Proximity and Branch Migration Mechanisms in HIV-1 Minus Strand Strong Stop DNA Transfer*

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Human immunodeficiency virus type 1 minus strand transfer was measured using a genomic donor-acceptor template system in vitro. Donor RNA D199, having the minimum region required for minus strong stop DNA synthesis, was previously shown to transfer with 35% efficiency to an acceptor RNA representing the 3′ repeat region. Donor D520, having an additional 321-nucleotide segment extending into gag, transferred at 75% efficiency. In this study each transfer step was analyzed to account for the difference. Measurement of terminal transfer indicated that the cDNA terminus is more accessible for transfer than that of D199. Nevertheless, acceptor competition experiments demonstrated that D520 has a greater preference for invasion-driven versus terminal transfer than D199. Competition mapping showed that the base of the transactivation response element is the primary invasion site for D520, important for efficient acceptor invasion. Acceptors complementary to the invasion and terminal transfer sites, but not the region between, allowed assessment of the significance of hybrid propagation by branch migration. These bipartite acceptors showed that with D520, invasion raises the local concentration of the acceptor for efficient terminal transfer by a proximity effect. However, with D199, invasion is relatively inefficient, and the cDNA 3′ terminus is not very accessible. For most transfers that occurred, the acceptor accessed the cDNA 3′ end by branch migration. Results suggest that both proximity and branch migration mechanisms contribute to transfers, with the proportion determined by donor-cDNA structure. D520 transfers better because it has greater accessibility for both invasion and terminus transfer.

Retroviruses, including human immunodeficiency virus type 1 (HIV-1),§ package two copies of single-stranded genomic RNA (1, 2). Reverse transcription, in which the genomic RNA is turned into a double-stranded DNA, is catalyzed by the viral enzyme reverse transcriptase (RT). RT possesses DNA polymerase activity that synthesizes DNA using either DNA or RNA as a template. RT also has RNase H activity, which cleaves the RNA within a DNA-RNA hybrid (3, 4). In HIV-1, reverse transcription is initiated by a tRNA Lys-3 primer annealed to the primer binding site (PBS). Synthesis to the 5′ end of the genome generates a minus strand strong stop DNA. RT-associated RNase H activity cleaves the copied region simultaneously. A 97-nt direct repeat (R) sequence is present at both ends of the genome, and allows the minus strand strong stop DNA to transfer from the 5′ end to the 3′ end of the genome via sequence complementarity. This step is termed minus strand strong stop transfer (1, 2).

Minus strand transfer involves shifting of the cDNA being synthesized from the initial RNA template, or donor, to the homologous region on another RNA template, or acceptor, and then continuing synthesis (1, 2). Two possible mechanisms were proposed. In the first, the strand would shift through a terminal transfer pathway (5, 6). The polymerase and RNase H active sites on the RT are separated spatially by a distance equivalent to 18 nucleotides on the template RNA (7). Consequently, when cDNA synthesis on the donor RNA is complete, the 3′ end of the cDNA is still annealed to the short segment of RNA. A slower RNase H function, called secondary cleavage, cuts this segment into fragments small enough to dissociate from the cDNA (8–12). The cDNA 3′ terminus would then transfer to the acceptor RNA (5, 6). In the second, transfer would occur through an acceptor-mediated invasion-driven pathway (6, 13–16). In this case, the cleavage of the RNA segment remaining at the donor 5′ end is not required (6). Instead, influenced by secondary structure, RT would pause synthesis 50–100 nucleotides from the donor 5′ end. There RT RNase H activity would clear a small gap that allows the acceptor RNA to invade and anneal to the cDNA (17). Although the RT continues extension of the cDNA, the acceptor-cDNA hybrid would propagate, displacing donor RNA oligomers remaining from RT RNase H cleavage. Eventually, the 3′ end of the cDNA would switch from donor to acceptor in a region favorable for transfer completion (14, 15). The acceptor invasion is proposed to be triggered by RT pausing at one or two strong pause sites on the donor, leading to a clustering of cuts (18–22), generating a gap for acceptor invasion (14).

