Mutations within the Primer Grip Region of HIV-1 Reverse Transcriptase Result in Loss of RNase H Function*

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Human immunodeficiency virus (HIV) DNA synthesis is accompanied by degradation of genomic RNA by the RNase H of reverse transcriptase (RT). Two different modes of RNase H activity appear necessary for complete RNA removal. In one, occurring during minus strand synthesis, positioning of the RNase H is determined by binding of the polymerase active site to the DNA 3'-end. In the other, used for removal of remaining RNA fragments, positioning of RT for RNase H-directed cleavage is determined by the RNA 5'-ends. We attempted to identify RT amino acids responsible for these modes of positioning. Twelve RT mutants, each with one alanine replacement in residues 224 to 235, known as the primer grip region, were examined for catalytic abilities. Six of the examined primer grip mutants, although distant from the RNase H active site were altered in their ability to cleave RNA. The mutants P226A, F227A, G231A, Y232A, E233A, and H235A failed to perform RNA 5'-end-directed RNase H cleavage in heparin-challenged reactions. The last four mutants also lacked DNA synthesis and RNA 3'-end-directed RNase H cleavage activities in challenged reactions. Since mutants P226A and F227A carried out these latter reactions normally, these two residues specifically influence 5'-RNA-directed RNase H catalysis.

Human immunodeficiency virus, type 1 (HIV-1)† is the causative agent of AIDS. During replication, the virally encoded reverse transcriptase catalyzes the conversion of the single-stranded RNA genome to a double-stranded DNA genome. HIV-RT catalyzes RNA-directed DNA synthesis, DNA-directed DNA synthesis, RNase H, strand displacement, and strand transfer activities (1). The RNA-directed polymerase activity is essential for the formation of minus strand DNA from plus strand genomic RNA. RNase H activity is required for the removal of the RNA portion of the RNA/DNA hybrid formed during minus strand DNA synthesis. Biochemical and structural measurements show that the DNA polymerase and RNase H active sites are separated by a distance of about 18 nt along the template (2–8). Estimates vary from 14 to 20 nt in biochemical studies depending on the sequence of the nucleic acid employed. When the polymerase active site was bound at the 3’-OH of a DNA primer on a RNA template, this positioning determined the first site of cleavage of the template at the distance separated by the active sites (2, 3). Hence, this was termed the polymerase-dependent mode of RNase H-directed cleavage. Cleavage at other positions was termed polymerase-independent. This latter class includes secondary cleavages that occur near the polymerase-dependent cleavage site but closer to the DNA 3'-end. Following the initial cleavage, the RT displays a 3’→5’ directional processive RNase H activity (9–12).

As suggested by our biochemical studies, cleavage of the plus strand RNA is not completed by the RT that synthesizes the minus DNA strand (13). Fragments of RNA 13–45 nt in length are left behind that stay annealed to the newly synthesized DNA. Two of these are the polypurine tracts that are relatively resistant to cleavage and prime plus strand DNA synthesis (14). The cleavage of all of these intermediates is by a polymerase-independent mechanism, since there is no nearby DNA 3’-end. Positioning of the RT for this process was found to be determined by the RNA 5’-end (12, 15, 16). We proposed earlier that this mode of cleavage would require an RT configuration as shown in Fig. 1B. In the work presented here, we identify amino acids involved in this RNA terminus-directed positioning of the RT.

The native form of HIV-1 RT is a heterodimer comprising p66 and p51 subunits. The polymerase and the RNase H activities reside in the amino- and carboxyl-terminal regions of the larger subunit alone, respectively. Except for the lack of a 15-kDa carboxyl-terminal domain (RNase H), the p51 subunit is identical in amino acid sequence to p66 (for a review, see Refs. 17 and 18). However, structure determination by x-ray crystallography has revealed that p51 does not assume a configuration similar to p66 and therefore lacks a nucleic acid binding cleft and polymerase active site (7, 8). The p66 in the native RT assumes a configuration with an anatomical resemblance to a right hand. Its five distinct domains have been named the finger, palm, thumb, connection, and RNase H. The folding pattern of p66 allows accommodation of the primer-template between the DNA polymerase and RNase H active sites. A region within the p66 palm subdomain comprising amino acids 227–235 is thought to be involved in the positioning of the primer terminus for accommodating the incoming nucleotide (8). Hence, it has been named the “primer grip.” These amino acids form the β12–β13 hairpin of the RT molecule. Interestingly, amino acids Phe-227, Trp-229, and Leu-234

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‡ The abbreviations used are: HIV-1, human immunodeficiency virus, type 1; RT, reverse transcriptase; nt, nucleotide(s).
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of this region also line the non-nucleoside drug binding pocket (19). Importantly, none of the known mutations that confer resistance to non-nucleoside inhibitors are mediated by changes in the amino acids of the primer grip. This suggests that the primer grip residues also have a critical architectural role.

