Steps of the Acceptor Invasion Mechanism for HIV-1 Minus Strand Strong Stop Transfer*

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Minus strand strong stop transfer is obligatory for completion of HIV-1 minus strand synthesis. We previously showed evidence for an acceptor invasion-initiated mechanism for minus strand transfer. In the present study, we examined the major acceptor invasion initiation site using a minus strand transfer system in vitro, containing the 97-nucleotide full-length R region. A series of DNA oligonucleotides complementary to different regions of the cDNA was designed to interfere with transfer. Oligomers covering the region around the base of the TAR hairpin were most effective in inhibiting transfer, suggesting that the hairpin base is a preferred site for acceptor invasion. The strong pausing of reverse transcriptase at the base of the TAR and the concomitant RNase H cleavages 10–19 nucleotides behind the pause site correlated with the location of the invasion site. Oligomers closer to the 5′-end of R also inhibited transfer, though less effectively, presumably by blocking strand exchange and branch migration. We propose that pausing of reverse transcriptase at the base of TAR increases RNase H cleavages, creating gaps for acceptor invasion and transfer initiation. Strand exchange then propagates by branch migration, displacing the fragmented donor RNA, including the fragment at the 5′ terminus. The primer terminus switches to the acceptor, completing the transfer. Nucleocapsid (NC) protein stimulated transfer efficiency by 3-7-fold. NC enhanced RNase H cleavages close to the TAR base, creating more efficient invasion sites for efficient transfer. Most likely, NC also stimulates transfer by promoting strand exchange invasion and branch migration.

In retrovirus replication, transfers of partially synthesized DNA strands from one template to another are obligatory for completion of reverse transcription and synthesis of integration-competent double-stranded viral DNA. The transfer process is mediated by the viral polymerase reverse transcriptase (RT), which can catalyze both RNA- and DNA-templated DNA synthesis. In HIV-1, minus strand DNA synthesis is primed from a tRNA5′-end primer annealed to the primer binding site located near the 5′-end of the genome. Synthesis proceeds to the 5′-end of the genome, creating a DNA fragment called minus strand strong stop DNA (−ssDNA). The direct repeat sequences located at the ends of the genome enable the −ssDNA to transfer to the 3′-end of either the same or the copackaged RNA. This step, called minus strand transfer (1, 2), occurs through both intermolecular and intramolecular pathways with similar probability (3–5). Failure to detect a significant amount of −ssDNA in the infected cell cytoplasm suggests that minus strand transfer is an efficient event in vivo (6, 7). Mutational studies have shown that minus strand transfer mostly takes place after RT extends to the 5′-end of the genome, even though a small amount of premature transfer was also detected (8–13).

HIV-1 RT exhibits two enzymatic activities, DNA polymerase and RNase H. Both active sites reside within the p66 subunit and are separated by a spatial distance of 18–19 nts. The RNase H active site can make a cut 18–19 nts behind the primer terminus on the RNA strand. Cleavage is most likely when the RT is transiently paused during synthesis (14, 15). Both the polymerase and RNase H activities of the RT are essential for completion of minus strand synthesis. Viruses with defective RNase H activity fail to complete reverse transcription, especially at the minus strand transfer step, highlighting the important role of RNase H cleavage for transfer (16–21).

In minus strong stop transfer, the cDNA transfers to the 3′-end of either the same or the second RNA molecule based on the repeat (R) homology. In HIV-1 the R sequence is 97 nts long and includes all of the TAR hairpin and part of the poly(A) hairpin. Mutational and deletion studies have shown that the entire length of the R region is not required for minus strand transfer (22–24). Within a certain range, longer homology has been shown to facilitate minus strand transfer (22–25). In addition to the primary sequence, RNA secondary structure and cis-acting elements have been implicated in facilitating minus strand transfer (11, 26–28).

