The mechanism of human immunodeficiency virus 1 (HIV-1) minus strand transfer was examined using a genomic RNA sequence-based donor-acceptor template system. The donor RNA, D199, was a 199 nt sequence from the 5' end of the genome to the primer binding site (PBS) and shared 97 nt of homology with the acceptor RNA. To investigate the influence of RNA structure on transfer, a second donor RNA, D520, was generated by extending the 3' end of D199 to include an additional 321 nt of the genome. The position of priming, length of homology with the acceptor, and length of cDNA synthesized were identical with the two donors. Interestingly, at 200% NC coating, donor D520 yielded a transfer efficiency of about 75% compared to about 35% with D199. A large proportion of the D520 promoted transfers occurred after the donor RNA was copied to the end. Analysis of donor RNA cleavage, the acceptor invasion site and R homology requirements indicated that transfers with D520 involved a similar but more efficient acceptor invasion mechanism compared to D199. RNA structure probing by RNase T1 and the RT pause profile during synthesis indicated conformational differences between D199 and D520 in the starting structure, and in dynamic structures formed during synthesis within the R region. Overall observations suggest that regions 3' of the PBS influence the conformation of the R region of D520 to facilitate steps that promote strand transfer.
strand transfer in vivo. Analysis of minus strand transfer using this system led to the proposal of an acceptor invasion initiated transfer mechanism. By this mechanism, synthesis of the –sssDNA is accompanied by RNase H cleavages of the donor template. As pausing of RT promotes local RNase H cleavages (22, 23), when synthesis stalls at the base of TAR, a structured site in the R region, RT pauses and makes more adjacent cuts, generating a gap to which the acceptor template can then invade and anneal to the cDNA. The acceptor-cDNA hybrid propagates by branch migration, displacing the remaining pieces of donor template. Transfer is completed by annealing the DNA primer terminus to the acceptor and continuing synthesis (21, 24).

This system shed light on the minus strand transfer mechanism, however, it also raised a question. In vivo, minus strand transfer occurs efficiently, with no significant accumulation of –sssDNA in the cytoplasm of infected cells (25-27). While in the D199 transfer system in vitro, although the acceptor, RT and NC were in excess over the donor, only about 35% of the –sssDNA transferred. What reduced the strand transfer?

The HIV-1 genomic RNA is about 9.2 kb long and highly structured (28). Its multiple structure motifs are important for different steps of viral replication (29-35, Ref. 36 and references therein). Both the homology and the structure of the R region are important determinants of minus strand transfer (37-41). Using varying lengths of genomic RNA transcribed in vitro, Berkhout and colleagues have shown that structure of the R region is influenced both by NC and by the length and sequence of the flanking regions (42, 43). Presumably, the flanking region could fold back and induce the R region to restructure. Therefore, we wanted to test the possibility that use of longer templates in vitro would produce a better model for RNA structures that promote highly efficient minus strand transfer.

In the present study, we have examined –sssDNA transfer in the context of two different length donor RNA templates, D199 (+1 through +199) and D520 (+1 through +520), derived from the viral genome. Although both templates contained the 5' R and U5 sequences and shared the same homology with the acceptor RNA, the D520 donor yielded twice the amount of transfers (75%) as D199 (35%). We have analyzed steps of the transfer mechanism, and both the initial and dynamic structures of the two donor templates, in an effort to delineate the reasons for the efficient transfers with D520.

**Experimental Procedures**

**Materials** - DNA oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). T7-MEGashortscript high yield transcription kit was purchased from Ambion, Inc. (Austin, TX). Pfu Turbo DNA polymerase was purchased from Stratagene (La Jolla, CA), and the Platinum Tag DNA polymerase was purchased from Invitrogen (Carlsbad, CA). The 32P isotopes were purchased from Perkin-Elmer Life Science. The pNL4-3 molecular clone (from Dr. Malcolm Martin) was obtained through the AIDS Research and Reference Reagent Program (Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health). HIV-1 NCp7 (72 amino acids) was chemically synthesized as described previously (44). HIV-1 reverse transcriptase (p66/p51 heterodimer) was purified as described previously (45, 46). All the other reagents were purchased from Roche Applied Science (Indianapolis, IN).