Alanine-scanning mutagenesis of the primer grip and thumb regions has previously been shown to result in the loss of both polymerase and RNase H activities, possibly from altered binding configurations (20–22). This suggested that the primer grip region could also participate in binding directed by the RNA 5’-end. The configuration of hybrid substrates, when bound to the RT, also suggested that determinants of binding at both RNA and DNA strand termini were likely to be near each other in the RT structure (Fig. 1). We have examined the consequences of alanine replacement of amino acid residues 224–235 lining the primer grip region. Our analysis defined amino acids of the primer grip region that appear to be specifically involved in 5’-end positioning. Others appear to influence more than one feature of binding and catalysis.

EXPERIMENTAL PROCEDURES

Materials

T4 polynucleotide kinase was from U.S. Biochemical Corp., and DNase I, dNTPs, alkaline phosphatase, rNTPs, RNase inhibitor, T7 RNA polymerase, and a quick spin gel filtration columns were purchased from Boehringer Mannheim. Radio nucleotides were from DuPont NEN. Plasmids pBS + and pBS + (Δ) have been previously described (23).

Methods

Generation of Primer Grip RT Mutants—Subunit-selective mutagenesis procedures and subsequent metal chelate affinity purification of wild type and mutant RT preparations are described elsewhere (20, 21, 24, 25). SDS-polyacrylamide gel electrophoresis analysis of all purified RT preparations followed by Coomassie Blue staining revealed an essentially homogenous preparation containing only the p51 and p66 subunits at stoichiometric levels (20, 21). However, the L234A mutant failed to dimerize (20) and therefore was not used in our experiments. All preparations of RT were free of detectable contaminating nucleases (data not shown). Except for the W229A mutant, the specific activities of the mutants determined using homopolymeric substrates were comparable with the wild type under conditions that would allow multiple interactions between RT and substrate (21). Protein concentration was determined according to Bradford (26) using bovine serum albumin as a standard.

Preparation of the RT-RNase H Mutant—An alteration of residue Glu-478 to Gln within the catalytic domain of the RNase H was previously demonstrated to result in an enzyme devoid of Mg2+–dependent RNase H function (27). The preparation of the RNase H mutant enzyme p66RT-Δ/p51 is described elsewhere (27).

Generation of RNA Templates—RNA template 1 (142-mer) was generated by run-off transcription by T7 RNA polymerase from plasmid pBS + (Δ) following digestion by BstNI. RNA primer 1 (41-mer) was prepared similarly from plasmid pBS + following digestion by AccI. Subsequently, the plasmid DNA was removed by DNase I digestion. The RNA samples were gel-purified and then isolated upon visualization by UV shadowing as described previously (28). Dephosphorylation of the RNA molecules was carried out using calf intestine alkaline phosphatase. When required, 5’-end labeling was carried out with [γ-32P]ATP (3000 Ci/mmol) in the presence of T4 polynucleotide kinase. RNA samples were quantitated by “shift-up” assays using labeled primers of known concentrations as described previously (16).

RNA-DNA Hybridization—Annalization of RNA and DNA was performed in 10 mm Tris-HCl (pH 8.0), 1 mm EDTA, and 80 mm KCl. For annealing, the DNA primer was present in 3-fold excess over the RNA template. Components were mixed, heated to 65 °C for 10 min, and slowly cooled over a 90-min period (12).

