Residues in the αH and αI Helices of the HIV-1 Reverse Transcriptase Thumb Subdomain Required for the Specificity of RNase H-catalyzed Removal of the Polypurine Tract Primer*

(Received for publication, February 2, 1999, and in revised form, April 26, 1999)

Michael D. Powell§§, William A. Beard†, Katarzyna Bebenek‡, Kathryn J. Howard*††, Stuart F. J. Le Grice*‡‡§§, Thomas A. Darden†‡, Thomas A. Kunkel†, Samuel H. Wilson‡, and Judith G. Levin†††

From the §Laboratory of Molecular Genetics, NICHHD, National Institutes of Health, Bethesda, Maryland 20892, the †Laboratory of Structural Biology and Laboratory of Molecular Genetics, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709, and the **Center for AIDS Research at Case Western Reserve University, Cleveland, Ohio 44106

During retrovirus replication, reverse transcriptase (RT) must specifically interact with the polypurine tract (PPT) to generate and subsequently remove the RNA primer for plus-strand DNA synthesis. We have investigated the role that human immunodeficiency virus-1 (HIV-1) RT residues in the αH and αI helices in the thumb subdomain play in specific RNase H cleavage at the 3'-end of the PPT; an in vitro assay modeling the primer removal step was used. Analysis of alanine-scanning mutants revealed that a subgroup exhibits an unusual phenotype in which the PPT is cleaved up to seven bases from its 3'-end. Further analysis of αI mutants (G262A, K263A, N265A, and W266A) with changes in residues in or near a structural motif known as the minor groove binding track showed that the RNase H activity of these mutants is more dramatically affected with PPT substrates than with non-PPT substrates. Vertical scan mutants at position 266 were all defective in specific RNase H cleavage, consistent with conservation of trypophan at this position among lentiviral RTs. Our results indicate that residues in the thumb subdomain and the minor groove binding track in particular, are crucial for unique interactions between RT and the PPT required for correct positioning and precise RNase H cleavage.

The virus-encoded enzyme reverse transcriptase (RT) of human immunodeficiency virus type 1 (HIV-1) and other retroviruses catalyzes the conversion of genomic RNA to a double-stranded DNA replicative intermediate. As minus-strand DNA is synthesized, the RNA template is degraded by the RNase H activity of RT, which cleaves the RNA strand in an RNA-DNA hybrid (Refs. 1 and 2; for reviews, see Refs. 3–5). This results in the production of many small RNA fragments, any one of which could potentially serve as a primer to initiate synthesis of plus-strand DNA (6). However, a short, purine-rich sequence known as the polypurine tract (PPT) is almost exclusively used as the primer for plus-strand initiation (Refs. 7–12, 61; for a review, see Ref. 6). Why the PPT sequence is selected from all of the other available primers has been the subject of much speculation.

One possibility is that the PPT sequence is intrinsically resistant to RNase H degradation and therefore survives as the sole RNA primer available for plus-strand initiation. However, experimental evidence from HIV-1 (13–15) and murine leukemia virus (MuLV) (9, 16, 17) model systems demonstrates that cleavages within the PPT can occur. The MuLV PPT can also be internally cleaved by the isolated RNase H domain from MuLV RT; furthermore, the specificity of cleavage at the 3'-end of the PPT is lost when the polymerase domain is removed (18, 19). Finally, *Escherichia coli* RNase H catalyzes cleavages within the MuLV PPT (9, 16, 17, 19) as well as within the HIV-1 PPT.3 A second possibility to explain selection of the PPT primer is related to its unique helical structure (12, 20) and the shape and width of the major groove, which is wider than that of other RNA-DNA hybrids (20, 21). These structural factors could cause binding of RT to the PPT sequence to differ from the way RT binds to other primer-templates (P/T), thereby precluding cleavage within the PPT. Binding of RT to short RNA primers annealed to longer DNA templates has been suggested to occur through interaction of the polymerase active site with the 5'-end of the RNA (Fig. 1, IA; Ref. 22; for a review, see Ref. 23). In this binding configuration, RNase H cleavage can occur; however, polymerization cannot. Interaction with the polymerase domain will direct RNase H cleavage to a site 14–18 nucleotides (nt) from the bound 5'-end (14–18 nt being the distance between the polymerase and RNase H active sites (17, 24–31).

In the case of PPT-containing sequences, it appears that the 3'-end of the RNA primer is bound to the polymerase active site (Fig. 1, IB) and that extension is favored over RNase H cleavage (32, 33). This suggests a model in which non-PPT-containing fragments are preferentially degraded, while PPT-containing fragments are preferentially extended (Fig. 1, IB; Ref. 33). These conclusions also imply that RT interacts with the PPT in a highly specific manner and that the polymerase domain is

* This work was supported in part by the National Institutes of Health Intramural Antiviral Program (separate awards to T. A. D., T. A. K., S. H. W., and J. G. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Present address: Dept. of Microbiology and Immunology, Morehouse School of Medicine, 720 Westview Dr. SW, Atlanta, GA 30310.

‡‡ Supported by National Institutes of Health Grant GM 52263.

§§ Present address: HIV Drug Resistance Program, NCI-Frederick Cancer Research and Development Center, Frederick, MD 21702.

†† To whom correspondence should be addressed: Laboratory of Molecular Genetics, NICHHD, Bldg. 6B, Room 216, National Institutes of Health, Bethesda, MD 20892. Tel.: 301-496-1970; Fax: 301-496-0243; E-mail: judith.levin@nih.gov.