**Generation of RNA templates** - Genomic sequences from the HIV-1 pNL4-3 strain were amplified by PCR using the Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA) to create double stranded DNA templates for generation of RNA transcripts. The donor RNA template D199 and acceptor RNA templates A97h, A82h and A70h were generated by run-off transcription in vitro as described previously (21). The D520 donor RNA template included the first 520 nt of the genomic RNA (+1 through +520). The double stranded DNA template for D520 RNA was generated using forward PCR primer donorT7+NL4-3+1 (5'-GGATCCCTAAATACGACTCACTATAGGGTCTCTCTGTTAGATCA-3'), and the reverse primer reverse-1D520 (5'-CCCAGTAGTCACTAGGCCTCTCTCTGTTAGATCA-3'). The acceptor RNA template mut-A97h was the same as A97h except that five point mutations were introduced into the 3' R region. The double stranded DNA template for mut-A97h was
generated using forward PCR primer U3-R-mut(+)
(5'-GGATCCTTAATACGACTCATATAGGGCTG
CTTTTGCCCTGTACTGCGCTCTCTCTGGTTGTA
GACGAGATCTGAGGC-3', and the reverse primer
R-mut(-)(5'-TGAGCACTCAAG
GCAAGCTTATTTAGGCTTAAGGCAGTGGC
TTCCCTAGTATTAGCGAGAGCCCC-3').
Positions where nucleotides were changed are
underlined. The T7 promoter is indicated in
italics. All RNA templates used were purified by
denaturing PAGE and resuspended in 10 mM
Tris-HCl (pH 8.0), 1 mM EDTA buffer. RNA
centrization was measured by Ribogreen assay
(Molecular Probes, Eugene, OR) or UV
absorbance (GE Healthcare, Piscataway, NJ).

Transfer Assay – The PBS DNA primer or RNA
templates were radio-labeled at the 5' end as
described previously for either primer extension
assay or donor and acceptor RNA templates
degradation assays, respectively (21, 24). Primer
was heat annealed to donor RNA by incubating at
95°C for 5 min and slowly cooling to room
temperature. Then acceptor RNA was added and
substrates were incubated with 200% NC (100%
NC = 7 nt/NC) at 37°C for 5 min. The ratio of
donor:acceptor:primer was 1:3:1.5. RT was then
pre-incubated with substrates at 37°C for 5 min
before the initiation of reactions by MgCl2 and
dNTPs. The final reactions contained 50 mM
Tris-HCl (pH 8.0), 50 mM KCl, 1 mM
dithiothreitol, 1 mM EDTA, 6 mM MgCl2 and 50
µM dNTPs. Reactions were incubated at 37°C,
and stopped at appropriate times by adding 1
volume of termination dye (10 mM EDTA pH 8.0,
90% formamide (v/v), and 0.1% each of xylene
cyanol, and bromphenol blue). Products were
then resolved by polyacrylamide-urea gels, and
analyzed using a PhosphorImager (GE Healthcare,
Piscataway, NJ) and ImageQuant software
(version 2.1). Sizes of DNA products were
estimated using 10 bp DNA ladders, and sizes of
RNA products were measured using RNA ladders
generated by RNase T1 digestion.

Distribution of Transfer Products – To determine
where the primer terminus switched between the
donor and acceptor templates, transfer products
were isolated and amplified as described
previously (47, 48). PCR amplification by
Platinum Taq DNA polymerase (Invitrogen,
Carlsbad, CA) was performed using the forward
primer 5'-CACGAGCTCTAGGCGCTG
TTTAGCTGTACTGGG-3' and reverse primer
5'-CTAGGATCCGTCCCTGTGGTTTGGGCGCCAC-
3'. Amplified products were cloned by TOPO
TA cloning (Invitrogen, Carlsbad, CA).
Individual colonies were picked and sequenced
using the M13 Reverse (-20) primer from
Integrated DNA Technologies (Coralville, IA).

