Recombination promotes retrovirus evolution. It involves transferring a growing DNA primer from one genomic RNA template in the virus to the other. Strand transfer results in vitro suggested that pausing of the reverse transcriptase during synthesis allows enhanced RNase H cleavage of the initial, or donor, RNA template that facilitates primer interaction with the acceptor template. Hairpins are common structures in retrovirus RNAs that induce pausing. Analyzing primer transfers in hairpins by base substitution markers showed transfer sites well beyond the site of pausing. We developed methods to distinguish the initial site of primer-acceptor template interaction from the site of primer terminus transfer. The strand transfer mechanism was confirmed to involve two steps. In the first, the acceptor template invades the primer-donor complex. However, the primer terminus continues elongation on the donor RNA. The interacting primer and acceptor strands then propagate by branch migration to catch the advancing primer terminus. Some distance downstream of the invasion site the primer terminus transfers, marking the genetic shift from donor to acceptor. Nucleocapsid protein (NC) is known to influence primer elongation and strand exchange. The presence of NC increased the efficiency of transfers but did not appear to alter the fundamental transfer mechanism.

Recombination is an essential cellular process that is catalyzed by enzymes that are encoded and regulated by the cell. In addition to providing a basis for genetic variation, recombination is perhaps more important for repairing chemical and radiation damage to DNA, for altering the level of expression of certain genes, and for creating new protein coding regions (1). In HIV and other retroviruses, recombination is dependent on the packaging of heterogeneous RNAs within a single virion, and it takes place during viral genome replication (7). During replication, reverse transcriptase (RT) converts the single-stranded RNA genome into double-stranded DNA. To carry out this process RT uses its polymerase as well as its RNase H catalytic activities, both of which reside within the same polypeptide chain (8). Recombination occurs when RT transfers an elongating primer from one position to another within the same template or to another co-packaged RNA, a process known as strand transfer or template switching. Although two obligatory strand transfers take place from terminal sequences to complete genomic replication (2, 9), template switching has also been shown in vivo to take place from internal regions (7, 10–12). Retroviral recombination events are mostly homology-driven; however, non-homologous transfers have also been shown to take place 1/100 to 1/1000 times less commonly (13). Although recombination has been proposed to occur both during minus and plus strand DNA synthesis (14, 15), most transfers occur during minus strand DNA synthesis (16–20). It has been suggested that RT makes a “forced copy choice” transfer when it encounters a break in the RNA template, thereby allowing completion of minus strand synthesis (14). This model was later modified to include transfers that take place in the absence of breaks in the RNA, by means of a “copy choice” (21). Recombination during plus strand DNA synthesis is described by the “strand displacement-assimilation” mechanism (15, 22). By this process information exchange would take place as a displaced cDNA fragment anneals and becomes incorporated into the co-packaged molecule.

In vitro systems designed to study recombination using purified nucleic acids and enzymes indicated that RNase H cleavages are important for transfers from RNA substrates (23, 24). Subsequent work suggested that pausing of RT during primer extension on the donor template promoted strand transfer (25, 26). Positions of transfer were found to correlate with sequences and structures that caused pausing (25–27). It was proposed that stalling of RT at the pause site would allow the RNase H to make a group of adjacent cuts in the template. This donor template degradation would then facilitate interaction of the primer with the acceptor template behind the RT leading to the initiation of the transfer process. In vivo work using the
deletion of direct repeat sequences to model template switching corroborate these early insights (28–30). The process of RT stalling and degradation of the template were found to be dynamic processes, in which an appropriate balance between the two catalytic activities lead to productive transfers (30). Also, the presence of secondary structures between the repeat sequences appeared to increase the efficiency of the process (29).

Despite recent progress toward an understanding of the many factors that lead to a template switching event, the relationship between the location of pause sites induced by secondary structures and the point of genetic shift, where the primer transfers from donor to acceptor, remains unclear. The highly structured HIV-1 TAR hairpin causes RT to stall as synthesis reaches the base of the hairpin (31). Template switching on such a template yielded the paradoxical result that more transfers took place in the loop region than at the pause site at the base of the hairpin. A model where interactions between complementary regions of the hairpins in the donor and acceptor would determine the location of transfers was proposed. In a related manner, stimulation of strand transfers in vitro by the dimerization initiation sequence hairpin, which promotes template-template interaction, was also consistent with a role for hairpin interactions in promoting transfers (32).

We set out to extend and clarify previous results by examining a template switching in the context of a stable hairpin containing template. We also explored the role of nucleoplasmin protein (NC) on the mechanism of transfer. NC functions as an RNA chaperone by promoting the formation of the more thermodynamically stable conformations of nucleic acids (33–35). NC also reduces the amount of nonspecific priming during reverse transcription (36–38) as well as enhancing minus and plus strand transfers (37, 39, 40). NC has been found to influence primer elongation efficiency and processivity (41–43). Also, NC has been reported to affect the location of transfers without altering the pausing profile during reverse transcription, an observation primarily attributed to its effects on RNA secondary structure (44).

