 1.8 and 0.9, respectively.

The data in Fig. 6C show that significant amounts of transfer product were not apparent before 10 min of incubation. At 10 min and at each succeeding time point, NC increased the amount of transfer product made in a dose-dependent manner. In addition, as the concentration of NC was increased, the rate at which the transfer product was synthesized was also increased. The greatest effect was achieved with an NC concentration of 3.2 μM. Other experiments indicated that extending the time of incubation with NC from 30 min to as long as 3 h resulted in only a small additional increase in the amount of transfer product made in the reaction (data not shown). Experiments in which the synthetic 131-nt (−) SSDNA was incubated with acceptor RNA also showed that NC increased strand transfer and inhibited self-priming; in this case, however, strand transfer was detected as early as 2 min in the presence of NC (data not shown).

The efficiency of strand transfer at 30 min in the standard assay system was estimated by using the data from the experiment whose results are shown in Fig. 6C. In the absence of NC, transfer efficiency was 3.3%. (In multiple experiments, a range of 1 to 3% was obtained with reaction mixtures containing wild-type templates and no NC [Fig. 4; data not shown].) In the presence of NC, this value was dramatically increased: at NC concentrations of 0.4, 1.6, and 3.2 μM, the transfer efficiencies were 25, 45, and 66%, respectively.

Taken together, the data of Fig. 6 demonstrate that an increase in the efficiency of strand transfer by NC is correlated with a drastic decrease in self-priming from (−) SSDNA. This conclusion is illustrated schematically in Fig. 7, which shows that in the absence of NC, self-priming is the predominant reaction, whereas in the presence of NC, (−) SSDNA is transferred to the acceptor RNA.

DISCUSSION

In the present study, we have investigated the effect of the TAR secondary structure on minus-strand DNA transfer in a model HIV-1 in vitro system (Fig. 1). We show that the presence of TAR in the donor and acceptor templates inhibits strand transfer, and we have addressed the following questions. (i) How does the TAR structure reduce strand transfer? (ii) If the structure is destabilized by mutation, is there an increase in strand transfer? (iii) How does HIV resolve the problem of achieving a high level of strand transfer during reverse transcription while maintaining an intact TAR structure (required at a later stage in virus replication [50])? Our results suggest that viral NC provides the solution to the problem created by the TAR structure by inhibiting self-priming from (−) SSDNA. This appears to be a new role for the nucleic acid chaperone activity of NC, which is critical for its ability to promote efficient strand transfer.

In the model system described by You and McHenry (73), it was shown that annealing of an 81-nt RNA containing the TAR structure (representing the acceptor RNA) to a complementary 81-nt DNA [representing (−) SSDNA] was complete only after 4 h of incubation. In the presence of NC, the rate of annealing was accelerated by as much as 3,000-fold (73). These findings led to the prediction that NC stimulates strand transfer because it overcomes the kinetic delay in the annealing of two nucleic acid molecules which each contain a large secondary structure (73).

Our results indicate that the presence of the TAR secondary structure does not merely lead to a kinetic problem. Thus, if the TAR structure only inhibited the annealing reaction, then a significant level of strand transfer would be expected to occur following extended incubation. In fact, the data of Fig. 4A and B indicate that with wild-type templates, synthesis of the transfer product shows only a minimal increase over a 3-h interval.