Structure Probing by RNase T1 – Structure
probing by RNase T1 was performed as described
previously with slight modifications (40). Thirty
fmls of 5' end-labeled D199 or D520 donor
templates were heat-annealed to unlabelled PBS
primer as described earlier. After incubating the
reaction mix with 200% NC at 37°C for 5 min, 1
µg of tRNA was added, and the volume was
brought up to 10 µl. The final reaction contained
50 mM Tris-HCl (pH 8.0), 50 mM KCl, 1 mM
dithiothreitol, and 1 mM EDTA. Digestion
reactions were initiated by adding 0.5 unit of
RNase T1, incubated at room temperature, and
stopped 5 min later by adding one volume of
termination dye. Products were separated on an
8% polyacrylamide-urea gel, and visualized as
described above.

Results
We considered whether the region of the
HIV-1 genome 3' of the PBS could influence the
efficiency of minus strand transfer. To test this
efficiency, we compared transfer efficiency using
templates having or lacking sequences in that
region.

D520 promotes better minus strand transfer than
D199

Compared to donor D199, the newly
designed donor RNA D520 included an additional
321 nt 3' of the PBS (Fig. 1A). It was similar to
D199 in that there was no change in homology
between the donor and acceptor, and the primer
still annealed to the PBS. The acceptor RNA
A97h was composed of the 3' R and 20 nt from
U3. Synthesis produced the same full length
products and transfer products in both reactions.
During the course of transfer reactions (Fig. 1B),
full length products were detected by 1 min with
both templates. Interestingly, minus strand
transfer products, which were not detected until about 4 min with D199, were detectable as early as 2 min with D520. The full length products accumulated to about 8 min, and then decreased as they were chased into transfer products with D520. However, with D199, full length products accumulated with time throughout the transfer reaction. There was a time-dependent increase in transfer efficiency with both D520 and D199 (Fig. 1C). However, the transfer efficiency of D520 reached 75% by 32 min, more than two-fold greater than the 35% with D199. Clearly D520 promotes more efficient transfer than D199.

**The improved transfer with D520 depends on NC**

In contrast to the large differences between D199 and D520 transfer profiles with NC, the formation of full length and transfer products was similar for the two templates without NC (Fig. 2A). Instead of fading out with time, full length products accumulated with D520 in the absence of NC, similar to D199. Moreover, the transfer efficiency was only about 10% for both donor templates after a 32 min reaction (Fig. 2B). Clearly, NC promotes transfer in both systems. Also, significantly, these results demonstrate that the stimulation of minus strand transfer with D520 is dependent on NC.

**Altered donor-acceptor homology results support a similar transfer mechanism for D199 and D520**

Previously we have shown that transfer with D199 was most efficient if the homology with acceptor included all of the R region (21). Three acceptor templates, A97h, A82h, and A70h, which shared a homology of 97, 82, and 70 nt with D199 and D520, were assessed for transfer (Fig. 3A). The acceptors differed at their 3' ends, so the effect of losing portions of the R homology segment would be reflected in their transfer ability.

The above three acceptors were employed in transfer reactions at a 200% NC coating level (Fig. 3B). For the D199 donor, transfer decreased from 35% with A97h to 31% with A82h, and 21% with A70h. Similarly, for donor D520, transfer decreased nearly proportionally from 75% with A97h to 68% with A82h, and 48% with A70h (Fig. 3C). Reducing acceptor homology had the effect of decreasing transfers with both the short and long donor templates. Clearly, both template systems involve a transfer mechanism that is better facilitated with longer homology.

**Donor cleavage in the transfer reaction**

We have proposed that the mechanism for –ssDNA transfer with D199 involves pausing of RT at the base of TAR, which then allows the RT-RNase H to clear a site for acceptor invasion (21, 24). The synthesis profile of the D520 transfer reaction also revealed a prominent RT pause at the same site (142 and 146 nt, Fig. 1B). When D199 was 5' end radio-labeled and analyzed at increasing times during the transfer reaction, we observed substantial RNase H cleavages (57-97nt, Fig. 4) near that pause site. Analysis of the degradation of 5' end radio-labeled D520 also showed similar cleavages in the region as was observed with D199 (Fig. 4). The cleavage at that site was distinctive among those observed throughout the region of R homology. This result is consistent with the interpretation that pausing at the 3' end of the TAR region results in RNA cleavage that allows acceptor invasion by a similar mechanism in both D199 and D520.