1 The abbreviations used are: RT, reverse transcriptase; HIV-1, human immunodeficiency virus type 1; PPT, polypurine tract; MuLV, murine leukemia virus; P/T, primer-template; nt, nucleotide(s); MGBT, minor groove binding track; WT, wild type.

2 K. Post and J. G. Levin, unpublished observations.

3 K. Post, M. D. Powell, and J. G. Levin, unpublished observations.
I. RNA Primer Selection
(A) non-PPT

(B) PPT

II. PPT Primer Removal
(A) WT RT

(B) Mutant RTs

FIG. 1. Schematic diagram showing positioning of RT on an RNA-DNA substrate during RNA primer selection and PPT primer removal. I, RNA primer selection. A, non-PPT substrate. The substrate consists of a short RNA primer and a long DNA template. The polymerase active site is centered on the 5′ terminus of the RNA. RNase H cleavage of the RNA occurs at the RNase H active site, which is 14–18 nt from the polymerase active site. B, PPT substrate. In this case, the polymerase active site is centered on the 3′ terminus of the PPT primer; extension of the PPT by RT is favored over RNase H cleavage. II, PPT primer removal. A, WT RT. With WT RT, the RNase H active site is centered on the 3′-end of the PPT primer. Cleavage occurs precisely between the 3′ rG in the PPT and the first deoxyribonucleoside (dA) incorporated into nascent DNA. B, mutant RTs. The mutant enzymes are, in many cases, unable to correctly position the RNase H site on the 3′ terminus of the PPT. As a result, cleavage occurs at multiple positions within the PPT. P, circled, polymerase active site; R, RNase H active site. The ellipse represents HIV-1 RT. The black lines represent the minus-strand DNA template and nascent plus-strand DNA; the gray lines represent the RNA oligonucleotide primers. The vertical arrows denote cleavage by RNase H; the horizontal arrow denotes PPT primer extension.

mainly responsible for this specificity.

In HIV-1 RT, two regions within the p66 subunit contribute substantially to the binding and positioning of the P/T (28). The first region, known as the “primer grip,” comprises residues in the β12-β13 hairpin in the palm subdomain (Fig. 2; Ref. 28). As we demonstrated earlier, primer grip mutations have a profound effect on the ability of RT to extend the PPT and minus-strand RNA primers but little or no effect on the extension of DNA versions of the same primers (37, 38). Differences in RNA and DNA primer usage by RT are likely to result from differences in the helical structures of hybrids having RNA or DNA in the primer strand (20, 38, 39). Mutations in the primer grip region can also affect RNase H function (37, 38, 40); for example, mutant Y232A is defective in utilization of an RNA primer and also exhibits altered cleavage specificity at the PPT (38).

A second region having a major role in binding of P/T is composed of portions of the antiparallel helices, αH and αI, in the p66 thumb subdomain (Fig. 2). It has been proposed that αH and αI residues form contacts that are important for holding the P/T in position during the translocation step in polymerization, and these residues have been collectively described as a “helix clamp” (41, 42). A refinement of the structure of the complex of HIV-1 RT with a double-stranded DNA P/T and the Fab fragment of a monoclonal antibody revealed the participation of αH and αI in a “translocation track” for bound P/T but showed no direct evidence of interactions in the minor groove (36).

Functional analysis in combination with molecular dynamics modeling performed in an earlier study (43) led to the identification of a track-like element in p66 consisting of five amino acids: Gln258, Gly262, and Trp266 in the αH helix in the thumb subdomain; Gln269, just outside of αH; and Ile94, in the palm subdomain. This motif, termed the minor groove binding track (MGBT), interacts in the minor groove over a distance from the second to sixth base pair from the 3′ terminus of the primer (43) in the region where a bend of about 41° occurs in the bound P/T and the DNA undergoes a transition from A- to B-form helical structure (28, 36). More recently, analysis of the x-ray crystal structure of a covalently trapped catalytic complex showed that site-specific cysteine mutations introduced into α-helix H residues Gln258, Gly262, and Trp266 could form specific cross-links with a single tethered thiol group placed in the minor groove of the bound double-stranded DNA substrate (44); these findings are consistent with the MGBT proposal.

In previous work, we conducted an extensive analysis of the effect of alanine-scanning mutations in α-helices H and I on polymerase activity and examined P/T binding, fidelity, and enzyme kinetics (43, 45–47). This analysis showed that changes in individual residues of αI do not affect P/T binding or fidelity, although a reduced amount of active enzyme in the mutant preparations was noted (46). In contrast, a number of
Experimental procedures

Materials—RNA oligonucleotides were purchased from Oligos Etc., Inc. (Wilsonville, OR). WT RT was purchased from Worthington. T4 DNA polymerase was purchased from Roche Molecular Biochemicals. Bacterial strains, phage, and other materials have been previously described (12, 49).

Construction and Purification of Alanine-scanning Mutants in the HIV-1 RT α-helices and α-helices, as well as the alternate substitutions at position 266, result in a loss of cleavage specificity with PPT-containing substrates. Further, the effects appear to be specific for PPT cleavage rather than more general RNase H-catalyzed cleavage.

RESULTS

Alanine-scanning Mutations in αH and αI—Mutant clones with single alanine substitutions in the entire region spanning the αH (residues 253–270) and αI (residues 277–287) helices of HIV-1 RT (see Fig. 2) were constructed and expressed (45, 46). The effect of these mutations on the removal of the PPT primer was tested with each of the mutant RTs in the standard primer removal assay (Fig. 3; Ref. 38). In these assays, a preformed double-stranded nucleic acid substrate molecule (Fig. 3) was tested with each of the mutant RTs in the standard primer removal assay. This made it possible to measure primer removal activity without having to depend on the widely variable polymerase activities of many of these enzymes (45–47). Thus, the assay reveals the ability of each enzyme to bind and specifically cleave the PPT from a 35-nt minus-strand DNA substrate (Fig. 4; for summary of results, see Table I).