RNase H Assays (Unchallenged Reactions)—Final reaction mixtures (25 μl) contained 50 mm Tris-HCl (pH 8.0), 1 mm dithiothreitol, 1.0 mm EDTA, 34 mm KCl, 4 nm substrate, and HIV-1 RT. RT:primer-template ratio was 10:1, unless otherwise stated. In all cases, the enzyme was allowed to prebind to the substrate for 5 min at 37 °C. The reaction was initiated with MgCl2 allowed to incubate for 15 min, and then terminated with 25 μl of 2 × termination mixture. For time course analysis, a mixture of all components except MgCl2 was prepared. Subsequent to the initiation of the reaction with MgCl2 at 37 °C, 25-μl aliquots were drawn at varying time intervals, and the reaction was terminated by adding those aliquots to an equal volume of the 2 × termination mixture (20 μl of 1% (v/v) 10 mm EDTA (pH 8.0), and 0.1% each of xylene cyanole and bromphenol blue). Eight-μl samples were then subjected to denaturing electrophoresis to resolve reaction products. The gels were then vacuum dried and subjected to autoradiography by employing standard protocols (29). Exposures were carried out using Kodak XAR-5 or Biomax films unless otherwise specified.

RNase H Assays (Challenged Reactions)—In some experiments, the products resulting from catalysis during a single binding event of the RT to the substrates were examined. This was accomplished by adding excess trapping polymer heparin (5 μg) at the start of the reaction (16, 28). RT was allowed to prebind to the substrate, and the reaction was initiated by the addition of MgCl2 along with heparin. Presence of excess heparin allows only a single round of RT action because it traps any RT molecule that dissociates from template-primer. A control reaction was always included to ensure the presence of adequate trap. Here, the RT was exposed to the substrate in the presence of excess trap prior to the initiation of reaction with MgCl2. Complete absence of synthesis verifies adequate trap.

DNA Polymerase Assay—Conditions were identical to those of RNase H assays except that all four dNTPs were present in the reaction mixture at 50 μM final concentration. Reactions were initiated by the addition of MgCl2 and dNTPs. 5’-Labeled DNA primer was employed to monitor DNA synthesis. Product analysis was carried out as above.

Preparation of Molecular Size Markers—RNA markers were made either by alkaline treatment of 5’-labeled RNA substrates to generate a nucleotide hydrolysis ladder or by RTase T7 treatment to generate a G-ladder as described in the protocols supplied with the Pharmacia RNA sequencing kit. MspI-digested fragments of plasmid pBR322 (Life Technologies, Inc.) that were labeled at the 5’-end were also run in each gel as additional size markers.

Gel Retardation Assays—The procedure was adapted from Guo et al. (6) with some modifications. MgCl2 was omitted from the reaction to avoid degradation of the RNA by RT-RNase H. Labeled RNA/DNA hybrid substrates A and B were prepared as described (Fig. 2). The final reaction mixture (10 μl) contained 50 nm Tris-HCl (pH 8.0), 1 mm dithiothreitol, 1.0 mm EDTA, 34 nm KCl, 4 nm substrate, and HIV-1 RT. The enzyme was present in a 10:1 ratio with respect to template-primer. Binding was allowed to proceed for a period of 5 min at 37 °C. In some experiments, heparin (8 μg) was added at this stage and the mixture was incubated for an additional 5 min. Samples were immediately placed on ice for 5 min. Following this, 2 μl of 50% glycerol was added to each reaction. Reaction products were then separated on 5% native polyacrylamide gels at room temperature or at 4 °C for varying lengths of time as indicated. The composition of electrophoresis buffer was as described previously (6).

Substrate Sequences—The nucleotide sequences of templates and primers employed were as follows: RNA template 1, 5’-GGGGGAAU UAGCUGUUUGG UCCCUUGAAG GACCGGCGU GCGUAAAUU GGCUUAAGCU GGCUUAAGCU GGCUUAAGCG CGCGGCGAC AACUUGACAG CCGGACCAU AAAGUGUAAA GCCU; DNA template 1, 5’-GGGACCTGAGGATCC CGCAGATTCC GATCCCCGCTT GTT, DNA template 2, 5’- CGACTCTAGAGGATCCCCGGGTACCGAGCTC CGCTCT, DNA template 3, 5’-TTGGATCCGCAGCGTTCGAGCTC CTGCGCTTGT, DNA template 4, 5’-CCTGATATCTTGGTCTGGA AAGCGGAGGATCG.