The R homology region is important for minus strand transfer, which has been shown to occur in vitro through either a terminal transfer pathway when the homology between donor
and acceptor was short or through the more efficient invasion-driven pathway when homology was long (6, 14). However, results in vivo suggest that the length of homology is not the only requirement for efficient transfer. Deletion or mutational studies demonstrated that strong stop transfer in viral replication could occur with only a portion of the R region (23–28). The R region in mouse mammary tumor virus is only 12 nucleotides long, suggesting an efficient terminal transfer pathway (29). The complex effect of R region on transfer was revealed by additional studies in vitro. Levin and co-workers (30) reported that truncating the R region of the acceptor actually led to efficient transfer. The explanation was that removal of secondary structures facilitated transfer. In some cases, the local secondary structure rather than the overall conformation of acceptor is more of a determinant of minus strand transfer efficiency (31). Considered together, the results suggest the length, structure, and sequence of the R region are all important factors in the transfer reaction.

Besides RT, the nucleocapsid protein (NC) is another important component of the viral replication system (32–34). NC is a nucleic acid chaperone. It coats the RNA genome and delivers it into the assembling virion (35, 36). In minus strand transfer, NC transiently destabilizes the secondary structure of substrates, alleviating RT pausing and facilitating the annealing of complementary regions between cDNA and acceptor (37–40). Self-priming product, which is non-transferable, is greatly reduced in the presence of NC (40–45). As a result, the transfer reaction is greatly stimulated by NC (32–34).

The region outside of the R homology is also likely to be important in regulating minus strand transfer in HIV-1. Berkhout and co-workers (46, 47) have examined the folding of the HIV-1 5'-untranslated region. Two alternative structures, branched multiple-hairpin and long-distance interaction were proposed by comparing the properties of RNAs sharing the same R homology. The proportion of each structure is influenced by sequences outside of R (46, 47). Presumably, each structure produces different patterns of RT pausing and RNase H cleavage, which could influence transfer. For the acceptor template, secondary structure predictions by mFold (48, 49) indicated the R region of the acceptor could form altered structures as varied lengths of U3 were attached, leading to different transfer efficiencies. This result suggests the structure and stability of the R region of the acceptor could also be influenced by regions outside of those directly involved in the base pairing associated with the long-transfer (30). Reverse transcription in HIV-1 is initiated by a tRNA15s-3 primer. Marquet and co-workers (50) have demonstrated that a long-distance interaction between the anticodon stem of tRNA15s-3 and a conserved nonanucleotide motif located in the U3 region greatly promoted minus strand transfer. This is another way that sequences outside of the region required for synthesis could be significant determinants of transfer.

We initially analyzed minus strand transfer in vitro using two donor templates. Donor RNA D199 was a 199-nt segment extending from the primer binding site to the 5’ end of the genome; donor D520 was identical to D199 with an additional 321-nt segment extending into gag. They both shared 97 nt of homology with the acceptor A97h. Significantly, at 200% NC coating, the transfer efficiency of D520 with A97h was ~75%, 2-fold greater than the ~35% observed with D199. We found that both systems performed at least some transfer through an invasion-driven pathway. We attributed more effective transfer with D520 to the transient folding of the donor template during synthesis and its effect on the pausing of RT, which in turn determines the RNase H cleavage that creates the invasion site (16). In the current study, to understand why D520 promoted more efficient transfer than D199, we dissected and analyzed each step involved in the transfer reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—HIV-1 reverse transcriptase (p66/p51 heterodimer) was purified as described previously (51, 52). HIV-1 NCp7 (72 amino acids) was chemically synthesized as described previously (53). DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). T7-MEGASHortscript high yield transcription kit was purchased from Ambion, Inc. (Austin, TX). The Platinum TaqDNA polymerase was purchased from Invitrogen. For 5’ end labeling, 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). All the other reagents were purchased from Roche Applied Science.

**Preparation of Substrates**—Genomic sequences from the HIV-1 pNL4-3 strain were amplified by PCR using TaqDNA polymerase to create DNA templates for the generation of RNA substrates. The donor RNA templates D199, D520, and acceptor RNA template A97h were generated by in vitro run-off transcription as described previously (16). The acceptor RNA templates A19h, A97h(30A), and A97h(30R) were transcribed in vitro from a synthetic double-stranded DNA fragment using the Ambion T7-MEGASHortscript kit. The sequence of the forward PCR primer for A19h was 5’-GGG CTT CTT TTT TTT GCC TGT ACT-3’, whereas the sequence of the reverse primers for A19h was 5’-GGT CTA ACC AGA GAG ACC CAC TAC AGG CA-3’. The sequence of the forward PCR primer for A97h(30A) was 5’-GGG GCC TCA GTG CAC TAC GTA CTA TGA GCC TGG TTT TTT TTT CCT GTA CTT GGT TCT TCT GGT TAC ACC AAA AAA AAA AAA AAA AA-3’, whereas the sequence of the reverse primer was 5’-TGA AGC ACT CAA GGC AAG CTT TAT TGA GCC TTA AGC AGT TTT TTT TTT TTT TTT TTT-3’. The sequence of the forward PCR primers for A97h(30R) was 5’-GGG GCC TCA TAC GAC TCA CTA TAG GCC TGC TTT CCT CCT GTA CTT GGT CTC TCT GGT TAC ACC TTA AAT TTT CCC ATT GCC CG-3’, whereas the sequence of the reverse primer was 5’-TGA AGC ACT CAA GGC AAG CTT TAT TGAGGC TTA AGC AGT AGT CTC AAT AGG ACT AAT GGG-3’. All RNA templates were purified by denaturing PAGE and resuspended in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA buffer. RNA concentration was measured by Ribogreen assay (Molecular Probes, Eugene, OR). The acceptor DNA templates dA19h, dA26h, dA35h, dA59h, dA70h, and dA80h were purchased from Integrated DNA Technologies, Inc. The sequence of dA19h was 5’-AAA AAA AAA AAG GGT CTC TCT GGT TAG ACC-3’. The sequence