WT HIV-1 RT catalyzed the removal of the RNA PPT primer, as evidenced by the conversion of the 35-nt chimeric RNA-DNA substrate (Fig. 4, lane T4) to a 20-nt labeled DNA product (Fig. 4, lane WT; also A267). This conversion involves a single cleavage at the RNA-DNA junction followed by release of an intact 15-nt PPT primer and a labeled 20-nt DNA with no RNA bases attached (Fig. 3; Ref. 12).

When the alanine-scanning mutants in αH and αI were tested in the same assay, several different cleavage patterns were observed (Fig. 4; for summary of results, see Table I). Many of the alanine-scanning mutants generated a cleavage pattern similar to that of WT RT (T253A, V254A, N255A, I257A, K259A, L260A, G262A, A267A (WT), S268A, R277A, Q278A, C280A, K281A, R284A, G285A, and K287A). Some of these enzymes cleaved the PPT substrate poorly, despite the fact that they catalyzed cleavage with the correct specificity (T253A, I257A, K259A, and R284A).

Another subgroup of nine of the alanine-scanning mutants (D256A, Q258A, K263A, N265A, W266A, Q269A, I270A, L279A, and R282A) had an unusual phenotype; they generated multiple products that varied in length from 21 to 27 nt (Fig. 4; Table I). These products were produced by cleavage at different positions of the PPT substrate (Fig. 3).
sites within the PPT primer, 1–7 nt from the RNA-DNA junction (Fig. 1, IIb), and resulted in incomplete primer removal. In these cases, the final products consisted of a labeled 20-nt DNA with from 1 to 7 nt of RNA attached. Many of the enzymes in this subgroup also produced varying amounts of the correct 20-nt DNA only (i.e. 21 or 22 nt) would tend to accumulate, and the proportion of signal in each band would change over time. To distinguish between these two possibilities, we analyzed the time course of RNase H cleavage with several of the mutants.

Fig. 5 shows a comparison of WT and W266A cleavage patterns seen at times ranging from 1 to 15 min. WT RT made one cleavage at the RNA-DNA junction, which resulted in the accumulation of a single 20-nt product over time; no intermediate size cleavage products were observed, in accord with previous results (38). In contrast, an assay of W266A activity shows that even at the earliest time points, there was a pattern of multiple cleavages. PhosphorImager analysis revealed that the relative proportion of radioactivity in each of four major bands produced by W266A remained roughly the same over the entire time course, with only a slight trend toward accumulation of band 4 DNA (Table II). Toward the end of the incubation, there appeared to be some secondary cleavage, leading to accumulation of a relatively small amount of a product 1 nt smaller than the DNA in band 4 (Fig. 5). This result is in accord with the observation that secondary cleavage normally occurs in the presence of excess enzyme (here, the ratio of RT to P/T was 10:1) and extended incubation (29, 50).

Thus, the data in Fig. 5 illustrate the dramatic difference between the RNase H cleavage patterns of WT and mutant W266A. The results are consistent with the possibility that for the mutant, multiple cleavages occur at the very earliest times and that each enzyme molecule is making one cleavage per P/T, each at a different position. Similar results were obtained with mutant K263A under the same conditions (data not shown).

**Analysis of the Effect of Primer-Template Geometry on RNase H Cleavage**—A group of four αH mutants (G262A, K263A, N265A, and W266A) and WT RT were selected for investigation of the effect(s) that changes in P/T geometry might have on PPT removal. The rationale for selecting these particular mutants was as follows. 1) In the alanine scan of αH and αI residues (Fig. 4), there was a rather stark change in the cleavage pattern between residues Gly262 and Lys263. Mutant G262A appeared to have an essentially WT phenotype, while K263A exhibited the unusual cleavage within the PPT. 2) Residues Gly262 and Trp266 are part of the MBGT (Ref. 43; also see Introduction), which plays an important role in DNA binding, processivity, and frameshift fidelity (43, 45, 47).

To analyze the influence of P/T geometry, the standard assay was modified (see the schematic diagrams of the substrates

---

**Table I**

| 20-nt DNA only | 20-nt DNA plus multiple bands | Multiple bands only |
|----------------|-----------------------------|---------------------|
| T253A          | D256A                       | W266A               |
| V254A          | Q258A                       |                     |
| N256A          | K263A                       |                     |
| G262A          | L260A                       |                     |
| L260A          | I270A                       |                     |
| G262A          | L279A                       |                     |
| A267A<sup>a</sup> | L282A                      |                     |
| S268A          |                             |                     |
| R277A          | Q278A                       |                     |
| C280A          | K281A                       |                     |
| E284A          | G285A                       |                     |
| K287A          |                             |                     |

