Rapid Kinetic Analysis of a Point Mutant of HIV-1 Reverse Transcriptase Lacking Ribonuclease H Activity†

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Received December 30, 1992; Revised Manuscript Received March 26, 1993

ABSTRACT: The comparative kinetics of RNA-dependent DNA polymerization catalyzed by wild-type HIV-1 reverse transcriptase and a point mutant specifically lacking RNase H activity were analyzed using a heteropolymeric substrate consisting of a 19-mer primer hybridized to a 345-nucleotide RNA template. The rapid-quench product distributions generated under single-turnover conditions, in which primer extension by the two enzymes was restricted to the incorporation of 5 nucleotides (N+5), were significantly different. Whereas the wild-type enzyme catalyzed synthesis of the N+5 product over the time course of the reaction (20 ms–10 s) with a relatively low degree of processivity, the extent of accumulation of the intermediate N+2 and N+3 products was grossly exaggerated in the parallel mutant-catalyzed time course. The observation of concomitant polymerase-dependent hydrolysis during the course of synthesis catalyzed by the wild-type enzyme suggested that the inability of the RNase H– mutant to hydrolyze the RNA template created blocks to further synthesis by reducing the rates of DNA polymerization at these intermediate positions, and hence impaired the ability of this mutant to complete cDNA synthesis.

We recently reported the construction and preliminary characterization of point mutants of HIV-1 reverse transcriptase (RT)[1] which specifically lacked RNase H activity (Mizrahi *et al.*, 1990). Although the RNase H– phenotype had no effect on the steady-state kinetics, product distribution, and processivity of reverse transcription of a homopolymer template, it did impair the ability of the enzyme to catalyze the further extension of pause-site polymerization products formed during the multiple-turnover reverse transcription of a 345-nt heteropolymeric RNA template (Dudding *et al.*, 1991). In an attempt to quantitatively investigate this functional impairment, we have used a rapid-quench technique to study the kinetics of extension of a 19-mer primer catalyzed by the WT and D443N enzymes under conditions in which the synthesis was limited to the incorporation of 5-nt residues immediately upstream of a previously identified major pause site within the HIV-1 *gag*-derived RNA template (Dudding *et al.*, 1991).

MATERIALS AND METHODS

Materials

Sources of commercial enzymes and radionucleotides were as previously described (Mizrahi *et al.*, 1990; Dudding *et al.*, 1991). High-grade TTP and dCTP (Pharmacia) were purified by HPLC prior to use. Recombinant HIV-1 RT enzymes (WT and D443N) were purified as previously described (Mizrahi *et al.*, 1990).[2] Protein concentrations were measured using the Bio-Rad protein assay (kit I, bovine γ-globulin standard). The 19-mer primer, GAG-3 (5′-ATAGAAACCGGTCTACATAG-3′), was synthesized using a Milligen/Biosearch Cyclone oligonucleotide synthesizer and was purified by denaturing gel electroporesis [20% polyacrylamide/7 M urea/TBE buffer (89 mM Tris base/89 mM boric acid/2 mM EDTA)].

Methods

RNA Template Preparation. Run-off transcription to produce the (+)-GAG^{345} template was carried out as previously described (Mizrahi *et al.*, 1990; Dudding *et al.*, 1991) using a final NTP concentration of 2 mM. [α-^{32}P]UTP was included in the transcription reactions designed to produce template either for the polymerase quench-flow assay (100 cpm/pmol) or for the RNase H assay (2000 cpm/pmol). The RNA was purified by gel electrophoresis (8% polyacrylamide/7 M urea/TBE buffer) followed by electroelution using an Elutrap elutor (Schleicher & Schuell), eluting at 200 V for 5 h. An aliquot of the purified RNA was quantitated by liquid scintillation counting.

5′-End-Labeling. Primer concentrations were measured spectrophotometrically at 260 nm using an extinction coefficient that was calculated on the basis of the nucleoside composition of the primer. GAG-3 was 5′-end-labeled as previously described (Mizrahi *et al.*, 1986).

Hybridization. Hybrids were formed by mixing 200–400 nM RNA and 200–600 nM primer in 40 mM Tris·HCl (pH 8.0), heating at 50 °C for 2 min, and cooling to room temperature over 1 h. Hybrid concentrations were corrected, where necessary, for unhybridized primer by measuring the amount of unextended GAG-3 remaining in prolonged polymerization reactions containing a 10–20-fold excess of WT enzyme over 3′-ends.