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RESULTS

D520 Promotes Better Terminal Transfer Than D199—Previously we have shown that transfer with D520 was more efficient than with D199 using acceptor A97h, which supports the acceptor invasion-driven transfer pathway (16). To analyze the transfer efficiencies of the cDNA 3′-terminal regions of the two donors, RNA acceptor template A19h was designed. A19h shared homology with the terminal 5′ 19 nt of the donor and, therefore, could only undergo terminal transfer (Fig. 1A). Minus strand transfer product, which did not appear until about 4 min with D199, was detectable as early as 2 min with D520. There was a time-dependent increase in transfer product for both D520 and D199. However, the transfer efficiency was about 40% with D520 at 32 min and only about 10% with D199 (Fig. 1B). A higher transfer efficiency of D520 with A19h than D199 indicated that the 3′ terminus of cDNA formed from D520 is more available for direct interaction with the acceptor.

D520 Facilitates Faster Invasion-driven Transfer Than D199—Although terminal transfer is more efficient with D520 than with D199, the efficiency does not approach the measured level when invasion-driven transfer is allowed using A97h. To determine the nature of the advantage conferred by invasion-driven transfer, we measured transfer rates. The transfer reaction occurs at a slower rate than primer extension on the donor template. This is evident because products of full-length extension of donor template appear much sooner than transfer products. Then transfers continue after donor extension is completed as described above with the following exceptions. The primer was heat-annealed to the donor in the absence of acceptor. The primer:donor duplex and the acceptor template were then incubated with NC separately before initiation of the extension reaction. After the donor extension reaction was allowed to proceed for 32 min, acceptor templates were added. The final ratio of donor:acceptor:primer was 1:3:1.5.

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FIGURE 1. Terminal transfer with D199 and D520. A, schematic of the donor and acceptor templates. Donor D199 only contains 5′-R, U5, and PBS. Donor D520 contains the 520 nt of the 5′ end of the genomic sequence. Acceptor RNA A19h is homologous to 19 nt at the 5′ terminus of the donor RNA and contains 20 nt of the U3. Synthesis is initiated by an 18-nt DNA primer complementary to the primer binding site. The star indicates the position of the 32P label. B, representative gel showing transfer reactions with A19h. Reactions were sampled at 0.5, 1, 2, 4, 8, 16, and 32 min. Self-priming product (SP), transfer product (TP), and donor extension product (DE) are indicated. Lane L is a 10-bp DNA ladder. Transfer efficiency was calculated as 100% × TP/(TP + SP + DE).

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A.

\[ S' \rightarrow 5' +1 +19 +197 +199 +520 \]

Donor

\[ S' \rightarrow 5' +1 +197 +199 +520 \]

A19h

\[ S' \rightarrow 5' +1 +199 +520 \]

A97h

B.

![Graph showing relative transfer efficiency over time for different acceptors](image)

C.

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in earlier time points than in reactions with a lower rate of transfer (54). Consequently, the slope of the initial part of the curve can be used to compare rates. Clearly, the transfer rate with A19h, allowing only terminal transfer, was similar on both D520 and D199. However, the rate with A97h, which allows invasion-driven transfers, was faster on D520 than that on D199. This result shows that allowing invasion increases the transfer rate on D520. Even though invasion is also allowed with D199, either fewer transfers use the invasion pathway or invasion is slow in the D199 system.

