Mechanistic Analysis of Pause Site-dependent and -independent Recombinogenic Strand Transfer from Structurally Diverse Regions of the HIV Genome*

Retroviral recombinants are generated by strand transfers occurring within internal regions of the viral genome and are a major source of genetic variability. Strand transfer has been linked to “pausing” occurring at secondary structures during synthesis by reverse transcriptase. Yet, weakly structured templates lacking strong pause sites also undergo efficient transfer. In this report, transfer crossover sites on high and low structured templates from the gag-pol frameshift region (GagPol) and the env (Env) regions, respectively, were determined by using a reconstituted in vitro strand transfer assay. The assay tested transfers occurring between a donor and acceptor template over a 150-nucleotide homologous region. The majority of crossovers were in a small 23-nucleotide region near a major pause site on GagPol, clearly indicating a pause-driven mechanism. In contrast, on Env, transfers were more dispersed clustering toward the end of the homologous region. Slowing down polymerization on Env by decreasing the dNTP concentration resulted in crossovers shifting toward the beginning of the homologous region. Removal of a small 38-nucleotide region at the 3'-end of the Env acceptor had a large effect on the level of strand transfer despite very few crossovers mapping to this region. This implicated this part of the acceptor in transfers occurring at downstream positions. For Env the results support a mechanism where the acceptor rapidly binds nascent DNA, then “zippers” downstream catching up with the donor-DNA hybrid and displacing the donor. Such a mechanism may be important to recombination in low structure regions of the HIV genome.

During reverse transcription, the human immunodeficiency virus (HIV)* undergoes extensive recombination. Jetzt and coworkers (1, 2) have shown that on average, HIV-1 recombines approximately two to three times in every cycle of replication. More recently results suggesting ~10 recombinations per infectious cycle in T cell lines and as many as 30 per cycle in macrophages have been reported (3, 4). By generating genetic diversity in the viral population recombination can allow some viruses to evade the host immune response and drug therapy (5–8). In addition the process is important in generating recombinants between different viral subtypes (intersubtype recombinants) (9–13). These can potentially lead to new stable circulating viruses (circulating recombinant forms (CRFs)) that can further complicate vaccine and drug approaches (9).

Most retroviral recombination occurs during synthesis of minus strand DNA using genomic RNA as template by a process called strand transfer (also referred to as strand jumping or template switching) (7, 14, 15). Strand transfer involves the switching of DNA being synthesized on one template (referred to as “donor”) to homologous regions on the same or on a second template (referred to as “acceptor”) where the synthesis continues. The resultant proviral DNA is capable of encoding a genomic RNA that is a chimera of the original parent templates. The first and second strand transfers (strong stop DNA transfers) that occur during reverse transcription are obligatory transfers without which the viral replication cannot proceed to completion. In addition to these vital events, the virus can also undergo internal strand transfers, which can potentially occur at any position along the genome (1, 2, 16). They also may increase the probability of successful DNA synthesis by providing a salvage pathway for broken or damaged genomes (17). Internal transfers can occur during plus strand DNA synthesis, albeit to a lesser extent than during minus strand DNA synthesis (7, 18).

The role of pausing by reverse transcriptase (RT) during these events has been studied extensively by several investigators (19–22).Pause sites are positions on the template where the rate of DNA synthesis temporarily slows resulting in a build up of products at that point. Pausing generally results from secondary structures that impede RT or specific sequences like runs of adenosine on DNA templates (23–28). The pause-driven transfer mechanism proposes that the paused DNA products are the substrates that ultimately are used to produce transfer products, or that pausing sets the stage for transfer events that occur immediately downstream of the pause site (20, 21). This mechanism involves a complex interplay of nucleic acids, nucleocapsid protein (NC), and RT. Previous reports have shown that the polymerase and the RNase H activities of RT are not strictly coupled, and the rate of polymerization is generally much faster than RNase H (29, 30). This means that during uninterrupted polymerase activity, the RNA template may not be extensively degraded. This situation changes at a pause site; here the RNase H activity takes precedence over synthesis (23). Degradation of the RNA template destabilizes the donor RNA template-nascent DNA interactions and also clears regions on the DNA that are free to bind.
to the acceptor template. Transfer can occur after the DNA dissociates from the donor or by active invasion of the donor-DNA complex by the acceptor template (acceptor-facilitated transfer) (20, 21). During invasion, there is a transient trimeric structure with the 3'-terminal region of the DNA bound to the donor and more upstream regions bound to the acceptor template. In vitro (31) and ex vivo (32) studies have shown evidence supporting the existence of a trimeric structure during strand transfer. There is in vitro evidence for the occurrence of the first mechanism also (21); hence, it is not yet known if minus strand DNA transfers occur preferentially through one of the two above mentioned mechanisms.