<sup>a</sup> The 20-nt DNA is the only product released by WT HIV-1 RT in this assay.
<sup>b</sup> The residue at position 267 in WT RT is alanine; thus, A267A is the WT enzyme.
used under each panel of Fig. 6; also see “PPT Primer Removal Assays”). First, we added 20 bases to the 3′-end of the template in all reactions (Fig. 6, bottom of A–C). This moves the 3′-end of the DNA template far enough away from the primer so that any possible effect on positioning of the bound RT is eliminated. Second, we modified the RNA primer used to generate the RNA-DNA chimeric substrate in two ways. 1) We added five RNA bases to the 3′-end of the 15-nt PPT primer to make a 20-nt RNA primer (Fig. 6, bottom of B). After extension by T4 DNA polymerase, the resulting RNA-DNA substrate is a chimeric 20-nt RNA/15-nt DNA annealed to a 55-nt DNA template. This modification allowed us to determine whether cleavage could occur further downstream, relative to the 3′-end of the PPT. 2) We added five bases to both the 5′- and 3′-ends of the PPT to make a 25-nt RNA primer (Fig. 6, bottom of C). After extension by T4 DNA polymerase, the resulting chimeric RNA-DNA is a 25-nt RNA/15-nt DNA annealed to the 55-nt DNA template. The additional bases at the 5′-end of the RNA primer should move the cleavage site if positioning is driven mainly by the location of the 5′-end of the RNA primer. In addition to these substrates, we also included two RNA primers (15 and 20 nt) (Fig. 6, D and E, respectively), which do not contain the PPT sequence. We reasoned that together, these substrates should allow us to determine whether the RT mutations have a specific effect on cleavage of the PPT or positioning in general.

In reactions containing WT RT, the predominant cleavage product from each of the three PPT-containing substrates was 20 nt in length (Fig. 6, A–C, lanes 1–3). When the RNA portion of the RNA-DNA chimera was increased in length, all of the mutants (including G262A) exhibited similar cleavage patterns (Fig. 6, B and C, lanes 2–5). In each case, the mutant enzymes cleaved 1 nt upstream from the RNA-DNA junction of the chimera. Since the labeled plus-strand DNA portion of the substrate was 15 nt in length, the final product consisted of a 15-nt DNA with one RNA base attached (see position of the 16-nt marker). Note that in each case, little or no specific 20-nt product was produced.

When the same enzymes were tested with substrates that do
Specific Interactions between the HIV-1 RT Thumb and PPT

In the present study, we have investigated the role that residues in the αH and αI helices in the HIV-1 RT thumb subdomain play in positioning the bound PPT for correct removal of the PPT. Precise recognition and cleavage of the PPT is a crucial event in retrovirus replication and ultimately determines the in vivo outcome of the viral life cycle. Therefore, precise understanding of the interactions necessary for PPT recognition, we analyzed vertical scan mutants at position 266. The WT tryptophan residue was changed to alanine, glutamic acid, phenylalanine, isoleucine, leucine, arginine, valine, and tyrosine. In a related study, many of these mutants were found to have altered interactions with the P/T, as manifested by an increased P/T dissociation rate constant and changes in AZTTP sensitivity, processivity, and frameshift fidelity (48).

In the standard assay with a 15-nt RNA PPT primer, all of the vertical-scan mutants had some effect on primer removal (Fig. 8). The total RNase H activities of these mutants relative to the WT control (set at 100%) were quantified by PhosphorImager analysis. The mutant activities varied greatly from a low value of 36% (Fig. 8, lane 7) of the total substrate cleaved in 20 min to a high value of 99% (Fig. 8, lane 1). More specifically, substitution of valine (lane 7), glutamic acid (lane 2), and isoleucine (lane 4) gave the lowest activities, while the activities obtained by substitution of alanine (lane 1), tyrosine (lane 8), and arginine (lane 6) were almost as high as that of WT RT.

Intermediate levels of activity were observed with the phenylalanine (lane 3) and leucine (lane 5) mutants. With the exception of W266A, all of the other mutants generated multiple cleavages within the PPT while still retaining the ability to cleave two bases from the RNA-DNA junction, resulting in a 22-nt product. The mutant W266Y with the most conservative substitution (Fig. 8, lane 8) was most like WT enzyme, having ~53% of the total cleavage products represented in the 20-nt product (as determined by PhosphorImager analysis); however, this mutant still made some spurious cleavages within the PPT. The most striking phenotype was that of the 266 residue in the MGBT and several neighboring residues are required for proper interactions with the PPT primer.

Vertical Scan of Residue Trp266—To further examine the interactions necessary for PPT recognition, we analyzed vertical-scan mutants at position 266. The WT tryptophan residue was changed to alanine, glutamic acid, phenylalanine, isoleucine, leucine, arginine, valine, and tyrosine. In a related study, many of these mutants were found to have altered interactions with the P/T, as manifested by an increased P/T dissociation rate constant and changes in AZTTP sensitivity, processivity, and frameshift fidelity (48).

In the standard assay with a 15-nt RNA PPT primer, all of the vertical-scan mutants had some effect on primer removal (Fig. 8). The total RNase H activities of these mutants relative to the WT control (set at 100%) were quantified by PhosphorImager analysis. The mutant activities varied greatly from a low value of 36% (Fig. 8, lane 7) of the total substrate cleaved in 20 min to a high value of 99% (Fig. 8, lane 1). More specifically, substitution of valine (lane 7), glutamic acid (lane 2), and isoleucine (lane 4) gave the lowest activities, while the activities obtained by substitution of alanine (lane 1), tyrosine (lane 8), and arginine (lane 6) were almost as high as that of WT RT. Intermediate levels of activity were observed with the phenylalanine (lane 3) and leucine (lane 5) mutants. With the exception of W266A, all of the other mutants generated multiple cleavages within the PPT while still retaining the ability to produce very small amounts of the specific 20-nt product (Fig. 8, lanes 2–8). There appeared to be a bias in all of these enzymes to cleave two bases from the RNA-DNA junction, resulting in a 22-nt product. The mutant W266Y with the most conservative substitution (Fig. 8, lane 8) was most like WT enzyme, having ~53% of the total cleavage products represented in the 20-nt product (as determined by PhosphorImager analysis); however, this mutant still made some spurious cleavages within the PPT. The most striking phenotype was that of mutant W266A, which cleaved the substrate as well as WT yet exhibited an aberrant cleavage pattern (Fig. 8, lane 1).