**Acceptor mapping indicates the site of acceptor invasion**

We recently developed an approach using a 5' end radio-labeled acceptor to detect acceptor-cDNA interaction during the transfer process (24). Acceptor-cDNA annealing creates a hybrid structure that is sensitive to RT-RNase H cleavage. By sampling the reaction over time, we could assess both the initial site of contact and the propagation of this hybrid. The earliest observed position of cleavage on the acceptor represents the site involved in invasion (Fig. 5A).

Fig. 5B shows the acceptor cleavage profile in transfer reactions with D199 and D520. With D199, the earliest cleavages were detectable by 2 min at a distance 80-110 nt from the 5' end of the acceptor. This was consistent with acceptor invasion in the region of homology behind TAR (21, 24). Subsequent cleavage products, 60-5 nt in size, suggest that the acceptor-cDNA hybrid then propagated from the invasion site to the 5' end of acceptor in a time-dependent manner. With D520, the earliest cleavages were detectable by 1 min at the same positions, 80-110 nt from the 5' end of the acceptor. These data indicate that the initial point of acceptor-cDNA interaction, the
principal invasion site, is the same with both D199 and D520.

Interestingly, the quantity of cleavage products formed at the invasion site in early time points was much higher with D520 than observed with D199. Furthermore, with D520, a higher relative abundance of cleavages at the 5′ end of the acceptor is consistent with a greater proportion of acceptor molecules being involved in successful completion of transfer than with D199. Overall these results support the interpretation that the invasion and hybrid propagation steps of transfer are occurring similarly with D199 and D520, however, with substantially higher efficiency with D520.

Transfer distribution analysis reveals more end transfer with D520

The final step of the invasion mechanism of transfer is the switch of the cDNA primer terminus from the donor to the acceptor template. To identify where transfer completions occur, five base substitution markers were introduced into the R region of the acceptor to generate the mut-A97h acceptor (Fig. 6A). The sequence of the transfer products could then be used to indicate the position of primer terminus transfer. Transfer efficiencies of D199 and D520 with mut-A97h were the same as with the wild-type A97h (data not shown).

Fig. 6B shows the percentage of transferred primers that switched from donor to acceptor in each marker-defined region. The values in Fig. 6B were generated by dividing the number of products showing transfer between two adjacent markers by the total number of transfer products sequenced. With D199, about 40% of primer termini transferred after completing synthesis to the end of the donor template, while it was about 56% with D520. The significant percentage of transfers occurring only after the 5′ end of D520 had been copied is in agreement with the time dependent conversion of full length products into transfer products in the transfer reaction profile of D520 (Fig. 1B). About 30% of the transfers with D199 were completed in the region just before and at the base of TAR (before marker 2), while with D520, that value was only about 13%.

Since segments between two adjacent markers are of different lengths, the distance corrected transfer distribution shows which segment is most recombinogenic (Fig. 6C). The values in Fig. 6C were generated by dividing the raw transfer distribution (Fig. 6B) by the number of nucleotides in each segment and multiplying by 10. The profile suggests that with both D199 and D520, the segment at the base of TAR, between markers 1 and 2, is the segment in which most internal recombination occurs. Interestingly, for D199, that region was twice as recombinogenic as for D520 (Fig. 6C).

As D520 promotes transfers more effectively than D199, to enable a direct comparison of the transfer events in the D199 and D520 systems, we corrected the raw transfer distribution (Fig. 6B) for transfer efficiency. Fig. 6D shows the distribution of the 75% and 35% transfers of D520 and D199, respectively, after multiplying the raw transfer distribution in each segment (Fig. 6B) by the transfer efficiency for each system. Results indicate that 42% of the minus strand DNA synthesized in the transfer reaction underwent end transfer with D520. This was three times the 14% observed with D199. Taken together, these results indicate that a substantial amount of the additional transfers that occur with D520 are completed at the end of the donor template.