We report evidence that during transfers from hairpin structures the point of invasion is separate from the point of primer terminus transfer. Our results are consistent with a model in which template switching proceeds through a two-step mechanism. We report evidence that during transfers from hairpin structures the point of invasion is separate from the point of primer terminus transfer. Transfers are initiated through cDNA-acceptor interactions well behind the primer terminus, followed by primer terminus transfer, which completes the process. NC protein, although it increased the efficiency of the transfer process, did not cause a redistribution of the location of transfers.

**EXPERIMENTAL PROCEDURES**

**Materials**—Recombinant HIV-1 RT (p66-p51 heterodimer, 40,000 units/mg) was provided by the Genetics Institute (Cambridge, MA). Chemically synthesized nucleoplasmin protein (1–72) NCP7 was generously provided by Dr. Bernard P. Roques (45). NC was stored at –72°C. A 2:1 volume of gel loading buffer (90% formamide, 10 mM sodium phosphate buffer, pH 8.0, 0.5 mM EDTA, 10 mM β-mercaptoethanol) was added to each well and heated to 95 °C for 1 min. The samples were then cooled to room temperature.

**Strand Transfer Assays**—In vitro strand transfer reactions were performed with T7 RNA polymerase, as per the manufacturer’s protocol. The pEIAV-Donor was linearized with NotI and BsaNI to generate DI and DL donor templates, respectively, whereas the pEIAV-A2 was linearized with NotI, HindIII, and HaeIII to generate the AL, AI-2, and AS acceptor templates, respectively. pEIAV-A1 was linearized with HindIII to generate the AI-2 acceptor. RNA substrates were gel-purified and integrity-tested.

**Labeling and Annealing of Strands**—DNA primers or RNA templates (RNA templates first calf intestine phosphatase-treated) were labeled at the 5'-end using T4 polynucleotide kinase and [γ-32P]ATP (3000 Ci/mmol). Unincorporated nucleotides were separated using 30 Micro Bio-Spin columns. 25 fmol of donor RNA (either labeled or unlabeled) and 50 fmol of primer (either labeled or unlabeled) were brought to a final volume of 10 μl in 50 mM Tris-HCl, pH 8.0, 80 mM KCl, 1 mM dithiothreitol, and 1 mM EDTA, heated for 2 min at 95 °C, and slow cooled to room temperature.

**RT Assays**—Donor DI was primed for reverse transcription with the DNA oligonucleotide SP6 (5'-TAGATATTTAGGGTAGCAGCAGTATAG) and donor DL with rr11 (5'-ACCATTTAGGATGTCGCATGACGACGCGGT). 10 fmol of each primer (50 fmol in total) was added to a final volume of 12 μl. Two units of HIV-1 reverse transcriptase (50 ng) were incubated for 5 min at 37 °C with 25/50 fmol of donor/primer of hybridized substrate. Reactions were started by adding dNTPs and MgCl₂ to a final concentration of 6 mM and 50 μM, respectively. RNA H assays were performed with 5'-end donor RNA and unlabeled primer, whereas extension reactions were performed with labeled primer. Reactions were incubated at 37 °C and stopped at the appropriate time by adding 1 volume of gel loading buffer (90% formamide, 10 mM EDTA, pH 8.0, and 0.1% each of xylene cyanole and bromphenol blue). Strand transfer reactions were carried out under the same conditions as extension assays, except that acceptor was included in the annealing reactions. For blocking oligonucleotide EIAV-3 (5'-CAGCAGTATACCTGGAACCTTAC) containing a 3' phosphate modification was added to the reaction mixture at the same time as the start mix. Reaction products were resolved on 8% polyacrylamide-urea gels, visualized using a FluorChem and ImageQuant software (Amer sham Biosciences).

**NC Assays**—NC was added to the reactions prior to the addition of RT and incubated at room temperature for 5 min. A 100% NC coating was calculated by assuming that each NC molecule would cover seven nucleotides and that all nucleic acids would be completely covered.
Titrations of NC as well as cloning and sequencing of transfer products were carried out using a 1:1 donor to acceptor ratio.

**Analysis of Transfer Products—**To determine the transfer distribution, the transfer products were isolated, amplified, cloned, and sequenced as described previously (31). Primers JK32b/EcoRI (5'-GACT-GGAATTCGCTAATATTGCTTGACGACTAG-3') and JK33XbaI (5'-TGGCGGCCGCTCTAGAACTAGGGATAAATATAA-3') were used in the PCR amplification. Amplified fragments were doubly digested, purified, and cloned into the EcoRI/XbaI sites of pBluescript II SK(+)<sup>+</sup>. Individual colonies were picked and sequenced by automated sequencing using the M13 Reverse (−27) primer from Integrated DNA Technologies. Each clone represented an individual transfer event, and its sequence was used to determine the point of crossover between donor and acceptor.