† This research was funded by grants from the Foundation for Research Development, The Medical Research Council of South Africa, and the South African Institute for Medical Research.

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[1] Abbreviations: HIV-1, human immunodeficiency virus type 1; RT, reverse transcriptase; RNase H, ribonuclease H; WT, wild-type; D443N, point mutant of HIV-1 RT in which Asp-443 was substituted by Asn; nt, nucleotide; p66, 66-kDa polypeptide of HIV-1 RT; p51, 51-kDa polypeptide of HIV-1 RT; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

[2] Enzymes: reverse transcriptase, deoxynucleoside-triphosphate:DNA deoxynucleotidyltransferase, RNA-directed (EC 2.7.7.49); RNase H (EC 3.1.26.4).

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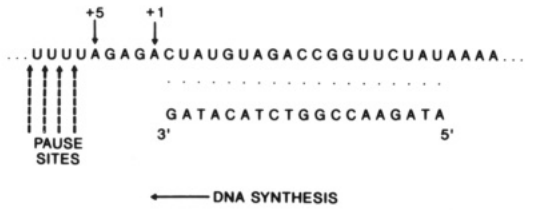

FIGURE 1: Structure of the hybrid substrate showing the nucleotide sequences of the RNA template and DNA primer in the region flanking the position of complementarity. The previously identified prominent pause site positions RVI–RIX (Dudding *et al.*, 1991) are indicated by the vertical arrows.

Quench-Flow Reverse Transcriptase Assay. Rapid-quench experiments (20 ms–10 s) were conducted using a quench-flow apparatus designed by Johnson (1986). The enzyme–hybrid solution was prepared by mixing equal volumes (200 μL) of hybrid (200 nM), 4× RT buffer [200 mM Tris·HCl (pH 8.0), 300 mM KCl, 0.4 mM EDTA, 4 mM DTT, and 0.4% Triton X-100], EDTA (4 mM), and enzyme (0.72 μM) and preincubating the mixture at 37 °C for 5–10 min. The Mg–dNTP solution was prepared by mixing equal volumes (200 μL) of $MgCl_2$ (60 mM), 4× RT buffer, TTP (0.8 mM), and dCTP (0.8 mM) and preincubating at 37 °C for 5–10 min. Reactions were initiated by mixing equal volumes (*ca.* 20 μL) of the two solutions and were quenched with 40 μL of EDTA (0.1 M) delivered from a third syringe. In challenged experiments, heparin was included in the Mg–dNTPsolution at a concentration of 2 mg/mL.[3] Samples were electrophoretically resolved on denaturing 15% polyacrylamide gels, and the individual products were quantitated as described by Carroll *et al.* (1991).

Steady-State Kinetics of N+2 Synthesis. The steady-state kinetics of *N*+2 synthesis were followed by preincubating hybrid (250 nM) with enzyme (1–3 nM) and initiating the reactions with Mg^{2+} and TTP/ddCTP (100 μM each). The final concentrations of the other assay components in the 40-μL reaction were as described for the rapid-quench assay above. Aliquots (4 μL) were quenched after 0–120 s at 37 °C, and the quenched samples were electrophoretically resolved and the excised *N*+2 product was quantitated by liquid scintillation counting.

RNase H Assay. The hybrid was prepared as above from 200 nM labeled RNA and 600 nM unlabeled GAG-3. Reactions were initiated by manually mixing equal volumes (35 μL) of the enzyme–hybrid solution (850 cpm/μL) and the Mg–dNTP solution at 37 °C. Aliquots (10 μL) were withdrawn after 5–60 s and were quenched into 10 μL of gel loading buffer. In the challenged experiments, heparin was included either in the enzyme–hybrid solution (control)[3] or in the Mg–dNTP solution at a concentration of 2 mg/mL. Aliquots (2–5 μL) were loaded onto a 8% acrylamide/8 M urea/TBE gel, and the products were visualized by autoradiography.

