Evidence That HIV-1 Reverse Transcriptase Employs the DNA 3’ End-directed Primary/Secondary RNase H Cleavage Mechanism during Synthesis and Strand Transfer*

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We previously analyzed strand transfers catalyzed by human immunodeficiency virus, type 1 reverse transcriptase (RT) in a hairpin-containing RNA template system. In this system, RT produces a series of adjacent RNase H cuts before the hairpin base on the first, or donor template that clears a region of the donor, facilitating invasion by the second, or acceptor RNA. Here we analyze characteristics of the prominent cuts before the hairpin base and their role in strand transfers. Analysis of the template cleavage pattern during synthesis suggested that the RT performs DNA 3’ end-directed primary and secondary cuts while paused at the hairpin base and that these cuts contribute to creation of the invasion site. RT catalyzed similar cleavages on a substrate representing a paused cDNA-template intermediate. DNA 3’ end-directed secondary cuts, which require positioning of the polymerase active site downstream of the primer terminus, had previously not been specifically identified during synthesis. Our findings indicate that during synthesis DNA 3’ end-directed primary and secondary cuts occur at pause sites. RT mutants with substitutions at the His539 residue in the RNase H active site were defective in secondary cleavages. Analysis of the template cleavage pattern generated by the His539 mutants during synthesis revealed inefficient cleavage at the invasion site, correlating with defects in strand transfer. Overall, results indicate RT can catalyze pause-associated DNA 3’ end-directed primary and secondary cuts during synthesis and these cuts can contribute to strand transfer by creation of an invasion site.

Human immunodeficiency virus, type 1 (HIV-1)3 reverse transcriptase (RT) is the virally encoded enzyme responsible for conversion of the viral RNA genome into double-stranded DNA. RT is a p66/p51 heterodimer that possesses both RNA- and DNA-dependent DNA polymerase activity and RNase H activity. The polymerase and RNase H active sites reside in the N-terminal and C-terminal portions of the p66 subdomain, respectively. Structural and biochemical studies indicate that the two active sites are separated by a distance corresponding to that covered by 18–19 nt (1–4). During minus strand synthesis, the polymerizing RT binds to the 3’ end of the primer and cleaves the RNA template concomitant with DNA synthesis (3). The relative rate of polymerization is 7–10 times faster than RNase H cleavage and the two activities are uncoupled (5). Biochemical studies indicate that the RNase H cuts generate various sized fragments of RNA some of which are left behind still bound to the newly synthesized cDNA during minus strand synthesis (6, 7).

In addition to binding to the 3’ terminus of a primer in an orientation for synthesis, RT has alternate modes of binding that are believed to be biologically relevant during reverse transcription. For example, an RNA oligonucleotide recessed on a longer DNA segment can represent the products that remain behind after the RT has synthesized a segment of minus strand DNA. On such substrates RT preferentially cleaves ~18 and 8 nt from the 5’ end of the RNA to produce primary and secondary cleavage products, respectively (8–10). Studies indicate that these RNA 5’ end-directed primary and secondary cleavages are independent of each other and have differing rates (9, 11). For the RT to make the secondary cut, it must bind ~10 nt closer to the 5’ end of the RNA relative to positioning for the primary cut. Finally, the RT has also been shown to make internal cleavages not directed by terminus alignment, but occurring with some sequence preference (12). In vivo, both RNA 5’ end-directed primary and secondary cleavages and internal cleavages are believed to occur on the RNA fragments that remain annealed to the DNA template following synthesis and cleavage by the polymerizing RT.

Similar to the primary/secondary cleavage pattern observed when RT utilizes an RNA 5’ end to direct binding, primary and secondary cleavages have also been observed roughly 18 and 8 nt from the DNA 3’ end, respectively (13–15). DNA 3’ end-directed primary cleavage arises from binding of the DNA primer terminus within the polymerase active site resulting in the positioning of the RNase H active site 18–19 nt upstream. Primary cleavages of this type occur concomitant with DNA synthesis. DNA 3’ end-directed secondary cleavage has also been observed (2, 13–17). However, these studies were performed in the absence of dNTPs, precluding synthesis. Secondary cuts of this type likely require movement of the polymerase active site away from the primer terminus to allow repositioning of the RNase H active site ~8 nt behind the primer terminus. Movement of the polymerase active site away from the primer terminus during synthesis is unanticipated, because DNA polymerases are thought to remain bound to the 3’ terminus of the primer during elongation. The possible roles and effects of DNA 3’ end-directed secondary cleavages have not been explored because these cleavages have only been analyzed and characterized as such in the absence of synthesis.

In the crystal structure of HIV-1 RT complexed to an RNA:DNA hybrid, the highly conserved histidine at position 539 is shown to interact with the scissile phosphate of the RNA (1) and is likely a key residue in the RNase H active site. Asparagine and aspartic acid substitutions of

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3 The abbreviations used are: HIV-1, human immunodeficiency virus; RT, reverse transcriptase; EIAV, equine infectious anemia virus; FL, full-length; SP, self-priming; TP, transfer product; WT, wild type; nt, nucleotide(s).
His539 produce RT mutants that are particularly defective in secondary cleavage (17). In addition, substitution of the His539 residue with phenylalanine was shown to reduce RNase H activity, however, inhibition of particular types of RNase H cleavage were not characterized (18, 19). Results from these and other studies have shown that mutation of the 539 position has little effect on the polymerase activity of RT, however, the RNase H activity and specificity were altered to varying extents (17, 18, 20, 21). Additionally, asparagine substitution of the 539 position produced an RT mutant with reduced efficiency of strand transfer in vivo (22).