The manifold effect of NC on pause-mediated transfers has been well studied (33–35). The main components of the NC nucleic acid chaperone activity are annealing and melting of nucleic acids. At pause sites, NC would be expected to melt out the obstructing secondary structures to facilitate continued synthesis on the donor template. Strand transfer experiments on a secondary structure from the polypurine tract of murine leukemia virus showed that NC did indeed reduce pausing and stimulate continued synthesis of nascent DNA on donor template (36). But in the presence of acceptor template, NC resolves pause sites with an accompanying increase in strand transfer (37). It has been proposed that the NC effect on pause-mediated transfer occurs via the following steps: (i) it causes enhancement of RNase H activity of RT (38), (ii) it promotes strand transfer (35), represents a new model for transfers in regions of structured regions, whereas, there is a high level of stimulation for structured regions.

In this report, two of the regions from the previous article, one highly structured (gag-pol frameshift region, denoted GagPol) and one with little structure (region from the env gene, denoted Env), were used to evaluate the different mechanisms of strand transfer. Various techniques including direct mapping of transfer crossover points under different conditions, and mutagenesis of the acceptor templates were used. Results supported the above hypothesis and showed that transfers were highly focused to the pause site region on GagPol. In contrast transfers on Env were more randomly dispersed and results supported a mechanism where the acceptor rapidly binds to the nascent DNA then displaces the donor at a downstream point as it “zippers” up the DNA. The second mechanism, which is similar to the “lock and dock” model for strand transfer (35), represents a new model for transfers in regions with relatively low secondary structure and little pausing.

**EXPERIMENTAL PROCEDURES**

**Materials**—All the mutation primers that were used to introduce mutations into the Env and GagPol acceptor templates are listed in Table I. The primers were obtained from Integrated DNA Technologies, Inc. QuikChange site-directed mutagenesis kit was obtained from Stratagene. The Pfu Turbo DNA polymerase used to introduce mutations into the pNL4–3 plasmid was from Stratagene. Plasmid pNL4–3, obtained from the National Institutes of Health AIDS Research and Reference Reagent Program contains a complete copy of the HIV-1 provirus derived from strains N/D and LAV (49). Top polymerase was from Eppendorf. SP6 RNA polymerase, DNase I (RNase-free and RNase/ DNase-free), was from Roche Diagnostics. RNase inhibitor was from Promega. T4 polynucleotide kinase was obtained from New England Biolabs. Proteinase K was obtained from Kodak. Radiolabeled compounds: [γ-32P]ATP was obtained from Amersham. Sephadex G-25 spin columns were from Amika Corp. Recombinant HIV-RT was provided to us by Genetics Institute (Cambridge, MA). This enzyme has a specific activity of ~40,000 units/mg (one unit of RT is defined as the amount required to incorporate 1 nmol of dTTP into nucleic acid product in 10 min at 37 °C using oligo(dT)-poly(rA) as primer template). The enzyme contained very low levels of single-stranded nuclease activity, which was found to be inhibited by including 5 mM AMP in the assays (21). At this concentration, the AMP did not affect the polymerase or RNase H activity of RT. Aliquots of HIV-RT were stored frozen at −70 °C, and a
donor RNA was hybridized to a complementary labeled primer by polynucleotide kinase according to the manufacturer's protocol. The inhibitor. Reactions were allowed to incubate for time points of 0, 2, 4, 6, and 8 h. Each reaction was terminated by mixing with 4 M of solution containing 25 mM EDTA (pH 8.0), 2.5 ng of RNase/DNase-free enzyme and allowed to digest for 20 min at 37 °C. 2 μl of proteinase K at 2 mg/ml in 1.25% SDS, 15 mM EDTA, pH 8.0, and 10 mM Tris, pH 8.0 was then added to the above mixture, which was placed at 65 °C for 1 h. Finally, 12 μl of 2× formamide dye (90% formamide, 10 mM EDTA, pH 8.0, 0.1% xylene cyanol, 0.1% bromphenol blue) was added to the mixture and the samples were resolved on an 8% denaturing polyacrylamide gel containing 7 M urea. Extended DNA products were quantified by phosphorimager analysis using a GS-525 phosphorimager from Bio-Rad. Strand transfer assays on the Env substrate were also conducted in the presence of suboptimal or saturating dNTP concentrations (1 or 100 μM, respectively).