**RESULTS**

Previous analyses of strand transfer from HIV-1 TAR RNA templates in vitro demonstrated that, although the helix region of hairpins induced strong pausing, most transfers occurred 20–80 nucleotides beyond the observed pause site (31). In this study we set out to dissect the transfer reaction on a different hairpin containing RNA template and specifically determine the basis for the observed distribution of internal transfers. We employed a recombination assay similar to that of Kim et al. (31) in which synthesis is initiated at a primer annealed upstream of the hairpin on the donor template. The primer could transfer to a longer acceptor template, which was homologous to the donor template in the region of the hairpin. Periodic base substitutions on the acceptor were used as markers to determine the points of transfer.

**HIV-1 RT Strongly Pauses and Cleaves at the Base of the EIAV PBS Hairpin—**To extend our studies to other substrates that contain stable secondary structures, a sequence from the Equine Infectious Anemia Virus (EIAV) genome was chosen based on its ability to form a stable hairpin structure. The viral sequence extended from nucleotide position +278 to +549 (46) and includes the primer binding site (PBS). The secondary structure for this region, shown in Fig. 1A, was predicted using mfold (47, 48) in combination with RNase T1 probing (data not shown). The hairpin sequences of all donor and acceptor molecules shown in Fig. 1B were predicted to fold in a similar manner. Primer extensions were carried out on the donor DI, in the absence of acceptor, to examine full-length (FL) and pause products (Fig. 2A). Full-length extension to the end of the donor gave rise to a 136-nucleotide-long product (Fig. 2A, lanes 2–6). In the presence of the acceptor template, a 149-nucleotide transfer product (TP) was also detected (Fig. 2A, lane 7). Because the full-length cDNA contains complementary hairpin stem sequences that can fold back on itself, it can self-prime to produce a 194-nucleotide long fold back (FB) product. Extension products were numbered starting with +1 as the nucleotide complementary to the 5′ nucleotide of the donor and increasing in the direction of the primer. In addition to these major extension products, RT paused briefly at other positions as evidenced by primers partially extended to those positions at the time of sampling (Fig. 2A). Two strong pause sites were seen corresponding to the cytosine residue at position +78 and the guanine at +79. The location of these two distinct pause products is consistent with slowing of synthesis caused by the need to strand displace the hairpin stem, an effect also seen by others (41, 49–51). Minor pause products were also seen at positions +57, +44, and +16. RNA folding predictions suggest that these pauses are at the bases of new hairpins that are formed transiently as the RNA refolds when RT opens up the original structure.

Using a 5′-end-labeled DI substrate and unlabeled primer, we determined the relationship between pausing and RNase H cleavage during reverse transcription (Fig. 2B). Two major sets of cleavage products were seen. The first set appeared as early as 1 min and was produced by cuts at positions +98, +94, and +91. The locations of these cuts are consistent with a concentration of RNase H-directed cleavages by RTs stalled with their polymerase active sites at +78 and +79. These products were eventually cleaved to smaller fragments, consistent with the progression of synthesis and periodic RNase H cuts. Later in the reaction, products appear from cleavages at positions +18, +13, and +11 and progressively increase in intensity (Fig. 2B, lanes 5–7). These cuts are characteristic of those observed when the RT reaches the 5′-end of a RNA template (24). Although numerous other minor bands appear, the transient appearance of the pause-related products and the accrual of terminal products are the main features of the reaction. A strong pause site at the base of the hairpin and concomitant RNase H cleavages were also observed with the HIV-1 TAR hairpin (31), consistent with the general ability of stable hairpins to promote pause-specific RNase H cleavages.

**Internal Transfers from the EIAV-derived Hairpin Show a Bimodal Distribution—**Transfer efficiency is a measure of how frequently switching is occurring between two templates. It is defined as the ratio of transfer product to the total amount of full-length products of reverse transcription, including fold-back product. An initial characterization of the transfer efficiency between DI and acceptor AI-1 yielded a value of 53% (Fig. 3A). To analyze the locations of template switching, the transfer products were gel-purified, PCR-amplified, and cloned. Individual colonies were sequenced and were assumed each to represent a single transfer event. Close to 70% of transfers took place after marker 7, and probably after RT synthesized to the end of the donor (Fig. 2C, black bars). The remaining 30% of the transfers were internal; 25% occurred within the loop (between markers 4 and 6) and 4% at the base of the hairpin. These results demonstrated that transfer within the EIAV-derived hairpin templates behaved in a similar manner to those observed in HIV-1 TAR, where 70% of transfers occurred from the end of the template (31). The distance between markers is not equal throughout the template. Therefore, we found it informative to normalize our distribution to the number of nucleotides between markers. To do so, we divided the percentage of transfers in each segment by the number of nucleotides within that segment and then multiplied that value by 10. The normalized data show that the loop of the hairpin supported more transfers on a per nucleotide than the base (white bars in Fig. 2, C and D).