RT RNase H activity is essential to several aspects of reverse transcription. One such process is RT-mediated strand transfer (23, 24). Several studies have suggested a correlation between pausing of the RT and strand transfer (24–29). Pause sites are positions where synthesis products accumulate because of a slow rate of synthesis, often as a result of an encounter with a secondary structure, such as a hairpin, on the template (30, 31). RT pausing is also correlated with increased RNase H cleavage, an anticipated result of increased residence time of the RT at one position (32). Recent studies from our laboratory have described a pause-mediated mechanism of strand transfer in which pausing of RT induces cleavage of the RNA template and promotes initial steps of strand transfer (28, 29, 33, 34).

We have previously analyzed pause-associated transfer by HIV-1 RT using templates containing the equine infectious anemia virus (EIAV) primer binding site hairpin sequence (Fig. 1 and see Fig. 7 in Ref. 28) (29). The steps in strand transfer with these templates were as follows. During primer extension, RT produced a series of cuts on the first, or donor template, upstream of the hairpin base. This generated an invasion site, a region where the nascent cDNA could interact with the second, or acceptor RNA, to facilitate transfer. Primer extension continued on the donor template and the acceptor cDNA hybrid propagated toward the primer terminus. At a site downstream of the invasion site, predominantly within the loop region, the primer terminus switched templates from the donor to the acceptor. In this two-step “dock and lock” model for strand transfer an important component of the transfer mechanism is clearing of a region of the donor template (creation of an invasion site) to allow acceptor interaction with the nascent cDNA. In this study we seek to further expand our understanding of strand transfer in this system by determining the characteristics of cleavages that promote efficient creation of an invasion site.

**EXPERIMENTAL PROCEDURES**

**Materials**—DNA oligonucleotides were obtained from Integrated DNA Technologies (Coralville, IA). NotI, HindIII, and Escherichia coli RNase H were purchased from Invitrogen. Poly(rA)-oligo(dT) was obtained from Amersham Biosciences. Radionucleotides were purchased from PerkinElmer Life Sciences. All other enzymes and dNTP solutions were obtained from Roche Applied Science.

**Preparation of WT RT and His539 Mutants**—Expression plasmids (HXB2 strain) pKK-p66(His6) and pKK-p51(His6) (35) were kindly supplied with the exception of codon 539. RT was purified as previously described (29, 36) with the minor variation that the p66 and p51 subunits were purified separately and mixed in equal amounts prior to dialysis. The specific activities of the RT preparations were determined by measuring the rate of [α-32P]dTTP incorporation using poly(rA)-oligo(dT) as substrates in a filter binding assay. The specific activities of the RTs were 18,000, 18,000, 21,000, and 24,000 units/mg for WT, H539F, H539D, and H539R RT, respectively. One unit of RT activity is defined as the amount required to incorporate 1 nmol of dTTP into nucleic acid product in a 10-min reaction at 37 °C using poly(rA)-oligo(dT) as template–primer.

**Generation of RNA Templates**—The donor and acceptor constructs pEIAV-Donor and pEIAV-A2 have been previously described (28). RNA templates were generated by in vitro run-off transcription from linearized plasmids using T7 RNA polymerase as previously described (28). RNA donor DI was generated from pEIAV-Donor linearized with NotI and RNA AI-2 was generated from pEIAV-A2 linearized with HindIII. Internally labeled donor DI was synthesized by adding [α-32P]CTP to the in vitro transcription reaction. All RNAs were purified by PAGE. To assess the purity of the isolated transcripts, all RNAs were treated with calf alkaline phosphatase, 5′ end radiolabeled with polynucleotide kinase and [γ-32P]ATP, and run on a polyacrylamide gel.

**Preparation of Substrates**—To prepare RNA templates for 5′ end labeling, the 5′ phosphate was removed by calf alkaline phosphatase as per the manufacturer’s protocol. RNA and DNA were radiolabeled with polynucleotide kinase as per the manufacturer’s protocol using [γ-32P]ATP. Unincorporated ATP was removed by a Bio-Rad P-30 Micro BioSpin size exclusion column. Substrates were prepared by mixing together the RNA and DNA in the appropriate ratio and heating at 95 °C for 5 min followed by slow cooling in a heat block to room temperature. The sequences of the primers used were: dp1, 5′-TACGATTAGGTCACAGTATAG-3′; dp2, 5′-ACACTATAGAATATGCACTACATGTAAGCTTCAGGTATGGTCTGC-3′; and dp3, 5′-GGCCGGCACATTTAGTGACACTATAGATATGCACTAC-3′. Primers ddP2 and ddP3 were the same sequence as dp2 and dp3, respectively, except that the 3′ terminal base was a 2′,3′-dideoxycytidine instead of 2′-deoxyadenosine. The 41-mer RNA and 77-mer DNA have been previously described (9).