**Fig. 6. Influence of P/T geometry on PPT removal.** WT RT and mutants G262A, K263A, N265A, and W266A were tested with PPT-containing substrates having three different P/T geometries (A–C) or with non-PPT-containing substrates with two different geometries (D and E). A schematic representation of each substrate is illustrated below each panel. The RNA portion of each PPT is shown in gray; plus-strand DNA synthesized by extension of the RNA by T4 DNA polymerase is shown in black. An asterisk at the 3′-end of the plus-strand DNA denotes incorporation of [α-32P]dATP. The boundaries of the original 15-nt PPT or non-PPT primers are marked by vertical lines. In each case, the DNA template strand (shown in black) was 55 nt in length. The template was designed so that there were 20 nt of flanking sequence on either side of the 15-nt RNA primers. **A**, the primer was a 15-nt RNA PPT sequence. **B**, five RNA bases were added to the 3′-end of the PPT primer. **C**, five RNA bases were added to both the 5′- and 3′-ends of the PPT primer. **D**, a 15-nt non-PPT primer was substituted for the PPT. **E**, five additional RNA bases were added to the 3′-end of the non-PPT primer. In each panel, lane **C** refers to a control reaction in which the RNA primer was extended by T4 DNA polymerase without further incubation with RT. The positions of the 35-nt substrate and the 20- and 16-nt products are shown to the left.

With WT, the mutant enzymes showed a significantly different pattern of cleavage with the three PPT-containing substrates. All of the mutant enzymes showed at least some cleavage within the PPT portion of the substrate. Thus, when mutants K263A, N265A, and W266A were assayed with the 15-nt PPT primer, the cleavages were exclusively within the PPT, with little or no specific cleavage at its 3′ terminus (Fig. 7, right side, line 1). When the RNA portion of the PPT substrates was lengthened, all of the mutant enzymes cleaved 1 nt from the RNA-DNA junction (Fig. 7, right side, lines 2 and 3). Once again, little or no cleavage occurred at the expected position for specific cleavage of the PPT. In the substrates that did not contain the PPT, all of the mutants tended to cleave toward the RNA-DNA junction (Fig. 7, lines 4 and 5). However, the mutant enzymes catalyzed additional cleavages closer to the 5′-end of the RNA primer (Fig. 7, right side, lines 4 and 5). Thus, from the results presented in Figs. 6 and 7, it appears that HIV-1 WT RT participates in specific interactions with the P/T that correctly position the RNase H domain for specific cleavage at the 3′ terminus of the PPT. The mutant enzymes appear to have lost the ability to recognize this site and preferentially cleave at positions either 5′ or 3′ of the WT cleavage site. Taken together, these results indicate that the Trp266 residue in the MGBT and several neighboring residues are required for proper interactions with the PPT primer.

**Fig. 7. Schematic representation of the cleavage patterns observed.** When WT enzyme was added to PPT-containing substrates, cleavage occurred primarily at the 3′-end of the PPT (Fig. 7, left side, lines 1–3), regardless of P/T geometry. Other minor cleavages occurred toward the RNA-DNA junction, but there was never a pattern of prominent cleavages within the PPT, as was observed with the mutant enzymes (Fig. 6, A–C, and Fig. 7, right side, lines 1–3). In fact, compared not contain the PPT, the results were more similar for the WT and mutant RTs (Fig. 6, D and E, lanes 1–5). In each case, cleavage was 14–18 nt from the 5′-end of the RNA (see Fig. 1, IA; Ref. 22). However, in contrast to WT RT (Fig. 6, D and E, lanes 1), all of the mutant enzymes had a greater tendency to make secondary cleavages toward the 5′-end of the RNA portion of the substrate (lanes 2–5).

Fig. 7 shows a schematic representation of the cleavage patterns observed. When WT enzyme was added to PPT-containing substrates, cleavage occurred primarily at the 3′-end of the PPT (Fig. 7, left side, lines 1–3), regardless of P/T geometry. Other minor cleavages occurred toward the RNA-DNA junction, but there was never a pattern of prominent cleavages within the PPT, as was observed with the mutant enzymes (Fig. 6, A–C, and Fig. 7, right side, lines 1–3). In fact, compared
mined the effect of two point mutations in the PPT primer.

The results of this study are not directly comparable with ours, since the substrates used were quite different; in their case, a probe annealed to a longer DNA template and was similar to the 5′-end of proviral DNA (for reviews, see Refs. 6, 59, and 60). Our approach was to test RNase H-catalyzed primer removal activity of mutant enzymes bearing single alanine substitutions in residues 253–270 and 277–287 (45, 46) and single amino acid replacements of Trp266 (48). Each mutant was tested in the standard PPT removal assay. The unusually high amount of products smaller than 20-nt generated by the W266F mutant RT (lane 7) may be due to trace amounts of DNase contamination in this preparation, which during the 20-min incubation could have degraded some of the 20-nt DNA. The lane marked Con is the substrate extended by T4 DNA polymerase without further addition of RT. To calculate the percentage of cleavage (shown below each lane), the sum of the radioactivity (expressed as “volume”) of all of the cleavage products generated by each enzyme was quantified by PhosphorImager analysis and compared with the value for the WT control (set as 100%). The positions of the 35-nt substrate and the 20-nt product generated by WT RT are shown to the right.