To clearly distinguish end transfers from internal ones, the AI-2 acceptor was prepared (Fig. 1B). Acceptor AI-2 did not contain marker 1a but had the two additional markers 2 and 8. They comprised the last nucleotide that is reverse-transcribed on the donor and its complementary nucleotide at the base of the hairpin (Fig. 1A). Thus, exclusion of this marker on any transfer product implies that template switching occurred after synthesis to the end of the donor. A maximum transfer efficiency of 20.4% was measured with the combination of AI-2 and DI (Fig. 3A). This was less than half the value obtained with AI-1. We reasoned that the presence of marker 8 was interfering with the end transfers by creating a situation where the transferred primer when annealed to the acceptor presented RT with a mismatched primer terminus. Sequence analysis of the transfer products on AI-2 supported this conclusion, where only 8% of transfers had occurred from the end (Fig. 2D, black bars). More importantly, the preferred sites of internal transfers remained similar to those of AI-1. Most transfers took place in the loop of the hairpin (43% from markers 4 through 6) and the base of the hairpin (20% from markers 1 through 3). Therefore, the marker allocation in acceptor AI-2 remained
suitable for addressing the distribution and mechanisms of internal strand transfers.

The normalized distribution showed marker segments 7–8 and 1–2 as preferred sites of transfer (Fig. 2D, white bars). This indicates that the large number of transfers at the loop of the hairpin is partly due to the distance between the markers. However, even after normalizing for length, segment 4–5 was still 75% as effective as segment 1–2 at the base of the hairpin in promoting transfers. Thus, although the strong pause site at the base of the hairpin promoted a fraction of the transfers, the
FIG. 2. Synthesis-related pausing RNase H cleavages on donor DI and transfer distributions by HIV-1 RT. A, 5’-end-labeled SP6 primer was annealed to DI donor and synthesis was started in the absence (lanes 1–6) or presence of acceptor AI (lane A). Full-length synthesis on the donor resulted in a 136-nucleotide product (FL), whereas strand transfer in the presence of acceptor yielded a 149-nucleotide product (TP). FB represents the cDNA fold back products. Reactions were stopped at the indicated times and products were resolved on an 8% polyacrylamide gel under denaturing conditions. Positions of the pause sites are indicated. Lane L, 10-base DNA ladder. B, RNase H cleavage of donor during synthesis was followed using 5’-end-labeled donor DI and unlabeled SP6 primer. Reaction times are indicated above each lane. The numbers indicate positions of major cleavage sites and correspond to the nucleotide positions on the donor. Lane C is a control reaction in absence of RT. Lane R, 0.5 unit of Escherichia coli RNase H was added after 30 min. Lane T, RNase T1 digestion of the 5’-end-labeled donor template. Transfer products were sequenced to determine the distribution of primer terminus transfers using the donor DI and acceptor (C) AI-1 and (D) AI-2. The transfer distribution (black bars) was obtained by counting the number of clones transferring between two specific markers and dividing it by the total number of clones sequenced times 100. This yields percent transfer/segment and the standard deviation (S.D.) as indicated. The normalized distribution is obtained by dividing %transfer/segment by the number of nucleotides in the segment and multiplying it by 10 yielding %transfer/segment/10 nucleotides. NA, not available: because end transfers do not take place over a segment, it was not possible to normalize such transfers over a distance.
majority occurred after the pause site and primarily in the loop. Overall, the distribution of transfers presented a bimodal distribution, which could only be partly explained by the uneven length of segments.

**Effects of Increased Homology between Donor and Acceptor in the Pause Region**—The lower than expected frequency of internal transfers near the base of the hairpin lead us to re-examine additional substrate features that could affect template switching at that location. We reasoned that short homology length prior to the pause site might be insufficient for primer-acceptor interaction to facilitate the transfer. If that were the case, then increasing homology in this region should lead to: (i) a shift to a greater probability of transfers at the pause site, and (ii) a higher transfer efficiency due to an increased opportunity for acceptor invasion.

To test this, the long donor DL and long acceptor AL templates were prepared (Fig. 1B) in which the homology in the section before the hairpin was increased from 16 to 61 nucleotides while maintaining the structure of the hairpin (as predicted by mfold). Both primer extension assays (Fig. 4A) and RNase H assays (Fig. 4B) showed only minor differences between the longer and shorter donor. These results suggested that the effects of the increased homology could be measured without the complications of changes in the hairpin structure or pause pattern.

