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# A Mechanism of AZT Resistance: An Increase in Nucleotide-Dependent Primer Unblocking by Mutant HIV-1 Reverse Transcriptase

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## Summary

**Mutations in HIV-1 reverse transcriptase (RT) give rise to 3′-azido-3′-deoxythymidine (AZT) resistance by a mechanism that has not been previously reproduced in vitro. We show that mutant RT has increased ability to remove AZTMP from blocked primers through a nucleotide-dependent reaction, producing dinucleoside polyphosphate and extendible primer. In the presence of physiological concentrations of ATP, mutant RT extended 12% to 15% of primers past multiple AZTMP termination sites versus less than 0.5% for wild type. Although mutant RT also unblocked ddAMP-terminated primers more efficiently than wild-type RT, the removal of ddAMP was effectively inhibited by the next complementary dNTP ($IC_{50} \approx 12$ μM). In contrast, the removal of AZTMP was not inhibited by dNTPs except at nonphysiological concentrations ($IC_{50} > 200$ μM).**

## Introduction

Unlike most cellular DNA polymerases, the reverse transcriptase (RT) of HIV-1 readily incorporates nucleotide analogs lacking a 3′OH group into the growing DNA chain (Furman et al., 1986; Cheng et al., 1987; St. Clair et al., 1987; Huang et al., 1990; Mitsuya et al., 1990; Ueno and Mitsuya, 1997). Once incorporated, these nucleotides block further elongation of the primer leading to inhibition of viral replication. Inhibition could be mitigated by an effective mechanism to remove the chain-terminating residue; however, HIV-1 RT and other viral RTs lack 3′–5′ exonuclease proofreading activity (Battula and Loeb, 1976; Roberts et al., 1988). Removal of chain-terminating residues could also occur through pyrophosphorolysis (Hsieh et al., 1993; Reardon, 1993; Carroll et al., 1994; Arion et al., 1998) or by transfer of the 3′ nucleotide from the primer to a nucleoside di- or triphosphate acceptor in a reaction recently described by our laboratory (Meyer et al., 1998).

The effectiveness of therapy with nucleoside analogs is limited by emergence of drug-resistant HIV-1 strains. Resistance to 3′-azido-3′-deoxythymidine (AZT) is acquired through the selection of amino acid substitutions at RT codons 41, 67, 70, 210, 215, and 219 (Emini and Fan, 1997). The biochemical mechanism of this resistance has been difficult to investigate because it cannot be reproduced in the in vitro assay systems that have been employed. These mutations cause high-level resistance to AZT in vivo (up to 120-fold increased $IC_{50}$ for AZT), with minimal cross-resistance to most other chain-terminating RT inhibitors. In assays with purified RTs, there are only subtle differences, if any, between the sensitivity of mutant and wild-type (WT) RT to AZTTP (Lacey et al., 1992; Carroll et al., 1994; Kerr and Anderson, 1997; Krebs et al., 1997) making it unlikely that AZT resistance is due to increased discrimination against AZTTP by the mutant enzyme. Canard et al. (1998) showed that AZT-resistant RT bound more tightly to AZTMP-terminated DNA chains than did WT RT and proposed that removal of the blocked primer terminus through PPi-dependent pyrophosphorolysis would be increased for the mutant enzyme because it would remain bound longer to the AZTMP-terminated primer. Both increased (Arion et al., 1998) and decreased (Carroll et al., 1994) rates of pyrophosphorolysis have been reported for the mutant enzyme.

In this report, we describe an in vitro assay that reproduces the essential in vivo properties of the AZT resistance mutants. HIV-1 RT containing the D67N, K70R, T215F, and K219Q amino acid substitutions (designated as 67/70/215/219 RT in this report) was much more efficient than WT RT at extending the primer past several potential termination sites in the presence of AZTTP when ATP was added to the reaction. Transfer of the AZTMP residue from the primer terminus to ATP to form dinucleoside polyphosphate and unblocked primer was enhanced in the 67/70/215/219 RT. Inhibition of this activity by the next complementary dNTP was reduced for the mutant enzyme, and removal of AZTMP from a blocked primer was uniquely insensitive to this inhibition for both mutant and WT RT.