Fig. 7. Schematic summary of the WT and mutant cleavage patterns observed with different P/T geometries. The summary is based on the gel data of Fig. 6. A schematic version of each substrate is shown. The DNA template is depicted in black, the RNA primer is shown in gray, and plus-strand DNA resulting from extension by T4 DNA polymerase is shown as a dashed line. Vertical arrows show the major sites of cleavage obtained with each substrate. Note that the mutant enzymes fail to cleave at the 3′-end of the PPT primer.

Fig. 8. Vertical scan of residue Trp266. Trp266 was replaced by alanine (A), glutamic acid (E), phenylalanine (F), isoleucine (I), leucine (L), arginine (R), valine (V), and tyrosine (Y) (48). Each mutant was tested in the standard PPT removal assay. The unusually high amount of products smaller than 20-nt generated by the W266F mutant RT (lane 7) may be due to trace amounts of DNase contamination in this preparation, which during the 20-min incubation could have degraded some of the 20-nt DNA. The lane marked Con is the substrate extended by T4 DNA polymerase without further addition of RT. To calculate the percentage of cleavage (shown below each lane), the sum of the radioactivity (expressed as “volume”) of all of the cleavage products generated by each enzyme was quantified by PhosphorImager analysis and compared with the value for the WT control (set as 100%). The positions of the 35-nt substrate and the 20-nt product generated by WT RT are shown to the right.

fines one end of proviral DNA (for reviews, see Refs. 6, 59, and 60). Our approach was to test RNase H-catalyzed primer removal activity of mutant enzymes bearing single alanine substitutions in residues 253–270 and 277–287 (45, 46) and single amino acid replacements of Trp266 (48). An in vitro assay was used that makes it possible to look specifically at PPT primer removal without regard to the polymerase activity of each enzyme (Fig. 3; Ref. 38).

Recently, a report by Gao et al. (15) appeared that determined the effect of two point mutations in aH, i.e. G262A and W266T, on RNase H cleavage of PPT-containing substrates. The results of this study are not directly comparable with ours, since the substrates used were quite different; in their case, a 20-nt DNA primer, consisting of sequences including or near the PPT, was annealed to an 81-nt RNA template. In this configuration, RNase H cleavage is dictated by the 3′ terminus of the DNA primer (13, 17, 24–31). By contrast, the PPT-containing substrates employed here were all short RNA primers annealed to a longer DNA template and were similar to intermediates expected to form during the course of reverse transcription. Thus, the types of cleavage patterns generated in the two studies are qualitatively different. Nevertheless, despite these differences, the overall conclusion of both studies is the same, namely that mutations in aH affect the specificity of RNase H cleavage. Interestingly, based on assays with RTs having the W266T mutation in either the p66 or p51 subunit, Gao et al. (15) also concluded that the thumb subdomains in both subunits play an important role in the selectivity, specificity, and efficiency of RNase H cleavage.

The mutant RTs analyzed here were extensively characterized in related work that focused on efforts to understand how the aH and αl residues contribute to DNA polymerase activity (45–47). Functional studies performed in conjunction with molecular dynamics modeling led to identification of the highly conserved MGBT structural motif, which contains three residues in αH, including Gly262 and Trp266, and is important for P/T binding, processive DNA synthesis, and frameshift fidelity (43). These results imply that αH residues play a role in positioning the P/T prior to incorporation of an incoming dNTP. Since the polymerase domain is also important for proper cleavage of PPT-containing substrates (15, 18, 19, 37, 38), it seemed likely that residues that are important for correct positioning of the P/T during polymerization are also involved in correct orientation of the RNase H domain during specific cleavage from nascent DNA.

In our initial survey of individual alanine substitutions in helices aH and αl (Fig. 4, Table I), we discovered a subgroup of the mutant RTs that exhibits an unusual phenotype. These enzymes cleave the PPT substrate at multiple positions within the PPT to produce products from 21 to 27 nt in length (Fig. 4, Table I). For this to occur, the 5′ terminus of the PPT must somehow “slip” from its normal binding position (Fig. 1, compare IIA and IIB). In every case, the limit to this slippage seems to be 7 nt, since there are no cleavage products greater than 27 nt in length. It may be that after moving 7 nt, there is insufficient primer left to efficiently bind RT and allow cleavage to occur.

Analysis of the time course of RNase H cleavage products with the W266A mutant demonstrated that the unusual pattern of cleavage was generated from the earliest times (Fig. 5). Moreover, the distribution of radioactivity in four major bands produced by the mutant remained essentially constant (Table II). This suggests that each band is the product of a single cleavage event. After the initial cleavage, only relatively small amounts of shorter products tend to accumulate over time (Table II, Fig. 5). This indicates that secondary cleavages are not a frequent event. The absence of substantial secondary cleavages may be the result of the RNA portion of the substrate being shortened to an extent that no longer permits rebinding to occur.

