Altering the RNase H Primer Grip of Human Immunodeficiency Virus Reverse Transcriptase Modifies Cleavage Specificity

Jason W. Rausch, Daniela Lener, Jennifer T. Miller, John G. Julias, Stephen H. Hughes, and Stuart F. J. Le Grice*

HIV Drug Resistance Program, National Cancer Institute−Frederick, Frederick, Maryland 21702

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ABSTRACT: Recent crystallographic data suggest that conserved residues in the connection subdomain and C-terminal ribonuclease H (RNase H) domain of human immunodeficiency virus type 1 reverse transcriptase (HIV-1 RT) contact the nascent DNA primer and modulate the trajectory of the template relative to the RNase H catalytic center. Within the RNase H domain, these residues include Thr473, Glu475, Lys476, Tyr501, and Ile505, while His539 and Asn474 interact with the scissile phosphate of the RNA template. Amino acid substitutions at several of these positions were evaluated in the context of hydrolysis of nonspecific RNA−DNA hybrids and substrates mimicking specific RNase H-mediated events. With the exception of mutant I505G, which exhibited a dimerization defect, substituting alanine at positions 473−476 and 501 had minimal consequences for DNA synthesis on duplex and hybrid DNA and RNA substrates. In contrast, the efficiency with which most mutants catalyzed polymerization-independent RNase H cleavage was sharply reduced. This deficiency was more pronounced when mutant enzymes were challenged to process the (+) strand polypurine tract (PPT) primer from either (+) RNA or a PPT/(+) DNA RNA/DNA chimera. Reduced polymerization-independent RNase H activity also significantly influenced the rate of DNA strand transfer, suggesting the donor template must be reduced in size below 13 nt before this event proceeds.

During retrovirus replication, one function of the ribonuclease H (RNase H)[1] domain of reverse transcriptase (RT) is the nonspecific hydrolysis that removes RNA from the RNA/DNA replication intermediate, making nascent DNA available for a second round of synthesis (*1*). However, there are also steps critical for synthesis of a replication-competent double-stranded viral DNA that require a considerable degree of cleavage specificity from the same domain. These include: (a) release of the cognate tRNA primer from (−) DNA prior to second strand transfer (*2, 3*); (b) excision of the polypurine tract (PPT) from the replication intermediate, providing the appropriate primer for (+) strand DNA synthesis; and (c) removal of the PPT primer from (+) strand DNA, which defines sequences critical for integration (*4*). Moreover, the PPT serves to prime (+) strand synthesis by virtue of its resistance to hydrolysis. An intriguing picture thus emerges of an enzyme with both relaxed and exquisite selectivity, and one that is required to ignore a particular RNA sequence while destroying over 99% of the viral genome. Interestingly, each specialized cleavage event requires that RT accommodate an unusual structural element within the replication intermediate, such as the RNA−DNA junctions separating duplex DNA from RNA/DNA hybrids encountered during removal of (+) and (−) strand primers, or the juxtaposed homopolymeric tracts within RNA/DNA duplexes that contain the PPTs. Because these elements must by accommodated differently by the retroviral polymerase, it is likely that they serve as structural determinants for specialized RNase H cleavage events. Furthermore, it is possible that by modulating contacts between these special structures and motifs within RT responsible for correctly positioning the nucleic acid substrate, cleavage specificity could be altered. A better understanding of these processes will require a detailed structural and biochemical evaluation of the manner in which RT interacts with such RNA−DNA hybrids. Unfortunately, despite extensive studies with *Escherichia coli* RNase H, on which many mechanistic aspects of its retroviral counterpart have been based (*5*−*11*), a cocrystal with an RNA/DNA hybrid is still unavailable.

For the HIV-1 enzyme, a high-resolution structure of the p66/p51 heterodimer complexed with a PPT-containing RNA/DNA hybrid (*12*) has complemented studies on unliganded (*13*), inhibitor-bound (*14*−*19*), and DNA-containing enzyme (*20, 21*). It also provides a major advance in understanding both RNase H specificity and resistance of the PPT to hydrolysis. Emerging from this structure is the suggestion that there is an "RNase H primer grip", a series of p66 residues within the connection subdomain and RNase H domain that interact with DNA primer strand and may help control the trajectory of the RNA template relative to the RNase H catalytic center. Amino acids of the RNase H domain contributing to the RNase H primer grip include the -Thr473-Asn474-Gln475-Lys476- quartet, Tyr501, and Ile505 (Figure 1). Conservation of these residues between retroviral and bacterial RNases H (*12*), together with the decreased catalytic activity of *E. coli* RNase H mutated at equivalent positions (*22*), suggests these residues are important. A second and more prominent feature of the cocrystal is distortion of the nucleic acid duplex in the vicinity of the

* Corresponding author. Tel: (301) 846 5256. Fax: (301) 846 6013. E-mail: slegrice@ncifcrf.gov.

[1] Abbreviations: HIV, human immunodeficiency virus; nt, nucleotide(s); PBS, primer binding site; PPT, polypurine tract; RNase H, ribonuclease H; RT, reverse transcriptase.

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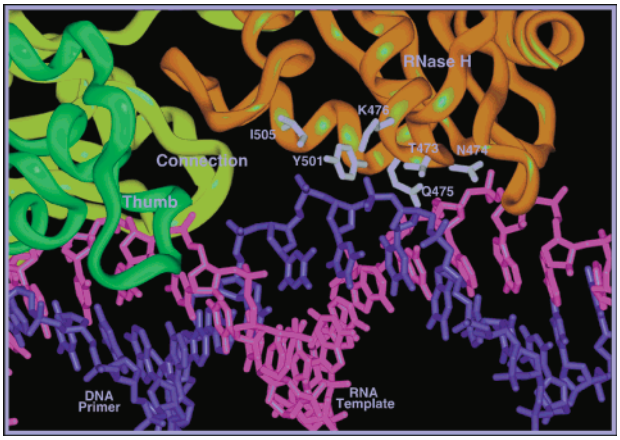

FIGURE 1: Ribbon diagram illustrating the RNase H primer grip residues of the HIV-1 RNase H domain. For simplicity, portions of the p51 subunit in the vicinity of the p66 RNase H domain have been omitted. The p66 thumb subdomain is indicated in green, the connection in yellow, and the C-terminal RNase H domain in orange. Residues of the RNase H domain contributing to the RNase H primed grip have been highlighted. Primer and template nucleotides of the RNA/DNA hybrid are indicated in blue and magenta, respectively.

RNase H catalytic center and a dramatic deviation from standard base pairing. Alterations in this region include a combination of unpaired, mispaired, and weakly paired bases, suggesting the possibility that the RNase H primer grip actively alters the nucleic acid structure in the vicinity of the active site. Although we recently demonstrated that significant structural deformations exist in a (+) PPT RNA/(−) DNA hybrid and assist in "guiding" HIV-1 RT to cleave at the PPT 3′ terminus (M. Kvaratskhelia, S. Budihas, and S. F. J. Le Grice, manuscript submitted for publication), the RNase H primer may also contribute to the structural distortion observed in the crystal.