Evidence for structural differences in the homology region

Although the 5′ 199 nt of D520 are identical to D199 in sequence, we considered the possibility that D520 assumes a different secondary structure than D199 because of the additional 3′ extension. This structure could influence transfer efficiency. During synthesis, the folded RNA template can assume different dynamic conformations as it is converted to an RNA-DNA hybrid. Such structural differences are reflected in the pause profile during a transfer reaction. Fig. 7A presents a magnified view of part of the same reaction profile shown in Fig. 1B, allowing comparison of pause sites on D199 and D520. Arrows indicate bands produced by RT pausing that are different in the profile of D199 compared to D520. In the TAR region, there was one dominant pause product at position 181, presumably resulting from a hairpin formed in the reaction with D199 (15). No such prominent pause products were observed with D520. In the poly(A) region, there were a series of specific pause products that were unique to either D199 or...
D520. These differences in the RT pause sites suggest that D199 and D520 form different dynamic structures during synthesis in both the TAR and poly(A) regions.

The particular value of the RT pause profile is that it provides structural information during synthesis. We additionally probed the structures of starting templates for both D199 and D520 before initiation of synthesis. We employed RNase T1, an endoribonuclease, which cuts RNA on the 3’ side of single stranded G residues. Fig. 7B shows a comparison of the T1 digestion profiles of D199 and D520 in the presence of 200% NC. Cleavage products, 31, 32 and 33 nt in length within the TAR region, appeared in reactions with both D199 and D520. The pattern suggests that these three Gs are single stranded, although the exact configuration of the local regions could not be determined from this data.

In the poly(A) region, there were a series of cleavage products ranging from 79 to 100 nt in digested D520, while 79 and 83 nt cleavage products were the only dominant cleavages in this region of D199, indicating a significant structural difference from D520. These combined observations further suggest that D520 differs from D199 in the structure of the R region.

Discussion

Our initial model of HIV-1 minus strand transfer in vitro used a 199 nt donor template D199, sufficiently long to generate the native –sssDNA (21, 24). However, we obtained only a maximum transfer efficiency of about 35% with 200% NC. To test whether template folding corresponding to a longer donor RNA influences transfer efficiency, we added native HIV-1 genomic sequence to the 3’ end of D199 to a length of 520 nucleotides. Transfer efficiency with the longer template rose to about 75%. Transfers appeared to employ the same mechanism with both templates, but were carried out more efficiently with the longer template. We present evidence that the folded conformations of the templates differ, and propose that this difference affects transfer efficiency.

Our previous analysis of the D199 template suggested that minus strand transfer in HIV-1 occurs through an acceptor invasion initiated multi-step pathway (21, 24). Acceptor mapping in the D520 system indicated that the initial interaction between cDNA and acceptor occurred within the region of homology behind TAR, similar to D199 (24). The time-dependent acceptor cleavages from this invasion site to the 5’ end of the acceptor suggest that transfers promoted by D520 occur through a similar acceptor invasion and hybrid propagation pathway.

The decrease in transfer efficiency observed in this study with decreasing donor-acceptor homology (Fig. 3B) supports the invasion model and suggests that efficient transfers in both systems are dependent on longer homology.

Levin and colleagues showed that changing the acceptor length also changes its structure and stability (40). Shorter acceptors may promote efficient transfer due to their favorable conformations in spite of the shorter homology.

Predictions by RNAstructure software package (version 4.3) (49) of our acceptor RNAs indicated that the shorter acceptor templates fold into structures with lower stability than the longer A97h. This may explain why there was not a larger drop in transfer efficiency when portions of the proposed invasion region of the acceptor were deleted in acceptor A70h.

In the acceptor invasion-initiated transfer mechanism, the donor template has to be cleaved by RT-RNase H to clear a site for invasion. Since D520 had more efficient acceptor invasion than D199, we predicted more extensive RNase H cleavages within the homology region behind TAR. However, we did not see any significant increase in cleavage of D520 at the invasion site as compared to D199. One possible explanation is that the cleavages on D199 around the invasion site may already be enough to clear a site for acceptor invasion. The reason why D520 can then promote more acceptor invasion may be that the conformations of some transient intermediates formed during synthesis allow for more efficient initial interaction of the acceptor with the cDNA.

