Substituting a Conserved Residue of the Ribonuclease H Domain Alters Substrate Hydrolysis by Retroviral Reverse Transcriptase*

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Jason W. Rausch‡§ and Stuart F. J. Le Grice¶¶
From the ‡Department of Biochemistry, ¶¶Division of Infectious Diseases, and ¶¶Center for AIDS Research, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106-4984

Altersations to the highly conserved Asp\(^{549}\) of the retroviral ribonuclease H (RNase H) domain were evaluated in the heterodimeric (p66/p51) reverse transcriptases of human immunodeficiency and equine infectious anemia viruses. In addition to the polymerization-dependent and -independent modes of template hydrolysis, mutants were evaluated via their ability to select and extend the 3′ polypurine tract (PPT) primers of these two lentiviruses into (+) strand DNA. Concerted and two-step reactions were designed to evaluate (+) strand priming, the latter of which allows discrimination between selection end extension events. In contrast to enzyme mutated at the highly conserved Glu\(^{478}\), substitution of Asp\(^{549}\) with Asn or Ala reduces, rather than completely eliminates, RNase H activity. When the requirement for RNase H function becomes more stringent, differences in activity are readily evident, most notably in the cleavage events liberating the 5′ terminus of the PPT primer. PPT selection thus appears to represent a specialized form of RNase H activity that is more sensitive to minor structural alterations within this domain and may provide a novel therapeutic target.

Although nonspecific hydrolysis of the RNA-DNA replication intermediate might be considered the primary function of the C-terminal ribonuclease H (RNase H)\(^1\) domain of retroviral reverse transcriptase (RT), increasing attention is being given to the extensive repertoire of highly specialized RNase H-mediated events necessary to synthesize a double-stranded provirus from the viral RNA genome. For example, polymerization-independent RNase H activity (which hydrolyzes the RNA template from the point of initial endonucleolytic cleavage within the C-terminal reverse transcriptase domain) has been demonstrated to be essential for efficient transfer of nascent (+) strand DNA between the 5′ and 3′ termini of the RNA genome (1, 2). In addition, (+), or second-strand DNA synthesis requires highly specific RNase H cleavage to release the 3′ OH of the polypurine tract (PPT) primer (3–5). Finally, the (+) and (–) strand RNA primers (the PPT and a host-derived tRNA, respectively) must be precisely removed from nascent DNA to preserve the integrity of the 5′ and 3′ terminal repeat termini of the provirus for recognition by the retroviral integration machinery (3, 6, 7). Each of these events can be considered candidates for therapeutic intervention by antiviral agents in the ongoing effort to stem the progression of human immunodeficiency virus (HIV) infection and acquired immunodeficiency syndrome. However, the success of such ventures would benefit from a more detailed analysis of RNase H-mediated events during retroviral replication.

Structural similarities between RNase H domains of retroviral and bacterial origin (8) make a strong case for metal ion co-ordination by the side chain carboxylates of Asp\(^{443}\), Glu\(^{478}\), and Asp\(^{498}\) (Asp\(^{110}\), Glu\(^{48}\), and Asp\(^{70}\) in Escherichia coli RNase H1). However, a fourth residue, Asp\(^{549}\) (Asp\(^{134}\) in E. coli) is also conserved among these enzymes, the significance of which is not immediately apparent. From the crystal structure of the isolated RNase H domain of HIV-1 RT, Davies et al. (8) have suggested that Asp\(^{549}\) could participate in binding a second metal ion, thereby invoking a two-metal ion-catalyzed mode of hydrolysis (9). Alternatively, hydrogen bonding between the backbone carbonyl oxygen of Asp\(^{498}\) and the side chain hydroxyl of Ser\(^{503}\) may have important architectural consequences for structural elements that aid in accommodating the template primer duplex within the C-terminal RNase H domain (α-helix E′ and the β2′-αE′ connecting loop (10, 11)). The latter possibility was initially supported by mutagenesis studies with HIV-1 RT, indicating that removing Ser\(^{503}\) via an eight-residue C-terminal deletion selectively eliminated polymerization-independent RNase H activity, concomitant with which was a substantial reduction in DNA strand transfer activity (12). However, when an equivalent enzyme from EIAV was evaluated, RNase H activity was unaffected but could be altered by removal of an additional two residues (13). In the enzyme from E. coli, Haruki et al. (14) have suggested that Asp\(^{134}\) is less likely to be involved in maintaining overall architecture, and that it might indirectly contribute toward catalysis by preserving active site geometry through negative charge repulsion. However, because Asp\(^{134}\) of E. coli RNase H forms a salt bridge with Arg\(^{138}\), an additional role in stabilizing α-helix V (counterpart of α-helix E′ in the HIV-1 enzyme) cannot be excluded. These observations prompted us to more precisely evaluate the role of Asp\(^{549}\) in two structurally related lentiviral enzymes (both HIV-1 and EIAV RTs are heterodimers of 66- and 51-kDa subunits) by site-directed mutagenesis.