Contrary to our expectations, the DL-AL template pair produced a transfer efficiency of 17.7%, slightly lower than the 20.4% supported by the initial substrates DI-AI-2 (Fig. 3B). To assess whether the lower transfer efficiency was a feature of the new long donor or acceptor we measured the transfer efficiency between the long donor and initial acceptor. The DL-AI-2 template pair gave a value of 25.8% (Fig. 3B). This suggested that a feature of the acceptor caused the reduction in template switching. Careful consideration of the most thermodynamically stable structure predicted by mfold for the long acceptor indicated that, although the hairpin sequences were folding in the same manner for all acceptors, the added sequences at the 3′-end of the AL acceptor formed a secondary hairpin with a calculated stability of 12.1 kcal/mol. In contrast, the 3′-region of the original AI-2 acceptor lacked the appropriate sequences for stable structures. We reasoned that this new motif 3′ to the hairpin was blocking the transfer process in the longer acceptor.

Analysis of the transfer products generated from the long template pair showed that a bimodal distribution was maintained despite the lower transfer efficiency (Fig. 4C). Of note, the number of transfers taking place closer to the 5′-region of the hairpin dropped significantly, showing a shift of transfers toward the early portions of the hairpin. The distance-corrected data indicated that the large number of transfers before marker 1 was mostly due to the longer distance between markers and that the peaks of transfers before the pause site (markers 1–2) and at the top of the hairpin (4–5) were not affected. The restoration of transfer efficiency of the longer donor by the initial acceptor (Fig. 3B) was also accompanied by a distribution in transfers that was very similar to that obtained with the initial templates (compare Figs. 4D and 2D). Thus, the increased homology within the longer donor did not substantially affect the distribution of products. However, the effect of this region on the transfer efficiency suggested to us that it plays a central role in facilitating the transfers, not just at the pause site, but throughout the template.

**Sequences within the Hairpin Are Not Sufficient to Induce Efficient Transfers**—The previous set of data suggested that the region before the hairpin was important for transfers. Therefore, eliminating the homology at the base of the hairpin should strongly suppress transfers. To test this hypothesis we constructed a short acceptor (AS) template that lacked the entire region of homology 3′ of the hairpin (Fig. 1B). The 3′-end of this template terminated two nucleotides from the pause site and into the hairpin. The hairpin structure was predicted to remain unchanged by mfold. Measurements of the transfer efficiency showed that, compared with the AI-2 acceptor, transfer efficiency with AS dropped ~10-fold when either donor DI or DL were used (Fig. 3, A and B). These results lead to the conclusion that most of the transfers in this system require, and presumably are initiated, in the region at the base of the hairpin. Only 10% of the transfers appear to be initiated within the hairpin. An analysis of the distribution of transfers (Fig. 5, A and B) indicated the ascending part of the stem (markers 3–4) was not able to support any transfers with either donor. The normalized data for segment 7–8 in both templates (Fig. 5, A and B) was inconclusive, because the short length of this segment (five nucleotides) tend to cause large standard deviations.

**Nucleocapsid Protein Increases Transfer Efficiency**—The viral NC has important nucleic acid chaperone function (34) and has been implicated to play an important role in transfers (37, 39, 40). Therefore, we decided to test whether NC influenced or altered the transfer mechanism. During extensions in the absence of acceptor (Fig. 6A, lane 1–4), NC increased the number of primers extended to the end of the RNA donors. The ratio of
fully extended primers to self-priming products was also increased. Quantitation of band intensities indicated that the ratio of aborted synthesis products at positions +78 and +79 to full-length products was slightly increased (data not shown). Furthermore, NC did not decrease the amount of pausing seen at the base of the hairpin, or at other sites throughout the template.

A sample titration using a 1:1 donor-to-acceptor ratio with AI-2 is shown in Fig. 6A (lanes 5–8). The efficiency of the transfer reaction was increased in the presence of NC for all acceptors tested (Fig. 6A and Table I). Table I shows the effect of NC on transfer efficiencies measured with the intermediate acceptors AI-1 and AI-2 as well as the short acceptor AS. Even though the short acceptor experienced the highest increase in transfers, 8-fold versus 3- to 4-fold for the AI templates, this was not enough to bring its transfer efficiency within the range of the other acceptors.

In the absence of homology in the strong pause site region, any increase in the absolute number of transfers experienced by the short acceptor would be promoted by an “unfolding” of the secondary structure of the hairpin, which would make its sequences more available for transfers. However, the transfer efficiencies indicate that the presence of a strong pause site over a region of homology substantially increases the

