Evidence for a Unique Mechanism of Strand Transfer from the Transactivation Response Region of HIV-1*

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We previously found that strand transfer by human immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) is promoted at sites where RT pauses during synthesis. In this report, strand transfer is measured within the 5′ transactivation response region (TAR) of HIV-1 RNA. We hypothesized that the stable hairpin structure of TAR would induce RT pausing, promoting RNase H-directed cleavage of the template and subsequent transfer at that site. We further predicted that HIV-1 nucleocapsid protein (NC), known to melt secondary structures, would decrease transfer. We show that TAR created a strong pause site for RT, but NC significantly promoted strand transfer. The effect of NC is specific, since other single strand binding proteins failed to stimulate transfer. In another unexpected outcome, preferred positions of internal transfer were not at the pause site but were in the upper stem and loop of TAR. Thus, we propose a new mechanism for transfer within TAR described by an interactive hairpin model, in which association between the donor and the acceptor templates within the TAR stem promotes transfer. The model is consistent with the observed stimulation of strand transfer by NC. The model is applicable to internal and replicative end transfer.

Strand transfer is an essential step in the replication of retroviruses, including that of the human immunodeficiency virus type 1 (HIV-1).1 It involves the movement of a primer from one position on the viral genome and reannealing to a different position. In addition to transfer reactions from the ends of the viral genome that are required for replication (1–3), there is evidence that strand transfer occurs from internal regions of the viral sequences resulting in recombination (4, 5). This recombination occurs with high frequency during viral replication (4–7). We have previously demonstrated a positive correlation between internal strand transfer and mutagenesis during synthesis by the HIV reverse transcriptase (HIV-RT) in vitro (8). It appears that strand transfer resulting in mutations or homologous recombination is a likely source of some of the genetic variation that produces quasispecies of HIV. This genomic alteration impacts the efficacy of antiviral therapy against HIV, presumably by initiating the emergence of drug-resistant viral strains carrying mutations in their genome.

Internal strand transfers require the RNase H activity of HIV-RT to degrade the RNA template, called the donor, originally hybridized to the extended primer (9). This degradation promotes release of the primer terminus from the donor template so that it can transfer to an acceptor (10, 11). We have previously shown that there are “hot spots” in the viral sequence that promote pausing of HIV-RT, leading to increased strand transfer (8).

The process of strand transfer entails orderly reactions of dehybridization and reannealing of the partially elongated primer. We had previously proposed that a secondary structure on a template may pause the RT, causing more extensive degradation of RNA template around the pause site, thus allowing invasion of acceptor templates and leading to transfer (12). Therefore, it would be of interest to measure strand transfer from a donor template with a large hairpin structure.

It was also important to explore the role of nucleocapsid protein (NC) in the reaction. NC is closely associated with the genomic RNA in virions (13) and was found to strongly stimulate hybridization of single-stranded RNA and complementary DNA (14). Yet, NC was also found to be capable of destabilizing the double helix region of oligonucleotides (15). Therefore, given the opposing functions of NC on nucleic acids, we postulated that NC would be a physiological modulator of strand transfer, especially for templates with extensive secondary structures.

We chose the TAR region of HIV as the substrate for these measurements. TAR is part of the repeat region (R region) of the HIV long terminal repeat duplicated at the 5′ and 3′ end of the viral RNA. TAR is 59 nucleotides long and forms a stable hairpin structure. The 3-nucleotide bulge on the upper stem of the hairpin binds an important viral transactivator, Tat (16). Upon binding TAR, Tat substantially increases the efficiency of transcription (17–19). Since the 5′ TAR constitutes the 5′ end of the genome, TAR participates in the first strand transfer reaction during reverse transcription. In this reaction, the nascent strong stop DNA strand containing TAR is dissociated from the 5′ end of the (+) strand RNA and rehybridized to the complementary sequence present at the 3′ end. There is also evidence that primers that have not fully copied TAR can transfer during viral replication, suggesting an internal strand transfer mechanism (20). The latter is only a minor pathway for replication (20, 21), but its frequency is high compared with recombination in other parts of the genome (5).

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1 The abbreviations used are: HIV, human immunodeficiency virus; RT, reverse transcriptase; NC, nucleocapsid protein; R region, repeat region; SSb, single-stranded DNA-binding protein; RPA, replication protein A; PCR, polymerase chain reaction; TAR, transactivation response region; nt, nucleotide(s); PBS, primer binding site.

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Strand Transfer within HIV-1 TAR

Previous studies of the effect of NC on templates containing the R region (14) demonstrated major effects of NC on the kinetics of annealing between a single-stranded DNA and a single-stranded RNA. Additionally, substrates with the partial sequence of TAR were shown to be stimulated for strand transfer (22). Thus, features of the TAR region make it an ideal substrate for investigation of the effects of secondary structure on strand transfer.