In this paper, the RNase H primer grip residues Thr473−Lys476 and Tyr501 were subjected to alanine scanning mutagenesis. At the same time, Ile505 was altered to Gly to match its *E. coli* counterpart. With the exception of p66 variant Ile505Gly, which failed to dimerize, all of the mutants could be reconstituted into stable heterodimers with minimal alterations to DNA polymerase function on A-form RNA, B-form DNA, and H-form template-primer duplexes. RNase H activity was evaluated on a nonspecific RNA/DNA hybrid and those mimicking either PPT processing or DNA strand transfer between RNA templates. Replacement of Lys476 caused minimal perturbation of RNase H function, while modifications at other residues led to significant alterations of cleavage specificity. Most notable was the $p66^{Y501A}$/p51 mutant that, despite retaining considerable levels of polymerization-dependent RNase H activity, had a dramatically reduced ability to excise the PPT primer from (+) RNA or (+) DNA. Finally, loss of DNA strand transfer function was found to correlate with (a) the failure of RNA fragments at the 5′ terminus of the donor genome to dissociate from nascent DNA and (b) reuse of these residual fragments as primers to copy the nascent DNA.

MATERIALS AND METHODS

Construction and Purification of HIV-1 RT RNase H Primer Grip Mutants. Generation of HIV-1 proviral clones containing RNase H primer grip mutations is described elsewhere (J. G. Julias, M. J. McWilliams, S. Sarafianos, E. Arnold, and S. H. Hughes, manuscript submitted for publication). The sequence coding for the portion of RT containing these mutations was amplified using the polymerase chain reaction (PCR) and ligated as a *Kpn*I/*Hin*dIII fragment into the previously described RT expression vector pRT (*23*) from which the homologous wild-type sequence was removed. Mutagenesis was verified by DNA sequencing. Mutant p66 subunits expressed from modified pRT vectors were reconstituted with wild-type p51 to form p66/p51 heterodimers (*24*). Reconstituted heterodimers were purified by metal chelate (Ni^{2+}-nitrilotriacetic acid-Sepharose) and ion exchange chromatography (Mono-S). The $p66^{I505G}$/p51 enzyme failed to dimerize and was not studied further.

Analysis of DNA-Dependent DNA Polymerase and RNase H Activities. DNA-dependent DNA-synthesis was measured on a 71 nt DNA template annealed to a 5′ end-labeled 36 nt DNA primer as previously described (*25*). Briefly, reactions were initiated by adding 10 nM enzyme to a reaction mixture containing 50 nM template/primer, 200 μM dNTPs, 10 mM Tris-HCl (pH 8.0), 80 mM NaCl, and 6 mM $MgCl_2$ at 37 °C. Aliquots were removed at intervals indicated in the text and mixed with urea-based gel-loading buffer to quench the reaction. RNase H activity was evaluated similarly on an in vitro transcribed, 5′ end-labeled 90 nt RNA template annealed to the same 36 nt DNA primer as above. Reaction conditions were identical to those of the DNA synthesis assay except that dNTPs were excluded from the mixture. Reaction products in both assays were fractionated by high-voltage electrophoresis through 10% (w/v) polyacrylamide gels containing 7 M urea in Tris/borate/EDTA buffer. After being dried, gels were subjected to autoradiography and/or phosphorimaging analysis using a Molecular Imager FX phosphorimager (BioRad, Hercules, CA).

PPT Selection and Removal. PPT selection and removal were evaluated as described previously (M. Kvaratskhelia, S. Budihas, and S. F. J. Le Grice, manuscript submitted for publication). A 5′ end-labeled 30 nt RNA or chimeric oligonucleotides containing the HIV-1 polypurine tract and flanking sequences annealed to a 55 nt DNA served as the substrates for PPT selection and removal, respectively. Based on recent studies that an r(U) tract 5′ to the PPT is important in virus replication (*26*−*28*), this sequence was included in the PPT-containing oligonucleotides. RT (10 nM) was added to a mixture containing one of these hybrid duplexes (final concentration 50 nM) in 10 mM Tris-HCl (pH 8.0), 80 mM NaCl, and 6 mM $MgCl_2$ at 37 °C to initiate cleavage. Reactions were quenched and hydrolysis products processed in a manner similar to that used to evaluate DNA synthesis and RNase H cleavage products, except that a 15% polyacrylamide/7 M urea gel was used for fractionation.

Model System for (−) Strand Transfer. The interdependence of RNase H and strand transfer activities was investigated using a model system similar to the one originally described (*29*). To evaluate minus strand transfer, RT was added to a reaction mixture containing a 5′ end-labeled 20 nt DNA primer annealed to the 3′ end of a 40 nt "donor" RNA and a 40 nt "acceptor" RNA, the 20 3′ nucleotides of which share sequence identity with the 5′ terminus of the donor (*3*). Buffer components were identical to those used in the DNA synthesis and RNase H cleavage

reactions described above. Concentrations of RT, primer/donor duplex, and acceptor templates were 250, 50, and 250 nM, respectively. Reactions were quenched at the times indicated in the text and reaction products fractionated and analyzed as previously described. RNase H cleavage during RNA-dependent DNA synthesis was evaluated similarly, except that the donor RNA rather than the primer DNA was 5′ end-labeled. To analyze secondary initiation products, reactions were terminated by extraction with phenol/chloroform/isoamyl alcohol (25:24:1), and the products were precipitated in the presence of glycogen, dried, and resuspended in 20 μL of RT reaction buffer (see above). Half of each reaction was subjected to treatment with 0.2 unit of RNase-free DNase I for 45 min at 37 °C, and the other half was stored at 4 °C. Each half was then mixed 1:1 with a urea-based gel loading buffer and fractionated and analyzed as above.

Minus-Strand DNA Synthesis. RNA-dependent DNA synthesis was evaluated on an in vitro transcribed, 5′ ^{32}P-end-labeled tRNA^{Lys,3} annealed to a 105 nt HIV-1 (HXB2) PBS RNA as previously described (*30*). Enzyme (83 nM) and template/primer (28 nM) were preincubated for 2 min at 37 °C in 10 mM Tris/HCl, pH 7.8, 10 mM $MgCl_2$, 100 mM KCl, 5 mM DTT, after which dNTPs were added to a final concentration of 200 μM to initiate the reaction. At 1 and 5 min after initiation, aliquots were removed, and RT was extracted using an equal volume of phenol/$CHCl_3$/isoamyl alcohol (25:24:1). Nucleic acid in the aqueous phase was precipitated in the presence of glycogen, and the dried sample was resuspended in urea-based gel loading buffer. Reaction products were fractionated by high-voltage electrophoresis through 6% (w/v) polyacrylamide gels, which were dried and subjected to autoradiography and phosphorimaging analysis.

Determination of Dissociation Constants. The affinity of wild-type and mutant RTs for duplex DNA was measured by electrophoretic mobility shift analysis. Enzymes were diluted to various concentrations (10−800 nM) in 50 mM Tris-HCl, pH 7.0, 25 mM NaCl, 1 mM EDTA, 0.5 mM EDTA, 0.5 mM DTT, 50% (v/v) glycerol. Then 0.1 volume of diluted enzyme was added to a mixture containing a 5′ ^{32}P-labeled 30 nt DNA primer annealed to a 40 nt DNA template in 10 mM HEPES, pH 7.5, 1 mM $MgCl_2$, 25 mM KCl, 1 mM DTT, 0.5 mM EDTA. After 5 min at 4 °C, 0.1 volume of 10× nondenaturing gel loading buffer (50% glycerol, 0.05% xylene cyanole, 0.05% bromophenol blue) was added, and the samples were applied to a nondenaturing 5% polyacrylamide gel (25 mM Tris, 192 mM glycine, 1 mM EDTA, pH 8.5). Following electrophoresis (at 4 °C), gels were dried and subjected to phosphorimaging analysis. Quantity One image analysis software (BioRad, Hercules, CA) was used to quantify shifted complexes, and K_d values were determined by curve fitting analysis using Delta Graph software (SSPS, Chicago, IL).