In addition to the polymerization-dependent and -independent modes of RNase H-mediated hydrolysis, we elected to investigate the consequences of amino acid alterations in the RNase H domain on PPT: (a) selection; (b) extension into (+) strand DNA; and (c) removal from nascent (+) strand DNA, using RNA-DNA duplexes within which the PPTs of HIV and EIAV were embedded. Recent reports have indicated that the r (purine)/d (pyrimidine) configuration confers considerable ther-
mal stability on the duplex compared with its d (purine/r (pyrimidine) counterpart (15). Furthermore, model hybrids mimicking the PPT demonstrate an unusual CD spectrum, suggestive of a novel conformation (16). Given these potentially unique features of the PPT, subtle alterations to the geometry of the RNase H domain may have significant impact on PPT selection and/or its subsequent release from nascent (+) strand DNA. In addition to highlighting differences in the modes of hydrolysis of HIV-1 and EIAV RT, we demonstrate in this communication that substituting Asp^{549} with Asn compromises selection of the HIV-1 3′ PPT and its release from (+) strand DNA. The ability of the same mutant to extend a preselected PPT into (+) strand DNA as efficiently as wild-type enzyme suggests that the mutation Asp^{549} → Asn does not have global structural consequences.

**EXPERIMENTAL PROCEDURES**

**Construction and Purification of EIAV and HIV RT Mutants**—The coding sequence for EIAV protease (17) was amplified from pEIAV (a gift of Dr. S. L. Payne) by the polymerase chain reaction and ligated into pDS56RBSII (18) as a BamH\-HindIII fragment. The resulting EIAV protease expression cassette was introduced to pDH EIAV RT (19) as an XhoI fragment, creating pEIAV PR-6H RT, which controls co-expression of p66 EIAV RT and protease. The analogous HIV vector, pHHRT- PROT, has been described (18). Mutation of HIV and EIAV RT genes at amino acid position 549 (D549N and D549A) was achieved using the USE mutagenesis kit (Pharmacia Biotech Inc.).

Purification of HIV RT has been described (18), whereas the EIAV enzyme was purified essentially as described by Rausch et al. (13), with the exception that cultures were harvested and cooled to 4 °C 30 min after induction to prevent overdigestion of EIAV RT by protease. Metal chelate (Ni^{2+}-NTA-Sepharose) and Mono-S ion exchange chromatography (Pharmacia Biotech Inc.) yielded highly pure enzymes free of contaminating nucleases, with a 1:1 stoichiometry of p66 and p51 subunits. Purified enzymes were stored at 20 °C in 50 mM Tris-HCl, 8 mM MgCl\(_2\), 0.1 mM EDTA, 25 mM NaCl, and 5 mM dithiothreitol.

**Preparation of HIV and EIAV 3′ PPT-containing RNAs**—The location of the HIV 3′ PPT has been determined (20), whereas location of the EIAV 3′ PPT was based upon homology to HIV. Polymerase chain reaction was used to generate fragments of the HIV and EIAV genomes containing 3′ sequences from pHab2 (21) or pEIAV, respectively. To standardize the positioning of PPTs on the H3 RNA template annealed to a 20-nt DNA primer, with the extreme 3′-G-A dinucleotide of the PPT was flanked by 50 and 70 bases of genomic sequence at the 5′ and 3′ termini, respectively. These products were introduced as XhoI/BglII fragments into pSP72 (Promega Corp.) to generate transcription vectors pH3 and pE3, respectively. 126 nt (+) strand DNTPs and [α-\(^{32}\)P]dATP in the presence of an “extending” enzyme. To evaluate (+) strand DNA synthesis, HIV-1 RNA-DNA hybrid (50 nM) was incubated with RT (85 nM), 50 μM dNTPs, and [α-\(^{32}\)P]dATP in reaction buffer for 60 min at 37 °C. Reactions were terminated by heat denaturation (90 °C for 2 min), after which one-half of each mixture was added to 0.3 volume 1 n NaOH and incubated at 65 °C for 20 min to achieve complete RNA hydrolysis (the remaining portion was stored at 4 °C). Reaction products were then precipitated (50% isopropanol and 3.5 mM NH\(_4\)Ac) and resuspended in a urea-based, gel-loading buffer.