RESULTS

The hybrid substrate used in the present study is illustrated in Figure 1. As previously described (Dudding *et al.*, 1991), the preferred termination of the mutants at the RVI–RIX positions was unaffected by relocating the original primer terminus (GAG-1) to a position 5 nt upstream of the RVI position, as indicated by the relative product distributions formed by polymerization of the (+)-GAG^{345}/GAG-3 substrate under conditions of multiple turnover. To analyze the kinetic basis of this functional dependence on the RNase H phenotype of the enzyme, we originally intended to compare the transient kinetics of polymerization of the WT and D443N enzymes under conditions in which the number of nucleotides incorporated was restricted to 1 or 5, by the inclusion either of TTP alone or of an equimolar mixture of TTP and dCTP, respectively. However, in the presence of TTP alone, the formation of considerable levels of *N*+2 and *N*+3 products was observed in addition to the expected *N*+1 product, indicating a high degree of TMP misinsertion opposite the template G at the +2 position, followed by extension of the mismatched 3′-terminus to yield the *N*+3 product (data not shown). The high frequency of mismatched 3′-terminus extension by HIV-1 RT is in accordance with the analogously high frequency of mismatch extension observed for DNA-dependent DNA polymerization by the WT enzyme (Perrino *et al.*, 1989). Since the misinsertion problem would complicate the comparative kinetic analysis of single nt incorporation at the *N*+1 position, we could only analyze the kinetics of *N*+5 synthesis under which conditions the formation of the terminally mismatched *N*+6 product was negligible (see Figure 2 below).

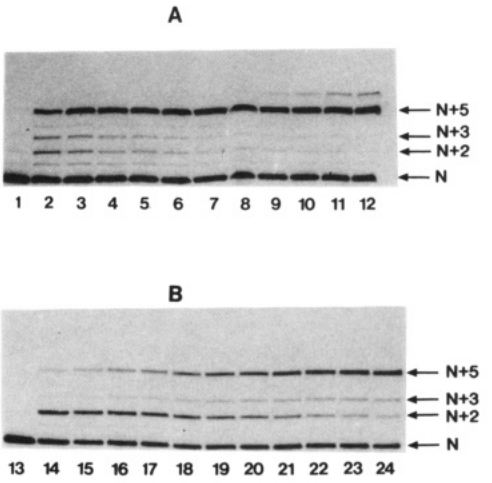

FIGURE 2: Rapid-quench time courses for *N*+5 synthesis. Reactions containing 90 nM WT (panel A) or D443N (panel B) enzyme, 25 nM 3′-OH, and 100 μM each of TTP and dCTP were quenched after 0 (lanes 1 and 13), 20 (lanes 2 and 14), 31 (lanes 3 and 15), 62 (lanes 4 and 16), 107 (lanes 5 and 17), 197 (lanes 6 and 18), 497 (lanes 7 and 19), 997 (lanes 8 and 20), 4000 (lanes 9 and 21), 6000 (lanes 10 and 22), 8000 (lanes 11 and 23), and 10 000 (lanes 12 and 24) ms, respectively, and were analyzed by denaturing gel electrophoresis.

The comparative rapid-quench time courses of *N*+5 synthesis catalyzed by the WT and D443N enzymes under conditions of a 3.6-fold molar excess of enzyme over 3′-ends are shown in Figure 2 (panels A and B, respectively). The data are graphically depicted in the time-dependence plots shown in Figure 3. The rates of disappearance of the unextended substrate (*N*) were similar in the WT and D443N time courses (80–85% of the primer extended within 20 ms), suggesting that the kinetic partitioning of this particular preformed E–hybrid complex between dissociation and extension was similar in both cases. However, as shown in Figure 3, the actual time-dependent distribution of products between *N*+2 and *N*+5 was noticeably different for the two enzymes. After 20 ms, the relative concentrations of the *N*+2, *N*+3, and *N*+5 products were 24, 17, and 46%, respectively, for the WT, as

[3] The efficacy of the heparin trap at a final concentration of 1 mg/mL was confirmed in control experiments in which the trap was included instead in the E–hybrid mixture (2 mg/mL). Under these conditions, initiation of the reaction by mixing equal volumes of the E–hybrid–heparin and Mg–dNTP solutions gave no detectable reaction over the experimental time courses (10 s for DNA synthesis, 60 s for hydrolysis).