**Primer Extension and Strand Transfer Assays**—Reactions were performed as previously described with slight modifications (28). DI donor and radiolabeled dP1 primer were mixed in a 1:2 ratio prior to annealing for primer extension experiments. For strand transfer reactions a 1:2 ratio of donor:acceptor primer was used. RT was preincubated with annealed primer and template for 5 min prior to initiation by addition of MgCl2 and dNTPs. The final reaction contained 4 nM primer, 2 nM donor, 4 units of RT, 50 mM Tris-HCl (pH 8.0), 6 mM MgCl2, 50 μM dNTPs, 50 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. Strand transfer reactions additionally contained 4 nM acceptor. Reactions were incubated at 37 °C and were terminated at appropriate time points by the addition of 2X termination dye (20 mM EDTA (pH 8.0), 90% formamide, and 0.1% each of bromphenol blue and xylene cyanol). The products were separated by denaturing PAGE and visualized and quantitated by Storm PhosphorImager (GE Healthcare) and ImageQuant software version 1.2 (GE Healthcare). A 10-bp DNA ladder, 5′ end radiolabeled with [γ-32P]ATP using polynucleotide kinase per the manufacturer’s protocol, was loaded to serve as a size marker (labeled L in the figures).

**RNase H Cleavage Assays**—Reactions were performed as described above with some minor variations. DNA and radiolabeled RNA were mixed in a 10:1 ratio prior to annealing for donor cleavage experiments. The final reaction contained 2 nM template, 20 nM primer, 4 units of RT, 50 mM Tris-HCl (pH 8.0), 6 mM MgCl2, 50 mM NaCl, 1 mM dithiothreitol, and 1 mM EDTA. The concentration of dNTPs was 50 μM unless otherwise described. To determine the exact position of the cleavage fragments, reaction samples were run alongside RNase T1 and alkaline hydrolysis ladders generated from the donor template. For polymerase
trap experiments, 1 μg of poly(rA)-oligo(dT) per 12.5-μl reaction was added when the reaction was initiated.

RESULTS

We reasoned that the ability of RT to generate a series of adjacent cuts at the primary invasion site influences the efficiency of acceptor invasion. Experiments were designed to determine the origin of these cuts and their relevance to the transfer process.

Primer Extension and Donor RNA Cleavage by WT HIV-1 RT on EIAV Templates—The hairpin-containing template system used in this study has been previously described (28) and is illustrated in Fig. 1. The donor template region from +1 to +78 folds into a stable hairpin as predicted by mfold (37, 38). The acceptor shares a 97-nt region of homology with the donor that begins 19 nt before the hairpin base and extends to the 5' end of the hairpin. Synthesis is initiated from the 22-nt primer, dP1, bound to 3' end of donor DI. 8, illustration of donor DI structure within the region of homology (+1 to +97) with acceptor as predicted by mfold (37, 38). Hairpin structure extends from +1 to +78 on the donor template. Underlined bases represent positions at which point mutations in the acceptor are located.

FIGURE 1. Sequence and structure of templates used in this study. A, schematic of substrates used in this study. The 140-nt RNA donor, designated DI, and the 110-nt acceptor, designated AI-2, share a 97-nt region of homology. The region of homology begins 19 nt before the base of the hairpin and extends to the 5' end of the hairpin. Synthesis is initiated from the 22-nt primer, dP1, bound to 3' end of donor DI. B, illustration of donor DI structure within the region of homology (+1 to +97) with acceptor as predicted by mfold (37, 38). Hairpin structure extends from +1 to +78 on the donor template. Underlined bases represent positions at which point mutations in the acceptor are located.

FIGURE 2. Primer extension on donor template. Schematic above the panel illustrates the generation of FL and SP products from dP1 primer extension on donor DI. Primer extension reactions using a 5' end-labeled primer. A, WT; B, H539F; C, H539D; and D, H539R. The reactions were sampled at the times indicated above the lanes, and the products resolved on an 8% denaturing polyacrylamide gel. The prominent pause site at the hairpin base (+58/+59), other pause sites along the donor template (+44, +80, +93, and +120), as well as the FL and SP products, are indicated to the right of the gel. Lane L, 10-bp DNA ladder.

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monitored using a 5' end-labeled DNA primer annealed to the donor template. Extension of the dP1 primer by WT RT resulted in a full-length product (FL) that was 136 nt long and a self-priming product (SP) that was 194 nt long (Fig. 2A). Early in the reaction (1–3 min) the majority of extended primers were stalled at the base of the hairpin (+58/+59). Over time, synthesis proceeded beyond the base of the hairpin and the amount of FL product increased. However, the +58/+59 pause products remained prominent in these reactions. Aside from the major pause site at the hairpin base, pausing of WT RT was also observed at +44, +80, +93, and +120, and numerous minor sites.