The viral nucleocapsid (NC) protein is required for minus strand transfer. HIV-1 NC coats the viral RNA genome in a sequence-independent mode, at a ratio of one NC to 7 nts (29, 30). As a chaperone protein, NC facilitates the melting and reannealing of nucleic acids, promoting them to fold into their most stable conformations (31–38). Numerous studies have shown that such properties of NC enhance the efficiency of minus strand transfer (25, 39–46). The ability of NC to promote strand annealing, particularly as it applies to interaction between the −ssDNA and RNA in the 3′-R (34), has been implicated in minus strand transfer. Studies done in vitro also suggest that NC prevents the −ssDNA from forming foldback products (25, 38, 42, 47–51), which otherwise would result in dead-end transfer intermediates. In addition, NC has also been

¶ The abbreviations used are: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; NC, nucleocapsid; nt(s), nucleotide(s); R, repeat; −ssDNA, minus strand strong stop DNA.
shown to facilitate strand transfer by stimulating RT RNase H activity (25, 40).

We recently showed by analysis in vitro that minus strand transfer proceeds through an acceptor invasion-initiated mechanism (25). A feature of this mechanism is that the first interactions of the −ssDNA with the acceptor RNA occur when the acceptor anneals to the cDNA donor well upstream of the DNA 3′-end. This is followed by a branch migration process in which expansion of the RNA-DNA hybrid region displaces the remaining RNA fragments, ultimately leading to the terminus transfer. In the present study we examine the location of invasions in a system of donor and acceptor RNAs that contain the full-length HIV-1 R region. Using a series of DNA oligomers designed to block invasion, we show that the region around the base of the TAR hairpin is a major acceptor invasion initiation site. The patterns of RT pausing and increased RNase H cleavage at the base of the TAR region support this conclusion. We also examine factors that influence the efficiency and location of the invasion step, including template homology and NC protein.

EXPERIMENTAL PROCEDURES

Materials—Purified HIV-1 RT (20,000 units/mg) was generously provided by Genetics Institute (Cambridge, MA). DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coraville, IA). The Pfu Turbo DNA polymerase was purchased from Stratagene (La Jolla, CA), and all other enzymes were from Roche Applied Science. The 32P isotope was purchased from PerkinElmer Life Science. The pNL4-3 molecular clone (from Dr. Malcolm Martin) was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, NIAID, National Institutes of Health). HIV-1 NCp7 (72 amino acids) was prepared by solid phase chemical synthesis as described by de Rocquigny et al. (52).

Preparation of RNA Templates—The donor RNA template D199 and acceptor RNA templates A70h, A82h, and A97h were generated by run-off transcription in vitro, from the corresponding PCR-amplified double-stranded DNA products using the Ambion T7-MEGAscript kit (Austin, TX) as per the manufacturer’s protocol. The PCR primers were designed to contain the T7 promoter sequence. The 5′-end of the D199 donor template corresponded to the +1 position on the genomic RNA, whereas its 3′-end corresponded to position +199 (primer binding site 3′-end), respectively. The acceptors A70h, A82h, and A97h started from the U3 region (position +9056) and extended into the 3′-R region (positions +9145, +9157, +9172, respectively). The sequence of the forward PCR primer for D199 was 5′-GGATCCTAATACGACTCACTATAGGGTCTCTCTGGTTAGA-3′ (A82h), 5′-ATACGACTCACTATAGGGCUGCUUUUUGCCU-3′ (A70h), and 5′-ATACGACTCACTATAGGGCUGCUUUUUGCCU-3′ (A97h). All RNA templates used in the study were purified by denaturing PAGE and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer. RNAs were quantitated using the RiboGreen assay (Molecular Probes, Eugene, OR).

Substrate Preparation—DNA primers were 5′-end labeled using γ-32P-ATP (6,000 Ci/mmol) and polynucleotide kinase. For reaction transfers, primer, donor, and acceptor were mixed at a 1:5:1:3 ratio, incubated at 95 °C for 5 min, and slow cooled to room temperature. Reaction mixtures contained 50 mM Tris-HCl (pH 8.0), 50 mM KCl, and 1 mM dithiothreitol. For use in RNase H assays, RNA templates were first dephosphorylated by calf intestine phosphatase, 5′-end labeled using γ-32P-ATP (6,000 Ci/mmol) and polynucleotide kinase and purified by denaturing PAGE as described previously (53).