RNAase Protection Assays—The structural features of the GagPol acceptor RNA was tested by RNAase protection assays. The two enzymes used were T1 RNAase and RNAse A. The recovered RNAs from the above transcription reactions were dephosphorylated using calf intestinal alkaline phosphatase according to the manufacturer’s protocol. Reactions were extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The RNA precipitates were labeled at the 5′-end with 32P using T4 polynucleotide kinase. The labeled RNAs were again gel-purified on 8% denaturing gels. The recovered labeled RNAs were quantified spectrophotometrically from optical density.

T1 RNAase enzyme analysis was conducted by digesting each labeled RNA from above in a 10–μl reaction mixture containing 0.2 pmol of RNA, 100 mM Tris-HCl, pH 8, 80 mM NaCl, and serially diluted T1 RNAase enzyme was digested at 50 °C for 500 units and 15 units. The reactions were incubated at room temperature for 4 min, and then 10 μl of 2× formamide dye were added. Reactions were placed on ice until gel electrophoresis. The samples were resolved on an 8% denaturing polyacrylamide gel containing 7 M urea. A base hydrolysis ladder and undigested control RNA were run along with the samples. The gels were dried and subjected to autoradiography.

RNAse A enzyme analysis was conducted by digesting the labeled RNA in a 10-μl reaction mixture containing 0.2 pmol of RNA, 50 mM sodium acetate, pH 5.2, and RNAse A enzyme. RNAse A was diluted to 0.1, 0.25, 0.5, 1, and 3 microunits. The reactions were incubated at room temperature for 4 min; they were stopped with 10 μl of 2× formamide dye and put on ice until gel electrophoresis. The samples were resolved on an 8% denaturing polyacrylamide gel containing 7 M urea. A base hydrolysis ladder and undigested control RNA were run along with the samples. The gels were dried and subjected to autoradiography.

Mutation of the Env and Gag Pol Acceptor Templates—The primers listed in Table I were used to introduce mutations into the previously use acceptor RNA templates from the env and gag-pol regions of the genome. A QuikChange site-directed mutagenesis kit from Stratagene was employed for this using the manufacturer’s protocol. Five point mutations were introduced in the env acceptor template. The mutations correspond to base numbers 32, 60, 91, 119, and 155 from the 3′-end of the Env acceptor template (Fig. 2). Similarly, four point mutations were introduced into the gag-pol region of the pNL4–3 plasmid that correspond to base numbers 45, 68, 89, and 105 from the 3′-end of the GagPol acceptor template (Fig. 2). After preparation of the mutated plasmid, the acceptor RNA was prepared as described above from the recovered PCR products.