Our results show that the structure of TAR causes a major pause in synthesis by RT, as we predicted. However, a surprising outcome from our experiments is that the pause site did not serve as a preferred transfer site, providing evidence for an alternative strand transfer mechanism. We offer an explanation for the stimulatory effect of NC and show that it cannot be produced by other single-stranded binding proteins. Based on the current data, we present a new model for the mechanism of strand transfer in TAR.

EXPERIMENTAL PROCEDURES

Materials

Recombinant HIV-RT (p66-p51 heterodimer) was provided by the Genetics Institute (Cambridge, MA). The enzyme had a specific activity of 40,000 units/mg. One unit is defined as the amount required to incorporate 1 mol of dTTP into nucleic acid product in 10 min at 37 °C using poly(rA)-oligo(dT)18 as template/primer. HIV-NC was chemically synthesized by the Louisiana State University Health Center Central Laboratories. The sequence of the mature NC used for synthesis was that of the first 55 amino acids of the NC precursor protein described by Khan and Giedroc (23). The peptide was kept under reducing conditions, and aliquots were stored in 10% β-mercaptoethanol. The peptide concentration was determined by amino acid analysis, performed by the Cornell University Peptide Facility. The identity of the peptide was confirmed by amino acid composition analysis. A molecular mass of 6444 daltons was determined by electrospray mass spectrometry. Aliquots of both HIV-RT and NC were stored at −80 °C, and a fresh aliquot was used for each experiment. Escherichia coli single-stranded binding protein (SSB) was obtained from U.S. Biochemical Corp. The recombinant human replication protein A (RPA) was expressed and purified as described previously (24). The Expand High Fidelity PCR system, used for all of our PCR reactions, was purchased from Boehringer Mannheim.

pUC-BS-WT plasmid, which contains the HIV-1 long terminal repeat sequences, was provided by Dr. Malcolm A. Martin (NIADDK, National Institutes of Health). T7 DNA ligase, T4 DNA polymerase, T7 RNA polymerase, plasental RNase inhibitor, RNase-free DNAase I, dNTPs, rNTPs, restriction enzymes, and G25 and G50 Sephadex (RNA) columns were obtained from Boehringer Mannheim. T4 polynucleotide kinase and the DNA sequencing kit were obtained from U.S. Biochemical Corp. Radiolabeled compounds were from NEN Life Science Products. Epicurian Coli® SURE2 Supercompetent Cells and pBluescript® II SK+ vectors were obtained from Stratagene (La Jolla, CA). pGEM vectors were from Promega (Madison, WI). The plasmid purification kit, and quantitated by spectrophotometry. The plasmid was purified, and quantitated by hybridizing with a labeled DNA primer of known quantity, as described previously (30).

Methods

Generation of Donor and Acceptor RNA Templates—The donor template, BS-TAR, was made as follows: The pOUl-HindIII fragment of pUC-BS-WT plasmid was ligated into pBluescript II SK+, which previously had been digested with XhoI followed by a fill-in reaction with Klenow fragment and then digested with HindIII. The resulting plasmid, pBS-TAR, was transformed into competent cells. White colonies were selected, and the plasmid was amplified, harvested with a Qiagen plasmid purification kit, and quantitated by spectrophotometry. The plasmid with the correct insert containing the wild type TAR was identified by sequencing: pBS-TAR then was linearized by digesting with HindIII and transcribed in vitro, according to the Promega Protocols and Applications Guide as described before (5). The 188-mer RNA was gel-purified and was quantitated by hybridizing with a labeled DNA primer of known quantity, as described previously (30).

The acceptor template, GEM-TAR, was created by ligating the pOUl-HindIII fragment into a SmaI- and HindIII-digested PGEM®-7z+ vector. The plasmid construct, pGEM-TAR, was sequenced, amplified, purified, and quantitated as described above. It then was transcribed in vitro to create a 155-mer RNA that contains the TAR sequence.

The second donor template, TAR-PBS, was created by first amplifying the plasmid, pbGUR, given generously by Dr. Stephen Goff, with two primers, JK8 and JK9. The sequences for the two primers are as follows: 5′-GGGCGA GCGGTCT ATGC-3′ and 5′-GGGTTTCG TCTTG TTGAG ACCAG GGC-3′. The amplified product contained a T7 promoter and the wild type viral sequence from +1 to +276. The PCR product then was digested with BssHII and cloned into BssHII-digested pBluescript-ISK+. The resulting plasmid, pTAR-PBS, was then amplified and harvested as described above. The template was designed such that the viral RNA to be used as a donor template, TAR-PBS, started with the natural 5′ end of TAR with no plasmid sequence present.