Transfer Assay—Reactions were performed as described previously (25) with slight modifications. Final reactions contained 2.5 nM primer-template, 7.5 nM acceptor RNA, 50 nM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM dithiothreitol, 1 mM EDTA, and 32 nM HIV-1 RT. RT was allowed to prebind to substrate at 37 °C for 2 min before reactions were initiated with MgCl2 and dNTPs at a final concentration of 6 mM and 50 μM, respectively. Reactions were incubated at 37 °C for 60 min and terminated using 2X termination dye (10 mM EDTA (pH 8.0), 90% formamide (v/v), and 0.1% each of xylene cyanol and bromphenol blue). For reactions performed in the presence of NC protein, an appropriate amount of NC was added to the mixture and incubated at 37 °C for 5 min prior to the addition of RT. The amount of NC protein required for 200% coating of templates was determined from the length and amount of template used and assuming that one NC molecule covers 7 nts (29, 30).

For reactions performed with the blocking oligomers, 75 nM oligomer was added to the reaction mixture immediately after initiating reactions with MgCl2 and dNTPs, as described previously (54). All blocking oligomers were synthesized with 3′-phosphorylation to inhibit priming. In reactions designed to assess donor cleavage, donor RNA was 5′-end labeled. Sizes of DNA products were measured using a 10-bp DNA ladder, whereas sizes of RNA product were measured using ladders made by RNase T1 digestion and RNA hydrolysis.

RESULTS

We have presented evidence previously for an acceptor invasion-initiated mechanism of minus strand strong stop transfer in HIV-1 (Fig. 1) (25). By this mechanism, RNase H cleavages within the internal regions of the donor RNA during −ssDNA synthesis are crucial in facilitating the transfer. The homologous acceptor RNA anneals to the cDNA at short gaps created within the donor RNA template and initiates the transfer well behind the primer terminus. First contact of acceptor RNA and cDNA is called invasion because adjacent RNA segments are displaced to allow stable annealing. The acceptor RNA template strand then continues to displace the fragmented donor RNA competitively. Strand exchange proceeds by a branch migration process, displacing the donor RNA fragment, including the one at the terminus. Transfer is eventually completed by the primer terminus switching to the genome 3′-end. A notable feature of this pathway is that it does not require extensive cleavages at the donor 5′-end to dissociate the primer terminus. In the present study we define the major site of acceptor invasion and factors that influence its use.

Analysis of Minus Strand Transfer Using Templates with Full-length R Regions—In our previous study, we used a simplified donor-acceptor template system based on the HIV-1 R sequence to examine the mechanism of minus strong stop transfer in vitro. The donor and acceptor RNA templates
Fig. 2. Effects of template homology and NC protein on minus strand transfer. A, schematic of the minus strand transfer system used in the study. Description of the schematic is the same as in Fig. 1. Synthesis is initiated from a 5’-end-labeled DNA primer annealed to the primer binding site region on the donor RNA template. Three acceptor RNA templates sharing different lengths of homology with the donor were used. The lengths of homology shared by the donor and acceptor are indicated across from each acceptor. Nucleotide positions at the ends of the templates correspond to those of the genomic RNA. B, extension assays were performed in the presence of acceptors A70h (lanes 1–6), A82h (lanes 7–12), and A97h (lanes 13–18) and in the absence of acceptor (lanes 19–24). Reactions were performed in the absence of NC (lanes 1, 7, 13, and 19) or in the presence of 12.5% (lanes 2, 8, 14, and 20), 25% (lanes 3, 9, 15, and 21), 50% (lanes 4, 10, 16, and 22), 100% (lanes 5, 11, 17, and 23), and 200% (lanes 6, 12, 18, and 24) NC protein. Lane L is a 10-bp DNA ladder. Transfer product (219 nts), self-priming product, full-length donor extension product (199 nts) and primer (20 nts) are indicated. Transfer efficiency was defined as: 100 × transfer product/100% donor product + self-priming product + transfer product.