RESULTS

DNA Polymerase Activity of RNase H Mutants. Alanine substitutions were introduced at positions 473−476 and 501, while Ile505 was altered to glycine. Restricting our analysis to these residues allowed us to explore mutations confined to the RNase H domain; i.e., additional residues highlighted by Sarafianos et al. (*12*) are present in both the p66 and p51 subunits. With the exception of the I505G substitution, all mutant enzymes could be purified as stable heterodimers using a previously described reconstitution protocol (*24*); the latter mutant was therefore eliminated from subsequent analysis. Previous studies have indicated that altering the RNase H domain of HIV-1 RT can impact significantly on the ability of mutant enzymes to catalyze DNA synthesis. A good example of this is the mutant p66/p51Δ13 which supports oligonucleotide-primed (−) strand strong-stop DNA synthesis on the viral RNA genome, yet was devoid of activity when natural or synthetic tRNA was substituted as primer (*31*−*33*). Based on these findings, we first investigated whether altering the RNase H primer grip influenced DNA polymerase activity.

Both B-form DNA and A-form RNA substrates were evaluated (Figure 2 [A],[B]). The duplex DNA substrate comprises a 71 nt template to which a 36 nt ^{32}P-end-labeled primer is hybridized. An inverted repeat sequence on the single-stranded template allows this to assume a short hairpin configuration, which we used to measure the ability of mutant enzymes to resolve intramolecular duplexes (*34*). As indicated in Figure 2[C], wild type HIV-1 RT transiently pauses at the hairpin, but can resolve this and proceed to the 5′ terminus of the template. While minor differences in the pausing patters are evident, all RNase H primer grip mutants showed little difference in their DNA-dependent DNA polymerase activity. Figure 2[D] illustrates tRNA-primed (−) strand strong-stop DNA synthesis profiles of the same mutants. Although the tRNA primer employed for these experiments was derived by *in vitro* transcription, we previously showed that this surrogate can be used to highlight deficiencies in RNA-dependent DNA synthesis (*31*). Once again we observed little difference in the levels of (−) strand strong-stop DNA, and in each case minor pausing was evident in the vicinity of the TAR loop. Thus, although the data of Figure 2[C],[D] are qualitative, there was little difference in the manner in which duplex RNA and DNA substrates were copied by enzymes altered in the RNase H primer grip. Data of a later section indicate this also holds true for an RNA/DNA hybrid. Finally, the affinity of each enzyme for duplex DNA, determined by electrophoretic mobility shift analysis (Table 1), indicates only modest alterations to the strength of the nucleoprotein complex.

RNase H Primer Grip Mutations Suppress Polymerization-Independent RNase H Cleavage. Preliminary characterization of RNase H function was undertaken with a 90 nt ^{32}P-end-labeled RNA to which a 36 nt DNA primer was hybridized at the 3′ terminus (Figure 3 [A]). In the absence of DNA synthesis, wild-type HIV-1 RT hydrolyzes the RNA/DNA hybrid in a two-step fashion. Location of the DNA polymerase catalytic center over the primer 3′-OH induces cleavage of the RNA template at a distance defined by the spatial separation of the polymerase and RNase H active sites (17 bp). After this, directional processing of the template proceeds to within 8 bp of the primer 3′ terminus, after which the relatively short RNA/DNA hybrid dissociates. On the substrate depicted in Figure 3[A], these two events are illustrated by the sequential appearance of 71 nt (−17 cleavage) and 62 nt (−8 cleavage) hydrolysis products. Analysis of the RNase H primer grip mutants indicates p66^{K476A}/p51 RT produces a cleavage pattern similar to the

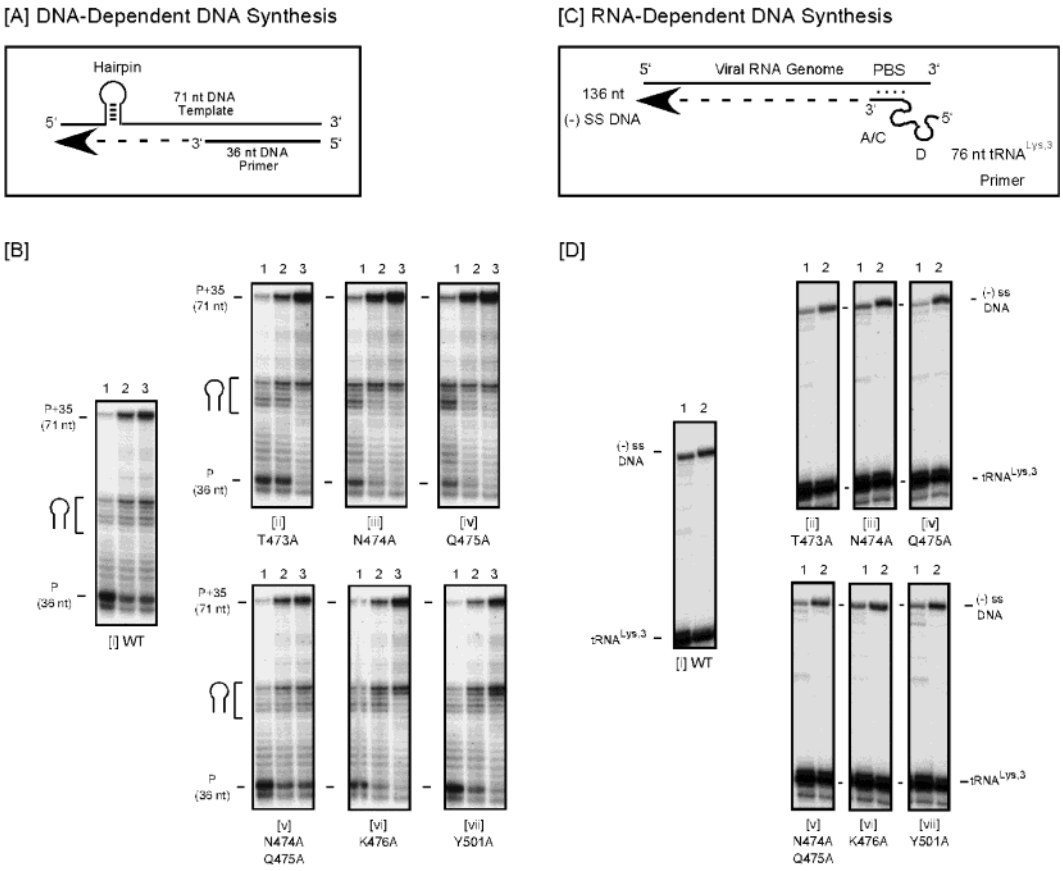

FIGURE 2: DNA polymerase activities of HIV-1 RNase H primer grip mutants. [A] DNA-dependent DNA polymerase, Substrate was a 71 nt template to which a 36 nt, ^{32}P-end-labeled primer was hybridized at the 3′ terminus. A inverted repeat allows a region of the single-stranded template to assume a short hairpin configuration (*54*). [B] DNA-dependent DNA polymerase activities. For each mutant, synthesis was evaluated after 1, 3, and 10 min (lanes 1−3, respectively). Migration positions of the unextended and fully extended primer, in addition to the region of transient pausing, have been indicated. [C] RNA-dependent DNA polymerase activity. Substrate was a 105 nt RNA fragment from the HIV-1 genome containing the primer binding site (PBS) and sufficient 5′ sequence to from the HIV-1 initiation complex (*55*, *56*), to which a 5′ ^{32}P-end-labeled tRNA^{Lys,3} primer was hybridized. Both template and primer were prepared by in vitro transcription. [D] Enzymatic analysis. tRNA-primed (−) strand strong-stop synthesis was evaluated after 5 (lanes 1) and 10 min (lanes 2). Migration positions of unextended tRNA and (−) strong-stop DNA have been indicated.