**Invasion-driven Transfer Dominates in D520 but Not D199**

To probe the ability of each donor template to utilize the invasion-driven *versus* terminal transfer pathway, an acceptor competition reaction was devised (Fig. 3A). A DNA acceptor dA19h was designed. Similar to the RNA acceptor A19h, it was homologous to the 19 nt of the 5’ terminus of the donor. Therefore, it was only available for terminal transfer. It contained an extra 11-nt overhang 5’ of the R region, so that when dA19h and A97h were added simultaneously into the reaction, transfer products generated through the invasion-driven pathway (TP-219) would be 219 nt in length and would be easily distinguished from those generated through the terminal transfer pathway (TP-210), which would be 210 nt in length. In addition, DNA primer termini that transferred to one acceptor could not relocate to the other since the sequence of the 11-nt overhang in A97h was not homologous to the longer overhang in A97h.

Fig. 3B shows how the transfer products were formed over a time course when A97h and dA19h were added either separately or simultaneously. Transfer efficiencies with each acceptor under the different reaction conditions are summarized in Fig. 3C. When only dA19h was added to the reaction, transfer efficiency was about 56% for D520 and 30% for D199. When A97h was added along with dA19h, for D520, transfer efficiency through the terminal transfer pathway using dA19h decreased dramatically from 56 to 26%, whereas for D199 there was almost no difference in terminal transfer utilizing dA19h regardless of the presence of A97h.

The ratio of transfer efficiencies with A97h *versus* dA19h in separate and competitive reactions are summarized in Fig. 3D. When A97h and dA19h were added separately, the ratios of transfer efficiency with A97h *versus* dA19h were similar, about 1.2–1.3, for both of the donors. When the acceptors were added simultaneously, however, the ratio of invasion-driven transfer *versus* terminal transfer was 1.1 for D199, significantly lower than D520 at 2.2. Clearly, under the conditions of competition, when both invasion-driven and terminal transfer pathways were available, for D520, A97h was much more effective at capturing the cDNA than dA19h. This suggests that compared with D199, D520 displays a much greater preference for invasion-driven transfer *versus* terminal transfer.

**The Base of TAR Is Important for Efficient Invasion of D520**

Use of dA19h and A97h in competitive reactions allowed us to determine the dominance of acceptor invasion as a transfer mechanism. The competition reaction also allowed us to perform fine mapping of the sequence that allows A97h to dominate transfers. This can be presumed to be the region critical for invasion-driven transfers, presumably the initial invasion site. A series of truncated DNA acceptor templates, dA19h, dA26h, A35h, dA45h, dA59h, dA70h, and dA80h, which shared a homology of 19, 26, 35, 45, 59, 70, and 80 nt, respectively, with D520 and D199, were constructed (Fig. 4A). These were used in competition reactions to finely map the sequence at the invasion site. Because all the truncated acceptors had the same 5’ end 11-nt overhang as dA19h, the size of transfer products generated through those acceptors (TP-210) was expected to be the same and 9 nt shorter than the transfer product through A97h (TP-219). The truncated acceptors differed in length at their 3’ ends, so their ability to compete with A97h would be a reflection of having or lacking the critical sequence for invasion.

Each truncated acceptor was added into the transfer reaction along with A97h in the ratio of 3:1 into the transfer reaction,
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**FIGURE 3. Analysis of invasion-driven transfer versus terminal transfer under competitive reaction conditions.** A, schematic of substrates used in this study. DNA acceptor dA19h is homologous to the 19 nt of the 5’ terminus of the donor RNA and contains an extra 11 nt overhang of random sequence 5’ of the R region. B, transfer reactions with only dA19h, or A97h, or both were sampled at 10, 20, and 30 min. Transfer products generated using A97h (TP-219) or dA19h (TP-210), donor extension product (DE), and self-primer product (SP) are indicated. C, quantitative data showing transfer efficiencies of 30-min reactions averaged from at least 3 independent experiments. When A97h and dA19h were added separately to the reaction (separate), transfer efficiency of A97h was calculated as 100% × (TP-210)/(DE + TP-210 + SP); transfer efficiency of dA19h was calculated as 100% × (TP-219)/(DE + TP-219 + SP). When A97h and dA19h were added simultaneously to the reaction (together), transfer efficiency of A97h was calculated as 100% × (TP-219)/(DE + TP-219 + TP-210 + SP); and transfer efficiency of dA19h was calculated as 100% × (TP-219)/(DE + TP-219 + TP-210 + SP). D, quantitative data showing the ratio of transfers to A97h versus dA19h when added separately (top row) and together (bottom row).