To further understand the effects of these mutations on P/T positioning, we performed a more detailed analysis of the activities of four of the alanine-scanning mutants: MGBT mutants G262A and W266A and two mutants with changes in neighboring residues, K263A and N265A. The substrates in the
standard assay were varied so that the relationship between the 5’-end of the RNA primer and the PPT was changed (see schematic diagrams at the bottom of Fig. 6, A–C). In one case, we added five RNA bases to the 3’-end of the PPT primer (Fig. 6B). WT RT was still able to recognize this substrate and make the appropriate cleavage at the 3’-end of the PPT (Fig. 6B). In contrast, the mutants cleaved this substrate in the vicinity of the RNA-DNA junction rather than at the 3’-end of the PPT (Fig. 6B). When we added an additional five RNA bases to both the 5’- and 3’-ends of the PPT, the mutant and WT cleavage patterns did not change (Fig. 6C). These results show that WT enzyme is recognizing some feature of the PPT primer that directs the cleavage to the appropriate position independent of P/T geometry (Fig. 7). The mutants have lost the ability to recognize the PPT cleavage site and prefer other cleavage sites over the normal one. This is evidenced by the fact that, regardless of which substrate we used, the specific 20-nt cleavage product was not produced by these mutants (Figs. 6 and 7). It is possible that under normal circumstances some unique aspect of the PPT is recognized to correctly position the RNase H domain for specific cleavage. The alanine-scanning mutations appear to block this interaction, allowing the enzyme to “slide” to one side or the other of the PPT to allow cleavage to occur.

Interestingly, mutational effects were less obvious with non-PPT-containing substrates: the WT and mutant cleavage patterns were more closely related, in contrast to the striking differences seen in assays with the PPT-containing substrates (Fig. 6, compare D and E with A and B; Fig. 7, lines 4 and 5).

The specificity of RT-PPT substrate interactions was also investigated by testing vertical scan mutants at position 266 (48); the mutations included changes of tryptophan to alanine, glutamic acid, phenylalanine, isoleucine, leucine, arginine, valine, and tyrosine (Fig. 8). Tryptophan was originally chosen for this scan, since of the five MGBT residues, it is most responsible for binding of RT to the PPT (43, 45, 48).

In our assay, mutants with alanine, arginine, and tyrosine substitutions were able to completely cleave the substrate, but all tended to “slip” on the template like mutant W266A; however, in each case, the detailed cleavage pattern was not exactly the same as that of W266A (Fig. 8). The alanine substitution gave the most homogeneous set of cleavage products, with each major product obtained in roughly equivalent amounts (Figs. 4, 5, and 8; Table II). It is interesting to note that although the total RNase H activity of mutant W266A was similar to that of WT (99% compared with 100%; Fig. 8), a barely detectable amount of the specific 20-nt cleavage product was produced. The arginine substitution produced some specific 20-nt product (20 nt) than the other mutants (the 20-nt DNA product was not produced by these mutants (Figs. 6 and 7). It is possible that under normal circumstances some unique aspect of the PPT is recognized to correctly position the RNase H domain for specific cleavage. The alanine-scanning mutations appear to block this interaction, allowing the enzyme to “slide” to one side or the other of the PPT to allow cleavage to occur.

Interestingly, mutational effects were less obvious with non-PPT-containing substrates: the WT and mutant cleavage patterns were more closely related, in contrast to the striking differences seen in assays with the PPT-containing substrates (Fig. 6, compare D and E with A and B; Fig. 7, lines 4 and 5).

The specificity of RT-PPT substrate interactions was also investigated by testing vertical scan mutants at position 266 (48); the mutations included changes of tryptophan to alanine, glutamic acid, phenylalanine, isoleucine, leucine, arginine, valine, and tyrosine (Fig. 8). Tryptophan was originally chosen for this scan, since of the five MGBT residues, it is most responsible for binding of RT to the PPT (43, 45, 48).

In conclusion, we have shown that mutations in the thumb subdomain of HIV-1 p66 RT can have a profound influence on specific RNase H cleavage at the 3’ terminus of the PPT. In all likelihood, this is due to an effect on positioning RT on PPT-containing substrates, which in one case (the substrate with the 15-nt PPT primer) models the intermediate present during viral reverse transcription. It is perhaps not too surprising that residues that are important for holding the P/T in place during polymerization would also play a significant role in positioning the P/T during RNase H cleavage. It is interesting, however, to see that some of these positioning effects appear to influence interactions with the PPT primer in a specific fashion. Since our current knowledge of how HIV-1 RT interacts with nucleic acids comes from analysis of co-crystals formed with DNA duplexes (28, 36, 44), this study underscores the need to investigate the structure of RT bound to P/Ts that consist of RNA-DNA hybrids and, more specifically, a hybrid containing the PPT.

Acknowledgment—We thank Dr. Tryun Wu for generous assistance in preparing the figures for final publication format.