Concomitant with primer extension, the RNase H activity of RT cleaves the donor template. Degradation of the donor template during primer extension was monitored by use of a 5' end radiolabeled donor template. Fig. 3A illustrates the 5' terminal donor fragments generated during synthesis by WT RT. By synthesis, we mean the overall primer extension reaction carried out by RT in the presence of primer-template and dNTPs, including primers that are extended, paused, or terminated. In the first minute of the reaction, extension of the primer to about 80 nt was detected. During this time, progressive cleavages on the donor gen-
End-directed Cleavages Using a Fixed Length Primer

Gao et al. (39) had reported that DNA 3′ end-directed secondary cleavages were suppressed in the presence of dNTPs. In our template system the 83-nt product thought to result from a secondary cut was observed during the course of synthesis in the presence of dNTPs (Fig. 3). For an analysis of DNA 3′ end-directed secondary cleavage in the presence of dNTPs, we designed the ddP2 primer, which was identical in sequence to dP2, but with a dideoxynucleotide at the 3′ terminus. No substantial change in the cleavage profile was observed as the concentration of dNTPs was varied from 0 to 300 μM using this primer (compare Fig. 4, C–E). The positioning of cuts generated with the ddP2/DI substrate were the same as those generated with the dP2/DI substrate (Fig. 4). This indicates that 3′ end-directed primary and secondary cleavages can occur while the RT is paused during synthesis.

His539 Mutants with Altered RNase H Cleavage Specificity—The analyses described above suggested that RT can catalyze DNA 3′ end-di-
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FIGURE 5. RNA 5' end-directed cleavage by WT and His539 mutant RTs. The substrate comprises a 5' end-labeled 41-nt RNA recessed on a 77-nt DNA. RNase H assay by WT, H539F, H539D, and H539R on 41-mer RNA recessed on a 77-nt DNA in the absence of dNTPs. The time points sampled are indicated above the lanes. Positions of cleavage are indicated in the schematic and to the right of the gel. The percentage of the primary (1°, 18 nt) and secondary (2°, 8 nt) cleavage products at the 30-min time point are: 13 (1°) and 36% (2°) for WT RT; 25 (1°) and 0% (2°) for H539F, 34 (1°) and 2% (2°) for H539D, and 26 (1°) and 11% (2°) for H539R. Lane C, control reaction containing all components except RT incubated for 30 min.

directed secondary cuts at a pause site and this can contribute to the creation of the primary invasion site in the EIAV template system. This observation implies that the ability of the RT to carry out primary and secondary cleavages is important in facilitating invasion and therefore strand transfer. An ideal test of this deduction would be to determine whether a mutant RT, incapable of secondary cleavage, produces an altered cleavage pattern at the primary invasion site, and is impaired for strand transfer. Whereas we could not make a mutant RT that uniquely lacks the secondary cleavage function, it was possible to study RT mutants in which the secondary cleavage is distinctly impaired. Because mutation of the His539 residue has been previously shown to be associated with secondary cut defects (17), we generated RT mutants with amino acid substitutions at this position. The polar, aromatic histidine at 539 was changed to the nonpolar, aromatic residue, phenylalanine (H539F), the polar, negatively charged aspartic acid (H539D), and finally the polar, positively charged arginine (H539R).

Characterization of RNA 5' End-directed Cleavages on a Recessed RNA Substrate—Because mutation of the His539 residue has been shown to impede secondary cuts, we first assessed the ability of the His539 mutants to perform RNA 5' end-directed primary and secondary cleavages. We examined RNase H cleavage on a substrate with an RNA primer recessed on a DNA template. WT RT produced a typical cleavage pattern with cuts positioned 18, 15, and 8 nt from the RNA 5' end (Fig. 5) (9). As time progressed the 8-nt secondary cleavage product predominated. For H539F, primary cleavage (18 nt) was less efficient compared with WT RT and the secondary cleavages (8 nt) were nearly absent. The cleavage pattern of the H539D and H539R mutants were similar. Both catalyzed efficient primary cleavage with secondary cleavage products detectable in lower amounts and only at later times. Although the efficiency of primary and secondary cleavage varied enormously, the position of cleavages were similar for all the RTs. The data indicate that the His539 mutants exhibit a particular defect in RNA 5' end-directed secondary cleavage, however, H539F also has considerable inhibition of primary cleavage.

Similar Characteristics of Synthesis on the EIAV Donor Template by WT and His539 Mutant RTs—Next, we compared the polymerase activities of the His539 mutants and WT RT by examining a time course of dP1 primer extension on the D1 template (Fig. 2). In agreement, with previous work (17, 18, 20, 21), mutation of the His539 residue had minimal effect on the polymerase activity. The synthesis profiles of H539F, H539D, and H539R were similar to the profile obtained with WT RT. At early time points there was significant pausing at the base of the hairpin and at later time points, there was a considerable amount of full-length product. As with WT RT, pausing at the hairpin base resulted in a prominent extension product throughout the reaction time course. The location and intensity of the pause site at the hairpin base and other minor pause sites were similar for the four RTs tested. Additionally, each of the RTs synthesized comparable levels of full-length product.

A unique feature of the synthesis profile of the His539 mutants is their inability to form the SP product. The SP product is generated from a full-length cDNA product in which the 3' end folds back onto itself to form a hairpin structure allowing the remaining 5' region of the cDNA to be used as a template for synthesis (see schematic in Fig. 2). Self-priming is inhibited when terminal cleavages at the 5' end of a hairpin template are inhibited (34, 40–42). Therefore, the absence of SP products is consistent with secondary cut defects in the mutants. These results indicate that the defects in the His539 mutants are largely confined to RNase H function.