and Fig. 4E shows the formation of transfer products in each reaction. For both D520 and D199, the transfer efficiencies of truncated acceptors increased in a homology length-dependent manner. As the truncated acceptors extended further into the R region, they were able to better compete with A97h for transfer. This change was gradual with D199. However, for D520, a distinct pattern emerged when the truncated acceptors dA59h, dA70h, and dA80h, which extended into the base of TAR, were compared. There was a significant decrease in invasion-driven transfer by A97h with D520 when the homology of truncated acceptor length was increased from 59 to 70 nt (Fig. 4C). Evidently, for both donors, increasing homology in the truncated acceptors resulted in their more effective competition with A97h. D520 was able to take greater advantage of the interaction of the base of TAR with A97h than D199 to promote efficient transfer. This advantage disappeared when that region was blocked by competition with dA70h and dA80h. This result shows that the base of TAR is important for the efficient transfer of D520 and suggests that it is the primary invasion site of D520.

Two-homology Acceptors Promote Terminal Transfer with D520 through a Proximity Effect—Invasion-driven transfer was initially envisioned to occur by several steps. These steps include contact of the acceptor at the invasion site, spreading of the hybrid by branch migration, strand exchange of the acceptor for oligonucleotides remaining from the donor RNA, and then completion of transfer as the branch migration captured the cDNA 3’ terminus (Fig. 5A) (14). If the cDNA 3’ terminus is available for direct interaction with the acceptor, as we measured above, we can envision an alternative mechanism of transfer (Fig. 5B). In this mechanism, initial interaction of the cDNA 30 A residues or 30 nt of random sequence, was 10 nt shorter than the sequence replaced from the wild-type A97h. Consequently, products derived from transfers within the invasion site (TP-209) could be easily resolved from those transferring within the end transfer site (TP-219).

Fig. 6B shows the transfer profile for each acceptor with D520 or D199. Interestingly, with D520, transfer efficiency increased significantly from 40% for A19h to 62% for A97h(30R) and 71% for A97h(30A), almost the same level observed with A97h. For D199, however, the bipartite acceptors produced no remarkable improvement in transfer efficiency compared with A19h. Transfer efficiency increased slightly from 10% for A19h to 15% for A97h(30R) and 17% for A97h(30A) (Fig. 6C). Evidently, D520 can very effectively support transfers through a proximity mechanism. Although transfer is generally poor with D199, the A97h acceptor is clearly superior to the bipartite acceptors. This suggests that the cDNA 3’ terminus is relatively inaccessible to direct transfer and, instead, requires hybrid propagation by branch migration for maximum transfer efficiency.

Branch Migration Allows More Effective Transfer Than Just Invasion after Primer Extension—The above results suggest the possibility that some cDNA ends become refractory to transfers during or after cDNA primer extension. Moreover, branch migration may be more effective than proximity in allowing the acceptor to gain access to the cDNA 3’ end. To determine whether there is a time-dependent inactivation of the cDNA end for transfer, each acceptor was added only after the donor extension product had been fully synthesized (Fig. 7A). Transfer efficiencies when acceptors were added during or after donor extension are summarized and compared in Fig. 7B. For
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A. Transfer through Branch Migration

B. Transfer through a Proximity Effect

FIGURE 4. Mapping the invasion site. A, schematic of the donor and acceptor templates used. Seven truncated DNA acceptor templates dA19h, dA26h, dA35h, dA45h, dA59h, dA70h, and dA80h, respectively, share 19, 26, 35, 45, 59, 70, and 80 nt of homology with both of the donor templates. They all have the same 11-nt random sequence 5' of the R region. B, a representative gel showing transfer reactions when both A97h and each of the truncated acceptors were added simultaneously to the reaction. Transfer reactions were sampled at 10, 20, and 30 min. Transfer product generated by A97h (TP-219), by truncated DNA acceptors (TP-210), donor extension product (DE), and self-primer product (SP) are indicated. Lane L, 10-bp DNA ladder. Control reaction (C) is the transfer reaction with only A97h. Schematic of the 5' end of the donor RNA indicating the region of homology with each truncated acceptor is shown to the right of the gels. C, quantitation of transfer efficiency. Transfer efficiency with A97h (black) and truncated acceptors (gray) was plotted for D520 and D199. Control reaction (C) is the transfer reaction with only A97h. Transfer efficiency for A97h was calculated as 100% × (TP-219)/(DE + TP-219 + TP-210 + SP). Transfer efficiency for truncated DNA acceptors was calculated as 100% × (TP-210)/(DE + TP-219 + TP-210 + SP). Values were averaged from a minimum of three independent experiments.