Table 1: Dissociation Constants for Wild-Type and Mutant p66/p51 RTs for Duplex DNA[a]

RT mutant	K_d (nM)	SD
p66/p51 WT	10.5	3.0
p66^{T473A}/p51	41.2	4.3
p66^{N474A}/p51	25.0	3.3
p66^{Q475A}/p51	12.9	2.9
p66^{N474A,Q475A}/p51	25.4	2.7
p66^{K476A}/p51	13.7	1.8
p66^{Y501A}/p51	57.0	8.4

[a] K_d values represent the average of three experiments. Standard deviations for each data set are indicated.

wild-type enzyme, albeit at a significantly slower rate. In contrast, enzymes harboring alterations at positions 473−475 and 501 retain −17 cleavage specificity, but are significantly impaired in −8 cleavage. At the same time, p66^{N474A}/p51, p66^{Q475A}/p51 RT, and the double mutant p66^{N474A,Q475A}/p51 cleave the RNA template 25 bp distal to the primer terminus, a phenotype also observed in previous studies of RT harboring a 13 residue C-terminal deletion of its p51 subunit (*31*−*33*). Two scenarios might account for this cleavage pattern. Conceivably, alterations to the geometry of the RNase H primer grip "lock" the enzyme in a conformation such that position −8 cannot be cleaved. Alternatively, following initial cleavage at template nucleotide −17, RT dissociates from the RNA/DNA hybrid, and fails to form a stable complex with the resulting hydrolysis intermediate. These possibilities will be discussed in more detail later.

Polypurine Tract Selection by RNase H Primer Grip Mutants. While the experiment of Figure 3 evaluated RNase H activity on a heteropolymeric RNA/DNA hybrid, several events in HIV replication require that RNase H cleave with considerable degree of precision. One example is selection of the (+) strand polypurine tract (PPT) primer from within the RNA/DNA replication intermediate for initiation of second or (+) strand synthesis. Indeed, the studies of this paper were based on the crystal structure of HIV-1 RT containing a polypurine tract RNA/DNA hybrid in an effort to understand its resistance to hydrolysis. The structure shows distortions of the RNA/DNA hybrid in the immediate vicinity of the RNase H primer grip, suggesting that this portion of the enzyme may contribute to the structural distortion. Consequently, the model substrate of Figure 4 [A] was prepared to investigate the precision with which RNase H primer grip mutants cleave the PPT primer from (+) RNA. Based on recent observations that a dA:rU duplex immediately preceding the PPT was important for virus replication (*26*−*28*), we retained this sequence in the PPT-containing oligonucleotides. The substrate of Figure 4[A]

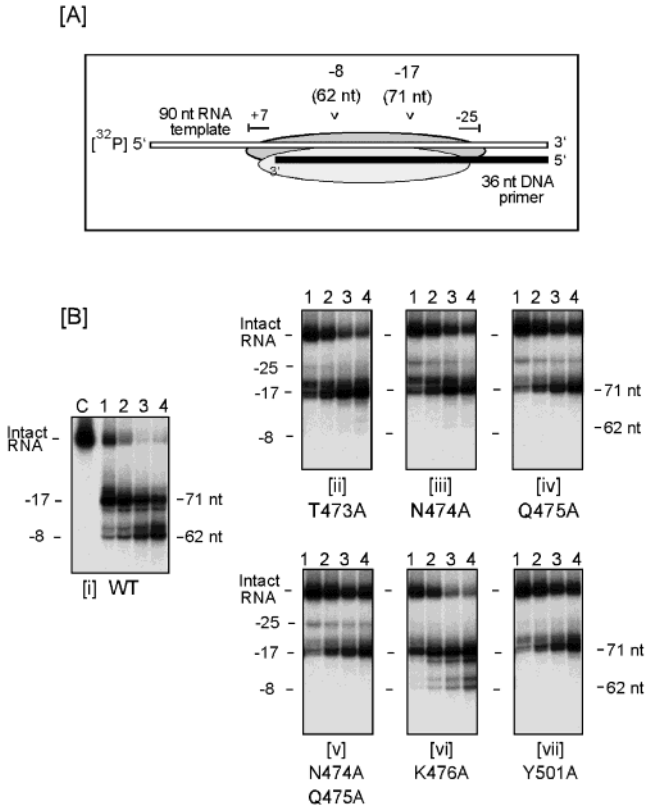

FIGURE 3: RNase H activity of primer grip mutants. Substrate for this experiment ([A]) was a 90 nt, 5′ ^{32}P-end-labeled RNA to which a 36 nt DNA was hybridized at the 3′ terminus. Location of p66/p51 RT over the template-primer duplex has been indicated. In the absence of DNA synthesis, hydrolysis occurs ~17 bp behind the DNA polymerase active center, liberating a 71 nt fragment. Subsequent directional processing at position −8 generates a 62 nt fragment. Nucleotide numbering designates −1 as the first base pair of the template/primer duplex in the polymerase catalytic center. [B] Analysis of RNase H primer grip mutants. Hydrolysis was evaluated after 1, 3, 10, and 30 min (lanes 1−4, respectively). The migration positions of RNase H hydrolysis products are indicated. In panel [i], lane C indicates the intact template.

was used to evaluate specific cleavage at the PPT−U3 RNA junction relative to nonspecific hydrolysis of the adjacent (+) U3 RNA/(−) U3 DNA hybrid.

A time course of these events with wild-type HIV-1 RT is provided in Figure 4[B], panel [i]. In keeping with recent data (M. Kvaratskhelia et al., unpublished experiments), cleavage at the PPT/U3 RNA junction is favored over hydrolysis of the adjacent (+) U3 RNA/(−) DNA hybrid. Although PPT nucleotides −2G/−3G can be hydrolyzed in the absence of polymerization and with extended incubation, as has been reported by others (*35, 36*), the (+) RNA/(−) DNA hybrid ahead of the PPT is clearly a less-favored substrate. A similar phenotype was observed for mutant p66^{K476A}/p51, which is in keeping with the data of Figure 3. In contrast, altering residues Thr473, Asn474, Gln475, and Tyr501 has a significant impact on the specificity of hydrolysis. Although in each case cleavage at the PPT 3′ terminus is still observed, equivalent (p66^{Q475A}/p51, panel [iv])) and enhanced cleavage (p66^{Y501A}/p51, panel [vii]) also occurs within the (+) U3 RNA/(−) DNA hybrid. This effect is more pronounced in Figure 4[C], which shows the extent of authentic PPT processing relative to total hydrolysis products. Thus, the data of Figure 4 suggest that alterations to the RNase H primer grip reduced the specificity of PPT cleavage from (+) U3 RNA.