PCR Amplification and Sequencing of Transfer DNA Products—Strand transfer assays were run for 32 min in the presence or absence of NC (see Fig. 1). Assay conditions were described above except that a 50-μl reaction was performed, and all the reaction material was used for a single time point. Reactions were processed, then electrophoresed on 8% polyacrylamide denaturing gels. Transfer DNAAs were located by autoradiography, excised, and eluted overnight in a TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The eluates were separated from the gel by centrifugation and subsequent filtration through a 0.45-micron disposable syringe filter. The DNAs were recovered by precipitation in ethanol with 300 mM sodium acetate (CH3COO), 10 mM EDTA, and 0.1% sodium dodecyl sulfate. The precipitated DNA was resuspended in 50 μl of RNaseA-free water, loaded onto two successive hydrolyzed Sephadex G-25 spin columns and processed according to the manufacturer’s directions. The amount of recovered DNAs was determined spectrophotometrically from optical density. RNA preparation were also examined on polyacrylamide gels to ensure that only full-length RNAs were present. DNA DNAs were excised and eluted overnight in a TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). The eluates were separated from the gel by centrifugation and subsequent filtration through a 0.45-micron disposable syringe filter. The DNAs were recovered by precipitation in ethanol with 300 mM sodium acetate. The recovered DNA was further PCR-amplified using the conditions described above with primers 5′-gattagttgctcactatatgataactgatcaagacccaaca-3′ and 5′-tattattaagataatcgactatag-3′ for Env products and 5′-gattagttgctcactatatgataactgatcaagacccaaca-3′ and 5′-tattattaagataatcgactatag-3′ for GagPol. The resultant DNA was ligated into a Topo vector (Invitrogen), which was used to transform Top10 E. coli competent cells (as per manufacturer’s protocol). Only the white and pale blue colonies were picked. Minipreps were prepared using a Bio-Rad mini prep kit, and DNA was sequenced using M13 reverse primer. The Env transfer products from 1 μM dNTP reactions were also PCR-amplified and sequenced.
RESULTS

Strand Transfer Assays—The general approach used in the earlier report (48) was employed to test for strand transfer as shown in Fig. 1A. This assay is designed to simulate internal strand transfer events occurring during minus strand DNA synthesis. DNA synthesis is initiated from a 5′-end-labeled DNA primer that was specifically designed to bind only to the 3′-end of the donor RNA. Strand transfer can occur over the region of homology between the donor and acceptor RNAs (solid lines). Primer extension to the end of the donor produces a 175-nucleotide full-length donor-directed DNA product. Strand transfers and subsequent extension on the acceptor produces a 195-nucleotide product. The donor also contained a 5-nucleotide non-homologous extension such that only internal strand transfers could occur in the assay.

Shown in Fig. 1B is an autoradiogram of a strand transfer assay with the GagPol and Env substrates performed in the presence or absence of NC protein. A graph of the results is shown in Fig. 1C. Consistent with the previous results (48) the efficiency of transfer on GagPol was highly reduced in the absence of NC, whereas a significantly lesser reduction occurred with Env. Transfer assays with the acceptors containing the point mutations were essentially identical (see below, data not shown). Notable in Fig. 1B is the strong pause site at position 77 on GagPol (Ref. 48 and see below). Other “minor” pause sites were also observed on this substrate and the Env substrate.