The His539 Mutant RTs Are Defective in Strand Transfer—Next, we characterized the ability of the His539 mutants to perform strand transfer using the EIAV donor and acceptor substrates illustrated in Fig. 6. The acceptor shares a 97-nt region of homology with the donor that starts 19 nt before the hairpin base and extends to the 5' end of the hairpin. If the primer were able to switch templates and initiate synthesis on the acceptor at any point within the region of homology, synthesis to the end of the acceptor would produce a transfer product (TP) 149 nt in length. Transfer efficiency, which is a measure of the amount of transfer product generated relative to the amount of all completed products, was determined for WT RT and the His539 mutants. The formula used to determine the transfer efficiency is TE = TP/(FL + TP + SP) x 100%. The transfer efficiency of WT RT at 30 min was 20%. In comparison, the transfer efficiency values of all the mutants were severely reduced. H539F was most defective with <1% transfer. H539D and H539R catalyzed about 3% transfer.

Cleavage Specificity of the His539 Mutant RTs during Synthesis—We next inquired whether inefficient strand transfer by the His539 mutants could be explained by their inability to generate the primary/secondary cleavages at the hairpin base that were important for creation of the primary invasion site. To test this idea, the patterns of donor degradation during primer extension using WT RT and the His539 mutants were compared using a 5' end-radiolabeled donor template (Fig. 3). As the primer is extended, regions closer to the 5' end of the RNA become susceptible to RNase H cleavage by RT, shifting the banding pattern from longer to shorter products over time. Within the first 3 min of the WT RT reaction, bands at +68, +50, +27, and +17 were produced. Significantly, cleavage fragments 93 and 83 nt long, which correspond to 15 and 5 nt before the hairpin base, were observed with WT RT (indicated by a bracket in Fig. 3). The cleavage patterns of the His539 mutants in this same region were noticeably different. For all three mutant RTs significant cleavage around +110 and +100 was observed. Additional cleavage bands around +96 were observed with H539D and H539R. Cleavage bands at +93 and +83, prominent with WT RT, were faint with the His539 mutants. These data indicate that during primer exten-
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Cleavages near the 5' end of the donor template were particularly evident at later times. Within the first 3 min of reaction, WT RT generated cleavage bands at +17 and +10, which correspond to the expected products of primary and secondary cleavages, respectively, after completion of full-length synthesis. For H539F even after 30 min, the prominent cleavage bands represented 110- and 100-nt products with some cleavage producing a smaller fragment of 21 nt. For H539D and H539R, significant amounts of the 21- and 17-nt fragments accumulated over time, with almost undetectable smaller products. For the H539 mutants, the absence of cleavage fragments less than 17 nt in length indicates that the mutants are particularly inhibited in secondary cleavage on a blunt end substrate. For each RT incubation, E. coli RNase H was added after the 30-min time point was taken and those control reactions are shown in Fig. 3E. The appearance of cleavage bands 10 nt and smaller in all these control reactions confirms that the primer was extended to the end of the donor template. Therefore the His539 mutants extended the primer to the end of the template but failed to make secondary cleavages at the RNA 5' end.

These results reveal cleavage alterations during synthesis for the His539 mutants. Compared with WT RT, the His539 mutants produced fewer prominent cuts within the invasion site. The +93 and +83 bands that correspond to primary and secondary cleavages when paused at the hairpin base were less significant with the His539 mutants than with WT RT. The defect in secondary cleavage displayed by the mutant RTs correlates with the absence of a prominent +83 band thought to be produced by secondary cleavage during synthesis. This is additional evidence that some cleavage in the primary invasion site is caused by secondary RNase H cleavage during synthesis. Additionally, the secondary cleavage defects of the His539 mutants are also manifested at the 5' region of the donor RNA by the absence of terminal fragments less than 17 nt in length. Overall, these results highlight the ineffectiveness of the His539 mutants in carrying out secondary RNase H cleavages while paused at the hairpin base and also at the 5' terminus of the RNA.

Defects in Donor Cleavage at the Hairpin Base—During synthesis, the His539 mutants did not produce prominent cleavage bands at +93 and +83 that we attribute to primary and secondary cleavages while paused at the hairpin base. To further characterize the altered specificity of the His539 mutants at the hairpin base, we examined their cleavage pattern using the dP2 and ddp2 primers previously described in Fig. 4.

The cleavage patterns of the His539 mutants using fixed length primer dP2 were similar to those observed during primer extension (compare Fig. 7A to Fig. 3). Prominent cleavage bands at 110 nt were observed for all three mutant RTs. For H539D and H539R, cleavage bands at +93 and +83 nt became more prominent at later time points. Cleavage fragments produced by the His539 mutants with the dP2/ΔI substrate were quite different from those made by WT RT. With the His539 mutants the band corresponding to a 3' end-directed primary cleavage product (93 nt) was observed, however, generation of this product was slower than with WT RT. Additionally, there was a striking absence of the secondary cleavage product (83 nt) for all three His539 mutants. The ability of the His539 mutants to make primary cleavages at the hairpin base was impaired, and secondary cleavages at the hairpin base were absent. Use of dP2/ΔI substrate in the presence of 50 μM dNTPs (Fig. 7B) produced a similar cleavage profile as dP2/ΔI, indicating that the presence of dNTPs did not significantly inhibit cleavage. These observations further link the deficiency of the His539 mutant RTs to carry out primary and secondary cuts at the hairpin base in the absence of synthesis with the banding pattern seen at the primary invasion site during synthesis.