RESULTS

Two modes of RT binding appear to be essential for the complete processing of plus strand RNA during HIV replication (13). The first causes positioning of RNase H at a fixed distance from the 3’-OH of the DNA primer for cleavage of the RNA template during minus strand synthesis (mode A; Fig. 1A). The second places the RNase H at a fixed distance from the 5’-end of fragments of RNA left immediately after the synthesis of minus strand DNA (mode B; Fig. 1B). We hypothesized that the amino acids responsible for recognition of the DNA 3’-end were in the same domain of the RT as those that recognize the RNA 5’-end, as suggested by the drawings in Fig. 1. If so, amino acid substitutions in the primer grip region, known to be involved in
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binding the DNA 3'-end, were likely candidates to influence binding the RNA 5'-ends.

**RNA 5'-End-directed RT-RNase H Cleavage by the Primer Grip Mutants**—Site-directed mutagenesis of the primer grip region and the purification of RT are as indicated under "Methods." For our measurements of RNase H cleavage, we first employed substrate A (Fig. 2). The RNA component of the RNA/DNA hybrid is recessed in this substrate. This would resemble a replication intermediate having segments of RNA remaining after synthesis of the minus strand DNA (Fig. 1B).

The 5'-end of the 41-nt RNA component of the hybrid was radiolabeled to allow detection of the cleaved product. To examine products resulting from a single binding event of RT to substrate, we carried out the experiments in the presence of a heparin challenger. In this case, the RT is prebound to the substrate, we carried out the experiments in the presence of a heparin challenger. In this case, the RT is prebound to the substrate, with which the heparin inactivates the RT.

The anticipated labeled 18-nt-long initial product, characteristic of cleavage determined from the RNA 5'-end, is seen with the wild type RT (Fig. 3, lane 12). Some products of the subsequent processive cleavage of RNA were also observed. Mutant RT's E224A, P225A, L228A, W229A, and M230A were able to form products similar to those generated by the wild type. However, lanes containing reactions with the mutants P226A, F227A, G231A, Y232A, E233A, and H235A were devoid of any cleavage products (lanes 3, 4, and 8–11). Results shown below demonstrate that the active site of the RNase H is still functional in the mutants P226A, F227A, G231A, and Y232A. In those cases, lack of catalysis is likely to have resulted from a defect in binding to the substrate. One possibility is that integrity of a structural element uniquely necessary for 5'-end positioning was altered by replacement of some of the residues with alanine. It is also possible that alanine replacement in some cases also altered the global configuration of the RT. A
more widespread structural change should also affect polymerase activity and the polymerase-dependent mode of RNase H cleavage.

**Polymerase-dependent Mode of RNase H Activity of Primer Grip Mutants**—The configuration of RT that would allow cleavage of the RNA during DNA synthesis is represented in Fig. 1A. We have employed substrate B to examine this mode of RT-RNase H cleavage. Unlike substrate A, the DNA component of the RNA/DNA duplex in substrate B is recessed. This resembles a replication intermediate during minus strand synthesis. As before, the 142-nt-long RNA component of the RNA/DNA duplex was 5'-end-labeled to allow detection of the RNase H cleavage product. Under conditions that allow multiple interactions of RT with substrate having a recessed DNA primer on an RNA template, we previously found that all primer grip mutants possess significant RNase H activity (20). In an effort to define products resulting from catalysis during a single enzyme binding event, reactions were performed in the presence of heparin challenger. They were also performed in the absence of dNTPs to prevent elongation of the DNA primer. This allowed us to measure the location of RNase H-directed cleavages with respect to the position of the DNA 3'-end. In this experiment, labeled products 102 and 94 nt long were generated by the wild type RT (Fig. 4, lane 12). The action of RT-RNase H on the RNA strand of RNA/DNA duplex about 18 nt from the 3'-OH of the DNA results in the formation of the 102-nt product. The smaller 94-nt product results from the subsequent 3'-to-5' directional nucleolytic activity of RT-RNase H.

Products of the cleavage reaction carried out by the primer grip mutants are in Fig. 4 (lanes 1–11). The relative efficiencies of the DNA polymerase dependent mode of cleavage by the mutants on substrate B generally paralleled those on substrate A, except in the case of two of the mutants. Conspicuously, the mutants P226A and F227A, which were devoid of 5'-RNA-dependent cleavage, displayed wild type levels of 3'-polymerase-dependent RNase H cleavage (compare Fig. 4, lanes 3 and 4, with Fig. 3, lanes 3 and 4). This demonstrated that the basic catalytic function of the RNase H in these two mutants is intact. From these observations it also appears that residues Pro-226 and Phe-227 interact differently with nucleic acids depending on whether the RNA or DNA component of the RNA/DNA duplex is recessed. This suggests that these two amino acids have a specific role in RT binding as directed from the RNA 5'-end.