FIG. 1. Strand transfer assays with the GagPol and Env substrate. A, schematic diagram of the strand transfer assay. The 5′-terminal nucleotide on the DNA primer is denoted number 1. Solid lines on the donor and acceptor RNAs indicate the region of homology between the two while dotted lines are non-homologous regions. The lengths of these various regions are indicated above the lines. Numbers running across the top of the figure indicate the length of DNA synthesis products extended to that point. Note that transfer products made on the acceptor are 20 bases longer (195 versus 175) than full-length donor-directed products. The position of the major pause site on GagPol is also indicated. B, autoradiogram of a strand transfer time course assay performed with GagPol or Env substrate in the presence or absence of NC (as indicated). The DNA primer was 5′-end labeled with P-32 for the assays. Positions of transfer (T) and full-length donor-directed (F) products as well as the strong pause site (Pause) on GagPol are indicated. Time points in each series were 0, 2, 4, 8, 16, 32, and 64 min and increased from left to right. C, graph of the % efficiency of transfer ((transfer products/(transfer + full length donor-directed products)) × 100 or (T/(T + D)) × 100) versus time for a typical strand transfer assay with GagPol or Env in the presence or absence of NC. Symbols are: open circles, GagPol – NC; filled circles, GagPol plus NC; open triangles, Env – NC; filled triangles, Env plus NC. For B and C, each assay was repeated several times, and a representative experiment is shown (also see Ref. 48).
Mapping of the Crossover Positions on the GagPol and Env Substrates—To map the positions of transfer on the Env substrate five approximately equally spaced mutations corresponding to base numbers 32, 60, 91, 119, and 155 from the 3'-end were introduced into the acceptor template. The mutations were carefully selected so as to retain the wild-type acceptor structure. For the GagPol substrate four point mutations that corresponded to base numbers 45, 68, 89, and 105 from the 3'-end of the acceptor template were introduced. These mutations were chosen to flank the strong secondary structure containing the major pause site on GagPol (see Fig. 2). Fig. 2 shows the structure and ∆G values of the GagPol and Env acceptors as predicted under “Experimental Procedures.” Positions of nucleotides that were mutated in acceptors used to map crossover points are indicated by arrows pointing from the base to the change that was made. The position of the base from the 3'-end of the acceptor is indicated in parentheses.

Mapping of the Crossover Positions on the GagPol and Env Substrates—To map the positions of transfer on the Env substrate five approximately equally spaced mutations corresponding to base numbers 32, 60, 91, 119, and 155 from the 3'-end were introduced into the acceptor template. The mutations were carefully selected so as to retain the wild-type acceptor structure. For the GagPol substrate four point mutations that corresponded to base numbers 45, 68, 89, and 105 from the 3'-end of the acceptor template were introduced. These mutations were chosen to flank the strong secondary structure containing the major pause site on GagPol (see Fig. 2). Fig. 2 shows the structure and ∆G values of the GagPol and Env acceptors as predicted using RNAstart (52). Mutation changes are indicated by arrows pointing from the nucleotide that was changed to the change that was made. For the GagPol acceptor positions of prominent T1 (boxed G residues) and RNase A (diamond U and C residues) cleavage are also indicated on the drawing. These were determined by gel analysis of 5'-end-labeled GagPol acceptor template (data not shown). In general the cleavages were in predicted single-stranded regions of the acceptor or within relatively weak portions of stems. Notable is the absence of cleavage in the strong stem loop (SL) structure containing the pause site (indicated on figure). Overall the enzymatic mapping results support the accuracy of the RNAstart program for GagPol. Refolding of the RNAs with the mutations inserted resulted in nearly identical structures to those shown.

In order to map crossover points from reactions with the mutated acceptors, DNA products (32-min time point) were excised from autoradiograms and processed as described under “Experimental Procedures.” Sequences of the cloned products were obtained and used to generate Fig. 3. Crossover points were determined from the sequences by looking for the mutations derived from the acceptor template. The first appearance of a mutation in the product indicated that crossover had occurred within the region encompassed by the recovered mu-
tation and the previous mutation toward the 3′-end of the acceptor. Products from reactions with or without NC were analyzed. For the Env substrate in the presence of NC in reactions with 100 μM dNTPs (Fig. 3A), 46% of transfers occurred between mutations at bases 119 and 155 (the last region where transfer could occur). Another 34% of transfers were between bases 91 and 119. In the absence of NC, crossover points were more evenly distributed with some skewing toward the more downstream regions. Results with GagPol were very different. Most of the crossovers occurred in the small 23-base region that contained the strong pause site (position 57 from the 3′-end of the acceptor, see Fig. 2) between bases 45 and 68 from the 3′-end of the acceptor (Fig. 3B). Some transfers also occurred in the last region between bases 105 and 150, and these increased somewhat in the absence of NC. Still there was a clear bias for the region containing the strong pause site, despite the fact that this segment represented only about 15% of the total homologous transfer zone. Overall the results support a pause site-driven mechanism of transfer for the GagPol substrate with the strong pause site essentially focusing strand transfer to a small region on the template. In contrast, crossovers in Env were more evenly distributed and did not correlate with a prominent pause site. This along with the absence of strong pausing indicated a different mechanism for transfer on Env.