The TAR-mtI acceptor template was made to contain point mutations within TAR to probe the point of strand transfer. The point mutations were designed such that the secondary structure of TAR was preserved. To make such a template, four single-stranded DNAs were synthesized. JK10 contains the T7 promoter, part of U3 and the first 34 nt of TAR (5′-CGGCC TTAA TA CACT CATTAGG TT GCCC GCTC-3′). JK12 has the following sequence: 3′-TCA GCCGG ATTAT GCTGA GTGAT ATGCC AAGCG ATAGTG CCCAG AGACATT ATCCG TACCT CGGAG AATGG TATTC TAGTG CGGTCA TAACT GGACT ACCG TC-5′. The underlined sequences indicate a region of complementarity between the two primers which were used to create a double-stranded DNA with two sticky ends. JK11 has the remaining TAR sequence and the additional 19 nt of the R region (5′-TCTGG TTAA C ATGCC AGC TCTGG TT AGG CCCA ATTA-3′). JK11 was then annealed to JK13 (3′-CTCGG ACCCT CGGAG AACATT ATCCG TACCT CGGAG AATGG TATTC TAGTG CGGTCA TAACT GGACT ACCG TC-5′), whose sequence is partially complementary to JK11. Again, the underlined regions indicate complementarity. The boldface letters are point mutations, and the italicized letters indicate a point insertion. The annealed JK10/JK12 and JK11/JK13 were ligated to each other, since the last 14 nt at the 3′ end of JK10 were complementary to the first 14 nt of the 3′ end of JK13. The annealed and ligated product formed a double-stranded DNA fragment that included a T7 promoter and the TAR region with point mutations, with 5′ and 3′ ends ready to be ligated to SfI and HindIII sites, respectively. The resulting DNA, dTAR-mt1, was inserted into a SfI- and HindIII-treated pGEM-7z+ plasmid. Then the ligated plasmid pTAR-mt1 was linearized by digesting with HindIII and then transcribed in vitro to produce the 93-nt-long acceptor RNA template with five specific point mutations.

5′ End Labeling of the DNA Primer—A DNA primer (5′-CTAGTG- GACCTCGGCCGCG-3′), named JK1, was labeled at the 5′ end by using T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmole). Free, unincorporated nucleotides were separated by using Quick Spin 228 G25 Sephadex RNA purification columns (Boehringer Mannheim).

Hybridization of the DNA Primer to the RNA Template—The labeled DNA primer was mixed with the RNA template at a 2:1 (primer/ template) molar ratio in 50 mM Tris-HCl, pH 9.0, 1 mM EDTA, and 80 mM NaCl RNA and template were kept at 37 °C for 10 min and then slowly cooled to room temperature for annealing.

Primer Extension and Internal Strand Transfer Assay—Primer extension was performed in a final volume of 12 μl. Two units (50 ng) of reverse transcriptase (final reaction concentration of 35.6 nM) was preincubated with 5 μl of dNTPs, 2 nM, in template termini, of primer-template, and the acceptor in the presence of NC in 50 mM Tris-HCl, pH 9.0, 80 mM KCl, and 1 mM dithiothreitol. The reaction was started by adding MgCl2 and dNTPs to a final concentration of 6 mM and 50 μM, respectively. It was terminated at various times by adding 1 volume of gel-loading buffer (90% formamide, 10 mM EDTA, pH 8.0, and 0.1% each of xylene cyanole and bromophenol blue).

Strand transfer reactions were also carried out in a final volume of 12 μl. The amount of primer-template and of RT was kept the same as in the primer extension reaction. The primer-template, and the acceptor in 40-fold excess, were preincubated for 5 min at 37 °C in the absence or the presence of NC in 50 mM Tris-HCl, pH 8.0, 80 mM KCl, 1 mM dithiothreitol. The amount of HIV-NC necessary to cost 100% of nucleic acids present in the reaction was calculated based on one molecule of NC binding to every seven nucleotides (25). HIV-RT was added and preincubated for 5 min for each reaction. The reaction was started, incubated for 20 min, and terminated as described above. In the reactions with RPA or SSB, the amount of RPA or SSB needed for 100% coating of templates in the reaction was calculated based on one molecule of SSB binding to every 65 nucleotides (26) and one molecule of RPA binding to every 30 nucleotides (24). The preincubation and reactions with SSB or RPA were carried out in the same way as with NC.
the presence of acceptor templates. Incidentally, we saw two
caused RT to pause, which should promote strand transfer in
5' and products prematurely halted at the base persisted (points
where the full-length products were formed, the short
formed by RT were those elongated up to the base of the TAR
At earlier time points during the extension, the major
major pause site near the base of the stem as we predicted. This
acceptor template needs to be destabilized.

The direction of the arrow indicates the direction of synthesis. The nt
numbering of the donor starts with the first nt complementary to the 5' end of the primer as 1. The nt at the 3' end of the acceptor, GEM-TAR, is
numbered 1. The TAR secondary structure is indicated by the stem
loop on both templates. The dotted box indicates the region of homology
between the two templates.