We were interested in whether the inability of the His539 mutants to perform 3' end-directed secondary cleavage was a general property of the mutants or was specific for a hairpin structure. To address this question, we examined the cleavages produced using a set of primers (dP3 and ddp3) having 3' termini within a region that was not predicted to form a hairpin structure and at which no pausing during primer extension was observed (Fig. 7). With these substrates, a set of primary and secondary cleavages were observed with WT RT (data not shown). However, whereas H539F, H539D, and H539R generated primary cleavage products, no secondary cleavage products were observed (data not shown). These data indicate that the His539 mutants are inhibited in their ability to perform secondary cleavage directed by the primer 3' end and this inhibition is not contingent upon the presence of a hairpin structure or the need to carry out strand displacement.

Characterization of Overall Donor Degradation during Synthesis—On a 5' end-labeled RNA, cleavage fragments generated during the course of synthesis present a view of progressive cuts as the cDNA-RNA
hybrid is formed. However, this does not allow detection of all fragments generated from multiple cleavage events. Use of an internally labeled donor for degradation experiments allows for detection of overall donor cleavage, including contributions from internal and 5’/3’ end-directed cleavage. To assess the overall effects of the 539 mutation on RNase H function, we compared donor degradation by WT RT and the His539 mutants with this approach. The degradation profile of the internally labeled donor during dP1 primer extension is shown in Fig. 8. The WT RT generated prominent bands at 93 and 83 nt, similar to the pattern observed in Fig. 3 where the donor is labeled at the 5’/3’ end. At early times, bands at 93 nt and less than 10 nt were predominant, indicating that WT RT had to have cleaved the template multiple times on the same donor RNA. With the WT RT, the cleavage pattern around the hairpin base was similar to that observed in Fig. 3 in which the donor was labeled at the 5’ end. For all three mutant RTs, a significant cleavage band at +110 was produced. Other cleavages around +100 were prominent with the His539 mutants.

The distribution of small size fragments (<25 nt) varied greatly among the RTs. WT RT produced fragments predominantly less than 10 nt in size even within the first min of reaction. In contrast, with H539D and H539R only trace amounts of cleavage fragments less than 25 nt long were observed within 1 min. At later time points, small fragments ranged in size from 20 nt to less than 10 nt. This range of products persisted for the duration of the reaction (30 min). For H539F, the prominent cleavage fragments were quite large indicating that much of the donor does not get degraded to fragments of small size, even at longer time points. These observations support the conclusion that the His539 mutants have diverse defects in RNase H function, including, but not limited to, their ineffectiveness at secondary cleavage at the hairpin base. It is very likely that the inability to effectively cleave the donor template affects the ability to promote strand transfer. However, the relative contribution of the different mechanisms of RNase H cleavage cannot be distinguished.

DISCUSSION

In previous work we determined that the predominant mechanism for strand transfer in an RNA hairpin template system is a pause-initiated two-step dock and lock process (28, 29). Evidence from other studies suggests that this mechanism is employed for strand transfer events...
associated with replication and recombination in retroviruses (33, 34, 43, 44). Pause initiated cleavage of the donor template at the docking or invasion site is a fundamental component of this mechanism. Several lines of evidence had pointed to the region before the hairpin, designated the invasion site, as the place where the first interaction of the acceptor with the cDNA occurs (28, 29). This evidence includes the following: (a) although transfer products in this template system were generated by primer terminus transfers well past the pause site, a short DNA oligomer of the same sequence as the donor at the region of extensive cleavage before the hairpin base effectively inhibited transfers. (b) Transfers decreased 10-fold when a shorter acceptor, lacking the homologous region before the hairpin base was used. (c) The ineffectiveness of EIAV RT at promoting transfers correlated with its inability to make extensive cleavages at the predicted invasion site. In the presence of nucleocapsid protein, EIAV RT made more extensive cleavages at the hairpin base and also promoted more efficient transfers.

Considered together, the evidence indicates that cuts on the donor template before the hairpin base are likely to contribute to creation of an invasion site and thereby facilitate strand transfer. Interestingly, although HIV-1 RT produced a single major pause (+58/+59) at the hairpin base during primer extension, it made a series of prominent cleavages between 15 and 5 nt before the base. In the current study, we investigated these cleavages that contribute to creation of the invasion site at the hairpin base. We hypothesized that the cleavages were DNA 3’ end-directed primary and secondary cleavages that occur while the RT is paused at the hairpin base. Such cleavages had been observed when the RT was bound to the 3’ end of a DNA-primed RNA template in the absence of synthesis (2, 13–17).