D199, when acceptors were added later in the reaction, the terminal transfer efficiency, through A19h, was a mere 5%. The addition of the bipartite acceptors A97h/30A or A97h/30R barely increased the transfer efficiency to 8–10%. Interestingly, adding back A97h to the completed extension reaction restored the efficiency to 30%. These results suggest the 3' end region of the cDNA formed from D199 was severely inactivated for transfer after full extension, and this defect could be overcome by branch migration after invasion but not by proximity effects alone. For D520, after donor extension, the terminal transfer efficiency through A19h was still as high at 28%, and adding back the bipartite acceptors restored the transfer significantly to 48–56%. These results suggest the 3' terminus of cDNA formed from D199 was inactivated to a larger degree than D520, and the requirement for hybrid propagation for D199 is more acute.

DISCUSSION

We previously described an HIV-1 minus strand transfer system in vitro in which the donor template included a 199-nt genomic RNA extending from the PBS to the natural 5' end (D199) (14, 16). Even though transfers in vivo occur with high efficiency (55–57), the maximum transfer efficiency achieved with D199 was only about 35% (14, 16). However, with a donor RNA that extended 520 nucleotides from the natural 3' end (D520), the transfer efficiency rose to 75%. Probing with RNases indicated that the two donor RNAs folded differently. We proposed that the region 3' of the PBS in D520 folds back to create an R-region structure that promotes transfer (16). Relating specific structures to the improved transfer efficiency is virtually impossible because the folding of the RNA changes dynamically as the cDNA is extended. Instead, we have addressed the consequences of this dynamic folding on specific steps in the transfer reaction that could...
analyze transfer reactions with two-homology acceptors. A, schematic of the experimental design. Two-homology acceptors A97h(30A) and A97h(30R) were generated by replacing the 40 nt region between the invasion site (positions +59 to +97) and 19-nt terminal transfer sites (positions +1 to +19) in A97h with either a segment of 30A residues or 30 nt of random sequence (patterned line). Transfer products including the 30 nt of base substitution are indicated as TP-209, and transfer products without are labeled as TP-219. B, transfer reactions with A97h, A19h, A97h(30A), or A97h(30R) were sampled at 0.5, 1, 2, 4, 8, 16, and 32 min. Donor extension product (DE) and self-priming product (SP) are indicated. C, quantitation of transfer products. Amount of transfer products formed at 32 min was plotted for each donor with A97h, A97h(30A), A97h(30R), or A19h. Transfer efficiency was calculated as 100% (DE) indicated as TP-209, and transfer products without are labeled as TP-219.

We first compared the accessibility of the extended cDNA 3’ end with each donor RNA for transfer. To accomplish this, we prepared A19h, a short acceptor that was only capable of interaction with the cDNA terminus (Fig. 1). For D199 and D520, there was some decrease in transfer efficiency using A19h compared with reactions with A97h. This suggests that for both donors, the additional homology in A97h that accounts for proposed invasion and hybrid propagation is a significant contributor to efficient transfer. It is of interest that at 200% NC coating, D520 transferred effectively at 40% efficiency with A19h, although the homology was only 19 nt. The effective terminal transfer of D520 with limited homology is consistent with earlier studies which demonstrate that full homology is not required for transfer (23–28, 58). We note that when the terminal transfer reaction of D199 was performed at 400% NC coating, which introduced the same NC concentration as that in the transfer reaction of D520 at 200% NC coating, the transfer efficiency of D199 was still a low value of 14% (gel not shown). Therefore, the different transfer efficiencies between two donor templates observed in Fig. 1 cannot be attributed to differences in the absolute NC concentrations but occur because of better cDNA 3’ end accessibility. Several observations argue that the key to effective transfer is the invasion mechanism but that mechanism also benefits from good cDNA 3’ end accessibility. Earlier results showed that transfer products measured in vitro always appear at a much slower rate than the donor extension products (12, 21, 59–61). We recently examined the reason for this slow appearance by dissecting the transfer reaction into its individual components and measuring the rates of each. Results suggested that creation of an invasion site controlled the overall reaction rate (54). Transfers with
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A19h do not involve invasion, so a different step controls the rate. Possibly that step is the gaining of access to the cDNA 3' end by the acceptor. We wondered whether transfer rates would suggest the role of invasion in transfers. We superimposed the transfer efficiencies measured over time, all normalized to 100% (Fig. 2C). The rates of transfer with A19h, estimated by initial slope, were similar on D199 and D520 and relatively slow. Transfers with D199 and A97h occurred at a similar rate as those with A19h. However, when A97h was used with D520, transfer occurred at a faster rate, i.e. with a steeper initial slope. We interpret these results to indicate that whatever invasion occurs with D199, it does not improve the rate at which the cDNA 3' end is accessed. However, with D520 it appears that invasion accelerates the interaction of the acceptor with the 3' end of cDNA. The effect is to both increase rate and shift the rate-controlling step to initial invasion.