Polypurine Tract Removal from (+) U3 DNA. Replacing (+) U3 RNA sequences 3′ to the PPT with their DNA counterparts provides a second model substrate mimicking the excision from nascent (+) DNA. Specific cleavage is required at this site during retroviral replication, since this event defines the sequences used by integrase during insertion of double-stranded viral DNA into the host genome (*37*). At the same time, the substrate of Figure 5 [A] challenges the RNase H domain with a novel structural element, namely, a PPT/(+) DNA RNA−DNA chimera hybridized to a DNA template. The results of this assay are summarized in Figure 5[B]. At early time points, both wild-type RT and mutant p66^{K476A}/p51 cleave preferentially at the PPT−(+) U3 DNA junction. As was noted for the RNA PPT primers shown in Figure 4, prolonged incubation leads to internal cleavage at −1G, −2G, and −7A. Although the overall rate of hydrolysis is affected, alteration of RNase H primer grip residues Thr473, Asn474, and Gln475 has little influence on cleavage specificity (panels [ii]−[v]). A clear exception to this is mutant p66^{Y501A}/p51, which is virtually incapable of cleaving at or behind the PPT−U3 (+) DNA junction. Quantitation of the hydrolysis profile for this mutant indicates that while hydrolysis within the PPT at position −7A is substantially reduced, this site is in fact preferred over the PPT−U3 DNA junction. Alteration of RNase H primer grip residue Tyr501 thus appears to have a direct impact on positioning of the scissile bond at the RNA−DNA junction within the RNase H catalytic center. Data in the accompanying section indicate that altering Tyr501 has more profound consequences for PPT removal than other specialized RNase H-dependent events.

Alterations to DNA Strand Transfer Activity. In retroviral replication, two mandatory DNA strand transfer events relocate nascent DNA either within or between acceptor templates. The first of these involves transfer of (−) strand strong-stop DNA within or between (+) strand RNA genomes exploiting homology between repeat (R) regions at their 5′ and 3′ termini. Once the growing point reaches the 5′ terminus of the donor RNA template, this must be degraded to a size permitting dissociation and annealing of nascent DNA to an acceptor (*29*). Mutants retaining only the polymerization-dependent RNase H function (i.e., cleavage of the RNA template 17 nt behind the primer terminus) fail to promote DNA strand transfer (*31, 33, 38*), suggesting a requirement for polymerization-independent RNase H activity in hydrolyzing the residual donor RNA template and promoting its release. Conceivably, DNA strand transfer could also represent an event where the polymerization machinery must interact with nascent DNA as well as the donor and acceptor template (*29*). It was thus of interest to determine how such a process might be influenced by alterations to the HIV-1 RNase H primer grip. The DNA strand transfer substrate is presented schematically in Figure 6 [A], and the efficiency with which transfer is carried out by the RNase H variants in Figure 6[B]. In all cases, the 20 nt DNA primer is efficiently extended to the 5′ terminus of the donor RNA template, generating a 40 nt strand transfer intermediate (STI). Thus, while the experiments of Figure 2 indicated that DNA synthesis was unaffected on duplex DNA

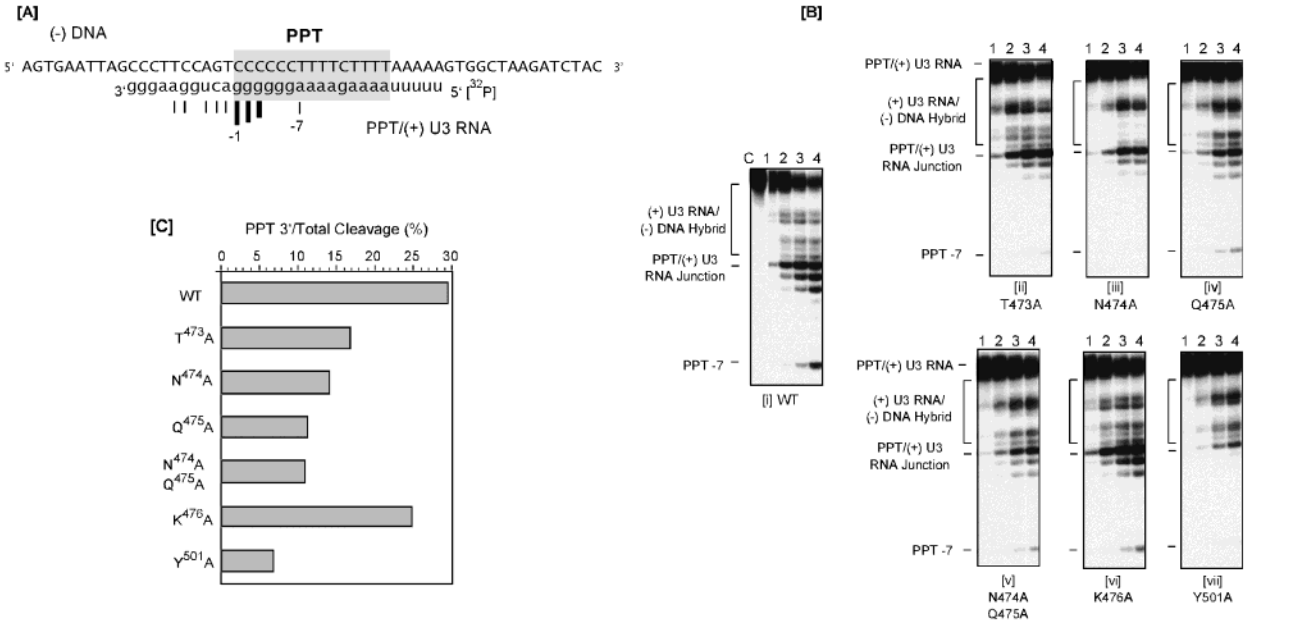

FIGURE 4: RNase H primer grip mutants display altered PPT processing activity. [A] Duplex RNA substrate. The HIV-1 PPT primer (shaded area) was flanked by 10 and 5 ribonucleotides at its 3′ and 5′ termini, respectively, and 5′ ^{32}P-end-labeled. Template was a 55 nt DNA complementary to the primer and extended at its 5′ and 3′ termini by 10 and 15 nt, respectively. Bars below the PPT-containing oligonucleotide represent major and minor positions of hydrolysis in the presence of wild-type enzyme, with the size of the bar representing the extent of cleavage. [B] RNase H hydrolysis profiles. For each enzyme, hydrolysis was evaluated after 1, 3, 10, and 30 min (lanes 1−4, respectively). The position of the PPT−U3 RNA junction, i.e., the authentic cleavage site, is indicated, in addition to PPT nucleotide −7. In panel [I], lane C indicates the intact PPT-containing oligoribonucleotide. [C] PPT selection expressed as a percent of total cleavage. Values were obtained by quantitative phosphorimaging analysis of products generated after 1 min.

and RNA, Figure 6[B] indicates that an RNA/DNA hybrid presents no impediment to the polymerizing enzymes. However, efficient DNA strand transfer, evidenced by accumulation of a 60 nt strand transfer product (STP), is efficiently catalyzed only by wild-type RT and mutant p66^{K476A}/p51 (Figure 6[B], panels [i] and [vi], respectively).