To distinguish between PPT selection from primer-extension, the RNA-DNA hybrid containing the HIV-1 3′ PPT (50 nM) was initially incubated with a “selecting” enzyme (85 nM each) in the absence of nt for 30 min at 37 °C, thus preventing DNA synthesis from occurring. The RNase H hydrolysis products were next extracted with phenol/chloroform, precipitated, resuspended in DNA synthesis buffer, and evaluated for their ability to support (+) strand synthesis by the addition of dNTPs and [α-\(^{32}\)P]dATP in the presence of an “extending” enzyme. After 30 min at 37 °C, DNA synthesis was terminated, and the products were treated as described above. Reaction products were fractionated by high-voltage gel electrophoresis through 10% (w/v) polyacrylamide gels containing 3 M urea and 0.1% SDS. The RNase H activities of all mutants were determined in three volumes of ethanol, 0.1 volume of 3 M NaOAc, pH 5, and dried to constant weight.

**Analysis of DNA- and RNA-dependent DNA Polymerase Activities**—DNA-dependent DNA polymerase activities were assessed on a 71-nt DNA template annealed to a 36-nt DNA primer as described by Rausch et al. (13). Enzyme (8 nM) and template/primer (8 nM) were incubated 1 min in buffer containing 10 mM Tris-Cl, pH 8.0, 6 mM MgCl\(_2\), 80 mM NaCl, and 5 mM dithiothreitol. DNA synthesis was initiated, poly(dNTPs) were added to a final concentration of 50 μM. Aliquots were removed at the times indicated in the text and mixed with urea-based, gel-loading buffer. RNA-dependent DNA synthesis was assessed in a similar manner on the H3 RNA template annealed to a 20-nt DNA primer, with the exception that DNA synthesis was terminated after 10 min and the enzyme/template-primer ratio was increased 2-fold. Single-round DNA synthesis conditions were achieved by adding heparin to a final concentration of 2 mg/ml, as described previously (13). Reaction products were fractionated by high-voltage gel electrophoresis through 10% (w/v) polyacrylamide gels containing 7 M urea in Tris borate/EDTA buffer. After drying, gels were subjected to autoradiography, using the DuPont X-ray film.

**Analysis of RNase H Activity by High-resolution Gel Electrophoresis**—A 5′-32P-end-labeled, heteropolymeric 90-nt RNA hybridized to a 36-nt DNA primer was used to evaluate RNase H activity in the presence of DNA synthesis (22). Enzymes were incubated with template/primer (final concentrations, 150 and 50 nM, respectively) in the absence of Mg\(^{2+}\), under conditions described previously (12, 21, 23). Hydrolysis was initiated by addition of MgCl\(_2\) to a final concentration of 6 mM and terminated at the times indicated in the text by the addition of Tris borate/EDTA buffer containing 100 mM Tris, pH 8.5, 100 mM borate, 2 mM EDTA, and 7 mM urea. Alternatively, the final concentration of MgCl\(_2\) was varied as described. RNase H activity was also examined on the same substrate, the 3′ terminus of which was end-labeled with [\(^{32}\)P]dCTP and RNA ligase (NEB) under conditions recommended by the manufacturer. Reaction mixtures containing enzyme and template-primer concentrations were 43 and 12 nM, respectively. Hydrolysis products were fractionated by denaturing high-voltage electrophoresis and analyzed by autoradiography. Product size was determined by co-electrophoresis of partial RNase A and alkaline hydrolases of the radiolabeled RNA template.

**RESULTS**

**DNA Polymerase Activities of RNase H Mutants**—p66/p51 heterodimers of HIV-1 and EIAV RT carrying the mutations Asp^{549} → Asn and Asp^{549} → Ala were evaluated in this study to assess structurally related enzymes. The conservative substitution with asparagine introduces a residue with similar polarity and volume; in contrast, substitution with alanine introduces a neutral side chain, while minimizing unfavorable steric contacts and avoiding imposition of new charge interactions or hydrogen bonds. To eliminate the possibility that major structural changes accompanied amino acid substitutions in the RNase H domain, the DNA polymerase activities of all mutants were first assessed on two heteropolymeric template-primer combinations, the results of which are illustrated in Fig. 1.

The 71-nt DNA template/36-nt DNA primer of our studies contains a short template hairpin 15 nt upstream from the primer terminus. This structure has the potential to trap the replication machinery but is eventually overcome, resulting in synthesis of a full-length, 72-nt cDNA (10, 13). In the time course of Fig. 1A, this pattern is demonstrated for the wild-type HIV-1 (panel ii) and EIAV enzymes (panel [iv]). Minor differences in processivity were observed when Asp^{549} was substituted with either Asn (panels [ii] and [iv]) or Ala (panels [iii] and [vi]), although this most
likely reflects experimental error. Taken together, the data of Fig. 1 suggest that DNA-dependent DNA polymerase activity of the HIV-1 and EIAV RT mutants was unaffected. This is strengthened by the data of Fig. 1B, which assesses the RNA-dependent DNA polymerase activity of each mutant on a 126-nt template derived from the HIV-1 genome. In this experiment, DNA synthesis was also evaluated in the presence of the competitor heparin, which restricts polymerization to a single binding event. For all enzymes tested, the addition of heparin highlighted dissociation of the replication complex within 10 nt of the primer terminus and subtle alterations in the stalling pattern further along the template. Collectively, the data of Fig. 1 thus indicates that the DNA polymerase catalytic center of all enzymes retained the appropriate structural integrity.