## Results

### Primer Extension by WT and 67/70/215/219 Mutant RT in the Presence of Chain-Terminating Nucleotides

Primer extension reactions were carried out under conditions where AZTMP incorporation and removal could occur in the same reaction mixture. Wild-type or mutant HIV-1 RT was incubated with a 5′-$^{32}$P-labeled oligodeoxyribonucleotide primer/template in the presence of all four dNTPs and AZTTP. At each nucleotide A position on the template, either the natural nucleotide, dTMP, or the chain-terminating residue, AZTMP, could be incorporated. The pattern of primer extension/termination products was determined by the ratio between AZTTP and dTTP and the ability of RT to discriminate against AZTTP. Addition of a ribonucleoside triphosphate (ATP) to the reaction mixture provided an acceptor for the nucleotide-dependent primer unblocking activity in which the AZTMP residue from the chain-terminated primer was transferred to ATP to form $Ap_4AZT$, and the primer was shortened by one residue and was no longer blocked to elongation (Meyer et al., 1998).

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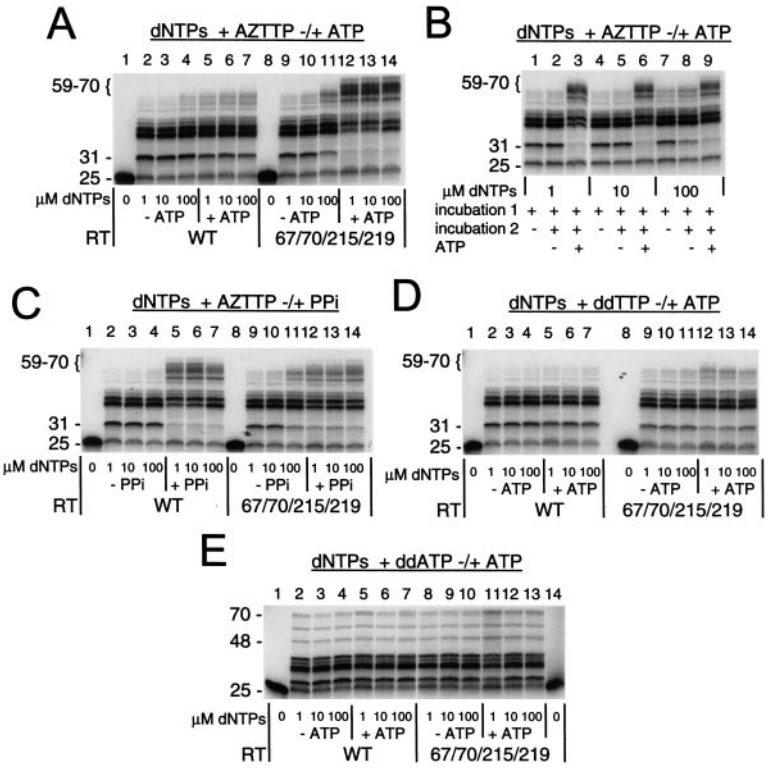


Figure 1. Primer Extension by WT and 67/70/215/219 Mutant RT in the Presence of Chain-Terminating Nucleoside Triphosphate

(A) Primer extension in the presence of AZTTP with or without ATP. The 5′-$^{32}$P-labeled D25 primer/D70 template (2.5 nM) was incubated for 30 min at 37°C with 100 nM WT RT (lanes 1–7) or 67/70/215/219 RT (lanes 8–14); 1, 10, or 100 μM of each of the four dNTPs; and AZTTP (AZTTP/dTTP = 2:1) in the absence (−ATP) or presence (+ATP) of 3.2 mM ATP. The synthetic DNA oligonucleotides D25 and D70 are described under the Experimental Procedures. The products were fractionated on a 10% denaturing polyacrylamide gel. Primer lengths are indicated in nucleotides.

(B) The effect of adding ATP after a preincubation with dNTPs and AZTTP. The 5′-[$^{32}$P]D25 primer/D70 template (2.5 nM) was incubated for 15 min at 37°C with 100 nM 67/70/215/219 RT, dNTPs at the indicated concentrations, and AZTTP (AZTTP/dTTP = 2:1) followed by no further incubation (lanes 1, 4, and 7) or incubation for an additional 30 min at 37°C in the absence (lanes 2, 5, and 8) or presence (lanes 3, 6, and 9) of 3.2 mM ATP.

(C) Primer extension in the presence of AZTTP with or without PPi. Incubation as in (A) except that ATP was replaced by 50 μM PPi.