Analysis of Reaction Products—Extended primers were separated
electrophoretically on 8% denaturing polyacrylamide gels (19:1 acryl
amide/bisacrylamide) with 7 M urea as described (27), visualized on a
Kodak BioMax film (Eastman Kodak Co., Rochester, NY), and quanti
tated by using a PhosphorImager with ImageQuant software (Molecu
lar Dynamics).

RESULTS

Experiments were designed to test the hypothesis that the unique secondary structure in the TAR region template influ
ences the position and efficiency of internal strand transfer. Additionally, they test whether NC protein, known to stimulate
annealing of TAR sequences, has a major impact on this process.

Primer Extension Catalyzed by HIV-RT—We have primed
the 166-nt-long donor template, BS-TAR (Fig. 1), with a 5’
end-labeled DNA primer and analyzed both the efficiency of
primer extension on the donor template and that of strand
transfer to the 155-nt-long acceptor template, GEM-TAR. Both
templates include the complete TAR sequence, which serves as
a region of homology between the two templates. Homologous
strand transfers are most likely to occur within this region. In
order for RT to synthesize a full-length donor-directed product,
it has to extend the primer through the double-stranded stem
loop of TAR. During that process, encountering the stable stem
loop structure was expected to make the RT pause, and subse
quently transfer the primer strand to the acceptor without distur
bling the stem region. Likewise, in order for the RT to
transfer the nascent DNA strand and to continue synthesis on
the acceptor template, the double-stranded region of TAR on
the acceptor template needs to be destabilized.

To examine whether the stem-loop structure of TAR affects
polymerization by RT, we analyzed the synthesis pattern by primer
extension, in which the 5’-end-labeled DNA primer is extended on the RNA template, BS-TAR, in the absence of the acceptor
(donor template in Fig. 1). Results in Fig. 2 show a
major pause site near the base of the stem as we predicted. This
was confirmed by sequencing with the same primer in parallel.
At earlier time points during the extension, the major products
formed by RT were those elongated up to the base of the TAR
hairpin loop (Fig. 2, P in lanes 1 and 2). Even at the later time
points where the full-length products were formed, the short
products prematurely halted at the base persisted (lanes 3, 4, and 5). We concluded that the secondary structure of TAR
caus[ed] RT to pause, which should promote strand transfer in
the presence of acceptor templates. Incidentally, we saw two
other strong pause products near the 166-nt full-length prod
uct. However, these pauses occurred in the plasmid-derived sequences near the 5’ end of the RNA template, far past the
region of homology containing the viral sequence. In addition,
the 5’ regions of the donor and acceptor template containing plasmid sequences do not share homology with each other.
Therefore, these pauses seen at the plasmid-derived sequences
were not likely to have affected the TAR-specific polymeriza
tion or the strand transfer reactions below, catalyzed by HIV-RT.

Remarkably, in the presence of the trapping polymer hepa
rin, which prevents rebinding of templates by RT, products
included one that was 166 nt long, representing full-length
synthesis (data not shown). This indicates that HIV-RT, al
though it usually pauses at the base of the helix stem and may
dissociate, can at times synthesize completely through the TAR
region at a single binding event.

Effect of NC on Strand Transfer—We then examined the
strand transfer reaction in the presence and absence of NC
(Fig. 3). NC coats the single-stranded RNA genome in a virion,
in a similar fashion as other single-stranded binding proteins.
It was shown to promote strand exchange, favoring the most
stable duplex (15); participate in dimerization of the viral RNA
(28); unwind tRNA^Lys (23); and anneal tRNA^Lys to viral RNA.
polymers.

Lane 5

for calculation of percentage of coating level of NC.

The experiment was repeated at least three times. See "Methods"

the 166-nt full-length donor-directed synthesis product, respectively.

level of NC.

acceptor templates in the presence of NC enough to coat 100% (calculated by dividing the amount of transfer product (Fig. 3,

tions, and calculated the transfer efficiency. The efficiency is
differentiated by gel mobility.

donor template. In this way, the two products could be readily

would be longer than the full-length extension product on the

ceptor template was designed so that the transfer product

primer mentioned above and the acceptor GEM-TAR. The ac-

tations using the donor BS-TAR primed with the same DNA

FIG.3 .

Lane 1–4

indicate strand transfer reactions with the TAR-derived donor and

acceptor templates in the presence of NC enough to coat 100% (lane 1), 50% (lane 2), 25% (lane 3), and 12.5% (lane 4) of the single stranded polymers. Lane 5 shows a reaction in the absence of NC. Lane 6 shows donor-directed synthesis in the absence of acceptors at a 100% coating level of NC. T and F designate a 192-nt full-length transfer product and the 166-nt full-length donor-directed synthesis product, respectively. P indicates the products elongated up to the pause site near the TAR stem. The experiment was repeated at least three times. See “Methods” for calculation of percentage of coating level of NC.