The dominance of invasion over direct terminal transfer was also revealed in acceptor competition assays (Fig. 3). When A97h and dA19h were both present with D520, A97h was pre-emptive in capturing the cDNA 3' end for transfer. This was evident by considerable suppression of transfers to dA19h in the presence of A97h, resulting in a high ratio of use of A97h over dA19h. At least one reason for this effect is that access to the cDNA 3' end on D520 is accelerated in reactions that can use the invasion mechanism. A likely explanation is that the faster rate of transfer promoted by the invasion mechanism allows cDNA strands that might have transferred by direct cDNA 3' end interaction over a longer time frame to transfer quickly by invasion. This interpretation is consistent with our previous observations using acceptor mapping. They showed that early in the reaction, a significant proportion of A97h was cleaved right at the invasion site of D520 (16). We interpreted this to mean that a high percentage of the cDNA was rapidly committed to the invasion mechanism.

The approach of setting up competitive reaction conditions was also applied to map the invasion site (Fig. 4). This type of mapping is conceptually similar to, but more sensitive than prior methods. Comparison of minus strand transfers with progressively longer homology acceptors previously indicated a role for homology 60–100 nucleotides from the template RNA 5' end in producing maximum transfer efficiency (14). Similarly, use of blocking oligonucleotides suggested that a sequence in that same region is significant for most effective transfer (14). However, by using progressively elongated DNA acceptors in the competitive approach, the difference in transfer between the neighboring truncated acceptors could be attributed to the addition of very short sequences. With D520, there was a major decrease in transfer with A97h when competition with dA70h was compared with that with dA59h. dA70h differs from dA59h only by an 11-nt extension. The extension has little secondary structure, and therefore, is not expected to influence the overall fold of the two acceptors. Evidently the 11-nt region was important for transfer and presumably represents a crucial part of the invasion site. This result, consistent with previous observations (16), identifies the invasion site as the base of TAR.

One important observation of this work is that D520 supported a transfer mechanism through proximity effect by using bipartite acceptors A97h(30A) and A97h(30R) (Fig. 6). A similar phenomenon was observed measuring transfers using templates containing the HIV-1 dimerization initiation signal (DIS). The functional structure motif DIS is important for the formation of the viral RNA dimers (62–64). When we analyzed transfer in vitro with natural HIV-1 sequences in the DIS region, the presence of the DIS sequence caused the donor and acceptor templates to dimerize. Compared with the template without DIS, about a 4-fold increase in transfer efficiency was observed using templates harboring DIS, and most new transfers in the substrates having DIS occurred near the DIS sequence. We concluded that dimerization promotes recombination through a proximity effect (60, 65).

The use of the bipartite acceptors indicated that acceptor invasion can promote transfers by a mechanism that does not allow hybrid propagation by branch migration. However, the design of the bipartite acceptor does not allow an exact quantitative comparison of transfer efficiencies of the bipartite acceptors with that of A97h. In A97h(30A) and A97h(30R), the last 40 nt of the TAR hairpin in wild-type A97h was substituted with a 30-nt non-homologous sequence. This change altered the over-

FIGURE 7. Analysis of transfers after donor extension. A, representative gel showing transfer reactions when A97h, A19h, A97h(30A), or A97h(30R) were added after 30 min of donor extension. Transfer reactions were then sampled at 10, 20, and 30 min after the addition of acceptor. The labeling of the products is the same as described in Fig. 6. Donor extension product (DE) and self-priming product (SP) are indicated. Lane L, 10-bp DNA ladder. B, quantitation of transfer efficiency. For both D520 and D199, transfer efficiencies were calculated from normal transfer reactions when each acceptor was added at the beginning of the reaction (during) or from reactions when each acceptor was added after 30 min of donor extension (after) are shown. Values were averaged from a minimum of three independent experiments.