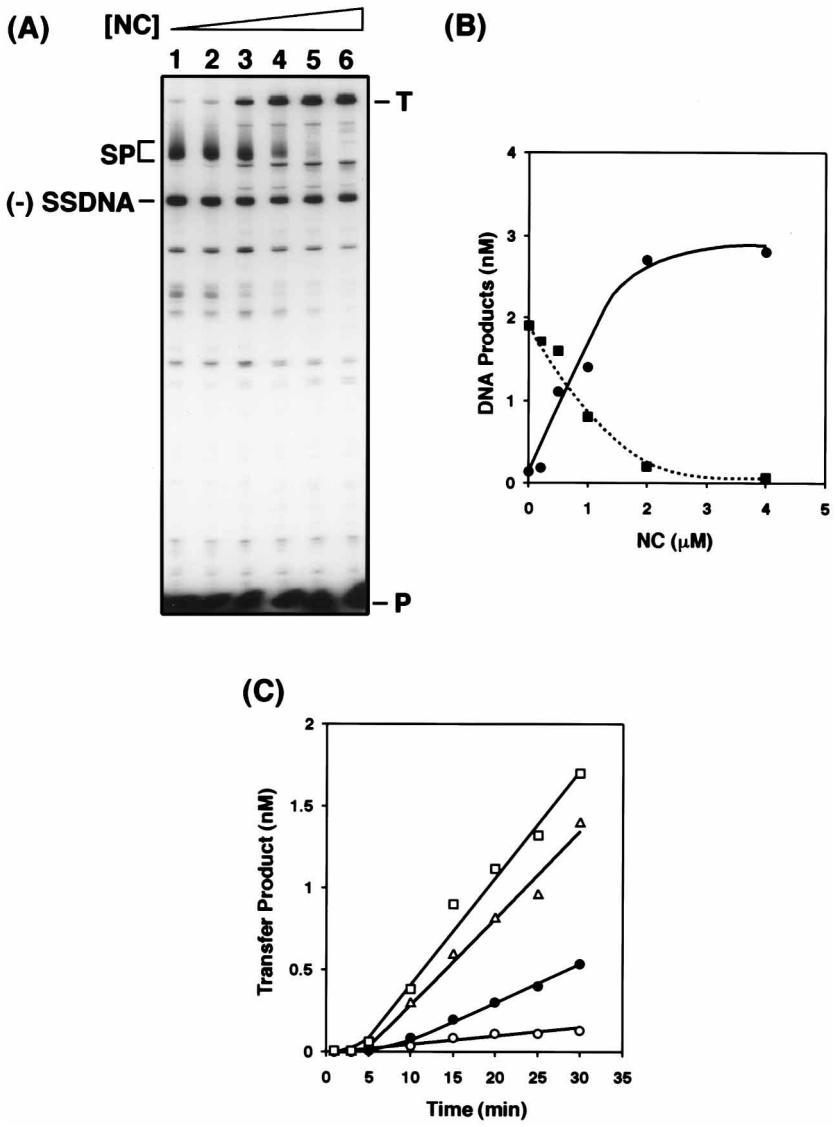

FIG. 6. Effect of HIV-1 NC on the rate and extent of the first strand transfer. (A and B) Dose response. (A) Reaction mixtures were incubated with increasing amounts of HIV-1 NC for 30 min at 37°C and were analyzed as described in Materials and Methods. The concentrations of NC were as follows: lane 1, no NC; lane 2, 0.2 μM; lane 3, 0.5 μM; lane 4, 1 μM; lane 5, 2 μM; and lane 6, 4 μM. The positions to which (−) SSDNA, SP, and transfer (T) products migrated in the gel are indicated. (B) The gel data in panel A were analyzed with a PhosphorImager, as described in Materials and Methods. The concentrations of SP DNAs and transfer product were plotted against NC concentration. Symbols: circles, transfer product; and squares, SP DNAs. (C) Time course. Reaction mixtures were incubated without NC or with three different concentrations of NC from 1 to 30 min. At each time point, a 20-μl aliquot was removed from each mixture and was processed and analyzed as described in Materials and Methods. The concentrations of transfer product in each reaction mixture were plotted against the time of incubation. Symbols: open circles, no NC; closed circles, 0.4 μM NC; triangles, 1.6 μM NC; and squares, 3.2 μM NC.

This suggests that the presence of TAR has an additional effect which cannot be overcome by longer incubations.