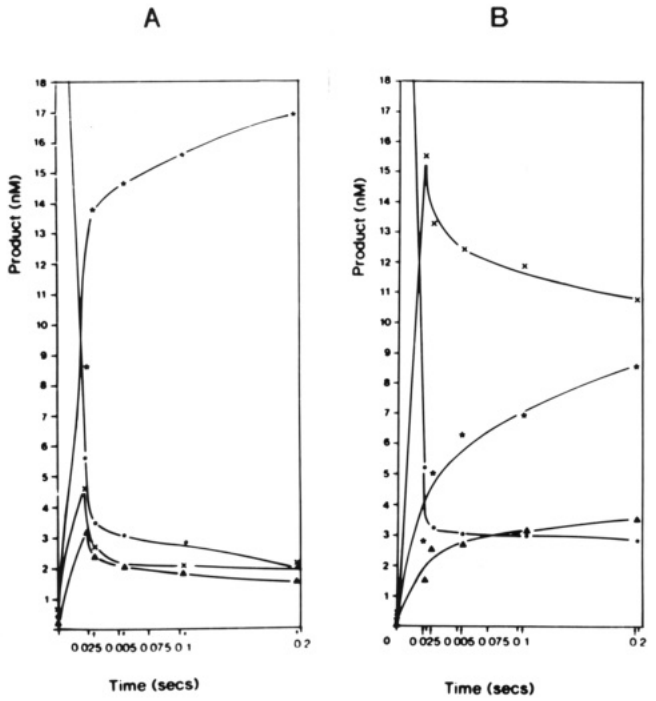

FIGURE 3: Rapid-quench polymerization product distributions. The concentrations of the substrate *N* (●) and *N*+2 (×), *N*+3 (▲), and *N*+5 (∗) products generated in the experiments shown in Figure 4 were individually quantitated as described under Materials and Methods, where *N* represents the unextended hybrid. Panel A, WT; panel B, D443N.

opposed to 77, 8, and 15%, respectively, for the D443N mutant (expressed as percent of total product). The *N*+2 and *N*+3 products represented significant blocks to full-length *N*+5 synthesis by the mutant, as indicated by their persistence at the latest time point (10 s). In contrast, full-length synthesis by the WT was essentially complete after 0.5 s.

The rapid-quench experiments of Figures 2 and 3 were repeated under conditions of substrate challenge which strictly limited the reactions to single-enzyme turnover. A heparin trap was included in the Mg–dNTP solution at a concentration that was sufficient to sequester free enzyme under the final reaction conditions (1 mg/mL; data not shown). The challenged 20-ms–10-s time courses for both the WT and the mutant enzymes were found to be quantitatively indistinguishable from the unchallenged reactions (Figures 2 and 3; ±7%), suggesting that the unchallenged time courses depicted in Figures 2 and 3 were insignificantly complicated by multiple turnover of the enzymes (data not shown).

In order to ascertain whether template cleavage occurred during the rapid-quench polymerization 10-s time course of the WT enzyme (Figures 2 and 3, panels A), the manually quenched 5–60-s RNase H time course of a hybrid consisting of uniformly labeled RNA and unlabeled primer, under otherwise identical assay conditions to those used in the corresponding *N*+5 polymerase assay, was followed by gel electrophoresis. As illustrated in Figure 4, extensive hydrolysis of the 345-nt RNA (*ca.* 70%) was observed at the earliest time points (50% hydrolysis after 5 s; 70% after 10 s), suggesting that concomitant template cleavage had occurred during the course of the rapid-quench polymerase assay of the WT enzyme shown in Figure 2. The inclusion of a heparin trap in the Mg–dNTP solution slightly reduced the extent of hydrolysis by the WT enzyme at the earliest measurable time point [*ca.* 35% hydrolysis after 5 s in the presence of trap (data not shown) *vs* 50% in the absence of trap (Figure 4, lane 7)]. However, in the trapped experiment, the extent of

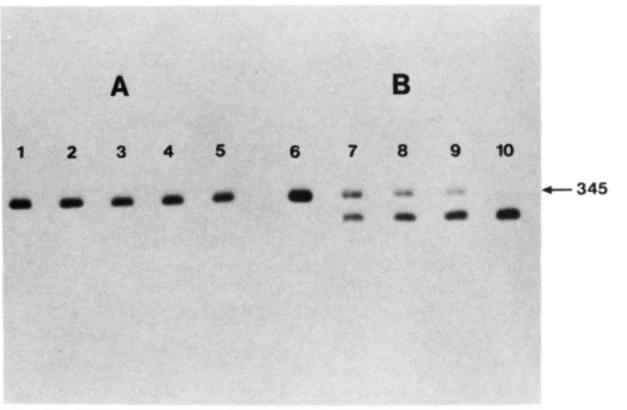

FIGURE 4: Analysis of the RNA template during *N*+5 synthesis. Reactions containing 25 nM hybrid [uniformly labeled (+)-GAG^{345}], 90 nM D443N (panel A), or WT (panel B) and 100 μM each of TTP and dCTP were manually quenched after 0 (lanes 1 and 6), 5 (lanes 2 and 7), 10 (lanes 3 and 8), 15 (lanes 4 and 9), and 60 s (lanes 5 and 10). The RNA was analyzed by denaturing gel electrophoresis in an 8% polyacrylamide/8 M urea gel. The positions of the substrate (345 nt) (arrow) and 5′-derived RNA products (*ca.* 320 nt) are indicated.