(29). We reasoned that the known ability of NC to unwind and melt the secondary structures would result in destabilization of the TAR hairpin. The melting of the stem-loop in TAR should diminish the pause. This would promote unhindered, continued synthesis on the donor, and consequently there would be less opportunity for transfer.

To test this hypothesis, we performed strand transfer reactions using the donor BS-TAR primed with the same DNA primer mentioned above and the acceptor GEM-TAR. The ac-

ceptor template was designed so that the transfer product would be longer than the full-length extension product on the
donor template. In this way, the two products could be readily differentiated by gel mobility.

We titrated the concentration of NC in strand transfer reactions, and calculated the transfer efficiency. The efficiency is calculated by dividing the amount of transfer product (Fig. 3, T) by the sum of products of strand transfer (T) and of full-length donor-directed synthesis without transfer (F): T/(T + F). At a high concentration of NC, formation of both F and T are inhibited (lane 1). This inhibition, observed at a level of NC greater than that necessary for 100% coating of the templates, has been observed previously (30). It is thought to result from co-aggre-
gation of excess NC and templates, which may impede primer association or polymerization by RT. As we predicted, the pause site at the base of TAR faded as the concentration of NC increased (compare lanes 2 and 5, e.g.). This indicated to us that NC was active in its role of altering the template structure. The observation is also supported by previous studies by other investigators, who reported reduction of pausing in the presence of NC (35, 36).

The efficiency of strand transfer was greatly elevated at low concentrations of NC (lanes 2–5). The highest transfer efficiency was achieved at the concentration of NC that would coat 50% of the templates present. Particularly striking in the data presented in Fig. 3 is the degree of strand transfer enhancement. In the previous study using templates lacking large secondary structures, the magnitude of increase in the transfer efficiency by NC, when compared with reactions in the absence of NC, was approximately 50% (30). The quantitation of the strand transfer efficiency using the TAR-containing templates showed that NC stimulated the transfer by 300% in comparison with transfer in the absence of NC.

In our original hypothesis, the mechanism of strand transfer was based on sequential events of pausing of RT at the base of stem and degradation of the donor template at the pause site, followed by reannealing of the nascent DNA onto the acceptor. Accordingly, diminished pausing of RT in the presence of NC should lead to continuation of the donor-directed synthesis downstream of the pause site, thereby reducing the probability of transfer. The unexpected outcome of the increased transfer concurrent with the fading of the pause site on the donor led us to reconsider the fate of the DNA products whose synthesis was prematurely halted at the hairpin base. We considered two interpretations of this interesting observation. 1) NC could have promoted extension of the paused primer through the hairpin on the donor template. This would suggest that the increase in transfer is not related to the pause. 2) NC could have facilitated transfer of the primer from the pause site. The presence of NC would then stimulate the transfer of the paused product at the base of the stem and continued extension on the acceptor. This would reconciliate the disappearance of the prematurely terminated products at the pause site with an accompanying increase in transfer.

Time Course of NC-stimulated Strand Transfer—We conducted a time course experiment of strand transfer in the presence and the absence of NC to further characterize the kinetics of the NC-mediated strand transfer within TAR (Fig. 4). The reaction conditions were kept the same as before, and the efficiency of strand transfer in the presence of NC was compared with that in the absence of NC. Throughout the time course, the amount of transfer product in the presence of NC was consistently higher than in its absence. The increase in the formation of transfer product in the presence of NC could be based on various effects of NC on the kinetics of the transfer reaction. The general stimulatory effect of NC on synthesis might be its predominant effect during the reaction. This would result in a general increase in the number of primers initiated and, accordingly, in the number of synthesis products overall, including the transfer product. In such a case, the ratio of T to T + F would remain the same, i.e. the efficiency of strand transfer would be unaffected with or without NC. However, we found that the efficiency of strand transfer in the presence of NC was significantly higher at all time points when compared with that in the absence of NC. Of the primers initiated for synthesis, more transferred onto the acceptor instead of com-

pleting synthesis on the donor template in the presence of NC throughout the time course.

An alternate explanation for the observed increase in the amount of the transfer product could be that the effect of NC was specific to the process of strand transfer, possibly facilitating the rate-limiting step(s) of strand transfer. If NC acceler-
ates specifically the process of the strand transfer, the stimulatory effect of NC should be the greatest in the earlier time points. At these times, in the absence of NC, full-length donor-directed product would appear before the transfer event and subsequent transfer product synthesis could be completed. If NC eliminates a delay in the transfer event, both donor- and acceptor-directed full-length products would appear at similar rates. However, at later time points, RT would have had a sufficient time to synthesize full-length transfer product, so the increase in transfer efficiency caused by NC would be less pronounced. In fact, the NC-mediated transfer enhancement was more pronounced at earlier time points. For example, at 5 min, the efficiency of transfer in the absence of NC (lane 8) was more than 7 times higher than that in the absence of NC (lane 18), whereas after 20 min the efficiency was 3-fold greater in the reaction with NC (lane 10) than without NC (lane 20). At the two earliest time points, 40 s and 2 min, the transfer product is barely formed; thus, the efficiency of strand transfer could not be accurately measured.