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**Fig. 4.** Synthesis-related pausing and RNase H cleavages on donor DL and transfer distributions by HIV-1 RT. The figure design is the same as in Fig. 2. A, 5′-labeled rr11 primer was annealed to DL and synthesis was started in the absence (lanes 1–5) or presence of acceptor AI (lane A). Full-length synthesis resulted in the production of a 192-nucleotide product, and strand transfer yielded a 208-nucleotide product. Reactions were stopped at the indicated time points, and products were resolved on an 8% polyacrylamide gel under denaturing conditions. Lane L, 10-base DNA ladder. B, RNase H cleavage of donor during synthesis was followed using 5′-labeled donor DL and unlabeled rr11 primer. Lane R, 0.5 unit of E. coli RNase H were added after 30 min. Lane T, RNase T1 digestion of the 5′-end-labeled donor template. Transfer products were sequenced to determine the distribution of primer terminus transfers using the donor DL and acceptor (C) AL and (D) AI-2. The transfer distribution (black bars) was obtained by counting the number of clones transferring between two specific markers and dividing it by the total number of clones sequenced times 100. This yields %transfer/segment and the standard deviation (S.D.) as indicated. The normalized distribution is obtained by dividing %transfer/segment by the number of nucleotides in the segment and multiplying it by 10 yielding %transfer/segment/10 nucleotides. NA, not available: because end transfers do not take place over a segment, it was not possible to normalize such transfers over a distance.
probability of starting a transfer event. Thus, even in the presence of NC, when a strong pause site is available, as with acceptor AI-2, 50% more transfers occur than when it is not, as in AS.

Because NC has been previously reported to alter the location of primer transfer, even when no change in the pausing pattern is observed (44), we decided to examine the transfer profile between the initial donor and AI-2 in the presence of 200% NC. The results from three independent experiments are presented in Fig. 6C, whereas the same results corrected for the distance between markers is shown in Fig. 6D. A comparison with the profile obtained in the absence of NC (Fig. 2D) shows no major change of the preferred positions of transfer.

Initiation of Transfers Can Be Blocked by a Short Oligomer—If the region before the hairpin is important for initiating transfers, then blocking DNA primer-acceptor interactions in this section should produce an effect similar to that of using a short acceptor. To accomplish this, a 21-nucleotide DNA oligomer identical in sequence to the donor from position 78, 79, and 80 was used. The oligomer was phosphorylated at its 5’-end so that it could not prime synthesis. The region between markers 4 and 6 was still the most common location for transfers. The presence of NC did not lead to an increase in transfers at the base of the hairpin. In fact a slight decrease was evident in two of the experiments (sets B and C). Finally, although data set A showed an increase in the number of end transfers, this was not reproducible over the next two experiments. Thus, NC increased the overall transfer efficiency without altering the dynamics of the extension and transfer reaction and without substantially altering the distribution of transfers.

Fig. 5. Transfer distributions from donor DL and DI to Short Acceptor AS by HIV-1 RT. Transfer products were sequenced to determine the distribution of primer terminus transfers using (A) the donor DL and (B) donor DI to short acceptor AS. The transfer distribution (black bars) was obtained by counting the number of clones transferring between two specific markers and dividing it by the total number of clones sequenced times 100. This yields %transfer/segment and the standard deviation (S.D.) as indicated. The normalized distribution is obtained by dividing %transfer/segment by the number of nucleotides in the segment and multiplying it by 10 yielding %transfer/segment/10 nucleotides. NA, not available: because end transfers do not take place over a segment, it was not possible to normalize such transfers over a distance.

DISCUSSION

Although a number of reports present evidence that pausing during reverse transcription drives strand transfer (25–27), a paradoxical outcome was observed when scoring the location of transfers from RNA structures. An in vitro analysis of transfers on an HIV-1 TAR hairpin template showed that most transfers occurred in the loop region or beyond, despite RT pausing predominantly as it transcribes through the base of the structure (31). Based on this distribution of template switching it was proposed that transfers within large hairpins, such as the TAR, were facilitated though complementary sequences in the donor
and acceptor template hairpins that interacted to bring the templates together in a way that promoted the transfer. The current work sets out to clarify the seemingly contradictory result that pause sites do not correlate with the location of transfers by extending the analysis to a different hairpin. Our data provide direct evidence supporting a two-step transfer mechanism in which the steps of primer terminus transfer and a pause-triggered invasion are separated in time and space.

We examined template switching using a section from the EIAV genome containing a hairpin structure. Extension reactions on the new substrate (Fig. 2A) demonstrated that RT paused strongly in the region at the base of the hairpin. Assays designed to follow RNase H cleavages on the EIAV-derived substrate (Fig. 2B) indicated that a large number of cuts occurred as a result of RT stalling at the base of the hairpin. It was important to know the location of these cuts, because