To test for synthesis related primary and secondary cleavage, we compared the cuts produced by WT RT during primer extension (dP1) to those generated in the absence of synthesis using a substrate that was designed to represent a primer paused at the hairpin base (dP2, Fig. 4). The presence of dNTPs in the synthesis reaction results in primers that are actively elongating plus those that are paused or terminated at the time the reaction is sampled. The formation of the 93- and 83-nucleotide cleavage products in the dP2/DI reaction confirmed that these cuts could be generated by DNA 3’ end-directed primary and secondary cuts at the pause site. However, Gao et al. (39) had previously observed that DNA 3’ end-directed secondary cleavages were suppressed in the presence of dNTPs. As a result we were concerned that the secondary cleavages observed with the dP2 primer in the absence of dNTPs may not accurately reflect the cleavage products generated by RT during synthesis.

To address this issue and more faithfully simulate conditions during synthesis, we generated a 3’ dideoxy-terminated primer (dD2P). This allowed us to compare the cleavages produced in the presence of dNTPs to those generated in its absence. The 93- and 83-nucleotide products continued to be observed even in the presence of dNTPs. In fact, the pattern and intensity of the cleavage bands remained similar as the dNTP levels were increased from 0 to 300 μM (Fig. 4). We cannot determine the reason for the difference between our findings and those of Gao et al. (39) regarding the dNTP dependence of 3’ end-directed secondary cleavage. However, we consider it likely that variations in template sequence account for the difference, as their experiments examined cuts generated within the PPT sequence of HIV-1. Considered together, the results presented in Fig. 4 show that the 93- and 83-nucleotide cuts observed under conditions of synthesis could be generated by 3’ end-directed primary and secondary cleavages at the pause site.

Primary cleavages occur at a distance behind the 3’ terminus corresponding to the physical separation of the polymerase and RNase H active sites within the RT. As such they would be expected during synthesis, and be most likely to occur when the RT is paused. However, secondary cleavages should require that the polymerase active site of the RT detach from the primer terminus. This is because secondary cleavages can only be made if the RNase H active site slides forward 8–10 nucleotides in comparison to positioning for primary cleavages. We have previously observed DNA 3’ end-directed primary and secondary cuts, although these studies were done in the absence of synthesis (no dNTPs). In this study we present evidence that during synthesis WT RT makes 3’ end-directed primary and secondary cuts while paused at the base of the hairpin.

So how is the enzyme able to move away from the terminus to make the secondary cut? Because polymerases in general are thought to have a high affinity for the primer terminus such a positioning would be unexpected during processive synthesis. This is likely the case for HIV-1 RT, as well. Conceivably, the observed process only occurs at a pause site, when synthesis is halted and the polymerase is not in a processive synthesis mode. In fact, the intense pausing at the hairpin base by the WT and mutant RTs indicates that reinitiation from the stalled primer terminus is not very efficient at this site. We envision a scenario in which inefficient primer extension at the pause site provides more opportunity for RT to reposition at the primer terminus, facilitating secondary cleavage. Based on the above interpretation, we predict secondary cuts to occur during the course of synthesis, but most likely only at pause sites. In support of this idea, studies examining minus strong stop transfer show significant cuts 18 and 8 nt behind the base of the TAR hairpin that are likely generated from 3’ end-directed primary and secondary cleavages, respectively, whereas the RT is paused at the base of TAR (33, 42). Furthermore, a series of templates with altered structures also showed a correlation between pausing during synthesis and cleavages on the donor template 18 and 8 nt behind (45). Experiments conducted in the presence of polymerase trap (poly(rA)-oligo(dT)) provide further insight into the nature of the secondary cleavages at the hairpin base. The secondary cleavage product (+83) was absent when trap is added to cleavage reactions using WT RT and dD2P/DI and ddP2/DI substrates (data not shown). These data indicate that secondary cuts at the hairpin base result from a rebounding event, rather than a reorientation of a bound RT.

Apparently, creation of the invasion site involves successive cuts while RT is paused at the hairpin base. Therefore, we predict that the ability of RT to make these cuts facilitates strand transfer in this system. In support of this concept, previous studies with EIAV RT using these templates showed that addition of nucleocapsid protein increased the efficiency and distribution of cuts at the hairpin base and also produced more transfer (29). If secondary cuts contribute to creating successive cleavages at the invasion site, an RT that lacks the ability to perform secondary cleavages would be expected to be impaired in strand transfer in the EIAV system. Previous studies have indicated that certain amino acid substitutions at position 539 of HIV-1 RT inhibit secondary cuts (17). This provided us with an opportunity to produce mutant RTs with the desired properties. For our studies we compared WT RT to three mutants with amino acid substitutions at position 539: H539F, H539D, and H539R. Because the effect of any mutation is pleotropic, the creation of three mutations offered a better opportunity to distinguish the consequences of inhibition of secondary cleavages from other effects of each mutation.

Analysis of RNA 5’ end-directed cleavages indicated that all three mutants are severely defective in RNA 5’ end-directed secondary cuts (Fig. 5). H539F also exhibited lower primary cleavage ability; however, primary cleavage with H539D and H539R was only slightly diminished relative to WT RT. In agreement with previous studies, mutations at the
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539 position had minimal effects on DNA polymerase activity, and all three mutants displayed similar characteristics as the WT RT for primer extension on the EIAV template (Fig. 2).