(D) Incubation as in (A) except that AZTTP was replaced by ddTTP (ddTTP/dTTP = 8:1).

(E) Incubation as in (A) except that AZTTP was replaced by ddATP (ddATP/dATP = 8:1).

In the absence of ATP, WT and 67/70/215/219 RT gave similar termination products (Figure 1A, compare lanes 2–4 with 9–11) in agreement with previous reports that the difference in the ability of these enzymes to discriminate between dTTP and AZTTP is minimal (Lacey et al., 1992; Carroll et al., 1994; Kerr and Anderson, 1997; Krebs et al., 1997; Arion et al., 1998). Addition of 3.2 mM ATP to the reaction mixture gave dramatically different results for WT and 67/70/215/219 RT. Mutant RT extended 12% to 15% of the primers to almost the full length of the template (Figure 1A, lanes 12–14), while only 0.2% to 0.5% of the products formed by the WT RT were extended to comparable lengths (Figure 1A, lanes 5–7). The chain termination was essentially complete after the first 15 min of incubation (Figure 1B, compare lanes 1, 4, and 7 with 2, 5, and 8), and addition of ATP after 15 min resulted in formation of longer products (Figure 1B, lanes 3, 6, and 9) indicating that the previously terminated chains have become unblocked as a result of the addition of ATP. Addition of 50 μM PPi to the reaction resulted in increased primer elongation by both WT and 67/70/215/219 RT (Figure 1C). In contrast to the results when ATP was added to the reaction, the increase in PPi-dependent extension was not greater for the mutant enzyme, suggesting that the AZT resistance is more readily accounted for by the nucleotide-dependent reaction.

While the 67/70/215/219 mutant RT readily overcame termination by AZTTP in the presence of ATP (Figure 1A), termination by ddTTP (Figure 1D) or ddATP (Figure 1E) was only slightly affected. Rescue and elongation of the AZTMP-terminated primers occurred at all three dNTP concentrations tested; however, rescue and elongation of the ddTMP- and ddAMP-terminated primers was most evident at the lowest dNTP concentration. In summary, by adding ATP at concentrations likely to be present in intact cells, we have established an in vitro system that reflects the in vivo properties of the 67/70/215/219 mutant virus. These primer extension assays have allowed us to investigate two characteristics of the 67/70/215/219 mutant virus that could not previously be addressed in vitro—namely, the high degree of resistance to AZT in comparison with wild-type virus and the lack of cross-resistance to other chain-terminating nucleotides (Larder et al., 1989, 1990).

### Unblocking of an AZTMP-Terminated Primer

The results of the primer extension assay could be explained if the 67/70/215/219 mutant RT has enhanced ability to remove AZTMP from blocked primer termini in the presence of ATP. We measured this activity directly by monitoring the synthesis of radioactive dinucleoside polyphosphate and indirectly by the extension of unblocked primer by added DNA polymerase. Figure 2A shows that HIV-1 RT can transfer a radioactive AZTMP residue from a 3′-labeled AZTMP-terminated primer into products containing ATP or GTP. The product of the reaction with ATP (Figure 2A, lanes 4 and 8) comigrated on a 20% denaturing polyacrylamide gel with authentic $Ap_4AZT$ synthesized by firefly luciferase (FL) (Sillero et al., 1997) (Figure 2A, compare lanes 3 and 4 or lanes 7 and 8), and the product of the reaction with GTP (Figure 2A, lanes 12 and 16) comigrated with authentic $Gp_4AZT$ (Figure 2A, compare lanes 11 and 12 or lanes 15 and 16). The labeled products made by HIV-1 RT were resistant to alkaline phosphatase (CIP), as were the corresponding products made by firefly luciferase. Therefore,