The viral protein NC is an important facilitator of efficient minus strand transfer (Ref. 9 and references therein). As expected, efficient transfers with both D199 and D520 were only observed in the presence of NC. The percentage of self-priming product in both template systems also decreased with NC (Fig. 1 and 2). Since NC promotes strand exchange (50, 51) as well as RNase H cleavage (18-20), its actions are
consistent with a transfer mechanism including acceptor invasion and branch migration. The presence of NC is key to observing a difference between the transfer efficiency of the two RNA templates (Fig. 1 and 2).

Both RNase T1 probing (Fig. 7B) as well as RNA structure predictions indicated differences in the initial conformations of D199 and D520 templates in the R region. Analysis using the RNA structure prediction software, RNAstructure (49), indicated that sequences 3' to the PBS region on D520 can fold back to interact with and restructure the TAR and poly(A) regions (Fig. 8). Sequences that form the poly(A) hairpin in D199, interact with sequences 3' to the PBS in D520. Also, several nucleotides at the base of TAR, usually considered part of the TAR stem helix, are in a different configuration in D520. This could explain the difference in pausing at +142/+146 between D199 and D520 (Figs 1 and 7A). As synthesis progresses along the RNA, it is subject to constant and dynamic refolding. Such structural changes can be inferred to some extent from the synthesis pause profiles. It is therefore not surprising that D199 and D520, each of which starts with a different conformation, also show different pause profiles within the R region during minus strand synthesis (Fig. 7A). It is likely that aspects of the D520 dynamic structure favor transfer, although it is not very apparent from the present studies how this might be facilitated.

Berkhout and colleagues have examined conformations assumed by the HIV-1 5'-untranslated region (5'- UTR), using RNAs of different lengths transcribed in vitro (42, 43, 52). A 5' terminal 290 nt segment of the 5' UTR was shown to assume two alternative structures (42, 43). In the branched multiple-hairpin (BMH) structure, the 5' region forms the TAR and poly(A) hairpins, while in the long-distance interaction (LDI) structure, the regions on either side of the PBS interact intimately, forming a long, mostly double stranded structure. The poly(A) and dimerization initiation signal (DIS) hairpins which are distinct in the BMH structure, become part of the long double-stranded region in the LDI structure (42, 43). The BMH conformation is strongly favored by the presence of magnesium and NC protein (42, 52) and is consistent with the expected availability of the DIS to dimerize the genome in the virus (53, 54). Structure probing of the RNA conformation in the virions also indicates a BMH-like structure (55). The structure of our D199 donor (not shown) as predicted from RNAstructure (49) combined with nuclease probing (Fig. 7B) resembles the 5' side of the BMH, while our D520 donor forms a structure similar to the LDI (Fig 8). However, unlike with the 290 nt segment in the Berkhout study (42), NC did not shift the D520 structure from the LDI-like to the BMH-like conformation, as indicated by RNase T1 digestion (Fig. 7B). The NC-promoted transfers in our system very likely occur from a template initially in an LDI-like conformation, but constantly changing in structure as synthesis of the cDNA progresses.

Components of the transfer reaction other than the donor RNA length also influence transfer efficiency. Berkhout et al. reported a high transfer efficiency in a minus strand transfer system with a donor resembling D199, but a different length acceptor RNA, although the conditions were not the same as we employed (39). Also, a high transfer efficiency using a synthetic 128 nt –sssDNA and a 148 nt acceptor RNA was observed with a high concentration of NC (13). These results suggest that a combination of features of the donor and acceptor templates and reaction conditions collaborate to produce the highly efficient transfer observed in vivo.

In summary, our results provide additional evidence for the clearly important effect of template structures on mediating strand transfer. The evidence in the present study combined with information from our recent studies with D199 (21, 24) supports the acceptor invasion mechanism as one possible pathway for –sssDNA transfer. This mechanism is promoted not only by properties of the RT and NC proteins, but apparently by structural features encompassing a large region at the 5' end of the HIV-1 genomic RNA.