hydrolysis remained unchanged by prolonged incubation (≤60 s; data not shown), in contrast to the continued hydrolysis that was observed in the absence of the trap (Figure 4, panel B, lanes 8–10). This result confirmed that the continued hydrolysis observed in the unchallenged experiment was attributable to secondary multiple-turnover hydrolysis.

The kinetic basis of the block to further extension of the *N*+2 product by the mutant enzyme was further investigated by measuring the relative rates of dissociation of the WT and mutant enzymes from the *N*+2 product. The rate-limiting step in DNA synthesis by HIV-1 RT under conditions of limited nucleotide incorporation has been shown to correspond to the dissociation rate of the enzyme from the hybrid product (Kati *et al.*, 1992; Reardon *et al.*, 1992). Therefore, the relative k_{off} values for the E–H_{N+2} complexes could be measured by comparing the steady-state rates of synthesis under conditions in which the number of nucleotides incorporated was limited to 2. This was achieved by following the comparative steady-state kinetics of synthesis in parallel assays containing a large excess of hybrid over enzyme (≥70-fold) and saturating levels of TTP and the chain-terminator ddCTP. Linear incorporation was observed for both enzymes over a 0–120-s time course. Under these conditions, the rate of *N*+2 synthesis by the D443N mutant was 1.7-fold faster than that of the WT.

DISCUSSION

In an attempt to create a biochemically useful system for probing the functional interrelationship between the DNA polymerase and RNase H activities of HIV-1 RT, we reported the construction (Mizrahi *et al.*, 1990) and preliminary enzymatic characterization (Dudding *et al.*, 1991) of point mutants of the p66/p51 form of the enzyme that specifically lacked RNase H activity. Under conditions of unlimited multiple enzyme turnover, the D443N mutant was found to be impaired in its ability to reverse-transcribe a heteropolymer template (Dudding *et al.*, 1991). In the present study, we further analyzed the RT activity of the mutant using an assay in which the synthesis was limited to the incorporation of 5 nt under conditions favoring single enzyme turnover. The high frequency of sequence-specific pausing by the mutant after the addition of 2 or 3 nt to the primer terminus contrasted the facile addition of 5 nt by the WT enzyme, albeit with a

relatively low processivity. The mutation thus adversely affected the efficiency of further extension of the N+2 and N+3 primers, as evidenced by the accumulation of these intermediates at the expense of full-length N+5 product.

The rates of disappearance of the original 19-mer primer into larger products were the same for both enzymes, suggesting that D443N had no effect on the rates of the individual steps up to, and including, the addition of the first TMP residue to the preformed E–H_N complex. The processivity of N+5 synthesis by the WT enzyme was relatively low, as evidenced by the accumulation of significant levels of the intermediates (particularly N+2 and N+3) under the conditions of the quench-flow experiment of Figure 2, in agreement with the results of Kati *et al.* (1992).

However, the rapid extension of all intermediates to the final N+5 product suggested that a relatively high k_{pol}/k_{off} ratio was nonetheless maintained at the subsequent positions along the template. In contrast, exaggerated stalling of the D443N mutant was observed after the addition of 1 or 2 nt to the initial N+1 intermediate. Since the rates of enzyme–hybrid dissociation and/or translocation of DNA polymerases are significantly slower than the other steps involved in the phosphodiester bond-forming reactions (Kuchta *et al.*, 1987; Reardon, 1992; Kati *et al.*, 1992), a possible explanation for the functional impairment of the D443N mutant was a selective increase in the dissociation rate of the enzyme from the hybrid at these template positions. This hypothesis was tested by measuring the relative rates of dissociation of the two enzymes from the N+2 product under conditions in which the synthesis was limited to the addition of two nucleotides by the use of the chain-terminating nucleotide analogue ddCTP. However, under these conditions, the steady-state rate of N+2 synthesis by the mutant was only 1.7-fold faster than the WT enzyme, suggesting that the functional impairment of the mutant in extending the N+2 intermediate could not be solely attributable to a relatively faster dissociation rate of the enzyme at this position, but must rather be coupled to a significant lowering of the corresponding k_{pol} value for N+2 extension.