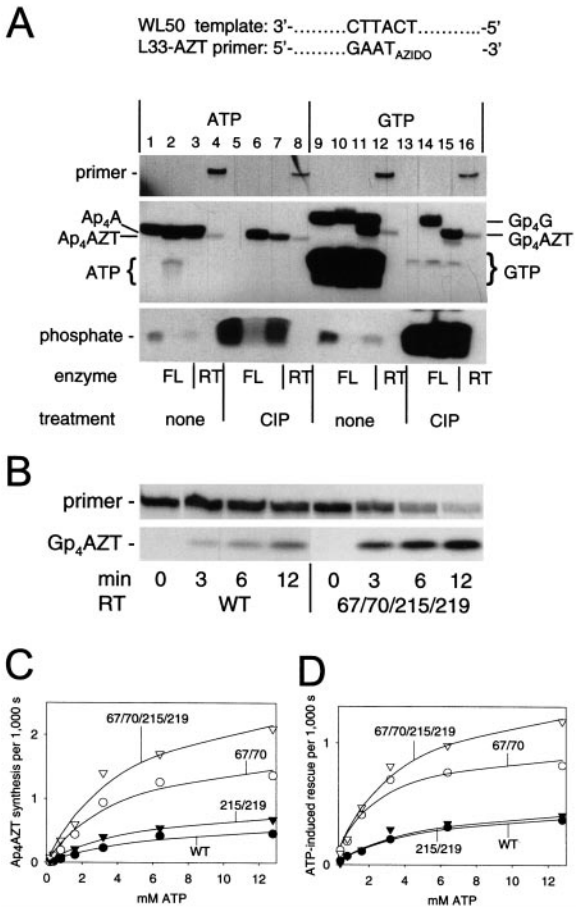


Figure 2. The Unblocking of AZTMP-Terminated Primer/Template through Dinucleoside Polyphosphate Synthesis

(A) The electrophoretic mobility and alkaline phosphatase sensitivity of AZT-containing products formed by WT RT (RT) and firefly luciferase (FL). The [$^{32}$P]AZTMP-terminated L33 primer/WL50 template (5 nM) was incubated with 200 nM WT RT and either 3.2 mM ATP (lanes 4 and 8) or 3.2 mM GTP (lanes 12 and 16). A partial sequence of the primer/template is shown at the top of the figure. The synthetic DNA oligonucleotides L33 and WL50 are described under the Experimental Procedures. FL was incubated with luciferin and 100 nM [$\alpha$-$^{32}$P]ATP in the absence of additional nucleotides (lanes 1 and 5) or with 3 mM ATP (lanes 2 and 6) or 3 mM AZTTP (lanes 3 and 7). FL was also incubated with luciferin and 100 nM [$\alpha$-$^{32}$P]GTP in the absence of additional nucleotides (lanes 9 and 13) or with 3 mM GTP (lanes 10 and 14) or 3 mM AZTTP (lanes 11 and 15). Portions of each reaction mixture were left untreated (lanes 1–4 and 9–12) or digested with calf intestinal alkaline phosphatase (CIP) (lanes 5–8 and 13–16). The reaction products were separated on a 20% denaturing polyacrylamide gel. All nucleotides were treated with PPase prior to use. The products made by FL that are sensitive to CIP are likely formed by breakdown of luciferin–AMP or luciferin–GMP intermediates.

(B) Gp$_4$AZT synthesis by WT and 67/70/215/219 RT. The [$^{32}$P]AZTMP-terminated L33 primer/WL50 template (5 nM) was incubated at 37°C for the times indicated with 3.2 mM GTP and 200 nM WT or 67/70/215/219 RT.

(C) The synthesis of Ap$_4$AZT as a function of ATP concentration. The incubation was carried out as in (B) except that 0.2–12.8 mM ATP was substituted for GTP and the incubation was for either 3 or 10 min at 37°C. The products were quantitated by phosphorimaging. For (C) and (D), the data points are as follows: closed circle, WT; open circle, 67/70; closed triangle, 215/219; open triangle, 67/70/215/219 RT. Each line represents the best fit to the Michaelis-Menten equation.

we conclude that HIV-1 RT is capable of removing the AZTMP residue from a blocked primer/template through the synthesis of dinucleoside polyphosphate.

Figure 2B shows time dependence for synthesis of Gp$_4$AZT by WT and 67/70/215/219 RT. The dependence of Ap$_4$AZT synthesis on ATP concentration is shown in Figure 2C. The apparent second order rate constants ($k_{cat}/K_m$) for synthesis of Ap$_4$AZT and Gp$_4$AZT were elevated 5.3- and 3.7-fold, respectively, for the 67/70/215/219 mutant RT in comparison with the WT RT (Table 1). All reactions were carried out in the presence of excess RT over primer/template to ensure that production of an increased amount of dinucleoside polyphosphate reflects a true difference in synthesis rates between the mutant and wild-type enzymes.