|          | D199 during | D199 after | D520 during | D520 after |
|----------|-------------|------------|-------------|------------|
| A97h     | 35 ± 4%     | 10 ± 2%    | 17 ± 2%     | 15 ± 2%    |
| A19h     | 30 ± 3%     | 5 ± 2%     | 10 ± 2%     | 8 ± 2%     |
| A97h(30A)| 75 ± 5%     | 40 ± 4%    | 71 ± 4%     | 62 ± 3%    |
| A97h(30R)| 68 ± 5%     | 28 ± 3%    | 56 ± 3%     | 48 ± 4%    |
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all structure of the TAR hairpin in the acceptors. Secondary structure predictions by mFold (48, 49) of A97h(30A) and A97h(30R) indicated that they folded into different, lower stability structures than the wild-type A97h. It has been reported that presence of a secondary structure in acceptor RNA reduces transfer efficiency, and removal of structure promotes transfer (19, 30). Therefore, it is likely that structural changes made to construct the bipartite acceptors made them more effective for transfer. If this were the case, a comparison of transfer efficiencies of A97h with those of the bipartite acceptors would underestimate the importance of hybrid propagation by branch migration to transfer efficiency of A97h. Predictions by mFold (48, 49) indicated a stretch of 30 nt of A residues formed a simpler structure than the 30 nt of the random sequence we used. Predictably, A97h(30A) transferred with higher efficiency than A97h(30R) with both D520 and D199. This observation lends further support to our conclusion that reduction of structure in the acceptor promotes transfer. Therefore, we can conclude that transfers with D520 or D199 are possible by both proximity and branch migration pathways, although transfer with D199 is more dependent on branch migration.

How might the faster transfer rate with D520 and A97h relate to the observed higher transfer efficiency? Transfers with both D199 and D520 do not approach 100% efficiency primarily because a portion of the cDNA primers achieve full-length extension on the donor template but never transfer. In fact, they never extend beyond the exact length of full synthesis on the donor template. This suggests the possibility that the extended cDNA-donor complex assumes a structural configuration that is inactive for transfer. This configuration is not simply fully annealed folded back cDNA, since that minor product is extended to a longer product clearly visible on the gel. To investigate the nature of the apparent inactivation, we asked whether the extended cDNA became progressively more inactive with time (Fig. 7). The approach was to allow cDNA extension without acceptor RNA. The acceptor would then be added later to determine how many cDNA ends could still be captured for transfer. Results showed that both the D199 and D520 systems inactivated progressively with time. However, D520 remained much more effective for transfer than D199. Significantly, the progressive inactivation was very evident with A19h and either donor template. However, very little time-dependent inactivation occurred with A97h. This suggests that the ability of the acceptor to engage in the invasion mechanism is a means of countering inactivation. This may be due in part to the higher transfer rate, which would capture the cDNA 3’ end before it could be inactivated. Significantly, the bipartite acceptors were able to transfer well with the D520 donor when added after primer extension but very poorly with the D199 donor. This shows that for D199 the branch migration mechanism of hybrid propagation is necessary to achieve any significant amount of transfer after the 3’ end of the cDNA has been fully extended. In fact, this result indicates that branch migration is a viable mechanism for multistep transfers and likely participates with the proximity mechanism in driving a portion of minus strand transfer.

Based on all of the results presented above, we propose a two-pathway acceptor invasion-driven model for minus strand transfer of HIV-1 (Fig. 5). The RT extends from the PBS toward the RNA 5’ end. When it reaches the base of TAR, a strong pause site, it will temporarily pause, concentrating RNase H cuts of the donor RNA and generating a potential invasion site for the acceptor. After initial interaction, the acceptor-cDNA hybrid propagates toward the 5’ end of the donor by branch migration, displacing the cleaved donor template fragments until the hybrid catches up with the cDNA 3’ terminus (Fig. 5A). Alternatively, acceptor invasion at the pause site raises the local concentration of the sequence in the acceptor that is complementary to the 3’ end region of the fully extended cDNA. If the 5’ end of the donor is highly accessible, a larger proportion of the acceptor could be facilitated to complete transfer to the cDNA, allowing the acceptor to be successfully transferred regardless of branch migration (Fig. 5B).

In summary, our results reinforce the idea that an invasion-driven multistep mechanism is important for the minus strand transfer reaction in HIV-1. We provide evidence that the initial invasion of the cDNA by the acceptor RNA can be propagated by either a branch migration or a proximity pathway. The branch migration pathway appears to be most effective for transferring extended cDNA that has assumed a conformation that limits acceptor accessibility. Comparison of the properties of D199 and D520 indicates that RNA folding occurring during cDNA extension on D520 but not D199 promotes efficient acceptor invasion and high accessibility of donor 5’ terminus, allowing efficient transfer.

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