Assays with Low dNTP Concentrations on the Env Substrate Support an Invasion and Zipper Mechanism—Previously we proposed a mechanism for transfer on Env in which the low structure of the acceptor allows it to rapidly bind to nascent DNA then zipper up the DNA and displace the donor template at a downstream point (48). Such a mechanism would require synthesis of DNA and removal of the RNA template to provide an area for initial acceptor binding. After this, downstream migration of the DNA-acceptor hybrid would ultimately lead to displacement of the donor (see Fig. 4 for model). This mechanism is consistent with most transfers occurring at more downstream positions within the region of homology as was found above. One prediction would be that slowing the progression of RT down the donor would allow the acceptor to “catch-up” more quickly with the DNA-donor hybrid and displace the donor earlier. To test this, strand transfer assays were performed at either 1 or 100 μM dNTP concentrations. Transfer products from the 1 μM reactions were recovered, cloned, and sequenced as above to determine where crossovers occurred. A typical transfer assay with NC is shown in Fig. 5A. In the absence of acceptor, fully extended products are clearly visible by 2 min in reactions with 100 μM dNTPs while these products appear at the 4-min time point in 1 μM reactions. This reflects the saturating level of dNTPs in the 100 μM reactions and the suboptimal conditions in the 1 μM reaction (21). In the presence of acceptor there were less total transfer products with 1 μM dNTPs, however, the efficiency of transfer ((Transfer products/ (Transfer + full-length donor-directed products)) × 100) was considerably greater with the lower dNTP concentration (see Fig. 5B). Also notable was the clear decrease in the level of full-length donor-directed products in reactions with the acceptor (plotted in Fig. 5C). This implies that products that would have been completed on the donor are transferred to the acceptor when it is present, a finding that is consistent with the mechanism proposed above. Sequencing of cloned transfer products from 1 μM reactions performed with the mutated acceptor used above yielded the results shown in Fig. 3 under 32 mins 1 μM reactions. In comparison to 100 μM reactions, crossover points clearly shifted upstream in 1 μM reactions. The penultimate region now showed the highest level of transfer at 44% whereas the last region went down from 46 to 28%. The region between bases 32 and 60 also increased from 8 to 20%. Overall, slowing down the polymerase resulted in earlier crossover points. This is consistent with the proposed mechanism where after binding the DNA the acceptor must zipper and catch up with the DNA-donor hybrid to displace the donor. Note that the mapped crossover points reflect where the donor was displaced, and synthesis began on the acceptor rather than where the acceptor associates with the DNA.

Transfer on Env with a 3′-Truncated Acceptor Support the Proposed Mechanism—In the presence of NC using 100 μM dNTPs, 80% of the recovered crossovers on the Env substrate occurred between bases 91 and 150 in the region of homology (see Fig. 3). However, the invasion and zipping mechanism that is proposed implies that the acceptor binds to the nascent DNA at a point upstream of the actual crossover. Therefore removing an upstream region of the acceptor that is distal from the major crossover regions could have a large effect on transfer. To test this, Env acceptor template with the 3′-end removed (–38 acceptor) was used in strand transfer assays. Results are shown in Fig. 6. There was about a 50% reduction in the efficiency of transfer with the –38 versus full-length acceptor at corresponding time points. Since 80% of the transfers with full-length acceptor occurred in a region that began 53 bases downstream of the truncation (91–38), such a dramatic effect on overall transfer would not be expected unless the upstream region is important to transfers occurring in the 91–150 region. This supports a mechanism where the acceptor binds the DNA well upstream of the point of transfer.