We next examined the effect of NC in the transfer reactions. Assuming one NC molecule/7 nt as 100% coating level (29, 30), the amount of NC was titrated to achieve from 12.5 to 200% coating of the substrates (Fig. 2B). NC did not alter the overall extension profile of the reaction but slightly decreased the overall intensity of the pause sites. However, increased levels of NC noticeably stimulated the transfer efficiency, as observed previously (25). At 200% coating level, transfer efficiency increased to 25% for A70h, 36% for A82h, and 35% for A97h. The effect of NC was observable at as low as 12.5% NC coating. This was in contrast to the results from our previous study using templates with short homology, in which NC stimulation of transfers was not obvious below 200% coating (25).

In addition to stimulating transfer, NC also inhibited self-priming. This inhibition was more distinct with the longer templates than observed previously with the short templates (25). At 200% NC coating level, self-priming product was greatly reduced from 25% (A70h and A97h) or 31% (A82h) to 3–5% on these templates. This suggests that the long homology system currently employed more effectively simulates the natural viral minus strand transfer.

Blocking Oligomers Reveal Initiation Sites for Strand Transfer—To determine the positions and characteristics of the invasion initiation sites during minus strand transfer, a series of
“blocking” DNA oligomers 20–30 nts in length was designed (Fig. 3A). These DNA oligomers had sequences representing specific sections of the RNA template and were therefore complementary to the ssDNA. The rationale is that the DNA oligomer will compete with the acceptor for annealing to the cDNA at the invasion site. This should block initiation of transfers from that site and result in a drop in transfer efficiency. Of course an oligomer could potentially also block the branch migration or the primer terminus switch steps, so the action of specific oligomers requires careful interpretation.

The oligomers were chosen to be complementary with the ssDNA and not the donor or acceptor RNAs so that their presence would have minimal effects on the structures of RNA which are relevant to the transfer mechanism. Their effects on transfer should be exerted, after the RNA-DNA hybrid is formed from the donor RNA, so that the region of invasion is largely double-stranded. They should displace fragments of the donor strand and take their place, protecting a specific invasion region.

To test their effects under conditions highly favorable for transfer, the oligomers were added to the transfer reactions at a 200% NC coating level. Transfers were first examined using the full-length homology acceptor A97h (Fig. 3B). To prevent priming of synthesis, the oligomers were phosphorylated at their 3'-ends. Presence of the oligomers did not change the primer extension pattern except in one important way. An intense band was observed at position 130, 172, 164, 160, 142, or 127, depending on the oligonucleotide present in the reaction (Fig. 3B, shown by arrows). These products corresponded to cDNA strands that transferred to the oligomer, extending to a final length at the 5'-end of the oligomer. This is a favorable observation indicating that the oligomers annealed to the cDNA to block invasion by the acceptor. The transfer of some DNA to the oligomers should not affect the accuracy of our transfer efficiency measurements. This is because transfer efficiency is determined from the ratio of primers that have been extended past the blocking oligomer, to either transfer or complete extension to the end of the donor RNA. All of the blocking oligomers inhibited transfers to some extent compared with a control reaction lacking any blocking oligomer (Fig. 3B). In addition, the oligomers also inhibited cDNA self-priming. This was because annealing of the DNA oligomer prevents the cDNA from folding back on itself. This was especially true for oligomers 36–80 and 40–69, which were complementary to the cDNA around the base of the cTAR hairpin.