Analysis of products of reactions performed in the presence or absence of acceptor RNA unexpectedly revealed that a majority of the DNA products was represented in a group of heterogeneous DNAs (termed SP DNAs), which migrate more slowly than (−) SSDNA (Fig. 3, 4, and 6). In the absence of acceptor, SP DNAs are detectable with a lag of ~10 min during synthesis of (−) SSDNA with donor RNA and a labeled DNA primer (Fig. 3A, lanes 1 to 6) and also accumulate in reaction mixtures containing only (−) SSDNA and no donor or primer (Fig. 3A, lanes 7 to 12). These findings strongly support the conclusion that SP DNAs are generated from (−) SSDNA by a self-priming mechanism. Formation of SP DNAs is inhibited by actinomycin D, as would be expected for a reaction involving synthesis of plus-strand DNA sequences by DNA-dependent RT activity (Fig. 3B). In addition, RNase H activity is required for synthesis of SP DNAs (Fig. 3C), presumably reflecting the fact that self-priming can occur only after donor RNA sequences are degraded and (−) SSDNA is free to form fold-back structures. Sequence analysis indicates that SP DNAs do not serve as precursors to the transfer product (data not shown). Thus, the consequence of self-priming is that it makes (−) SSDNA unavailable to anneal to acceptor RNA and thereby reduces the possibility that strand transfer will occur (Fig. 4).

To demonstrate that a TAR-related secondary structure in (−) SSDNA is responsible for self-priming, we studied the

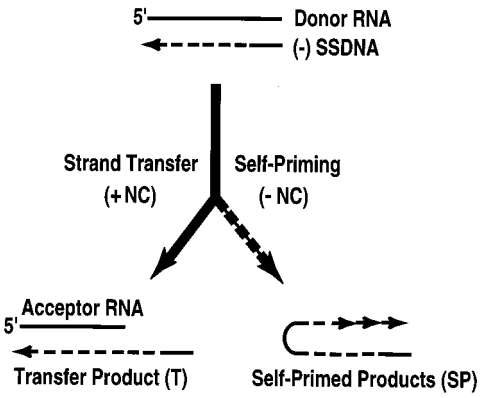

FIG. 7. Schematic diagram illustrating the fate of (−) SSDNA in the presence or absence of HIV-1 NC. In the presence of NC, (−) SSDNA gives rise to the transfer product (acceptor RNA-dependent reaction). In the absence of NC, most of the (−) SSDNA undergoes a self-priming event, which gives rise to SP products (acceptor RNA-independent reaction).

effects of two mutations in the RNA templates which destroy a large portion of the TAR stem (Fig. 2). The complement of the mutant sequence in the donor will be present in (−) SSDNA. When the mutant templates were used, self-priming was significantly reduced in one case (substitution mutation) and virtually eliminated in the other (16-nt deletion), while the rate, extent, and efficiency of strand transfer were markedly increased (Fig. 4).

Self-priming was not observed in several previous studies of HIV-1 in vitro strand transfer, presumably because the viral templates used did not contain the entire R region. These templates included short HIV-1 transcripts which were missing a large portion of R and hence were lacking any secondary structure (53, 54) or longer transcripts containing the primer binding site, all of U5, and R with a deletion of the first 22 nt (3a). A high level of strand transfer was observed with these templates, in accord with our findings with the Δ4-19 mutant RNAs (Fig. 4). (It should be noted that more-extensive deletions in the R region of the longer templates reduced the efficiency of strand transfer [3a].) In one study where the HIV donor and acceptor RNAs contained the complete R region, the presence of SP DNAs was not noted (64). Analysis of the DNA products in an agarose gel may have precluded resolution of SP DNAs in the large area of radioactivity identified as (−) SSDNA (64).

It is clear from the data presented in this report that if self-priming were to occur in vivo, virus replication would be adversely affected. Destruction of the TAR secondary structure certainly limits the capacity for self-priming (Fig. 4) but is not compatible with the requirement for an intact TAR structure during transcriptional transactivation by the Tat protein (50) or with other possible roles of TAR in virus replication (36). Our inability to detect SP DNAs in endogenous reactions with detergent-treated HIV virions (Fig. 5) demonstrates that HIV has indeed overcome the potential problem which would be caused by self-priming and suggests that a component(s) present in the virus is sufficient for achieving efficient strand transfer. On the basis of these considerations and the known properties of viral NC, it seemed likely that this protein might be important for preventing self-priming induced by the TAR structure. Recently, Li et al. (47) reported that NC also abolishes self-priming from an HIV-1 RNA template during synthesis of (−) SSDNA initiated with $tRNA_3^{Lys}$.