Analysis of RNase H Activity on a 3′-labeled Substrate—Initial characterization of RNase H activity of the HIV-1 and EIAV mutants made use of a 90-nt RNA template/36-nt DNA primer described previously (12, 13), with the exception that radiolabel was introduced at the 3′ end of the template. By doing so, it was possible to distinguish products of the first endonucleolytic cut from those defined by polymerization-independent (27, 28) or directional processing RNase H activity (29). Under these conditions, the data of Fig. 2 indicate considerable differences in the manner in which HIV-1 and EIAV RT hydrolyze the model RNA-DNA hybrid. For HIV-1 RT, the primary hydrolysis products span template nt 216 to 221, with the most prominent events at positions 220 and 221 after 5 s. This hydrolysis profile indicates relaxed cleavage specificity of the HIV-1 enzymes but is essentially in keeping with reports suggesting that the RNase H and DNA polymerase catalytic centers are separated by a distance of 17–20 base pairs (27, 28, 30). Under the same conditions, template hydrolysis by the HIV-1 mutant p66D549N/p51 was only slightly reduced, while activity with mutant p66D549A/p51 was virtually undetectable. Under equivalent conditions, a different hydrolysis profile was generated by the EIAV enzyme.
position of hydrolysis, which was restricted predominantly to template nt –16 and –17. Once again, the overall level of hydrolysis was reduced with EIAV mutant p66D549N/p51 and absent in mutant p66D549A/p51. Because both the HIV-1 and EIAV enzymes used in this study are heterodimers of 66- and 51-kDa subunits sharing ~50% amino acid homology, differences in their RNase H hydrolysis profiles are unlikely to reflect major structural differences between the two enzymes. Although features of the two enzymes contributing to the different hydrolysis profiles will be addressed later, the data of Fig. 2 suggest that substituting Asp549 of either retroviral enzyme with Asn appears to have little effect on initial endonucleolytic cleavage of the template, whereas introducing Ala at the same position has severe consequences.

Upon prolonged incubation with wild-type HIV-1 and EIAV enzymes, considerably shorter RNA fragments predominate, indicating RNase H activity cleaving toward the radiolabeled template 3′ terminus. Results of this nature are consistent with recent reports that, under the appropriate conditions, the RNA 5′ terminal can direct the position of RNase H hydrolysis (31, 32). This concept is outlined schematically in the lower portion of Fig. 2B. Because RT binding is initially controlled by the DNA primer 3′ terminus, the combination of synthesis-dependent and -independent RNase H activities will have the effect of producing a “gapped” template. In doing so, this creates an RNA-DNA hybrid with a readily available RNA 5′ terminus, which can serve to re-direct RT to cleave further 3′, i.e. toward the radiolabel. This activity is virtually absent from HIV-1 and EIAV RT harboring the Asp549 → Asn mutation, reflecting either: (a) loss of RNA-5′ directed RNase H activity; or (b) an inability of Asp549 → Asn mutants to create a “gapped” duplex, effectively shielding the RNA 5′ terminus. Experiments of the following section suggest that the latter explanation is more likely. Equally important is the observation that, despite almost complete template hydrolysis, the hydrolysis profile of EIAV p66D549N/p51 RT is still distinguishable from its HIV-1 counterpart. The possibility that both the HIV and EIAV enzymes work in a similar manner but that the EIAV enzyme is simply slower can thus be discounted. Finally, although the extent of hydrolysis is substantially reduced, both Asp549 → Ala mutants generate the same hydrolysis pattern as the Asp549 → Asn enzymes.

**Alterations to Asp549 Influence Synthesis-independent RNase H Activity**—To monitor the 3′-5′ processing RNase H activity, mutants were next investigated on the same RNA-DNA hybrid within which the RNA component was uniquely end-labeled at the 5′ terminus (Fig. 3A). In keeping with the data of Fig. 2, a broader distribution of endonucleolytic cleavage products were derived with wild-type p66/p51 HIV-1 RT, suggesting a more relaxed specificity than its EIAV counterpart (Fig. 3B). In contrast, both enzymes catalyze qualitatively identical polymerization-independent cleavage, because the hybrid is hydrolyzed as far as template nt –8. Differences in endonuclease activity are subtle but reproducible, possibly reflecting minor differences in the manner in which the two enzymes contact the template-primer duplex previously alluded to by enzymatic footprinting experiments (10, 13).