The formation of primer molecules that were no longer blocked to extension by DNA polymerase was detected by the primer rescue assay previously described by Meyer et al. (1998). The 5′-labeled AZT-terminated primer/template was incubated with wild-type or mutant RT in the presence of various concentrations of ATP (Figure 2D). Similar results were obtained with GTP (data not shown). The RT was heat inactivated, and the unblocked primer was extended by addition of exonuclease-free Klenow fragment of *E. coli* DNA polymerase I and dNTPs. The increase in the fraction of radioactivity in molecules at least one nucleotide longer than the original primer was determined by phosphorimaging. The apparent second order rate constants ($k_{cat}/K_m$) for ATP- and GTP-dependent primer rescue activity (Table 1) were increased about 4-fold for the 67/70/215/219 mutant RT over the WT RT, in good agreement with the differences in dinucleoside polyphosphate synthesis activity. We conclude that the 67/70/215/219 RT has 4- to 5-fold higher dinucleoside polyphosphate synthesis activity than the WT RT leading to increased ability to unblock AZTMP-terminated primers. This translates into a much larger difference between the mutant and WT RTs under conditions where multiple chain termination and rescue events can take place.

### Unblocking of a ddAMP-Terminated Primer and Incorporation of the ddAMP Residue into Dinucleoside Polyphosphate Are Also Enhanced in the 67/70/215/219 Mutant RT

Dinucleoside polyphosphate synthesis and primer rescue assays were also carried out with a 3′-[$^{32}$P]ddAMP-terminated L32 primer. Figure 3A shows Gp$_4$ddA synthesis as a function of GTP concentration for WT and 67/70/215/219 RT. Figure 3B shows quantitative data for Ap$_4$ddA synthesis. For either nucleotide acceptor, dinucleoside polyphosphate synthesis was substantially greater for the 67/70/215/219 mutant than for WT RT.

(D) The rescue of AZTMP-terminated primer by WT and mutant RTs as a function of ATP concentration. The AZTMP-terminated, 5′-[$^{32}$P]L33 primer/WL50 template (5 nM) was incubated with 200 nM WT or mutant RT and 0.2–12.8 mM ATP for either 3 or 10 min at 37°C. The RT was inactivated by heat treatment and the primer was extended by incubation with the exonuclease-free Klenow fragment of *E. coli* DNA polymerase I and all four dNTPs. The amount of unblocked, extendible primer was determined by measuring the radioactivity in products greater than 34 nucleotides in length.

Primer rescue, carried out with a 5′-labeled, ddAMP-terminated primer/template, was also increased with the 67/70/215/219 mutant RT (Figure 3C). The apparent second order rate constants ($k_{cat}/K_m$) are given in Table 1. ATP- and GTP-dependent dinucleoside polyphosphate synthesis were increased 9.2-fold and 6.7-fold, respectively. ATP- and GTP-dependent primer rescue were increased to a similar or greater extent. Interestingly, all nucleoside triphosphates induced removal of ddAMP through dinucleoside polyphosphate synthesis to a much larger extent with 67/70/215/219 RT compared to WT RT, whereas nucleoside diphosphates induced similar amounts of removal with either enzyme (Figure 3D).

We conclude that removal of the terminal residue from both ddAMP- and AZTMP-terminated primers is enhanced in the 67/70/215/219 mutant RT. This result appears to disagree with the results in Figure 1 showing that the mutant RT could overcome the chain termination by AZTMP but had minimal ability to overcome the chain termination by ddAMP. This apparent discrepancy is resolved by results shown below, which demonstrate that removal of the AZTMP residue from an AZTMP-terminated primer is much less sensitive to inhibition by the next complementary dNTP than is removal of the ddAMP residue from a ddAMP-terminated primer.

### Inhibition of Dinucleoside Polyphosphate Synthesis by the Next Complementary dNTP and Formation of a Dead-End Complex with RT and a Chain-Terminated Primer/Template

Our laboratory has previously shown that dinucleoside polyphosphate synthesis by HIV-1 RT is inhibited by the next complementary dNTP (Meyer et al., 1998), and we have also shown that binding of the next complementary dNTP to HIV-1 RT and a chain-terminated primer/template results in formation of a dead-end complex (DEC) that is stable enough to be detected by electrophoretic mobility retardation assays (Tong et al., 1997). Concentrations of dNTP required for DEC formation and inhibition of dinucleoside polyphosphate synthesis are similar (Meyer et al., 1998), leading us to propose that dNTP inhibits dinucleoside polyphosphate synthesis by capturing the enzyme in DEC, which cannot carry out the transfer reaction to form dinucleoside polyphosphate. We therefore compared the ability of the complementary dNTP to induce DEC formation and to inhibit dinucleoside polyphosphate synthesis by mutant and WT RTs.