A number of investigators have found that NC stimulates strand transfer in model systems with viral templates (3, 53, 64) as well in systems using nonviral heteropolymeric templates (18, 19, 60). In some cases, the observed stimulation has been attributed to an effect on annealing of (−) SSDNA and acceptor RNA (3, 73). Peliska et al. (53) also noted that NC changed the specificity of RNase H cleavage and enhanced the rate of RNase H cleavage in their strand transfer system; these findings led to a proposal that NC interacts with the RNase H domain of RT.

The data presented here provide compelling evidence that NC has an additional function in HIV-1 strand transfer. Thus, a critical component of NC activity during strand transfer is its ability to prevent self-priming from (−) SSDNA, so that (−) SSDNA can anneal to the acceptor and undergo further elongation (Fig. 6 and 7). NC reduces self-priming in a dose-dependent manner and dramatically increases the rate, extent, and efficiency of strand transfer (Fig. 6). If the synthetic (−) SSDNA oligonucleotide is preannealed to the acceptor by incubation at 65°C, strand transfer proceeds with a high degree of efficiency in the absence of NC, and under these conditions, self-priming is not detected (data not shown).

We attribute the effect of NC on self-priming to its activity as a nucleic acid chaperone. One may ask, however, why NC favors the annealing reaction required for strand transfer over stabilization of the TAR structure (which would lead to increased self-priming). The findings of Tsuchihashi and Brown (66) are relevant to this question. They showed that in the presence of NC, annealing of DNA strands with longer base-paired regions, which would lead to more-stable duplex formation, was favored over annealing between strands which had a smaller number of complementary base pairs. Similarly, we suggest that NC promotes the annealing reaction in strand transfer since annealing of the larger number of base pairs in the entire R region results in formation of more hydrogen bonds and a more thermodynamically stable structure than is achieved by base-pairing of nucleotides in the TAR structure. It seems possible that this potential stabilization may provide a rationale for the presence of additional non-TAR sequences in the HIV-1 R region.

It is of interest to consider the possibility that reduction of self-priming induced by the TAR structure may be only one example of a more general NC activity during viral DNA synthesis. In the experiment whose results are illustrated in Fig. 3, DNA products smaller than (−) SSDNA (95 to 102 nt) with the characteristics of SP DNAs can be seen. When synthesis of these small SP DNAs is inhibited by incubation with actinomycin D (Fig. 3B) or RNase H-minus RT (Fig. 3C), RT pausing at a position near the 3′ end of the TAR stem in the donor becomes more prominent and a 77-nt DNA product accumulates. Interestingly, inspection of the gel shown in Fig. 6A indicates that the amounts of the pause product and the small SP DNAs are reduced in a dose-dependent manner by NC. Thus, the ability of NC to destabilize stem-loop structures in viral RNA which are responsible for pausing (41, 71) prevents the DNA pause product from undergoing a self-priming reaction. This allows RT to continue to traverse the template and facilitates further elongation of the DNA.

In summary, we have presented a new perspective on NC function and its role as an accessory factor to increase the efficiency and specificity of viral DNA synthesis during reverse transcription. We show that the increased efficiency of strand transfer promoted by NC is due not only to its stimulation of nucleic acid annealing (17, 46, 56, 66, 73), but also to a more general activity which allows it to inhibit self-priming. On the basis of the in vitro data on viral DNA synthesis, it appears that NC function represents an important element in assuring effi-

cient DNA synthesis and virus replication. Further support for involvement of NC in reverse transcription comes from in vivo data indicating that NC has other functions in addition to RNA packaging (31, 33, 74; reviewed in references 16 and 58) and also from studies with NC and RT inhibitors. Thus, synergistic antiviral effects are observed in a cytopathicity assay with HIV-1-infected lymphocyte-derived cell lines when compounds which inhibit HIV-1 NC activity are added together with chain-terminating (e.g., 3′-azido-2′,3′-dideoxythymidine) or non-nucleoside (e.g., nevirapine) RT inhibitors (59). Taken together, these considerations suggest that inactivation of NC may provide a novel approach for targeting HIV RT function in antiviral therapy. Such a strategy would be particularly valuable for combating infection with HIV-1 virions resistant to RT inhibitors.

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