Fig. 3C suggests that substituting Asp549 with Asn has a differential effect on RNase H activity of both the HIV-1 and EIAV enzymes. Although the rate of initial endonucleolytic cleavage does not appear significantly compromised, the products of this reaction were found to accumulate, apparently...

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**Fig. 2.** A, RNase H activity of HIV-1 (lanes 1–3) and EIAV RT variants (lanes 4–6). Enzymes were preincubated with 3′ end-labeled substrate described as described above. Hydrolysis was initiated by adding MgCl₂, and terminated after either 5 s (left) or 10 min (right). For both panels: lanes W, wt p66/p51 RT; lanes N, p66D549N/p51 RT. A, p66D549A/p51 RT product sizes were determined by comparison to NaOH and RNase H hydrolysis profiles of template RNA (data not shown). Cleavage locations corresponding to each product are also indicated. B, top schematic illustrates the template-primer combination used to assess RNase H activity. A 90-nt heteropolymeric, 3′ end-labeled RNA template annealed to a 36-nt DNA primer is shown. Endonuclease cleavage of the template occurs 17 bases behind the primer 3′ terminus and is defined as position –17. The size of the expected radiolabeled cleavage product relative to the 3′ terminus is shown. Endonucleolytic cleavage of the template RNA 5′-directed template hydrolysis by the RNase H domain of RT. Initially, endonuclease and directional processing activities are directed by the primer 3′ terminus. The resulting “gapped” template-primer subsequently makes the internal RNA 5′ terminus available for enzyme binding, which now directs hydrolysis toward the 3′ label, resulting in short oligonucleotides following prolonged incubation.
serving as poor substrates for polymerization-independent hydrolysis to template nt −8. These observations can be likened to data recently presented on HIV-1 RT mutants containing a deletion of either their p66 (p66Δ8/p51 (12)) or p51 subunit (p66/p51Δ13 (33)), although in the present case we do observe limited template cleavage. Previous studies on RNase H activity in the presence of the competitor heparin indicate predominant cleavage at template nt 217 (12), suggesting the same or a second enzyme must re-bind this “nicked” intermediate to achieve cleavage as far as template nt 28. Invoking this scenario, the data of Fig. 3 suggest that subtle alterations to the geometry of the RNase H domain may have serious consequences for the manner in which the retroviral polymerase accommodates the more relaxed structure of a nicked RNA-DNA hybrid. If under these conditions the RNA 5′-terminal is not accessible to RT, this would account for loss of RNA 5′-directed hydrolysis in Fig. 2.

The hydrolysis profiles of Fig. 3D indicate that although the highly conserved Asp549 is clearly important for RNase H function, it is not absolutely critical, because low-level hydrolysis could still be achieved with both p66D549A/p51 mutants. Although slightly prolonged autoradiographic exposure was necessary to highlight the hydrolysis products from each enzyme, retention of function can be contrasted with an HIV-1 RT mutant containing the substitution Glu478Gln, which completely abolishes Mg2+−dependent RNase H activity (24, 25). A second feature of both Asp549Ala mutants is relaxed specificity of endonucleolytic cleavage. With p66D549A/p51 HIV-1 RT, hydrolysis products reflecting cleavage between positions 216 and 224 are evident, whereas its EIAV counterpart hydrolyzes between positions 216 and 222. Reasons for this relaxed specificity are not immediately apparent, although data of a later section suggest this may reflect a hitherto unnoticed feature of the parental enzyme. If Asp549 were involved in Mg2+ co-ordination, this predicts that replacement with Asn or Ala would influence affinity for the divalent cation. To test this experimentally, the RNA-DNA hybrid of Fig. 2A was incubated with wild-type, p66D549N/p51, and p66D549A/p51 HIV RT, in the presence of increasing concentrations of Mg2+. The results of Fig. 3E indicate that both Asp549 mutants are unaffected at elevated Mg2+ concentrations. In fact, the increased ionic strength of the buffer appears to have a slight inhibitory effect on the wild-type enzyme. Other experiments indicate that the RNase H activity of p66D549A/p51 HIV RT, in contrast to that of p66D549A/p51 (23), is not restored in the presence of Mn2+ (data not shown).
and incubated with the enzymes indicated for the presence of dNTPs were prepared as described under "Experimental Procedures" and in-
or 1 strand DNA. The results of this evaluation of radiolabel into strand primer length. PPT sequences, as well as sites of (+) strand initiation, have been indicated.

Hence, Asp\textsuperscript{549} does not appear to be directly involved in metal ion coordination.