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Footnotes

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The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; PBS, primer binding site; NC, nucleocapsid; nt, nucleotide; R, repeat; RT, reverse transcriptase; -sssDNA, minus strand strong stop DNA; BMH, branched multiple-hairpin; LDI, long-distance interaction; UTR, untranslated region; DIS, dimerization initiation signal.

Figure Legends

**Figure 1.** Minus strand transfer reactions using D199 and D520.  
*A*, schematic of the donor and acceptor templates.  Donor D199 contains 5' R, U5 and PBS.  Donor D520 includes these regions and 321 nt of genomic sequences 3' of the PBS.  Acceptor A97h is comprised of 3' R and 20 nt of U3, and shares 97 nt of homology with the donor templates.  An 18 nt DNA primer complementary to the PBS was used as the primer.  Star indicates the position of the $^{32}$P label.  
*B*, representative gel showing minus strand transfer assays of D199 and D520 performed with 200% NC.  Reactions were sampled at 1, 2, 4, 8, 16, 32 and 64 min.  Self-priming product (SP), transfer product (T), full length product (FL), and two pause products formed at the 3' end of the TAR region, 142 and 146 nt in length, are indicated.  
*C*, quantitation of transfer products.  Amount of transfer products formed over time was plotted for the D199 (black square), and D520 (grey diamond) template systems.  Transfer efficiency was calculated as 100% X transfer product/(self-priming product + transfer product +full length product).  Values were averaged from a minimum of three independent experiments.

**Figure 2.** Transfer assays of D199 and D520 performed in the absence of NC.  
*A*, representative gel showing minus strand transfer assays using D199 and D520 template systems in the absence of NC.  Experimental set up was essentially the same as described in Fig. 1, except that NC was excluded.  Reactions were sampled at 1, 2, 4, 8, 16 and 32 min.  Self-priming product (SP), transfer product (T), and full length product (FL) are indicated.  
*C*, quantitation of transfer products.  Amount of transfer products formed over time was plotted for the D199 (black square), and D520 (grey diamond) template systems.  Values were averaged from a minimum of three independent experiments.

**Figure 3.** Effect of template homology on minus strand transfer.  
*A*, schematic of the donor and acceptor templates used.  The three acceptor RNA templates A70h, A82h and A97h share 70, 82 and 97 nt of homology with each of the donor templates.  They have the same 5' end but differ at the 3' end.  Numbers at the ends of the templates correspond to nucleotide position within the HIV-1 genomic RNA.  
*B*, representative gel showing transfer reactions with each of the acceptors.  Transfer reactions were performed in the presence of acceptors A97h, A82h or A70h with both D199
and D520 at 200% NC coating for 32 min. Self-priming product (SP), transfer product (T), and full length product (FL) are indicated. Lane L, 10 bp DNA ladder. C, quantitation of transfer products with each acceptor. Transfer efficiency with each of the three acceptors was plotted for D199 (white) and D520 (grey). Values were averaged from a minimum of three independent experiments.

**Figure 4. Analysis of donor RNA degradation during minus strand transfer.** RT catalyzed transfer reactions were performed using either D199 or D520 with acceptor A97h, at 200% NC coating. The donor RNAs were 5' end radio-labeled to examine their degradation during the course of the reaction. Reactions were terminated at 0.5, 1, 2, 4, 8, 16 and 32 min. Lane C, control reactions without RT. Labels to the right of the gel mark the positions of donor 5' end cleavages, the 3' end of TAR (57 nt), the 3' end of homology (97 nt), and the starting material D199 (199 nt) and D520 (520 nt).

**Figure 5. Analysis of acceptor RNA cleavage during minus strand transfer.** A, schematic of experimental design for analysis of acceptor cleavage in the transfer reaction. Strand transfer reactions with D199 or D520 were performed with 200% NC coating. The acceptor template, A97h, was 5' end radio-labeled to keep track of its interaction with cDNA during the transfer reaction. B, representative gel showing acceptor cleavage products resulting from RT-RNase H. Reactions were sampled at 1, 2, 4, 8, 16 and 32 min. Schematic of the acceptor is shown alongside the gel. The position of intense (black arrows), and moderate cleavages (grey arrows) are indicated. Lane C, control reactions without RT. Lane L, 10 bp DNA ladder.