**RNase H Activity of HIV-1 RT on TAR-derived Templates during Polymerization**—We have shown that the secondary structure of TAR pauses the HIV-RT during polymerization and that NC specifically increases the efficiency of strand transfer catalyzed by HIV-RT. If transfer occurs at the base of the hairpin, there should be enhanced RNase H-directed cleavage at this site. The coupled RNase H activity of the RT would cleave approximately 18 nucleotides upstream from the polymerase active site (31). Assuming the polymerase contacts and pauses adjacent to the base of the TAR structure, a primary cleavage product should be formed that is approximately 125 nucleotides long, from the 5'-end of the donor template (Fig. 5A).

Fig. 5B shows a result consistent with our prediction. In fact, the band corresponding to a 123-nt-long segment was the major product of RNase H activity. The reaction conditions were unchanged from the strand transfer reactions, except that the radioactive label was on the 5' end of the donor RNA instead of the DNA primer. One can see from the data that the template
The results showed that neither RPA nor SSB stimulated strand transfer within TAR. The percentage of coating level of each protein during the 20-min reaction was 100% (lanes 1 and 7), 50% (lanes 2 and 8), 25% (lanes 3 and 9), or 12.5% (lanes 4 and 10). Lanes 5 and 11 indicate reactions in the absence of each protein tested. Lanes 6 and 12 represent reactions in the absence of acceptors. Lane 13 shows a control reaction with no RT present. For the percentage of coating calculation of each protein, see “Methods.” At a 100% coating level, the final reaction concentration of each protein was 350 nM for RPA and 163 nM for SSB. The strand transfer reaction conditions were kept the same as those with NC.

in the presence of NC is degraded faster than in the absence of NC. This finding is consistent with a previous report by Benkovic and colleagues (22), who showed that NC enhanced the RNase H activity of the RT during strand transfer. However, in contrast to their result regarding the specificity of RNase H activity, we did not see any change in the cleavage pattern in the presence of NC, measured within the limits of our assay system.

Comparison of NC with Other Single-stranded Binding Proteins—The increase of strand transfer in the presence of NC might be due solely to the helix destabilization effects exhibited by all single-stranded binding proteins or a result of binding features unique to NC. To distinguish these possibilities, we measured the effects of two other well characterized single-stranded DNA binding proteins, E. coli SSB and human RPA, in the strand transfer reaction (Fig. 6). The concentrations of the proteins were titrated down from 100 to 0% coating level. The efficiency of strand transfer was then quantitated and compared with that of strand transfer in the presence of NC. The results showed that neither RPA nor SSB stimulated strand transfer (lanes 1–6 and 7–12). In fact, the presence of either protein was inhibitory to strand transfer. As the concentration of each protein was decreased, the transfer efficiency improved. However, the amount of primers fully synthesized to the end of the donor template remained little affected or slightly increased, since the single-stranded binding proteins are generally known to be stimulatory to DNA synthesis. It is also interesting to note that the pause site near the base of the stem remained unchanged at different coating levels of RPA or SSB, whereas the increase in the NC concentration corresponded with the decreased pause site. This may be due to specific interactions of NC with the viral sequence, affecting the secondary structures of TAR, that are not exhibited by RPA or SSB. Although RPA and SSB were reported to enhance strand displacement from templates containing the HIV-1 long terminal repeat (32), the two proteins were unable to increase the strand transfer reaction of TAR. The inability of the either protein to affect the transfer process indicates that a simple destabilization or displacement of hybrid strands within the TAR structure cannot be solely responsible for the observed enhanced rate of transfer by NC.

Determination of Points of Transfer—It was important that we define the molecular basis for strand transfer reactions within TAR to further elucidate the positive effect of NC on the reaction. In an attempt to understand how the transfer actually occurs, we mapped the sites of transfer. Results described above were consistent with transfer driven by the structure-related pausing of RT, followed by degradation of the donor and invasion of the acceptor at the cleavage site. To demonstrate that the base of TAR at which HIV-RT paused during synthesis correlated with the point of transfer of the elongated primers, we modified and applied the system used by Wu and colleagues, in which one could determine where the transfer occurred by sequencing the transfer product (8). First, we created a two-template system, similar to the one used for the experiments above, but the new acceptor now contained point mutations throughout the template, which would allow us to determine the point of transfer by examining the sequence of the hybrid transfer product, as described under “Experimental Procedures” (Fig. 7A). The mutations were carefully chosen so that the overall secondary structure of TAR could be maintained (Fig. 7B).