Relative transfer efficiencies of reactions with blocking oligomers were plotted, with the efficiency of the unblocked transfer reaction set at 100% (Fig. 3C). The oligomers 36–80 and 40–69 were most effective in inhibiting transfer, causing a 60–70% inhibition. Each of these spanned a region that included about half of the TAR hairpin and half of the poly(A) hairpin. The effectiveness of these oligomers in blocking transfer when considered with their specific location suggested that these oligomers inhibited transfers by blocking invasion by the acceptor. Oligomers 28–57 and 1–30 were somewhat less inhibitory to transfers. Because the mechanism predicts that cDNA-acceptor interaction must propagate toward the 3'-end of the ssDNA, these oligomers most likely inhibited transfers by interfering with branch migration and primer terminus transfer. Oligomers corresponding to the 3'-end of the R homology region were the least inhibitory, causing only a 30–35% inhibition of transfer. We interpreted this to mean that they bind primarily outside of the region most relevant to minus strand transfer.

The effects of the blocking oligomers were also measured in transfer reactions with acceptors A82h and A70h that had reduced R homology. An extension pattern was observed similar to that with acceptor A97h in Fig. 3B (data not shown). The blocking oligomers produced a profile of transfer inhibition similar to reactions with the full homology acceptor, A97h (Fig. 3C). Once again, oligomers 36–80 and 40–69 were most inhibitory to transfers, causing a 70–75% drop in transfer efficiency, whereas oligomers 1–30 and 28–57 produced a 50–60% inhibition. In comparison oligomers corresponding to the 3'-end of the region of homology were the least inhibitory to transfer (20–30%). With acceptor A70h, oligomer 73–102 falls outside the region of homology and as expected had no effect on transfer. The profile of transfer inhibition by the blocking oligomers therefore suggests that the region around the base of the TAR hairpin is the primary site for acceptor invasion initiation.

NC Protein Does Not Alter the Invasion Site or the General Transfer Mechanism—NC was observed to increase transfer efficiency by 5–7-fold at a 200% coating level, suggesting that it stimulates one or more steps in the overall transfer. To examine the effects of NC in the invasion and branch migration steps of the transfer, we next performed transfer assays with the blocking oligomers and the A97h acceptor, in the absence of NC protein (Fig. 4). Again, the blocking oligomers did not change the extension pattern, except for the appearance of the band resulting from cDNA transfer to the oligomer (data not shown). Comparison of the transfer efficiencies showed that NC did not alter the transfer inhibition profile of the blocking oligomers (Fig. 4). However, the overall inhibition by the oligomers was not as great as that in the presence of NC protein. Oligomer 36–80 was most inhibitory, causing a 50% drop in transfer efficiency. Oligomers 1–30 and 28–57 each produced a 30% inhibition of transfer, possibly by inhibiting strand exchange and branch migration. No significant inhibition was observed with oligomers 58–78 and 73–102. Similar results were also observed for transfer with A82h or A70h (data not shown). These results are consistent with the interpretation that NC protein does not change the transfer mechanism but does magnify the inhibitory effect of the blocking oligomers. The analyses uniformly point to the base of the TAR hairpin as the site of most acceptor invasions.

The Acceptor Invasion Site Correlates with a Strong Pause Site at the Base of the TAR Hairpin and Increased RT-RNase H Cleavages—The strand transfer model predicts that the primary site of invasion should have experienced prominent RNase H cleavage. Additionally, the base of the TAR hairpin induces strong RT pausing, known to correlate with RNase H cleavage. This prompted us to examine the cleavage profile of the donor RNA template during minus strand transfer. Transfer assays were performed in parallel. A 5'-end-labeled primer binding site DNA primer and an unlabeled donor RNA template were used to follow synthesis (Fig. 5A), whereas cleavage of the donor RNA was followed using a 5'-end-labeled D199 donor RNA and an unlabeled DNA primer (Fig. 5B). The reactions were sampled over time in the absence or presence (200%) of NC protein. In the synthesis assay prominent pause sites were observed at positions 142–146, which decreased with time (Fig. 5A). These corresponded to RT pausing at the base of the TAR hairpin, and at the stretch of 3Gs, 4 nts into the hairpin. The presence of NC did not change the overall pausing pattern, although the pausing intensity was decreased. NC inhibited self-priming while stimulating transfer. Transfer product first appeared prior to the self-priming product both in the absence and presence of NC protein, indicating that transfers occurred before cleavage of the 5'-terminal segment of the donor RNA, as discussed previously (25).