**RNA-dependent DNA Polymerase Activity of Primer Grip Mutants**—As shown above, alanine replacement of some of the primer grip residues resulted in the loss of both RNA 5'-end-directed cleavage and the DNA polymerase-dependent mode of RNase H cleavage. As illustrated in Fig. 1, this was not surprising, since we had expected to see at least some shared elements required for both types of RT positioning. One possible explanation for the lack of RNase H activity with some mutants is that the RT is distorted to such a degree that no catalysis of any sort is possible. This would include polymerase activity. To test this possibility, we measured DNA synthesis on substrate B. Again, measurements were made in a heparin-challenged reaction. The DNA portion of the RNA/DNA duplex was 5'-end-labeled to allow detection of extension products. The mutants E224A, P225A, P226A, F227A, L228A, and W229A also showed wild type levels of 3'-polymerase-dependent RNase H cleavage (20, 21). This demonstrated that the basic catalytic function of RNase H in these two mutants is intact. From these observations it also appears that residues Pro-226 and Phe-227 interact differently with nucleic acids depending on whether the RNA or DNA component of the RNA/DNA duplex is recessed. This suggests that these two amino acids have a specific role in RT binding as directed from the RNA 5'-end.

**TABLE I**

*Summary of the catalytic properties of the primer grip mutants*

Catalytic activities for the wild type and the primer-grip mutants were measured using substrates A or B in the presence or absence of heparin challenger as described under “Methods.” Alanine replacements were made for individual residues on the p66 subunit alone (20, 21).

| HIV-1 RT genotype | Challenged reaction | Unchallenged reaction |
|-------------------|---------------------|-----------------------|
|                   | RNA-dependent DNA polymerase activity | RNA 5'-end-directed, RNase H cleavage | DNA 3'-end-directed, RNase H cleavage | RNA-dependent DNA polymerase activity | RNA 5'-end-directed, RNase H cleavage | DNA 3'-end-directed, RNase H cleavage |
| HIV-1 RT genotype | 5' | 3' | 5' | 3' | 5' | 3' |
| Wild type         | X | X | X | X | X | X |
| E224A             | X | X | X | X | X | X |
| P225A             | X | X | X | X | X | X |
| P226A             | X | X | X | X | X | X |
| F227A             | X | X | X | X | X | X |
| L228A             | X | X | X | X | X | X |
| W229A             | X | X | X | X | X | X |
| M230A             | X | X | X | X | X | X |
| G231A             | X | X | X | X | X | X |
| Y232A             | X | X | X | X | X | X |
| E233A             | X | X | X | X | X | X |
| H235A             | X | X | X | X | X | X |

* X, catalytically inactive; all others are catalytically active.
* a, Defective in elongation.
* b, Partially defective.
M230A were relatively active for DNA synthesis compared with the wild type RT (Fig. 5). However, the mutants G231A, Y232A, E233A, and H235A were severely inhibited in DNA synthesis. Note that these latter mutants were also devoid of 5′-RNA-directed processing and the DNA polymerase-dependent mode of RNase H processing in challenged reactions (Figs. 3 and 5). However, when experiments were performed in the absence of heparin, they did not display distinguishable differences in catalytic activities from the wild type (Table 1). This implies that these mutants either have reduced affinity for the template-primer or are positioned in a conformation that would allow accessibility to heparin even when the RT is bound to the substrate.

As was shown previously (21), the W229A mutant was defective in elongation but had levels of initiation comparable with those of the wild type, as demonstrated by presence of only very short elongation products (Fig. 5 and data not shown).

**Gel Shift Analysis of Primer Grip Mutants P226A and F227A—**Results presented so far show that mutants P226A and F227A are distinctive. In heparin-challenged assays, they were defective in RNA 5′-end-directed RNase H processing but retained normal DNA polymerase-dependent RNase H processing and DNA polymerase activities. Therefore, it can be inferred that the binding configuration of the mutants P226A and F227A on substrate B, having a recessed DNA, was normal. It is possible that these two mutants were incapable of binding to substrate A, weakly bound, or bound with an altered configuration that did not allow RNase H action. Binding parameters could also differ among mutants and wild type depending on the presence or absence of metal ion. In our reactions, magnesium is not present until initiation of catalysis. Any one or more of these possibilities could have resulted in the observed defect in RNA 5′-end-directed RNase H activity. We used gel shift analysis to further explore the possibilities (Fig. 6). The gel shift assay was performed as described under “Experimental Procedures.” The RNA/DNA duplex was allowed to react with RT in the absence of MgCl2. The mixture tested in the gel shift had all of the components of the challenged assay just before initiation of the reaction by the addition of MgCl2 and the heparin challenger. Therefore, if the RT were bound to the substrate when the challenged assay was initiated, a complex should be observed in the shift assay.