Using the new templates, we performed strand transfer and isolated the transfer product. The single-stranded DNA product was converted to double-stranded DNA, amplified by PCR, and cloned into a vector, according to procedures previously reported (8). To decrease possible mutations made by the Taq polymerase during PCR, a commercially available “high fidelity” PCR set of Taq combined with a 3′ → 5′ exonuclease was used. Thirty clones representing individual strand transfer products were sequenced. We had expected that most of the transfer products would be chimeras consisting of the donor sequence from the 5′ end of the product up to the base of TAR and then the acceptor sequence from the base of TAR to the 3′ end, as a result of transfer at the pause site near the base.

However, sequencing of the transfer products revealed yet another set of surprising outcomes (Table I). Out of 30 transfer products sequenced, a majority (22 clones) were a result of transfer between positions 4 and 5, suggesting an end transfer. This is a reaction that occurs during viral replication and is expected to be efficient (2, 20, 21). Despite the presence of the secondary structure at the 5′ end of the donor, RT seems to transfer very efficiently after synthesizing up to the end. The remaining seven clones, representing about 23% of the transfer products, were a result of internal transfer. Unexpectedly, there was no strong correlation between the major pause site and the point of transfer. If the pause site were the site of transfer, then we would have seen most of the transfer products with marker mutations at positions 2, 3, 4, and 5. However, such a product was seen in only 1 of 30 clones. Most of the
internal transfer products were found to contain markers at positions 3, 4, and 5. These data tell us that although the stem-loop of TAR created a very strong pause site for the RT, most of the internal transfer occurred beyond that site and well into the TAR structure, spanning the region around the loop and the upper stem. This result suggested a different mechanism altogether for the internal transfer of the TAR-derived templates. Based on the new findings, a mechanistic model for the transfer within TAR is proposed and discussed below.

**DISCUSSION**

We previously found a positive correlation between sequence-dependent pausing of RT and the frequency of strand transfer, using HIV-nef-derived templates (8). Based on this observation, our original hypothesis for the mechanism of internal strand transfer in the HIV TAR region was that the stable secondary structure halts the RT, which then extensively degrades the RNA donor around the pause site, freeing the nascent DNA primer for transfer. In this report, we demonstrate that strand transfer involving the TAR structure is not correlated with the pausing of RT. Most likely, pausing-related transfer still occurs but accounts for a minority of transfer events in this case. This surprising and revealing outcome suggests that an alternative mechanism is driving most strand transfer from this region.

When transfer positions were determined in TAR by point mutant analysis, only 1 of 30 clones represented the transfer product derived from internal transfer occurring near the pause site. Most of the internal transfer took place in the upper stem region of the prominent hairpin. The new finding suggests that the main impetus for strand transfer is most likely a direct interaction between the donor and the acceptor templates via complementarity of their stem structures. This assertion is also consistent with the decreased intensity of the product P in parallel with the increased efficiency of strand transfer in the presence of NC, as we observed above. The known ability of NC to melt and reanneal nucleic acids may help association between the complementary regions of the stem in the two templates. Upon binding of the acceptor to the donor, the local concentration of the acceptor is greatly increased, promoting the transfer.

Additionally, our results show that the dominant position of strand transfer in the R region is at or near the very end of the donor template, in accordance with the accepted model of reverse transcription (2). This observation is consistent with recent results from the cell culture-based study of the minus strand strong stop transfer of Moloney murine leukemia virus (21), showing the great majority of transfer from the very 5' end.

Based on our sequencing data and the observations we have made with NC above, we propose a model for the mechanism of
displaced acceptor strand binds this nascent DNA region via complementarity (step E). Binding of DNA and the acceptor RNA results in the transfer of the growing end of the nascent strand (step F). Thus, because of the extensive intermolecular binding between the two hairpins, the internal transfer site mainly occurs within the upper stem region of TAR.

The modest stimulation of strand transfer of about 50% by NC on substrates lacking large hairpins was interpreted to be the result of two opposing phenomena (30). NC stimulated binding of the tRNA primer to PBS. A similar type of activity in promoting RNA-RNA association can be at work during step C, in which the donor and the acceptor bind. In addition, You and McHenry (14) have reported that NC also greatly enhances hybridization between the DNA and RNA strands containing TAR. The transition from step D to E, where the first association between the nascent DNA and the acceptor RNA occurs, can be facilitated by the same type of annealing activity. In addition, it is possible that NC stimulates step C to precede step B; in other words, the equilibration and base pairing of the donor with the acceptor may occur before RT reaches the stem, leading to the displaced 3′ segment of the donor stem, and facilitating synthesis on the donor up to the loop region. Therefore, the contribution of NC in this model is that it reinforces the central feature of the strand transfer reaction, namely the interaction between the hairpins.