**Fig. 6. Effects of NC on strand transfer.** A, donor extensions were carried as described in the Fig. 2 legend on donor DI in the absence of acceptor. Lanes 1–4 contain 0, 125, 250, and 500% NC coating. Lanes 5–8 all contain 25 fmol of acceptor Al-2 and 0, 125, 250, and 500% NC coating, respectively. B, lanes 1–8 contain 25 fmol of donor DI and 125 fmol of acceptor Al-2. Lanes 1–4 have no NC, while lanes 5–8 contain 250% coating NC. Lanes 1 and 5 have no blocking oligomer while lanes 2 and 6, 3 and 7, and 4 and 8 contain 125, 500, and 2000 fmol of blocking oligomer, respectively. C, transfer reactions were carried out in the presence of 200% NC coating and cloned and sequenced. The transfer distributions for three independent experiments (sets A, B, and C) were obtained. The %transfer/segment was calculated by counting the number of clones transferring between two specific markers and dividing it by the total number of clones sequenced times 100. D, normalized distribution for all three sets shown as %transfer/segment/10 nucleotides was obtained by dividing %transfer/segment by the number of nucleotides in the segment and multiplying it by 10. NA, not available; because end transfers do not take place over a segment, it was not possible to normalize such transfers over a distance.
models predicting transfers at pause sites suggest that concomitant with pausing there should be increased RNase H cleavages (25–27). The degradation of the donor then creates a region over which the acceptor and primer can interact. In addition, results obtained in vivo indicate that RNase H cuts made by a polymerizing RT create a higher affinity substrate for other RTs to bind in a non-polymerizing mode and further degrade RNA bound to DNA (29). This increases the exposure of the primer to the acceptor. Similarly, the deletion of repeated sequences, a process involving intramolecular template switching, is also dependent on RNase H activity (28, 29). Taken together, the results presented in Fig. 2 (A and B) suggested that the region of sequence prior to the base of the hairpin should be a preferred site for transfers. However, as with the HIV-1 TAR hairpin, most transfers (>70%) to acceptor Al-1 occurred following synthesis to the end of the donor (Fig. 2C) regardless of the presence of a strong pause site at the base (Fig. 2A). Studies with mutated viral genomes have shown that in most cases minus strong stop transfers are completed after the whole 5’-region was copied. Our results therefore correlate well with features of the TAR-associated minus strong stop transfers observed in vivo (52–55). The combined data from the HIV-1 TAR and EIAV hairpins suggest that, even in the presence of highly stable structures that induce strong pausing, RT is still most likely to copy until the end of the template before transferring.

To examine internal transfers in more detail acceptor Al-2 was used. An additional pair of markers at the hairpin base suppressed end transfers in Al-2. The profile of internal primer terminus transfers within the EIAV template was similar to that observed for the HIV-1 TAR substrates (Fig. 2D). Notably, transfers were not selectively clustered around the major pause site. The lower proportion of transfers at the base could not be attributed to a mismatch after transfer to marker 2. Even in the absence of marker 2 in Al-1, 27% of template switching happened in the loop versus only 3% at the base. Normalizing the transfers for the distance between markers had the effect of making the region at the base of the hairpin slightly more recombinogenic. However, the rest of the segments still supported a significant amount of transfers, such that overall most of them took place beyond the pause site.

Our attempts to increase the proportion of internal transfers in the region of pausing led to some surprising results. We tried to increase the likelihood of an invasion event prior to the pause site by increasing the homology before that site, only to find that the transfer efficiency was slightly reduced (compare initial versus long template titrations in Fig. 3, A and B). However, the profile of the primer terminus transfers was not substantially changed (Fig. 4, C and D). The increment in transfer efficiency observed when replacing the initial acceptor with the longer one made it clear that a feature of the long acceptor and not of the long donor was blocking transfers. The possibility of the formation of a smaller hairpin by the added sequence upstream of the primary hairpin suggested that the secondary structure of the acceptor interfered with an invasion step. Analysis of the cleavage pattern on both donors (Figs. 2B and 4B) had shown that the region just upstream of the helical hairpin stem could be a preferred site for invasion, because a large amount of RNase H activity was concentrated there. Thus, the coincidence of donor cleavage, over an acceptor region of strong secondary structure, led to a lower affinity of primer annealing to acceptor. Similarly, work done by the Berkhout group (56) on minus strong stop transfer suggests that primer-acceptor interactions within the repeated sequences behind the TAR hairpin play an important role in the efficiency of transfers (56). Consistent with our observation, they also found that the RNA structure was an important factor and that modifications that over-stabilized the poly-A hairpin at the 5’-end of the genome led to both a reduction in virus production (57) and a lowering of transfer efficiency in vivo (56).

To assess whether the section before the pause site was indispensable for an invasion event and for transfers throughout the hairpin, we used an acceptor template that lacked the entire pause site region. Transfer efficiency with the shorter acceptor was reduced 10-fold (Fig. 5, A and B). We concluded that, although donor degradation at the strong pause site created an ideal location for an invasion at the base of the hairpin, the closed structure of the acceptor did not provide any other regions to initiate such an interaction. The concentration of cleavages at the pause site as well as the availability of an unstructured region within an acceptor appear to be important requirements for an efficient invasion step. Negroni and Buc (44, 58) propose that transfers proceed through a two-step process, involving an initial “dock” step at which the DNA primer-RNA acceptor interactions are formed, followed by an “invasion” step involving primer terminus transfer to the acceptor through branch migration. Our results demonstrate that, in the absence of an efficient invasion point behind the RT, transfers throughout the whole hairpin were blocked. This is clear evidence of a spatial separation between the initial point of interaction and the place of genetic shift.