In the absence of heparin, the complex was, in fact, observed with substrate A, having a recessed RNA, and wild type RT (Fig. 6A, lane 4). Mutants P226A and F227A also formed a band shift complex (Fig. 6A, lanes 2 and 3). These results indicate that the mutant RTs bind to a substrate with recessed RNA even in the absence of metal ion. However, when gel shift assays were performed in the presence of a heparin challenge, the wild type RT formed a complex with substrate A, but the P226A and F227A mutants did not (Fig. 6B, lanes 2–4).

For controls we carried out similar experiments with substrate B. Since we were able to detect the polymerase-dependent mode of cleavage with these two mutants, we expected them to form a complex with substrate B even in the presence of heparin. As expected, a band shift was seen with substrate B and the two mutants regardless of the presence or absence of heparin (Fig. 6C and D). These results place residues Pro-226 and Phe-227 in the architecture of structural elements necessary for 5′-RNA-directed RNase H function. However, we cannot exclude the possibility that replacement of Pro-226 and Phe-227 with other residues would have some consequences in the DNA polymerase mode of cleavage as well.

**RNase H Catalysis by the P226A and F227A Mutants in Reactions Allowing Multiple Interactions of RT and Substrate—**RNase H activity on substrate A was measured in the absence of heparin (Fig. 7). Within a 15-min reaction time, the wild type RT made the expected measured cleavage at a distance of 18 nt from the RNA 5′-end. Subsequent directional cleavage, which results in further shortening of the RNA, also occurred efficiently. Unlike in the challenged reaction, the mutants P226A and F227A were able to make RNA 5′-end-directed RNase H cleavage products (compare Fig. 7, lanes 1 and 2, with Fig. 3, lanes 3 and 4, respectively). The points of cleavage are similar to the measured cleavage by the wild type RT. The greater accumulation of secondary cleavage product with P226A and F227A relative to the wild type results from relatively faster cleavage kinetics (data not shown). The combined data from Figs. 3, 6, and 7 show that the P226A and F227A mutants are bound in a relaxed configuration, which might have promoted their rapid dissociation from the template-primer, or that the loose conformation promoted accessibility to heparin even when bound to the substrate.

The P226A and F227A Mutants Have Defective RT-RNase H Activity on a Substrate with a 5′-Unannealed Tail—The dominant influence of RNA 5′-end-directed positioning of the RT was previously shown using novel RNA/DNA duplexes (12). Substrates that had short unannealed 5′-RNA tails were still cleaved at the normal fixed distance from the 5′-end. However, substrates with a tail larger than the 18-nt span from 5′-end to cleavage site were resistant to cleavage. We carried out exper-
imments under conditions allowing multiple RT-substrate inter-
actions using substrate C. The 5’-end of the recessed RNA on
this substrate has a 10-nt unannealed tail. Consistent with our
previous findings, the initial cleavage products made by the
wild type RT were about 18 nt long (Fig. 8, lane 3). This
demonstrates the strong influence of the RNA 5’-end on the
positioning of the RT. Significantly, the mutants P226A and
F227A were unable to perform any cleavages on substrate C
(Fig. 8, lanes 1 and 2). This further illustrates that RNA 5’-end
positioning by the mutants P226A and F227A is altered.

**DISCUSSION**

The complete degradation of the plus strand RNA is believed
to be accomplished by two mechanistically different modes of
HIV-1 RT-RNase H-directed cleavage (13). The polymerase and
RNase H activities reside on the p66 subunit of RT, and their
active sites are separated by about 18 nt. During minus strand
DNA synthesis, RT cuts the RNA at a measured distance of
about 14–20 nt from the 3’-OH of DNA. This mechanism
is referred to as the DNA polymerase-dependent mode of RNase
H activity (2, 30). Cleavage by this mechanism is not adequate
for complete removal of RNA. RNA segments left behind fol-
lowing a single round of RT minus strand synthesis are cleaved
by a different RNase H processing mechanism. This cleavage is
directed by RT positioning from the 5’-end of the RNA. The two
mechanisms are represented diagrammatically in Fig. 1. The
goal of the work presented here has been to define the residues
and structural features in the RT that participate in these
mechanisms of cleavage. We identified two residues in the
primer grip region of the RT that specifically influence RNA
5’-end-directed RNase H activity.