In an attempt to define the rate-limiting RNase H-mediated cleavage event(s) during DNA strand transfer, a second experiment was performed where the donor template was 5′ end-labeled and RNase H activity evaluated in the context of DNA synthesis. The results of this experiment are presented in Figure 6[C]. From the data of Figure 6[B], we had anticipated efficient and rapid RNA-dependent DNA synthesis would position the polymerization machinery at the 5′ terminus of the donor template where RNase H-mediated events would be initiated. The most prominent products arising early in the time course are 15 and 12 nt. However, while the strand transfer intermediate accumulates over the same period, strand transfer and continued synthesis are delayed (Figure 6[B], panel [I]). Such observations suggest that a residual 12 nt fragment of the donor template remains stably bound to nascent DNA, preventing its association with the acceptor. With prolonged incubation, a 9 nt fragment from the donor template accumulates, concomitant with a substantial rise in strand transfer activity (see Figure 6[B]). Our findings thus support previous studies (*29*, *39*), indicating that reduction of the donor template to ~9 nt by polymerization-independent RNase H function is the rate-limiting step in DNA strand transfer. The hydrolysis profiles of mutants p66^{T473A}/p51, p66^{N474A}/p51, p66^{Q475A}/p51, p66$^{N474A,\ Q475A}$/p51, and p66^{Y501A}/p51 (Figure 7, panels [ii], [iii], [iv], [v], and [vii], respectively) show accumulation of a hydrolysis products from 15 to 12 nt with little to no strand transfer product, supporting the notion that these fragments remain stably bound to nascent DNA and impair strand transfer. Based on the data with wild-type enzyme, the low levels of 9 nt hydrolysis product generated by these mutants correlate with the reduced strand transfer activity. One point worth noting is the ability of mutant RTs to cleave closer to the RNA 5′ terminus in a polymerization-independent fashion (i.e., −12) than was observed for the heteropolymeric RNA/DNA hybrid of Figure 2, where negligible hydrolysis beyond template nucleotide −15 was observed. Although speculative, this could infer distinct binding modes in the absence and presence of a template overhang.

Finally, an unusual feature of the RNase H hydrolysis profiles of Figure 6[B] is accumulation of a product of original template length over time with those mutants that displayed low levels of strand transfer. We speculated that since RNase H hydrolysis products generated by RNase H-deficient mutants are larger than those left by wild-type enzyme, they may remain stably associated with nascent DNA and serve as primers for a renewed round of RNA-primed, DNA-dependent DNA synthesis. This, in turn, may contribute to the failure of these mutants to efficiently catalyze strand transfer.

To test this hypothesis, the experiments depicted in Figure 6[C] were repeated for the wild-type and Y501A enzymes. However, half of each reaction was subjected to hydrolysis by RNase-free DNase I prior to fractionation by denaturing polyacrylamide gel electrophoresis. The results of this experiment are shown in Figure 7. As anticipated, the ~40 nt product generated by the Y501A enzyme disappears following treatment with DNase I, suggesting that it is a product of DNA synthesis. It is also worth noting that the hydrolysis profile generated by the wild-type enzyme appears unaffected by DNase I treatment, indicating that these

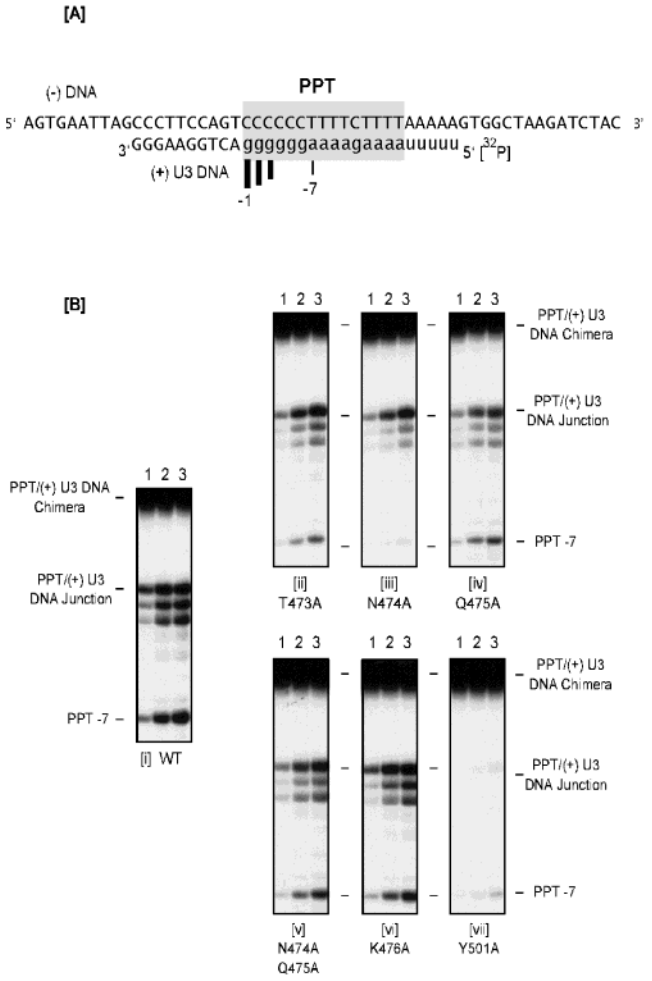

FIGURE 5: RNase H-mediated removal of the PPT from (+) strand DNA. [A] Experimental system. PPT removal from (+) U3 DNA sequences used a variation of the substrate of Figure 4[A], differing in that a decadeoxynucleotide sequence was placed downstream of the PPT 3′ terminus. Major cleavage sites at the PPT/U3 junction and within the PPT are indicated. [B] Activity of RNase H primer grip mutants. For each enzyme, PPT processing was evaluated after 3, 10, and 30 min (lanes 1−3, respectively).

cleavage fragments may be too small to serve as DNA synthesis primers.

DISCUSSION

Crystallographic analysis has identified motifs of the DNA polymerase and RNase H domains of HIV-1 RT critical in maintaining the appropriate geometry of the DNA primer for two distinct processes. Within the DNA polymerase domain, Jacobo-Molina et al. (*20*) proposed the $\beta12-\beta13$ hairpin, or primer grip, maintains the primer terminus in an orientation appropriate for nucleophilic attack on the incoming dNTP. Subsequently, Sarafianos et al. (*12*) have named residues within α-helices A′ and B′ of the RNase H domain the "RNase H primer grip", which, by contacting the DNA primer, may "guide" the RNA template into its catalytic center. Given the pleiotropic effects DNA polymerase primer grip mutations (*40*−*43*), defining the role of its counterpart in the RNase H domain is clearly important. Moreover, as biochemical studies have shown roles for polymerization-dependent and -independent modes of RNase H-mediated hydrolysis, it is important to understand how modulating these activities influences specific RNase H-mediated events such as DNA strand transfer and PPT selection. In this paper, alterations to the Thr473−Lys476 quartet (α-helix A′) and Tyr501 and Ile505 (α-helix B′) were evaluated. During purification, mutant I505G did not dimerize efficiently and was eliminated from our study. This defect likely reflects altered contacts between α-helix B′ and both the p51 subunit and other p66 subdomains. These contacts may be absent in the *E. coli* enzyme, where the counterpart is Gly77. Such a drastic consequence is not without precedent, as substituting Leu234 with Ala in the DNA polymerase domain likewise inhibits dimerization (*40*, *44*).