The interactive hairpin model can also be offered to provide mechanistic details for the first jump in the endogenous reverse transcription of HIV-1 in an infected cell. This replicative end transfer is the dominant reaction in vivo (20, 21). One of the advantages of the second set of our substrates, TAR-PBS and TAR-mtI, is that they simulate the parts of the viral genome participating in the first strand transfer. TAR-PBS as the donor represents the 5′ end of the viral RNA from which the minus strand strong stop DNA is synthesized, and TAR-mtI as the acceptor represents, despite the five marker mutations, the viral RNA results in the transfer of the growing end of the nascent strand (step F). Thus, because of the extensive intermolecular binding between the two hairpins, the internal transfer site mainly occurs within the upper stem region of TAR.

The TAR region has a central role in the viral life cycle. First, TAR RNA acts as a cis-acting regulatory element that binds the viral transactivator, Tat (16). As the cellular RNA polymerase transcribes the 5′ TAR, the binding of Tat to the stem-loop

**Fig. 8. The interactive hairpin model.** The hairpin on the left at step A is the acceptor template. The hairpin on the right is the donor template. The concave and convex shapes represent complementarity of the stem sequences. The lengthening arrow (top to bottom) indicates the DNA primer being elongated. The broken lines of the donor indicate degradation by RNase H activity. The lightly shaded stem is of the nascent DNA. The donor and the acceptor are shaded with solid black and with oblique lines, respectively. Step A shows the primed donor and the acceptor. At step B, the 5′ segment of the donor stem is displaced by the polymerase, which is synthesizing through the TAR stem region. At step C, the displaced donor segment and the complementary sequence of the acceptor hybridize, elevating the local concentration of the acceptor template available for transfer. Step D shows the unwinding of the acceptor-donor hybrid caused by the growing DNA end elongated through the region. Step E shows that the displaced acceptor stem segment subsequently binds to the nascent DNA via complementarity after the donor RNA is degraded and dissociates from the DNA strand. At step F, the physical binding between the DNA and the acceptor RNA facilitates the translocation of the growing end of the nascent DNA onto the acceptor. Step G shows that the transferred DNA continues to be synthesized on the acceptor RNA.

strand transfer in TAR (Fig. 8). In this “interactive hairpin model” the intimate interaction between the two hairpins is the driving force facilitating the transfer. As the primer is elongated through the TAR region, the double strand of the stem opens up (Fig. 8, step B). The displaced 5′ single-stranded region of the donor hairpin then binds the complementary sequence on the acceptor stem (step C). The association between the donor and the acceptor templates increases the local concentration of the acceptor. As the growing DNA primer unwinds the hybrid between the two templates (step D), the
dramatically increases the rate of transcription of the viral genome (34). Second, the TAR region is involved in the first strand transfer in the early steps of the viral replication. It is located within the R regions that are duplicated in the 5’ and 3’ end of the genome. During the first strand transfer, the nascent DNA is relocated from the 5’ end of the genome. During the first strand transfer, the nascent DNA is relocated from the 5’ end of the genome. During the first strand transfer, the nascent DNA is relocated from the 5’ end of the genome. It is located within the R regions that are duplicated in the 5’ strand transfer in the early steps of the viral replication. It is remarkable that TAR consists of a stem-loop structure at both ends of the viral DNA. Given the vital roles of TAR, it is interesting to see whether the model can predict the site of pausing and transfer in other substrates with similar secondary structure. Variation of the stem size, thus allowing for a stepwise association between the templates and the nascent DNA, should titrate the efficiency of transfer and subsequent viral replication by orderly base pairing among the involved strands.

Construed from the model is that this stepwise association between the templates and the nascent DNA is further expedited by specific functions of NC. The specificity of the role of NC in the transfer reaction is supported by the observations that other well characterized single-stranded binding proteins fail to produce a similar stimulatory effect on the efficiency of strand transfer (Fig. 6). E. coli SSB and its human homolog, RPA, are known to melt secondary structures and stimulate polymerases. However, strand transfer of TAR does not seem to depend simply on helix destabilization. There must be an active interaction between the two templates, where there are large hairpins. Although fully consistent with our results, the model is new and requires additional testing to verify the proposed steps. We are currently devising experiments to detect intermediate recombination structures predicted by the model. We are also attempting to dissect the contribution of the TAR sequence and that of the TAR structure in determination of pause sites and the efficiency of strand transfer. It would be interesting to see whether the model can predict the site of pausing and transfer in other substrates with similar secondary structure. Variation of the stem size, thus modulating the degree of interaction between the donor, the acceptor, and the nascent DNA, should titrate the efficiency and sites of transfer.

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