The primer grip region of RT is implicated in the positioning
of the primer 3’-OH for DNA synthesis (8, 31, 32). The residues
of the primer grip region are part of the p66 palm subdomain
and are in close proximity to the DNA polymerase active site.
The residues Asp-110, Asp-185, and Asp-186 involved in metal
ion coordination for DNA polymerization are in the p66, p9, and
p10 strands, respectively, and located near residues contribut-
ing to primer grip architecture. In addition, residues of the RT
fingers subdomain make contacts with the template sequence
ahead of the primer. Likewise, the residues of the p66 thumb
also contact primer and template, presumably playing a role in
the translocation of the template-primer. RT residues 227–235
make up the primer grip region (31). Residues 224–226 consti-
tute the β11b-β12 connecting loop, residues 227–229 constitute
the β12 strand, residues 230 and 231 form the β12-β13 con-
necting loop, and residues 232–235 form the β13 strand.

It is also documented that the larger subunit of RT alone
provides the structural framework for binding the primer, and
for DNA polymerase and RNase H catalytic functions. The
relative orientations of fingers, thumb, palm, and connection
subdomains of the p51 subunit are different from those in p66
(32). The p51 connection lies between its fingers and thumb,
thus excluding the nucleic acid duplex from the palm.

Residues in the primer grip region are highly conserved
among retroviral RTs (33). Alanine replacement of primer grip
residues was previously shown to impair both DNA-dependent
DNA polymerase and RNA-dependent DNA polymerase activ-
ities (20, 21). Although near the DNA polymerase active site,
the primer grip region is distal to the RNase H active site. How-
ever, mutations within this region were also previously
found to influence RNase H catalysis (20, 21). This influence
could be anticipated, since RT binding at the 3’-OH of the DNA
determines the position of RNase H-directed cleavage (Fig. 1A).
As drawn in Fig. 1B, one would also expect that the primer grip
region would influence RNA 5’-end-directed cleavage. This is
because after binding and positioning of the RT to a recessed
RNA on a longer DNA, the 5’-end of the RNA should lie near
the polymerase active site. Any amino acid in the primer grip
region might influence both modes of positioning or participate
uniquely in one.

Here we have examined the effects of each of 12 amino acid
substitutions with alanine in positions 224–235 of the primer
grip region. We have previously shown that the L234A muta-
tion resulted in the inability of RT to dimerize (20). By employ-
ing various reaction conditions and substrates we have been
able to define residues within the primer grip region that
influence the catalytic and binding activities of the RT. As was
expected, there were several mutants that had multiple defects
in catalysis (Table I). However, two amino acid substitutions,
P226A and F227A, were of special interest because they af-
fected the stability of RT positioning from the 5’-ends of RNA
segments recessed on longer DNA templates.

These mutants display normal RNA 5’-end-directed cleavage
also.
in the standard assay, demonstrating that the active site of the RNase H is not disrupted. A defect in 5′-end-directed cleavage was evident under two specialized reaction conditions. In the first, heparin was present as a challenger. Here no cleavage was observed. Gel retardation assays showed that the RT could bind the 5′-end of the RNA primer in the absence of heparin but would dissociate in its presence. While this observation may be a reflection of a lower binding affinity of the mutants for a substrate with a recessed RNA primer on a DNA template, an alternate explanation is that the 5′-end binding configuration is destabilized sufficiently that the heparin can access the bound RT in a way that induces dissociation from the substrate. In the second, the 5′-end of the RNA primer had an unannealed tail. The wild type RT binds this substrate and makes a measured cut the expected distance from the RNA 5′-end (12). We have previously hypothesized that the RT holds the unannealed tail in a configuration similar to that of an annealed primer so that the measurement can be made. This process does not destabilize the binding of the wild type RT; presumably, it is too difficult for the already defective mutant. Therefore, use of reaction conditions that apply a destabilizing feature to 5′-end positioning has magnified the effects of the mutations. This approach has effectively revealed that the P226A and F227A mutations each specifically affect positioning from the RNA 5′-end.