**DISCUSSION**

The mechanism of strand transfer on two structurally different substrates from the gag-pol and env regions of the HIV...
genome was investigated. A previous report demonstrated that several strongly structured regions of the HIV genome, including the GagPol region tested here, were highly stimulated by NC in vitro strand transfer reactions. In contrast, weakly folded substrates were only modestly stimulated and transferred at least as well as the more strongly folded substrates (48). Transfers on the highly structured substrates appeared to be driven by one or more strong DNA synthesis pause site evident during synthesis with HIV-RT. For GagPol the mapping result presented here (Fig. 3) clearly demonstrate a pause site-driven mechanism. The majority of crossovers on this substrate occurred in a small 23-nucleotide region that contained the strong pause site. Pause site-driven strand transfers with RT have been demonstrated on many substrates, and crossover to the acceptor generally occurs at or just downstream of the pause site (20, 21, 35). Other non-pause-dependent mechanisms for recombinogenic transfer have also been proposed, and in this case transfers seemed to involve interactions between the transferring DNA and a specific structure on the RNA acceptor (53, 54). In the case of the weakly structured Env substrate used in the current work, no strong pause sites were evident during RT synthesis. This was also the case for a second substrate from the pol-vif genome region (48). The weak folding and lack of strong pausing on these substrates suggests that transfer occurs by a different mechanism that does not involve pausing or structures on the acceptor. We propose an acceptor-mediated mechanism that involves invasion of the acceptor upstream of the transfer point followed by zippering and displacement of the donor. Such a mechanism has been...
proposed for transfers in the highly structured TAR region of the genome, although DNA synthesis pausing played a role in this mechanism (20, 35). We propose that strong pausing need not occur on weakly structured substrates for this mechanism to be active.

Results shown here support a mechanism where the acceptor rapidly binds the nascent DNA then displaces the DNA from the donor at a downstream point. Rapid binding would be enhanced by the weak structure of the DNA and acceptor. Transfer would tend to occur at more downstream regions on the acceptor for several reasons: 1) RNase H activity of RT is required to remove the RNA template from the nascent DNA to provide a region for the acceptor to bind. Sufficient RNase H cleavage may not always be carried out by the RT molecule performing synthesis, especially on a template with little pausing (29, 55). This would require other RTs to complete cleavage and provide a region for the acceptor to bind. On a template with little pausing the 3’ DNA terminus may be far downstream by the time the acceptor can bind the DNA. 2) It is easier for the acceptor to bind to longer regions of DNA with more complementary bases. Longer DNAs will lead to crossovers at downstream regions. 3) After binding, the acceptor-DNA hybrid must zipper toward the DNA-donor hybrid and displace the DNA. On a substrate with strong pause sites the DNA-donor hybrid is essentially stalled at the pause site allowing the acceptor time to catch up and displace the donor. On a substrate with weak pausing the acceptor must “catch” the DNA-donor hybrid for displacement to occur. Note that NC can essentially accelerate all three of the steps listed above so it is not surprising that transfer is greater with NC. The fact the strongly folded substrates like GagPol are stimulated more by NC probably reflects the greater requirement for the helix destabilizing activity of NC on these substrates.