Unlike $\beta12-\beta13$ hairpin mutations in the DNA polymerase domain, which can simultaneously affect polymerase and RNase H activity, alanine substitution in the RNase H primer grip only modestly affects RNA- or DNA-dependent DNA synthesis. This finding is supported by the observation that, with the exception of the T473A substitution, viruses carrying RNase H primer grip mutations still replicate reasonably efficiently (J. G. Julias, M. J. McWilliams, S. Sarafianos, E. Arnold, and S. H. Hughes, manuscript submitted for publication). Arion et al. (*45*), however, have reported that recombinant $p66^{Y501A}$/p51 retains 5% of wild-type DNA polymerase and no RNase H activity. One explanation for this apparent discrepancy may be the use of a synthetic homopolymeric substrate [poly r(A)/oligo d(T)] in these studies to evaluate DNA synthesis, and that the unique structure of this duplex may be poorly tolerated by RTs containing mutations in the RNase H primer grip. Interestingly, the structure of this homopolymeric duplex resembles that of an extended PPT, the native version of which we show here is inefficiently processed by mutant enzymes. Furthermore, the deoxynucleotide concentrations used in the DNA synthesis assays of Arion et al. are 10-fold lower than those used in the present study. Since it has been demonstrated previously that the processivity of HIV-1 RT is reduced at lower nucleotide concentrations, it is possible that the effect of moderately reduced affinity for template-primer (Table 1) on DNA synthesis is accentuated under these conditions. Stated differently, because DNA synthesis occurs more slowly under the conditions used by Arion et al., this reaction is rendered more sensitive to subtle changes in the stability of the enzyme−template-primer complex. Differences in reaction rates may also partially explain why, in general, the RNase H activities of these mutants are affected to a greater extent than their DNA synthesis activities. A more complete understanding of the factors that affect the DNA synthesis activity of these enzymes requires additional study.

With the exception of mutant K476A, altering the RNase H primer grip severely reduced polymerization-independent RNase H activity on a heteropolymeric RNA/DNA hybrid (Figure 3[B]) while cleavage specificity defined by spatial separation of the polymerase and RNase H catalytic centers is retained. A similar phenotype was noted for p66 HIV-1 RT harboring a 13-residue C-terminal deletion of its p51 subunit (*31*−*33*) and a second mutant lacking 8 residues of its RNase H domain (*38*). It is therefore important to consider the loss of polymerase-independent RNase H activity in the context of enzymes containing this wide spectrum of alterations.

While cleavage at position −17 is clearly related to the distance between active centers, cleavage at position −8 is

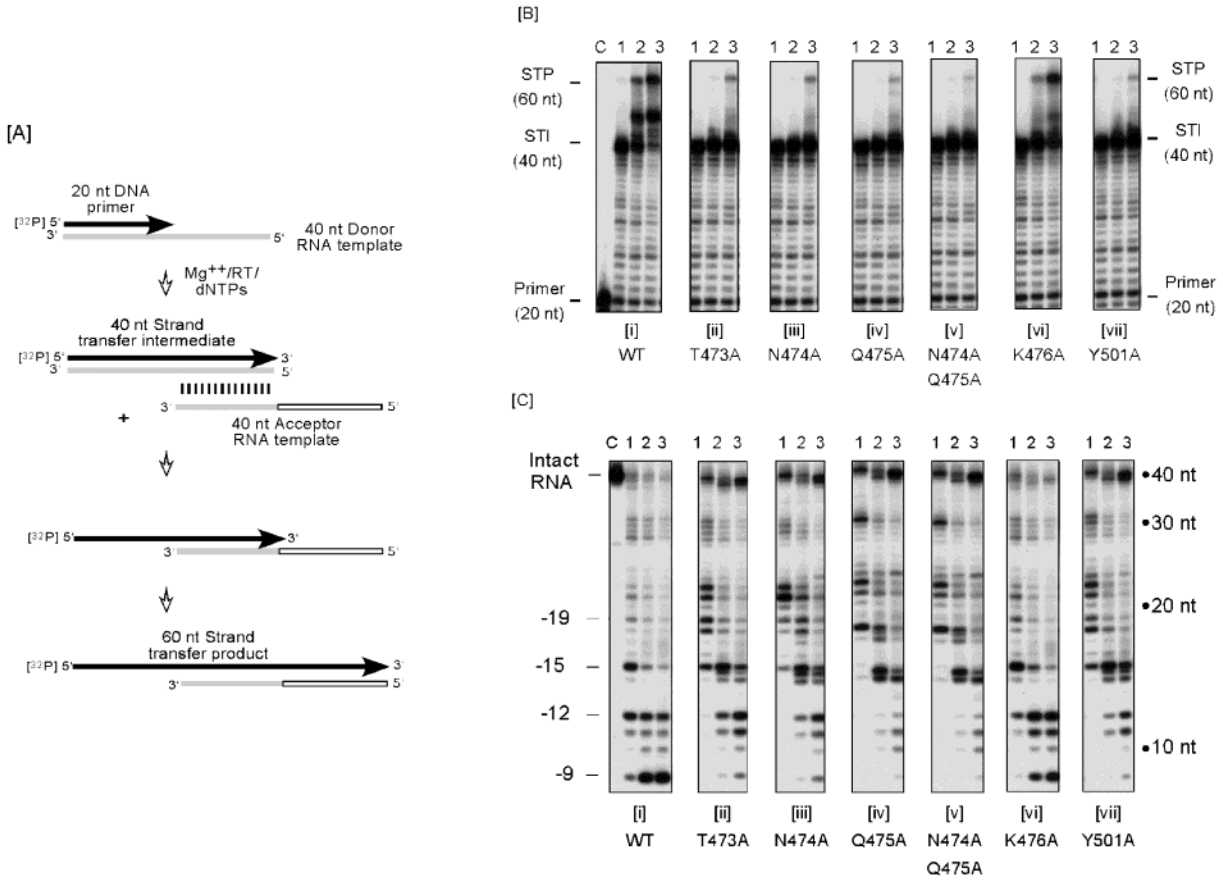

FIGURE 6: RNase H primer grip mutants alter the efficiency of DNA strand transfer. The model system ([A]) comprises a 40 nt donor RNA template to which a 5′ ^{32}P-end-labeled template is hybridized at the 3′ terminus and an acceptor template sharing 20 nt of homology to the donor at its 3′ terminus. Initial RNA-dependent DNA synthesis results in a 40 nt strand transfer intermediate, while successful strand transfer and continued DNA synthesis generate a 60 nt strand transfer product. [B] Strand transfer assays. Migration positions of the 20 nt DNA primer, 40 nt strand transfer intermediate (STI), and 60 nt strand transfer product (STP) have been indicated. For each enzyme, lanes 1−3 represent 1, 5, and 30 min time-points, respectively. [C] Analysis of RNase H activity concomitant with RNA-dependent DNA synthesis. The 40 nt RNA template/20 nt DNA primer of the DNA strand transfer assay depicted in [A] was modified in that (a) radiolabel was relocated to the 5′ terminus of the RNA donor template and (b) acceptor template was withheld. Conditions for RNA-dependent DNA synthesis were identical to those described for strand transfer. −18, −14, −11, and −8 hydrolysis products are relative to enzyme that has extended the DNA primer to the 5′ terminus of the RNA template, creating a blunt-ended duplex. RNase H activity was evaluated at equivalent time-points to the DNA strand transfer assay of [B].

not so easily explained. More specifically, if polymerase-independent cleavage reflects movement of RT along the RNA/DNA, why is cleavage at intervening sequences rarely observed? One explanation may lie in single-molecule fluorescence studies on RNA polymerase β, which show the enzyme sliding linearly along a DNA duplex when searching for a promoter (*46*, *47*). Thus, in the absence of DNA synthesis, HIV-1 RT initially positioned at the 3′ terminus of the DNA primer may slide forward or backward, with cleavage occurring only when the appropriate trajectory of the RNA is assumed at the RNase H active site. This hypothesis predicts that cleavage should occur at −17 and sites separated from that position by approximately one helical turn of the RNA/DNA hybrid. Data of this paper indicate that all enzymes cleave primarily around position −17, while secondary cleavage occurs around positions −8 and/or −25 (Figure 3). Given this scenario, it can be envisaged that in the RT configuration inducing −8 cleavage, the polymerase catalytic center is located over the single-stranded template. Since the preferred binding site for this domain is the primer 3′-OH, it seems likely that the contribution of the RNase H toward binding is greater relative to that of the polymerase domain in this alternate configuration. Accordingly, cleavage at position −8 should be particularly sensitive to alterations in the RNase H domain. This hypothesis is consistent with the data of Figure 3, where −8 cleavage is almost completely ablated by alanine substitution at positions 473−475 or 501 within the RNase H primer grip. Similarly, C-terminal truncation of p66 or p51 may subtly affect the geometry of the RNase H primer grip (p66Δ8/p51) or the orientation of the entire RNase H domain (p66/p51Δ13) such that the configuration of RT favoring polymerization-independent RNase H activity is compromised.