Measurements of RNA 5′-end-directed cleavage were made using substrate A. It is interesting to note that the polypurine tract RNA primers on the minus strand DNA also constitute a duplex structure similar to substrate A. It seems likely that 5′-end-directed RNase H activity is involved in their removal. Consistent with that notion, Powell et al. have recently found that the P226A and F227A mutant RTs were incapable of polypurine tract primer formation and processing (34). The Pro-226 residue is invariant among retroviral RTs that form a configuration similar to HIV-1 RT. However, the Phe-227 residue is naturally variable but replaced only by residues with a bulky side chain (21). Therefore, we can infer that alanine replacement of these residues alters critical primer grip functions.

Among primer grip residues, Phe-227, Trp-229, and Leu-234 line the hydrophobic nonnucleoside drug nevirapine binding pocket along with several other residues, including Tyr-181, Tyr-188, Leu-100, and Lys-103 (19). However, mutations conferring resistance to nevirapine have resulted from changes in residues such as Leu-100, Lys-103, Tyr-181, and Tyr-188, whereas none derive from changes in Phe-227, Trp-229, and Leu-234 (19). Therefore, it appears that mutations in any of these primer grip residues would not be tolerated for viral replication, presumably because they alter RT enough to cause a competitive disadvantage. Consistent with this notion, we previously observed defective DNA synthesis in vitro and loss of infectivity of virus harboring the W229A mutation and an inability to dimerize in vitro plus loss of viral infectivity with the L234A mutation (20).

The ability of P226A and F227A mutants to support wild type levels of DNA synthesis and the DNA 3′-end-dependent mode of RNase H cleavage was demonstrated using a DNA/RNA primer-template nucleic acid structure, substrate B. This substrate differs from substrate A in that the polymerase active site thumb, and primer grip region on the DNA are preserved. These may have compensated for instability caused by the mutations. On substrate A, the primer grip region is not near a DNA 3′-end. Binding stability appears to depend more heavily on contacts made by the mutated residues.

The accumulation of greater secondary cleavage products on substrate A in unchallenged reactions by mutants P226A and F227A with respect to the wild type RT may be indicative of low affinity binding to the primary cleavage site, allowing more frequent sliding and relocation to the secondary site.

Structural determinations of RT complexes with substrates similar to A should clarify the exact roles of the mutant residues in binding stability. Information on viral infectivity of P226A and F227A is presently unavailable. We might anticipate interference in steps leading to complete plus strand RNA removal.

In addition to the above two mutations, we noticed that the W229A mutation was defective in DNA synthesis. This observation is consistent with our previous reports (21). Recently, Ghosh et al. (35) obtained similar results for W229Y and W229F substitutions as well. The W229A mutation was shown to affect primer elongation. We had expected that the same factors that cause binding instability accompanying synthesis would destabilize modes of binding that are necessary for the RNase H activity. Surprisingly, in contrast to synthesis, both modes of RNase H cleavage were normal for W229A, irrespective of the reaction conditions employed. Possibly conditions of synthesis destabilize binding. These could include the presence of dNTPs or the encountering of certain sequences or structures on the template as the primer is elongated. Since RNase H measurements were made in the absence of dNTPs, movement related effects would not be evident.

In previous reports, mutations within the DNA polymerase active site of RT have been shown to abolish RNase H activity (for a review, see Ref. 18). The p15 RNase H domain of HIV-1 exhibits structural similarity to *Escherichia coli* RNase H (36). But unlike the *E. coli* RNase H, which cleaves the RNA component of RNA/DNA duplexes randomly, the isolated p15 of RT has been shown to be devoid of RNase H function (36, 37). In combination with the data available in the literature (18, 20, 21, 36–42), our results strongly support the notion that in the absence of structural elements responsible for RT positioning on the polynucleotide substrate, the RNase H active site is rendered inactive.

In summary, we conclude that HIV-1 RT-RNase H and DNA polymerase activities are strongly dependent on proper positioning of the RT on the primer-template. Alterations within the RT that result in improper primer positioning cause impairment or failure of the RT to perform DNA synthesis or cleave RNA. These would disrupt essential steps in viral replication. This interpretation is consistent with the observed conservation among residues that line the primer grip region of the RT.

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