The results from mapping experiments at 1 or 100 \( \mu \text{M} \) dNTPs (Fig. 5) as well as the acceptor 3’ truncation experiment (Fig. 6) were in agreement with an invasion then zipperping type of mechanism. However, they do not completely rule out other alternatives. For example, transfer could occur as a result of DNAs that are only partially completed on the donor being released from the donor by RNase H activity then transferring to the acceptor. Reactions typically showed some incompletely synthesized DNA extension products after long incubations (21, 48). Addition of more enzyme to these reactions does not lead to further extension indicating that the products are dissociated from the donor or irreversibly blocked (21). Relatively few incompletely synthesized products were observed with Env perhaps because of the lack of strong secondary structures. A newly proposed mechanism for pausing indicates that the transferred products at certain secondary structure-induced pause sites (similar to the one observed on GagPol) are difficult to extend because refolding of the RNA template at the pause site can displace the 3’ DNA terminus (27). This could explain the irreversible block that is observed with some pause sites. Pause site-driven transfer can occur by the paused DNA dissociating from the donor and then transferring to the acceptor for subsequent extension (21). Some of the transfers on Env probably resulted from these types of transfers occurring at weak pause sites. However, results in Fig. 5, A and C showed that the level of full-length donor-directed products decreased when acceptor was present. This argues that some of the transfer products are derived from DNAs that would have been completed on the donor. These DNAs were stolen from the donor by the acceptor. If all transfers were occurring from previously released DNAs then acceptor should not affect the level of fully extended donor-directed products. In addition, mapping results showed that 80% of transfers with NC occurred between position 91 and 150 on the Env substrate (Fig. 3), yet truncation of 3’-bases far removed from this region substantially decreased transfer (Fig. 6). If transfers resulted from binding of previously dissociated DNAs to acceptor then those extended to the region between 91 and 150 should still bind the truncated acceptor with high efficiency. Even if the DNAs had dissociated at position 91, they could still form a 53 base pair hybrid with the acceptor. Clearly the 3’ 38 base truncation would be expected to eliminate most of the transfers in map region one (bases 1–32) and possibly two (bases 32–60). There could even be some effect on the third region (bases 60–91). However, these regions only accounted for 20% of the observed transfers with 100 \( \mu \text{M} \) dNTPs. The ~50% decrease (Fig. 6) argues that many of the transfers occurring in the last two regions are dependent on acceptor interactions at upstream positions on the DNA.

Experiments with 1 \( \text{versus} \) 100 \( \mu \text{M} \) dNTPs also showed that the efficiency of transfer was greater with the suboptimal nucleotide concentration (Fig. 5B). This result is in agreement with results that examined recombination rates in tissue culture cells (56). In this case, depletion of the dNTP pool in cells by addition of hydroxyurea lead to an increase in the rate of recombination. The authors proposed a dynamic copy-choice model for recombination. Because the RNase H and polymerase activity of RT are essentially uncoupled (29, 30), slowing polymerization by lowering the concentration of dNTPs would lead to more extensive degradation of the RNA template. This in turn leads to more recombination. The increase in recombination could result from an enhancement in the steps required for acceptor invasion and zipperping, or an increase in the amount of DNAs that dissociate from the donor as a result of more extensive RNase H cleavage.

Overall our results support an acceptor-mediated invasion and zipperping type mechanism for genome regions with low structure that are devoid of strong pause site. Such a mechanism can also occur at a strong pause site as well and in this case pausing serves to stall DNA synthesis to allow acceptor binding and zippering. This results in transfer points that are focused at or just downstream of the pause site as shown for GagPol. This type of mechanism (termed “lock and dock”) has been proposed by other for pause site-driven transfers (20, 35). In contrast crossovers in low structure regions are more dispersed as demonstrated for the Env substrate. It is possible that they may focus to weak transient pauses that occur frequently on all heteropolymeric templates, although this was not tested. The stalling effect served by a pause site is abrogated on the low structure substrate possibly because acceptor-DNA binding and zipperping are highly efficient due to the lack of structure in the templates. We propose that genome regions with low structure could serve as hot regions for recombination and that pausing or intricate structural interactions are not required for efficient recombination in such regions. In conclusion there appears to be several different mechanisms by which recombination can occur in retroviruses. The particular mechanism used would be highly dependent on the structural properties of the genome in that region. The extent to which each of these contributes to recombination \textit{in vivo} remains to be determined.

Acknowledgments—We thank the Genetics Institute for the kind gift of HIV-RT and the AIDS Research and Reference Reagent Program for plasmid pNL4-3. We would also like to thank Dr. Charles McHenry from the University of Colorado for the plasmid clone for wild-type NC overexpression.

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