PPT selection and its removal from nascent (+) DNA may also be less dependent on positioning of RT by terminal nucleotides of the template or primer. Although the substrates depicted in Figures 4 and 5 were designed to place the PPT 3′ terminus at a suboptimal distance from the template termini, wild-type RT specifically cleaves this from downstream (+) RNA and DNA sequences. Although mutant enzymes still cleave the PPT from (+) RNA, the data of Figure 4[B],[C] indicate that there is a shift toward nonspecific cleavage of the adjacent RNA/DNA hybrid. This is particularly evident for mutant p66^{Y501A}/p51, which shows an almost 6-fold increase in its preference for the non-PPT

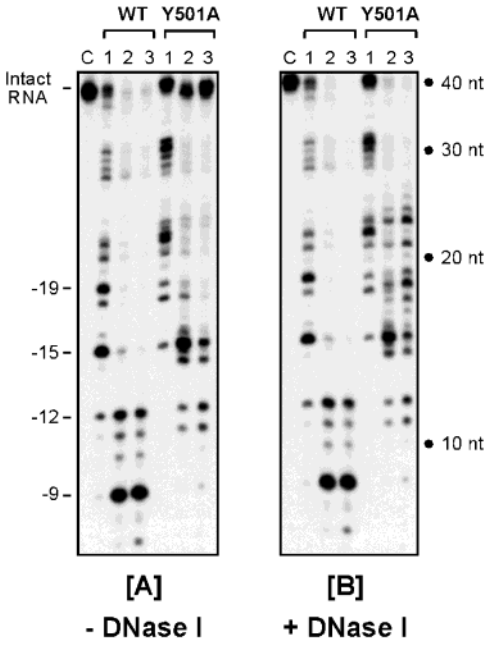

FIGURE 7: DNA synthesis initiates from cleaved donor RNA. RNase H cleavage of donor template evaluated as in Figure 6[C] for WT and Y501A RTs ([A]), except that half the reaction products were treated with RNase-free DNase I ([B]). Note that DNase I treatment removes the 40 nt product present after 5 and 30 min incubation with the Y501A mutant.

RNA/DNA hybrid and virtually incapable of removing (+) DNA from the PPT (Figure 5[B]). What then defines PPT selection, and how might this be affected by alterations to the RNase H primer grip? A clue to cleavage specificity might lie in our recent report of structural anomalies at both the PPT 3′ terminus (i.e., the cleavage site) and distal of the two $r(A)_4$:$d(T)_4$ tract in the absence of enzyme (M. Kvaratskhelia, S. Budihas, and S. F. J. Le Grice, manuscript submitted for publication). At the PPT/U3 junction, reactivity of template thymine +1 to $KMnO_4$ modification, despite base pairing with expended primers, suggests a considerable degree of unstacking. Stated differently, two structural anomalies exist within the PPT separated by 12−14 bp. Locating the RNase H catalytic center at the scissile bond 3′ to the PPT 3′ would position the thumb subdomain in the immediate vicinity of the distal $r(A)_4$:$d(T)_4$ tract whose structure is significantly distorted. Since we have shown biochemically an interdependence between these structural anomalies, we propose that an interaction of the p66 thumb subdomain with distorted/mispaired nucleic acid ~13 bp upstream of the PPT/U3 junction correctly positions the RNase H catalytic center over the scissile bond, guided in part by the +1 distortion. Altering the RNase H primer grip would not affect p66 thumb contacts to upstream PPT sequences, but rather the correct positioning of the PPT/U3 junction in the RNase H domain. Altered or lost contacts around position +1 may result in the relaxed specificity observed in Figure 4[B]. Interestingly, cleavage of the PPT at or close to the junction with (+) DNA is virtually eliminated with mutant $p66^{Y501A}$/p51 (Figure 5[B]), possibly reflecting imprecise positioning over duplex DNA. This notion is currently under investigation.

In summary, alanine substitution at positions 473−475 or 501 of HIV-1 RT markedly alters cleavage specificity, while altering position 476 has minimal effect. These results complement in vivo studies with recombinant viruses (J. G. Julias, M. J. McWilliams, S. Sarafianos, E. Arnold, and S. H. Hughes, manuscript submitted for publication). Using a construct restricting replication to a single round, these authors demonstrated that alanine substitutions at positions 473−475 or 501 of RT gave variations in virus titer ranging from a 30% reduction to completely inactive, while the virus titer of virus containing the K476A mutation was reduced by less than 10%. Moreover, sequencing of two-LTR circles demonstrated altered processing of viral RNA at the termini of preintegrative nucleic acid by RT containing the N474A+Q475A, Q475A, and Y501A substitutions. However, the in vivo and in vitro analyses were not entirely in agreement. For example, Julias et al. report a substantial reduction in (−) strand strong-stop DNA synthesis by mutant RTs, while we observe only minimal deficits in this activity of recombinant enzyme. Though puzzling, this discrepancy might be explained by amino acid substitutions within the RNase H primer grip region affecting features of virus replication not addressed in the current study. For example, gag-pol precursors containing mutant RTs may be processed inappropriately by HIV-1 protease, as observed upon substituting residue 234 of the DNA polymerase primer grip (*48*). Alternatively, substitutions in the RNase H primer grip may result in reduced or inappropriate placement of $tRNA^{Lys,3}$ onto the PBS.

Altering the primer grip also appears to have different effects on strand transfer in vivo and in vitro. While RT mutants in the current study exhibited a reduction in strand transfer activity, the N474A+Q475A mutant had no measurable effect on minus strand transfer during viral replication, and the Y501A mutation showed only a modest reduction. This can most likely be explained by the absence of nucleocapsid protein (NC) in the in vitro reaction and/or by the excess of RNase H activity present in HIV-1 virions (*49*). Since NC has been shown to increase the efficiency of (−) strand transfer (*50*−*52*), defects in RNase H activity may be compensated for by NC in vivo. In addition, the repeat (R) region of HIV-1, upon which minus strand transfer is critically dependent, is much longer than we have used in vitro. Given the correlation between the efficiency of strand transfer and the length of the homology region (*53*), differences in in vivo and in vitro strand transfer rates might not be surprising. Despite this, the two approaches demonstrate the importance of conducting complementary in vivo and in vitro analyses. Whereas the former demonstrates the consequences of altering the RNase H primer grip in the context of virus replication, the latter clarifies the nature and extent of the functional deficits of these enzymes and their mechanistic basis.

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