The degree of enzymatic coupling between the polymerase and the RNase H activities during reverse transcription by HIV-1 RT remains an intriguing point of debate. While some evidence supports an uncoupled mechanism (DeStephano *et al.*, 1991), other data convincingly argue in favor of a tight intramolecular coupling in which polymerase-dependent template degradation 15–20 nt upstream accompanies primer extension (Oyama *et al.*, 1989; Schatz *et al.*, 1990; Wöhrl & Moelling, 1990; Furfine & Reardon, 1991; Gopalakrishnan *et al.*, 1992). In the experiments described herein, concomitant template cleavage was observed over the time course of synthesis by the WT enzyme (≤10 s). However, since the unchallenged experiments were conducted under conditions of an excess of enzyme over 3′-ends, template cleavage may have occurred *via* both polymerase-dependent and polymerase-independent cleavage in this case. When the reaction was initiated instead in the presence of a heparin trap designed to sequester the enzyme not productively bound in a polymerase mode (Gopalakrishnan *et al.*, 1992), and hence eliminate the possible complication caused by polymerase-independent cleavage by the excess enzyme, the degree of template hydrolysis observed after 5 s was only slightly reduced. Therefore, although the extent of template cleavage under challenged conditions was not quantitative (*ca.* 35%), the hydrolysis observed under the enforced single-turnover conditions must have occurred in a polymerization-dependent (*i.e.*, coupled) fashion. The combined data presented herein are therefore consistent with a model involving the transient pausing of the WT enzyme at the N+2 and N+3 positions which allowed hydrolysis to occur to yield a product(s) with a higher k_{pol}/k_{off} ratio than that of the unhydrolyzed precursor. In terms of this model, the relative rates of polymerization, hydrolysis, and dissociation at a particular template position would determine whether or not coupled hydrolysis could kinetically compete with the forward polymerization process during reverse transcription.

The polymerization defect caused by the D443N mutation was only observed during heteropolymer reverse transcription (Dudding *et al.*, 1991). The lack of an effect of this mutation on poly(A) replication (Dudding *et al.*, 1991) may be attributable to the unusual properties of homopolymers which allow phenomena such as template slippage to occur (Kornberg & Baker, 1992). It is conceivable that during poly(dT) synthesis by the WT enzyme, the newly synthesized poly(A)·poly(dT) duplex product located upstream of the primer terminus is incorrectly positioned for precise passage through the RNase H active site (a prerequisite for polymerase-dependent cleavage), resulting in insignificant coupled template cleavage by WT HIV-1 RT under the conditions of limiting enzyme concentration over available 3′-ends used in our original comparative assays (Dudding *et al.*, 1991). In this respect, the peculiarities of this substrate might allow the WT enzyme to behave like its RNase H– counterpart within a processive cycle of synthesis, with the predicted consequence of negligible template cleavage accompanying poly(A) replication by WT HIV-1 RT. Alternatively, the data may be explained on kinetic grounds by assuming an unusually low rate of hydrolysis of the poly(A) template, in accordance with the well-documented resistance of the polypurine tract of HIV-1 to RNase H cleavage which allows for the accumulation of a relatively large cleavage product which serves as a primer for minus-strand DNA synthesis *in vivo* (Wöhrl & Moelling, 1990). In terms of the kinetic competition model described above, hydrolysis might be unable to kinetically compete with DNA synthesis on this template, thereby allowing extensive DNA synthesis to occur with negligible accompanying hydrolysis.

In conclusion, the presence of a catalytically functional RNase H domain located at the carboxy terminus of the polymerase domain increases the processivity of reverse transcription by HIV-1 RT. Conversely, the RNase H domain, which lacks hydrolytic activity in the absence of an associated polymerase domain, relies on the polymerase domain for hybrid binding (Hostomsky *et al.*, 1991; Davies *et al.*, 1991). Therefore, the activites of this versatile enzyme have evolved an important mutual functional interdependence for optimal activity.

ACKNOWLEDGMENTS

We are grateful to S. J. Benkovic, in whose laboratory the rapid-quench experiments were performed, for critically reading the manuscript. We thank J. Peliska, V. Gopalakrishnan, B. Eger, and C. Nkabinde for advice and assistance.

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