When donor degradation was followed during transfer in the absence of NC (Fig. 5B, left), we observed a prominent cleavage
**Fig. 3.** Effect of blocking oligonucleotides on minus strand transfer. **A**, schematic of the minus strand transfer system and the blocking oligonucleotides. Description of the templates is the same as in Fig. 2. The donor and acceptor RNAs share a homology of 97 nts, comprising all of the R region (gray box). The TAR and poly(A) hairpin regions are indicated. The blocking DNA oligomers represent short regions of the template and are aligned along the donor RNA template. The oligomers are numbered based on the template region to which they corresponded. Structure of the 5'-R is shown in the inset. **B**, transfer assays with the D199 donor and A97h acceptor were performed in the presence of the different blocking oligonucleotides. The blocking oligonucleotide used is indicated above the lane. Control (Ctrl) is a reaction done in the absence of oligonucleotides. Products resulting from the cDNA transferring and completing synthesis on the blocking oligonucleotide are indicated by short arrows in each lane. Assays were performed in the presence of 200% coating level NC protein. **C**, quantitation of assays presented in B. Transfer efficiencies were measured in the absence (Ctrl) and presence of various blocking oligomers. Assays were performed with each of the three acceptors A70h (gray bars), A82h (white bars), and A97h (black bars). Transfer efficiency in the absence of blocking oligonucleotide was taken as 100% (Ctrl), and relative transfer efficiency in the presence of each blocking oligonucleotide was plotted. Quantitation was based on at least three independent experiments.
product of 75 nts. This product disappeared gradually with time while a 65-nt product accumulated over time. The 75-nt product would result from RT pausing at the base of the TAR hairpin and making a cleavage 18–19 nts behind the DNA primer terminus on the donor RNA. RT then appeared to move 8–10 nts toward the RNA 5′-end direction to make a second cleavage, resulting in the 65-nt cleavage product. In the presence of 200% NC protein, increased disappearance of full-length donor RNA was observed, indicating that NC protein increased overall RNase H activity, as shown previously (25).

In a response opposite the disappearance of the 75-nt product observed in the absence of NC, the 75-nt cleavage product gradually increased over time, indicating more RNase H cleavage at this site. In addition, other cleavage products of 81, 85, and 95–96 nt in length accumulated over time. The increased internal RNase H cleavages in the presence of NC protein, especially around the base of the hairpin, should create more gaps for acceptor invasion, accounting for the improved transfer efficiency (Fig. 5B). A greater concentration of invasions at the hairpin base would also explain the greater inhibitory effect of base-blocking oligomers in the presence of NC protein. In addition, the increased cleavage at positions 81, 85, and 95–96 in the presence of NC may result in the observed 30% inhibition in the presence of NC but almost no inhibition in the absence of NC by oligomers 58–78 and 73–102.

These assays were also done using a second donor template comprising the first 130 nts from the genome 5′-end and all three acceptors. The same results were obtained showing strong pausing at the base of the TAR hairpin, increased cleavage, and the oligomer covering the TAR base region inhibiting transfer the most (data not shown).

Overall, time sampling of the transfer reaction indicates that when RT pauses at the base of the TAR hairpin and cleaves the template, the resulting gaps improve invasion and transfer. NC enhances these TAR cleavages and also allows cleavages in and near the poly(A) hairpin, improving acceptor invasion in both regions. This suggests the same invasion-propagation mechanism.