**PPT Selection by RNase H Mutants**—Initiation of (+) strand synthesis in retroviruses represents an event requiring considerably more precision of the RNase H domain to: (a) select the PPT 3' OH; (b) initiate DNA synthesis; and (c) remove PPT sequences from nascent (+) strand DNA. Using purified RNA-DNA hybrids, within which the 3' PPTs of HIV and EIAV were embedded, these multiple events were evaluated via incorporation of radiolabel into (+) strand DNA. The results of this analysis are presented in Fig. 4. (+) strand DNA products were analyzed prior to and following alkali treatment, the latter of which liberates newly synthesized DNA from the RNA primer.

In keeping with previous reports (5, 20), HIV-1 RT selects the appropriate 3' PPT primer to initiate (+) strand synthesis immediately downstream of a contiguous stretch of G residues (G\textsubscript{6}). The presence of correctly sized (+) strand DNA in the absence of alkali treatment also indicates that this enzyme efficiently removes the PPT primer. Under the same conditions, several differences were noted with mutant p66D549N/p51: (a) the overall amount of (+) strand product is considerably reduced, despite near wild-type levels of DNA-dependent DNA polymerase activity on the model substrate of Fig. 1; (b) although (+) strand synthesis initiates from the correct position, the primer selected contains the entire PPT and an additional five U residues 5' to this; and (c) alkali treatment is required to free the extended PPT from (+) strand DNA. Subtle alterations to RNase H activity demonstrated in Fig. 3 thus appear to have more serious implications for 3' PPT selection. Wild-type EIAV RT was found to initiate (+) strand synthesis at the equivalent position as its HIV-1 counterpart, whereas the activity of mutant p66D549N/p51 was also substantially reduced. For both lentiviral enzymes, (+) strand DNA could not be detected in reactions supported by p66D549A/p51 mutants.

In contrast to the HIV-1 3' PPT, wild-type HIV-1 and EIAV RT respond differently to the EIAV 3' PPT. The EIAV enzyme displays the expected precision to initiate exactly at the 3' end of the PPT, whereas its HIV-1 counterpart initiates at several positions within the 3' G\textsubscript{6} sequence. A slight reduction in (+) strand DNA was evident in reactions containing p66D549N/p51 EIAV RT; surprisingly, (+) strand products were virtually undetectable in reactions supported by p66D549N/p51 HIV-1 RT. Finally, in keeping with results from the HIV-1 3' PPT, enzymes containing the Asp\textsuperscript{549} \rightarrow Ala mutation failed to generate (+) strand product.

**Two-step Reactions Delineating PPT Selection and Extension**—Experiments of Fig. 4 cannot exclude the possibility that PPT selection is unimpaired in our HIV-1 and EIAV RNase H mutants, but DNA synthesis was affected by unique structural features of the (+) strand initiation complex, where an RNA-DNA hybrid is gradually replaced in the nucleic acid binding cleft by duplex DNA. This notion is not without precedent, evidenced by recent documentation that intermolecular base pairing between genomic RNA sequences outside the primer binding site and the tRNA primer influences initiation of (-) strand synthesis in lentiviruses and the transition from initiation to elongation (34, 35). We, therefore, sought to develop a strategy capable of independently assessing PPT primer selection and (+) strand synthesis. The approach we adopted is illustrated in Fig. 5A, and results with the HIV-1 and EIAV enzymes are presented in Fig. 5B.

The strategy of Fig. 5A takes advantage of the HIV-1 RNase H mutant p66E478Q/p51 (24, 25), which is completely devoid of Mg\textsuperscript{2+}-dependent RNase H activity (Fig. 3D) but retains full DNA polymerase activity. Initially, wild-type HIV-1 RT is permitted to select the PPT primer (in this experiment only, the HIV-1 enzymes and 3' PPT were used), after which the nucleic acid substrate is recovered by phenol extraction and ethanol precipitation. Precleaved substrate is subsequently offered to each RNase H mutant in the presence of dNTPs, and (+) strand synthesis was determined. In the converse experiment, the HIV enzymes are required to select the 3' PPT, which is recovered and offered as substrate to p66E478Q/p51 RT. The RNase deficiency in this mutant ensures that it cannot contribute to PPT selection.