**Figure 6. Transfer distribution analysis.** A, schematic of the mutant acceptor RNA template mut-A97h. Single nucleotide substitutions were introduced into A97h at 5 positions (underlined), indicated as markers #1 to #5, to generate acceptor mut-A97h. Transfer reactions were performed under standard reaction conditions as described in Fig. 1B, with the mutant acceptor being substituted for wild type A97h. B, distribution of transfers with the D199 (white) and D520 (grey) template systems. Transfer distribution was obtained by plotting the percentage of clones that transferred in each marker defined segment of the template. This yields % transfer/segment. C, transfer distribution corrected for marker distance. Normalized distribution was obtained by dividing % transfer within a segment (B) by the number of nucleotides in that segment and multiplying it by 10. This yields % transfer/10 nucleotides. NA, not available: end transfer does not occur over a segment, so it was not possible to correct it for marker distance. D, transfer distribution normalized for transfer efficiency. Normalized distribution was obtained by multiplying % transfer within a segment (B) by the transfer efficiency, which was taken as 75% for D520, and 35% for D99. This indicates the absolute number of fully extended primers that transferred within any given segment.

**Figure 7. Secondary structure analysis of D199 and D520.** A, RT pause profile on D199 and D520 donor templates. Transfer reaction profiles are the same as shown in Fig. 1B with only the R region highlighted. Arrows indicate positions of RT pausing that are unique to D199 and D520 templates. Self-priming product (SP), transfer product (T) and full length product (FL) are indicated. B, donor secondary structural probing by RNase T1. 5' end radio-labeled D199 and D520 were either undigested (-) or digested by RNase T1 (+). Cleavage products of interest are labeled with arrows. Lane L, 10 bp DNA ladder.
Figure 8. Secondary structure model of donor D520. Structure prediction of D520 was performed using the RNAstructure software package (49), version 4.3, with default parameters. The secondary structure of D520 shown here was one of the potential structures generated, which is consistent with the RNase T1 analysis. The presented figure was prepared using the XRNA software package (http://rna.ucsc.edu/rnacenter/xrna/xrna.html). This structure predicts base pairing of the region 3' of the primer binding site to the R region.
Figure 1

A.

B.

C.

Transfer Efficiency (%) vs Time (min)

D199 and D520
Figure 2

A.

B.
Figure 3

A.

Donor RNA

Primer

PBS +199 +520

Accepter RNAs

A70h
A82h
A97h

B.

[Image: Gel electrophoresis with gels labeled D199 and D520, with bands at 220 and 200, and lanes labeled A97h, A82h, A70h.]

C.

Transfer Efficiency (%)

|         | A97h | A82h | A70h |
|---------|------|------|------|
| D199    | 90   | 80   | 70   |
| D520    | 85   | 75   | 65   |

[Graph showing transfer efficiency with bars for D520 and D199.]
Figure 4

Donor cleavages

A97h

Primer

PBS +199 +520

5' *+1 R +97 Primer

5' 199 520

5'Donor 5' end cleavages

D199 D520

Time (min)

0.5 1 2 4 8 16 32

0.5 1 2 4 8 16 32

C C C C C C

- - - - - -

* C C C C C C

- - - - - -

C C C C C C

- - - - - -

C C C C C C

- - - - - -

C C C C C C

- - - - - -

C C C C C C

- - - - - -

C C C C C C

- - - - - -

C C C C C C

- - - - - -
Figure 5

A. Detection of cDNA-Acceptor Interaction

Sample Reactions at Different Time Points

Cleavage by RT-RNase H

B. D199 and D520

A97h

PBS +199

RT + dNTPs

Donor

Primer

5'-12 14 - 20 20 - 35 35 - 60 80 - 110

Time (min)

5'-1 2 4 8 16 32

5'-14 20 35

5'-12 14 20 35

80 - 110

35 - 60

17
Figure 6

A.

B.

C.

D.
Figure 7

A.

B.