REFERENCES
1. Collett, M. S., Dierks, P., Parsons, J. T., and Faras, A. J. (1978) Nature 272, 181–184.
2. Gilboa, E., Mitra, S. W., Goff, S., and Baltimore, D. (1979) Cell 18, 93–100.
3. Katz, R. A., and Shalaka, A. M. (1994) Annu. Rev. Biochem. 63, 133–173.
4. Arts, E. J., and Wainberg, M. A. (1996) Adv. Virus Res. 46, 97–163.
5. Telschow, A., and Goff, S. P. (1997) in Reverse Transcriptase (Skalka, A. M., and Goff, S. P., eds) pp. 103–117, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
6. Huber, H. E., and Richardson, C. C. (1990) J. Biol. Chem. 265, 10565–10573.
7. Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Barbbara, R. A. (1993) J. Biol. Chem. 270, 28169–28176.
8. Randolph, C. A., and Champoux, J. J. (1994) J. Biol. Chem. 269, 19207–19215.
9. Miller, M. D., Wang, B., and Bushman, F. D. (1995) J. Virol. 69, 3938–3944.
10. Bowman, E. H., Pathak, V. K., and Hu, W. S. (1996) J. Virol. 70, 1687–1694.
11. Powell, M. D., and Levin, J. G. (1996) J. Virol. 70, 5286–5296.
12. Wöhr, B. M., and Moelling, K. (1996) Biochemistry 29, 10141–10147.
13. Wöhr, B. M., Volkman, S., and Moelling, K. (1997) J. Mol. Biol. 220, 801–818.
14. Gao, H.-J., Boyer, P. L., Arnold, E., and Hughes, S. H. (1998) J. Mol. Biol. 277, 559–572.
15. Luo, G., Sharment, L., and Taylor, J. (1990) J. Virol. 64, 592–597.
16. Quo, J., Wu, Y., Yuan, Z. Y., Post, K., and Levin, J. G. (1995) Biochemistry 34, 5018–5029.
17. Schultz, S. J., and Champoux, J. J. (1996) J. Virol. 70, 8630–8638.
18. Zhan, X., and Crouch, R. J. (1997) J. Biol. Chem. 272, 22023–22029.
19. Fedoroff, O. Y., Salazar, M., and Reid, B. R. (1997) J. Mol. Biol. 265, 225–239.
20. Fedoroff, O. Y., Salazar, M., and Reid, B. R. (1993) J. Mol. Biol. 233, 509–523.
21. DeStefano, J. J. (1995) Nucleic Acids Res. 23, 3901–3908.
22. Hughes, S. H., Housman, Z., Le Grice, S. F. J., Lenz, K., and Arnold, E. (1996) J. Virol. 70, 2679–2683.
23. Furfine, E. S., and Reardon, J. E. (1991) J. Biol. Chem. 266, 406–412.
24. Doe, T. B., and Taylor, J. (1992) J. Virol. 66, 4281–4289.
25. Gopalakrishnan, V., Neluka, A., and Benkovics, S. J. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10763–10767.
26. Kohlschütz, L. A., Wang, J., Friedman, J. M., Rice, P. A., and Stitz, T. A. (1992) Science 256, 1785–1786.
27. Jacobsen, A., Ding, J., Nanni, R. G., Clark A. D., Jr., Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H., and Arnold, E. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6320–6324.
29. Post, K., Guo, J., Kalam, E., Uchida, T., Crouch, R. J., and Levin J. G. (1993) *Biochemistry* **32**, 5508–5517.
30. Ben-Artzi, H., Zeelon, E., Amit, B., Wortzel, A., Gorecki, M., and Penet, A. (1993) *Biochemistry* **32**, 5508–5517.
31. Goette, M., Fackler, S., Hermann, T., Perola, E., Cellini, L., Gross, H. J., Le Grice, S. F. J., and Heumann, H. (1995) *EMBO J.* **14**, 833–841.
32. Palaniappan, C., Fuentes, G. M., Rodriguez-Rodriguez, L., Fay, P. J., and Bambara, R. A. (1996) *J. Biol. Chem.* **271**, 3850–3856.
33. Palaniappan, C., Kim, J. K., Wisniewski, M., Fay, P. J., and Bambara, R. A. (1998) *J. Biol. Chem.* **273**, 3808–3816.
34. Kraulis, P. (1991) *J. Appl. Crystallogr.* **24**, 946–950.
35. Merritt, E. A., and Bacon, D. J. (1997) *Methods Enzymol.* **277**, 505–524.
36. Ding, J., Das, K., Hsiou, Y., Sarafianos, S. G., Clark, A. D., Jr., Jacobo-Molina, A., Tantillo, C., Hughes, S. H., and Arnold, E. (1998) *J. Mol. Biol.* **284**, 1669–1675.
37. Beard, W. A., Stahl, S. J., Kim, H.-R., Bebenek, K., Kumar, A., Strub, M.-P., Becerra, S. P., Kunkel, T. A., and Wilson, S. H. (1994) *J. Biol. Chem.* **269**, 28091–28097.
38. Beard, W. A., Minnick, D. T., Wade, C. L., Prasad, R., Won, R. J., Prasad, R., Kunkel, T. A., and Wilson, S. H. (1996) *J. Biol. Chem.* **271**, 12213–12220.
39. Kulkosky, J., Katz, R. A., and Skalka, A. M. (1990) *J. Acquired Immune Defic. Syndr.* **3**, 852–858.
40. Whitcomb, J. M., Kumar, R., and Hughes, S. H. (1990) *J. Virol.* **64**, 4903–4906.
41. Smith, J. S., Kim, S., and Roth, M. J. (1990) *J. Virol.* **64**, 6286–6290.
42. Pullen, K. A., Rattray, A. J., and Champoux, J. J. (1993) *J. Biol. Chem.* **268**, 6221–6227.
43. Varma, H., and Brown, P. (1997) in *Retroviruses* (Coffin, J. M., Hughes, S. H., and Varmus, H. E., eds) pp. 161–203, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
44. Klarmann, G. J., Yu, H., Chen, X., Dougherty, J. P., and Preston, B. D. (1997) *J. Virol.* **71**, 9259–9269.
Residues in the $\alpha$H and $\alpha$I Helices of the HIV-1 Reverse Transcriptase Thumb Subdomain Required for the Specificity of RNase H-catalyzed Removal of the Polypurine Tract Primer

Michael D. Powell, William A. Beard, Katarzyna Bebenek, Kathryn J. Howard, Stuart F. J. Le Grice, Thomas A. Darden, Thomas A. Kunkel, Samuel H. Wilson and Judith G. Levin

J. Biol. Chem. 1999, 274:19885-19893.
doi: 10.1074/jbc.274.28.19885

Access the most updated version of this article at http://www.jbc.org/content/274/28/19885

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 57 references, 33 of which can be accessed free at http://www.jbc.org/content/274/28/19885.full.html#ref-list-1