Fig. 5B, panel (i), assesses the ability of each mutant to initiate (+) strand synthesis from a PPT selected by wild-type HIV-1 RT. Following alkali treatment, all enzymes showed identical levels of DNA synthesis, which is in keeping with the data of Fig. 1, illustrating that neither substitution affects initiation from the PPT. However, when p66E478Q/p51 RT is
required to extend (+) strand primers selected by Asp649 mutants, major differences become apparent (Fig. 5B, panel [ii]). As expected, PPT extension into (+) strand DNA is highly efficient with wild-type p66/p51. In contrast, the efficiency of synthesis drops approximately 10-fold when p66E478Q/p51 RT is required to extend the PPT selected by mutant p66D549N/p51, and no product was found when p66D549A/p51 RT was required to select the PPT. Fig. 5C, panels [i] and [ii], represent equivalent reactions as Fig. 5B, with the exception that alkali treatment was eliminated, allowing visualization of the PPT/ (+) strand DNA chimera and its removal by the retroviral enzyme. In Fig. 5C, panel [i], wild-type RT is clearly efficient in PPT removal, with almost 90% of the (+) strand product represented by (+) strand DNA. These proportions are reversed with mutant p66D549N/p51, i.e. the (+) strand product is predominantly the PPT-DNA chimera, whereas (+) strand DNA
synthesized by p66D549Ap51 RT is exclusively an RNA/DNA chimera. Data of Fig. 5C, panel [i], also indicates that the (+) strand primer selected by wild-type RT is restricted almost exclusively to PPT sequences, i.e. through precise RNase H-mediated hydrolysis at both its 5' and 3' extremities. This is not the case in Fig. 5C, panel [ii], where p66E478Ap51 RT extends PPT sequences preselected by Asp549 mutants. Under these circumstances, a unique PPT primer is selected by p66D549N/p51 RT but is some 6–7 nt longer at its 5' terminus, corresponding to a stretch of contiguous Us immediately 5' to the PPT (Fig. 5D).

Finally, it is interesting to note that the difference between this novel cleavage site and the PPT 5' terminus is seven to eight nt, which is consistent with the extremity of the "directional processing" RNase H activity relative to the position of initial endonucleolytic cleavage (23, 27, 28). Furthermore, wild-type RT cleaves at several positions immediately 5' to the PPT (Fig. 5C, [iii]) which cannot be resolved by Asp549 mutants deficient in directional processing activity (Fig. 5C, [i], and Fig. 3). These observations suggest that PPT selection may be subject to fine control by either sequences or structural elements of the RNA/DNA replication intermediate in the immediate 5' vicinity. This model would envision 5' RNA-directed RNase H activity of RT (31, 32) as a mechanism allowing the replication machinery to "walk" along and hydrolyze the fragmented RNA genome by a series of endonucleolytic and directional processing steps until it encounters the 3' PPT. Once the last of these combined hydrolysis events is accomplished, the free PPT 5' terminus directs a final RNase H cleavage event to liberate the 3' terminus for extension into (+) strand DNA. Such a model remains speculative and is currently under evaluation.

DISCUSSION

An improved understanding of RNase H-mediated events is critical to defining novel therapeutic targets to combat HIV infection and acquired immunodeficiency syndrome. However, despite the wealth of data available on the DNA polymerase domain of HIV RT, the RNase H domain has received surprisingly little attention, despite documentation of its absolute requirement for replication (36, 37). One contributing factor may have been a lack of defined heteropolymeric substrates, reflecting critical steps in retroviral replication that demand considerable precision of the RNase H domain. This problem has largely been resolved with the advent of chemically synthesized oligonucleotides, which have provided model systems to evaluate events such as DNA strand transfer, PPT selection, and RNA primer removal. In combination with a program of site-directed mutagenesis, these strategies should provide a fine dissection of RNase H-mediated events and their potential for therapeutic intervention. In this study, we elected to evaluate the consequences of altering a highly conserved residue of the retroviral RNase H domain, Asp549, on overall catalysis, as well as selection and utilization of the 3' PPT primer. Analyzing two structurally related lentiviral RTs has also provided insights into subtle differences in RNase H-mediated hydrolysis.

A clear difference when comparing p66/p51 heterodimers of HIV-1 and ELAV RT is the stringency of RNase H cleavage (Figs. 2 and 3). This is best exemplified by use of a 3' labeled substrate, which reveals that HIV-1 RT cleaves the RNA-DNA hybrid at multiple sites centered around −20/−21, whereas its ELAV counterpart cleaves with considerably greater precision at positions defined by the spatial separation of the DNA polymerase and RNase H catalytic centers (30). One explanation for these differences might lie in observations of Palanippan et al. (32) that when presented with an RNA-DNA hybrid within which the DNA 3' end is unannealed, the position of RT (and hence the RNase H domain) is defined by the first hybrid base pair, which is upstream from the primer terminus. Partial hybridization or "breathing" of the duplex at the primer terminus could therefore lead to variability in enzyme localization, thereby inducing a corresponding change in RNase H cleavage specificity. In contrast, the ELAV enzyme may bind and stabilize the unannealed primer terminus, resulting in predominant cleavage around positions −16/−17. Alternatively, both enzymes may locate themselves in a similar manner at the DNA polymerase catalytic center but differ in the manner in which the RNA-DNA hybrid is accommodated within the RNase H domain.