The mutant RTs displayed important characteristics with respect to DNA 3′ end-directed cleavages. In comparing donor cleavage during extension, the prominent series of cleavages that were attributed to creation of an invasion site with WT RT were greatly reduced with the His539 mutants (Fig. 3). As predicted, the band at +83, caused by secondary cleavage during the major pause, was faint. Consistent with our initial conclusions, this result showed that RTs that are defective in secondary cleavage fail to make the cut that we had attributed to secondary cleavage during synthesis. The 93-nt product from DNA 3′ end-directed primary cleavage at the hairpin base was also not very prominent.

Analysis of 3′ end-directed cuts using the fixed length primers dP2 and ddP2 confirmed that all three mutants displayed altered cleavage specificity at the hairpin base (Fig. 7). They were inhibited in primary cleavage and particularly defective in secondary cleavage. Cleavage by WT RT resulted in products predominantly in the +93 to +83 region, and was indicative of RT cleavage directed by the DNA 3′ terminus. However, at early time points all of the mutants produced a prominent cleavage product at +110, which was not directed by the DNA 3′ terminus. Two plausible explanations for this difference are that the mutants do not bind efficiently at the hairpin base or prefer the 110-nt site for binding. At later time points, H539D and H539R generated +94 and +93 cleavage products indicative of DNA 3′ end-directed primary cleavage, however, at a rate slower than WT RT. The H539D and H539R mutants were unable to generate further cleavages within the primary invasion site. Furthermore, H539F primary and secondary cleavages at the hairpin base were severely inhibited, as indicated by the presence of the 110-nt fragment even after a 30-min incubation. These results establish that the mutant RTs have altered cleavage specificity at the hairpin base. The mutants have reduced primary cleavage and produce no bands corresponding to secondary cleavage products. Moreover, these defects are evident within the structure and sequence context of the major pause site in the EIAV hairpin system.

As expected, the mutant RTs were inefficient at promoting strand transfers, compared with WT RT. As revealed from the donor cleavage profile (Fig. 3), the mutants were inefficient at making the series of cleavages within the 19-nt region of homology before the hairpin base. A simple interpretation is that the His539 mutants were unable to generate sufficient cleavage to promote transfer at the invasion site. This may, in fact, be the main reason for the reduced transfer efficiency. However, the mutations also cause other effects on the properties of RNase H activity that could affect transfer efficiency. For example, the inability to make 5′ end-directed secondary cuts can impede polymerase-independent RNase H cleavages. This activity is thought to allow further degradation of the genomic RNA after cuts made during synthesis. In our in vitro system, such cleavages may contribute to clearing away large RNA fragments (7, 8). In fact the RNase H cleavage profiles produced by the His539 mutants with the internally labeled donor template support this idea (Fig. 8). The H539F was extremely inefficient in RNase H cleavage, producing negligible amounts of small fragments (<10), even after 30 min. However, H539D and H539R generate small fragments, although at a slower rate compared with WT RT. These defects associated with the His539 mutants also may affect the transfer efficiency by slowing the hybrid propagation and terminus transfer steps of the transfer mechanism.

Finally, during primer extension the His539 mutants show an inability to cleave the 5′ terminus of donor template to fragments less than 17 nt in size (Fig. 3). In contrast, WT RT generated terminal fragments 8–12 nt long. However, the differences in 5′ terminal cleavages are unlikely to greatly influence strand transfer, as determination of the transfer product distribution obtained with WT RT revealed less than 10% of the transfers occurred at the end of the donor template (28).

Our analyses reveal various RNase H defects in the His539 mutants. Prominent among them is the inability to catalyze secondary cuts, which can impede various aspects of the overall RNA template degradation process. Therefore, we conclude that the inability of the His539 mutants to perform DNA 3′ end-directed primary and secondary cleavages alters the cleavage pattern at the invasion site in a way that could inhibit strand transfer. However, the other deficiencies of RNase H activity exhibited by the mutant RTs are also likely contributors to the observed transfer defects.

The dynamic copy choice model presented by Pathak and co-workers (46) proposes that the efficiency of strand transfer is determined by a balance between the polymerase and RNase H activities of RT. Decreasing the amounts of overall RNase H activity decreased strand transfer. Particularly of interest, the H539N mutant of HIV-1 RT, previously described as having reduced secondary cleavage properties similar to H539D (17), showed a 2-fold reduction in transfer efficiency in vivo compared with WT RT (22). Our studies further these ideas by addressing the nature of the RNase H cleavages at pause sites and their contribution to strand transfer. Our data suggest that the specificity of RNase H cleavage may also influence the efficiency of strand transfer. These findings may have implications for the screening of RT inhibitors and future drug design.

In summary, we show evidence that some of the cleavages made in the viral RNA genome by the HIV-1 RT during minus strand synthesis occur by pause-related DNA 3′ end-directed primary and secondary cuts. DNA 3′ end-directed secondary cleavages indicate a departure of the RT from the primer terminus and, as such, were unexpected during synthesis. In our test system these secondary cleavages are part of the prominent cleavages that clear the primary invasion site for strand transfer. The results presented suggest that the ability of the HIV-1 RT to perform DNA 3′ end-directed primary and secondary cleavage during synthesis is a contributor to strand transfer that occurs during viral replication.

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