The features of this separation between primer invasion and primer terminus transfer were explored in the presence of NC, a component of the system that is known to affect strand exchange. NC increased transfer efficiency in our system (Table 1), as reported in other studies (37, 39, 40). Measurements indicated that in the presence of NC the short acceptor was able to overcome the severe drop in transfer efficiency. However, it was still 50% less efficient than the initial acceptor Al-2 during template switching. Thus, although NC was able to compensate for the absence of a preferred invasion region in the short acceptor, the presence of the strong pause site still made template switching much more efficient.

NC has also been reported to affect the pausing pattern and the processivity of primer elongation (41–43, 59). In vivo anal-
ysis of NC mutants suggests that its main impact during template switching in vivo is related to its effect on processivity (60). We observed only minor effects of NC on the pausing patterns (Fig. 6A) in our template systems, an observation also made by others (44, 58). However, we were still able to measure a substantial increase in transfer efficiency. Thus we conclude that the measured transfer enhancement is a consequence of the reported nucleic acid chaperone capacity (33–35) or enhancement of RNase H activity of RT (39, 61) by NC.

The use of a blocking oligomer partially clarified the manner in which NC affected strand transfer in the hairpin structure. We envisioned the oligomer as a way of detecting invasion by interference. Because we expected invasion to take place behind the major pause site, the oligomer was made complementary to the primer just upstream of the hairpin. Indeed, blocking invasion just upstream of the hairpin decreased transfer efficiency by 75% (Table I) in the absence of NC. However, in the presence of nucleocapsid, the oligomer was only 50% as effective (Table I). Although we expected such an effect in light of the strand exchange properties of NC, the combination of increased transfer efficiency of the short acceptor, and the lower blocking ability of the oligomer suggested an alternative explanation. Because the short acceptor should not be able to support any invasion at all at the base of the hairpin but still undergoes substantial transfer when NC is present, NC chaperone activity might be allowing the closed regions of the hairpin to be more accessible for invasion. Apparently, with NC the specific blocking oligomer only interferes with the fraction of invasion events at the base of the hairpin and, consequently, is less effective.

Analysis of the transfer distribution of AI-2 (Fig. 6, B and C) demonstrated that it was not significantly changed by NC. It has been proposed that NC could affect the distribution of transfer products by affecting the ability of the elongating primer terminus to anneal to the acceptor (44). We did not see such an effect. It appears that the mechanisms that determine the eventual transfer of the primer terminus are not affected in the same manner as those that allow for invasion. It is also possible that such effects of NC on the primer terminus transfers are not immediately revealed with the relatively short template system used in the current study and would require an environment with larger, more complex secondary structures.

Our data show that interference with the invasion step at the base of the hairpin in a number of ways, including structural block, absence of homologous sequences, and use of a blocking oligomer, leads to a reduction of transfers throughout the template. This strongly supports the conclusion that the invasion step is critical to the transfer process. The presence of NC had the effect of increasing the possible locations of such a step by “melting” the hairpin without disturbing the location of primer terminus transfer. We believe that the underlying separation between the point of invasion and the location of primer terminus transfer we observed in the absence of NC also holds in its presence. However, the location of the invasion site is altered in the presence of the RNA chaperon.

We propose a description of retrovirus strand transfer that we have named the “Dock and Lock” model (Fig. 7). It is based on the concept that most transfers initiate at pause sites where RNase H cleavage of the donor template allows the acceptor template to invade the donor-primer and bind to the primer. This is the previously proposed “docking” step (44), a term that we have retained (Fig. 7C). This step is pivotal, such that removal of the site of invasion strongly suppresses transfer near and downstream of the site, and the presence of an RNA chaperon increases the number of sites available and hence the transfer efficiency. The hybrid region of primer and acceptor would then race by branch migration toward the advancing primer terminus. Primer terminus release from the donor could then be triggered by the subsequent pause sites the RT encounters. The point at which the primer terminus eventually anneals to the acceptor will be the place and time of the “locking” step. Locking will be detectable experimentally as defined by the change in markers from donor to acceptor. The basis of the locking step is currently under investigation.

The model can also explain the characteristics of transfers in the dimer initiation sheet system (32). Donor-acceptor dimerization facilitated through the dimer initiation sheet hairpin would be expected to promote docking at the base of the hairpin. The extending primer terminus could break the templatematrix interactions, but the transfer had already been initiated. The rate between hybrid and primer could then end well beyond the hairpin, resulting in the observed transfer peak after the dimerization site. The model also agrees with results obtained by the Berkhout group on minus strong stop transfer
in the presence of NC (56). Their data show that either complete deletion of the poly(A) hairpin, which is immediately before TAR, or its presence lead to a reduction in transfers. However, this block is partially attenuated at higher temperatures or by partially deleting the poly(A) sequences, suggesting that its structure interferes with the invasion step. Overall, we propose the Dock and Lock model as the best explanation for the characteristics of strand transfer in hairpins and as a model applicable to mechanisms of recombination throughout the retroviral genome.

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