The experiment of Fig. 2, which restricts RNase H activity to visualization of the primary site of endonuclease activity, indicates little difference between wild-type RT and enzymes carrying the Asp549 → Asn mutation, which would be in keeping with a recent observation of Hostomsky et al. However, we have documented several HIV-1 mutants that support efficient endonuclease or synthesis-dependent RNase H activity but are severely compromised for the subsequent stepwise template hydrolysis as far as nt −8 (12, 29, 34). This scenario is again apparent following extended hydrolysis of a 3' labeled hybrid or through use of a 5' labeled RNA template, the latter of which monitors events subsequent to initial endonucleolytic cleavage. Markedly slower hydrolysis kinetics of p66D549Ap51 HIV-1 RT also highlight the reduced stringency of cleavage, where products corresponding to cleavage between positions −16 and −24 are clearly evident. The highly conserved Asp549 thus appears to make an important contribution to RNase H function, although retention of low-level activity in Asp549 → Ala mutants of HIV-1 and ELAV RT suggests that, in contrast to Glu478, it is not absolutely required for metal ion co-ordination (24, 25). A plausible alternative might be assisting in maintaining the integrity of α-helix E of the RNase H domain, which spans Gly544–Gly555 in the HIV-1 enzyme (30). Structural data from the isolated RNase H polypeptide (8) indicates hydrogen bonding between side chain residues of Asp549 and Ser555 in HIV-1 RT. Loss of hydrogen bonding function may conceivably alter the geometry of α-helix E’, which, together with β-strand 5’, is proposed to constitute a "floor and wall" to accommodate RNA-DNA hybrids in the RNase H domain (10, 11). Interestingly, when the equivalent residue of the duck hepatitis B virus polymerase gene (Asp755) is altered to Glu, a substantial difference in DNA synthesis was observed (38), suggesting that alterations to this RNase H domain mutant can also impact upon the DNA polymerase catalytic center.

The importance of the synthesis-independent or directional processing RNase H activity has been illustrated by the inability of mutants lacking this property to support DNA strand transfer (12, 29). A second feature of impaired processing function can be highlighted when another key step in retroviral replication is evaluated, i.e. initiation of (+) strand synthesis from the 3' PPT primer. Data of Fig. 5 indicate p66D549N/p51 and p66D549Ap51 HIV-1 RT catalyze equivalent levels of (+) strand synthesis as wild-type enzyme from a preselected PPT. However, when these mutants are required to select the PPT for extension by a second enzyme, major reductions in (+) strand product are evident. Furthermore the HIV-1 mutants are virtually unable to remove the PPT RNA primer from nascent (+) strand DNA, an event the parental enzyme accomplishes efficiently. Recent studies have indicated that the RNA-DNA PPT-containing hybrid has unusual structural features that may contribute to its resistance to RNase H-mediated hydrolysis. Given the importance for accurate initiation of (+)

2 S. H. Hughes, personal communication.
strand synthesis in defining sequences at the 5′ long terminal repeat terminus to be recognized by the retroviral integration machinery, it is not unreasonable that RT has adapted to sequence and/or structural features of the PPT. Should the manner in which the PPT RNA(+) strand DNA hybrid is accommodated be affected by perturbations to the geometry of the RNase H domain, this would be magnified in a PPT selection/extension assay. Stated differently, PPT selection/extension assays of Figs. 4 and 5 provide an additional example of the necessity for highly selective assay systems to finely dissect multiple functions of the retroviral polymerase.

Data with the HIV-1 mutant p66D549N/p51 may also provide clues to the mechanism of PPT selection. Fig. 5C, [ii], indicates that the major 3′ strand products with identical 3′ termini places the 5′ terminus of these primers within one to seven nt of the -A-A-A-A sequence at the 5′ end of the PPT. The presence of additional (+) strand products with identical 3′ termini places the 5′ terminus of these primers within one to seven nt of the PPT. Invoking the model of DeStefano et al. (31), RNA 5′-directed RNase H cleavage might be a means of directing the replication machinery to within seven nt 5′ of the PPT. At this stage, although RT located over the RNA 5′ terminus is unable to cleave within the PPT, the spatial distance between its catalytic centers would permit cleavage at the PPT 3′ terminus (a distance of ~20 nt). A conformational rearrangement, possibly promoted by unusual structural features of the PPT RNA-DNA duplex, would relocate RT with its polymerase catalytic center over the PPT 3′ OH. Finally, the directional processing activity of this enzyme would remove extraneous sequences 5′ to the PBS. Mutant p66D549N/p51 would follow the same route but simply make a series of endonucleolytic cuts. However, when this mutant selects the PPT 3′ OH and undergoes a conformational rearrangement, a lack of processing activity would leave these additional seven nt on the PPT, as we observed experimentally.

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Substituting a Conserved Residue of the Ribonuclease H Domain Alters Substrate Hydrolysis by Retroviral Reverse Transcriptase

Jason W. Rausch and Stuart F. J. Le Grice

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