**DISCUSSION**

Understanding the mechanism of minus strand strong stop transfer has been the focus of numerous studies. It has been speculated that minus strand transfer is initiated at regions upstream of the genome 5′ terminus (55). We recently showed that minus strand strong stop transfer in HIV-1 is facilitated through an acceptor invasion-initiated pathway that does not involve cleavage of the terminal 14-nt RNA at the genome 5′-end (25). Instead, transfer is dependent on cleavages within internal regions of the donor RNA. In this pathway transfer is initiated through annealing of the acceptor at internal sites in the cDNA. This annealing is an invasion process because it involves partial displacement of RNA template strands at the site of first interaction. Strand exchange would proceed by a branch migration process, displacing the cleaved donor RNA fragments including the 14-nt fragment at the 5′ terminus. However, two major questions remained to be answered. 1) What is the exact mechanism of acceptor invasion? 2) Where and why does the acceptor initiate invasion? In the current study we examine these issues using a transfer system in vitro, employing the 97-nt HIV-1 R sequence.

Using blocking oligomers designed to interfere with transfer in specific regions of the template, we showed that an oligomer corresponding to the region around the base of the TAR hairpin was most effective in inhibiting transfer. This suggested that the base region is a preferred site for acceptor invasion. Blocking oligomers covering the region closer to the 5′-end of the genome also inhibited transfer, though less effectively, presumably by blocking strand exchange and branch migration. Oligomers that bound toward the 3′-region of R beyond the TAR hairpin were inefficient at blocking transfer. We then time-sampled primer extension and donor degradation during transfer. We found that the strong pausing of RT at the base of the TAR hairpin correlated with increased RNase H cleavage on the donor RNA 10–19 nts behind the position of the RT polymerase active site during the pause. Overall, our data suggest that RT pauses at the base of the TAR hairpin and cleaves the donor RNA with the cDNA-donor hybrid. This creates opportunities for the acceptor RNA to interact with the cDNA and initiate transfer. NC protein increased transfer efficiency by 5–7-fold. NC noticeably increased overall donor RNA cleavages and in particular cleavages close to the base of the TAR hairpin. Most likely, the increased RNase H cleavages in the presence of NC created more effective invasion sites resulting in efficient transfer.

Some alternative explanations of the blocking primer results should be considered. We presume that invasions leading to −ssDNA transfer occur with basal frequency at any site in the TAR and poly(A) hairpin region. This is the probable reason for the observation that every blocking primer we used inhibited transfers to some extent. Aside from the influence of hairpin structure on invasion, it should be true that invasions initiated further from the RNA 5′-end become progressively less effective at leading to −ssDNA transfer. This is because there is a likely limit on the distance of efficient propagation during the period of the reaction. This expectation is consistent with the general ineffectiveness of primers bound furthest from the 5′-RNA end. We have interpreted the middle level inhibition by oligomers that bind in the region between the TAR base and the 5′-RNA end to mean that these oligomers inhibit invasion propagation. However, propagation, once initiated, may not be susceptible to oligomer blockage. The observed inhibition may represent blockage of invasions that occur at moderately effective invasion sites within the TAR. Because these sites are near the 5′-RNA end, invasions may almost always lead to completion of transfer. As such, blockage of the invasions would have a noticeable inhibitory effect. This alternative interpretation is still consistent with the conclusion that the −ssDNA transfer is driven by an invasion-induced transfer process.

Because the blocking oligomers contained self-complementary sequences from hairpin regions, they had the potential to anneal to complementary regions in the RNA templates, creating substrates for RNase H. Control RNase H assays were therefore performed in which the 5′-end-labeled donor or acceptor RNA was heat-denatured and refolded in the presence of different oligomers. No obvious (<10%) RNase H cleavage of

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**FIG. 4. Effect of the blocking oligonucleotides on minus strand transfer with A97h, in the absence of NC protein.** Description is the same as that in Fig. 3C. Quantitation was based on at least three independent experiments.
the donor or acceptor RNA was observed, indicating that the inhibition of transfers was not caused by degradation of the templates (data not shown). Although the oligomer 36–80 was very effective in inhibiting transfer, the two shorter oligomers, 28–57 and 58–78, which roughly span the same region of the template, were less effective in inhibiting transfer (data not shown). Such differences cannot be explained because many aspects of RNA structure are likely to influence primer effectiveness moderately.

RT pausing during synthesis has been proposed as an important factor in the strand transfer process (14, 56–59). In the current study, we found that acceptor invasion site correlated with RT pausing at the base of the TAR hairpin and increased RNase H cleavages. Pausing of RT during minus strand synthesis may be induced by the RNA template structure (58, 60), or homopolymer stretches of A/G in the template (61). Pausing effectively increases the ratio of RNase H to polymerase activity (14, 15, 58), and likely creates effective acceptor invasion sites. However, not all RT pausing cannot be explained because many aspects of RNA structure are likely to influence primer effectiveness moderately.

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Several studies have highlighted the importance of native structure and folding of the leader sequence in facilitating minus strand transfer. In support of this, when the native RNA genome is heat-denatured, it loses its ability to support efficient transfer (26). Berkhoit et al. (28) propose that the structural features in the HIV-1 R region facilitate minus strand transfer through “loop-loop kissing” interactions between the cTAR and cpoly(A) hairpins in the −ssDNA and the corresponding hairpin.

**Fig. 5.** Time course assays of primer extension and donor template cleavage during minus strand transfer. Assays were performed in the absence of NC (−NC) or in the presence of 200% coating level NC (+NC). Lane L is a 10-bp DNA ladder. Reactions were sampled at 30 s, and 1, 2, 4, 8, 16, 32, and 64 min as indicated above the lanes. A, time course of primer extension during minus strand transfer using acceptor A97h. Primer, full-length, transfer, and self-priming products are indicated. B, RNase H cleavage of donor RNA during the course of the transfer reaction. Arrows indicate positions of increased RNase H cleavage in the presence of NC. Sizes of RNA products were measured using ladders made by RNase T1 digestion and RNA hydrolysis.
pins (3′-TAR and 3′-poly(A)) in the 3′-R. We and others have shown that favorable acceptor conformation is necessary for productive invasion and annealing to cDNA (25, 46, 59).

The viral NC protein is another important factor for minus strand transfer. We showed here and previously that NC stimulates overall RT-RNase H activity, as observed by the rapid disappearance of the full-length donor RNA template. In the present study we show that NC increases the accumulation of cleavage products 95–96, 85, 81, 75, and 65 nt in length. The 75- and 65-nt cleavage products are likely to arise from pausing near the base of the TAR hairpin because a corresponding strong pausing band was observed. Increased RNase H cuts within the donor RNA expose the cDNA, thereby creating more opportunity for acceptor invasion. Interestingly, although NC within the donor RNA expose the cDNA, thereby creating more favorable for cleavage.

In addition to the stimulation of RNase H cleavages, NC also very likely facilitates strand transfer through its strand annealing properties. In the acceptor invasion mechanism of transfer, the nucleic acid annealing and strand exchange functions of NC are very likely to facilitate the acceptor invasion and branch migration steps in the transfer process. NC has also been proposed to interact with acceptor RNA creating optimum acceptor conformation for invasion (46). The stimulatory effects of NC on minus strong stop transfer are therefore manifested by its effects on different components of the reaction.

In summary, our results with blocking oligomers strongly support our earlier hypothesis that –ssDNA transfer occurs by a two-step invasion-initiated mechanism. The earlier work employed region-specific O-methylation of the donor RNA to show that RNA cleavage well upstream of the 5′-RNA terminus is required for efficient transfer (25). The current work employed blocking oligonucleotides to define the base of the TAR hairpin as the primary site for acceptor invasion leading to terminus transfer. Although current results have revealed the fundamentals of the transfer mechanism, the components of the system that produce the 100% efficient transfer observed in vivo remain to be determined.

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