DEC formation was tested on the AZTMP-L33/WL50 primer/template as a function of dGTP, the next complementary nucleotide (Figures 4A and 4C, closed symbols), and on the ddAMP-L32/WL50 primer/template as a function of the next complementary nucleotide, which is dTTP (Figures 4B and 4C, open symbols). Partial nucleotide sequences for these chain-terminated primer/templates are shown in Figures 2 and 3, respectively. $K_{d,app}$'s for these reactions are shown in Table 2. For both mutant and WT RT, as well as the other mutants shown in Table 2, the $K_{d,app}$ was much higher when the primer was terminated with AZTMP than with ddAMP. This suggests that the azido group in the 3′ terminal position on the primer inhibits the interaction of the RT/primer/template complex with the next complementary dNTP and agrees with results previously reported from this laboratory (Tong et al., 1997). DEC was formed less readily by 67/70/215/219 mutant RT than by WT RT for both the AZTMP- and ddAMP-terminated primers (Figure 4C and Table 2).

Concentrations of the next complementary dNTP required to inhibit dinucleoside polyphosphate synthesis were similar to those required for DEC formation (Figure 4D and Table 2). AZTMP-terminated primer/templates were much less sensitive to this inhibition (Figure 4D, closed symbols) than primers terminated with ddAMP (open symbols), and 67/70/215/219 RT was less sensitive to this inhibition than WT RT for both AZTMP- and ddAMP-terminated primers.

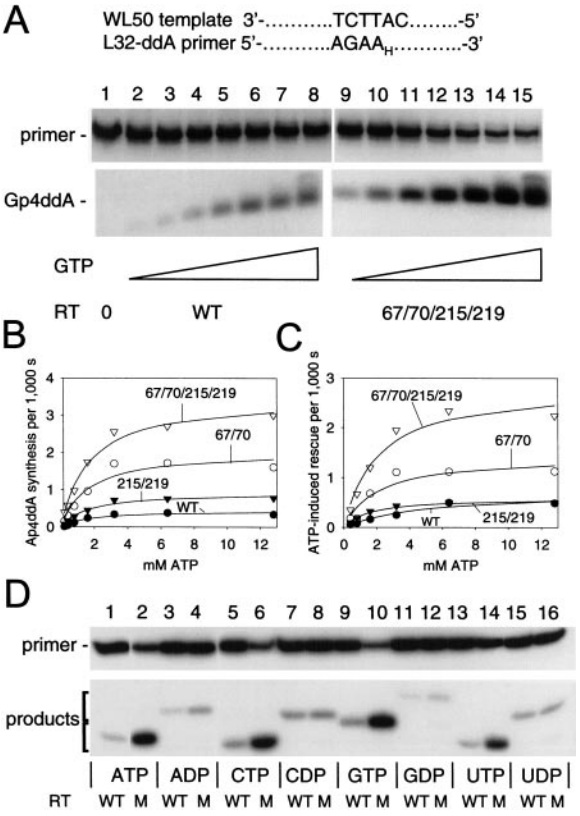


Figure 3. The Unblocking of ddAMP-Terminated Primer/Template through Dinucleoside Polyphosphate Synthesis

(A) $Gp_4ddA$ synthesis by WT and 67/70/215/219 RT. The [$^{32}$P]ddAMP-terminated L32 primer/WL50 template (5 nM) was incubated for 3 min at 37°C with 200 nM WT (lanes 2–8) or 67/70/215/219 RT (lanes 9–15) and GTP concentrations increasing from 0.2 mM to 12.8 mM in two-fold increments. A partial sequence of the primer/template is shown at the top of the figure. The products were separated on a 20% denaturing polyacrylamide gel.

(B) $Ap_4ddA$ synthesis as a function of ATP concentration. The reactions were carried out as in (A) with ATP substituted for GTP. The amount of $Ap_4ddA$ formed was determined by phosphorimaging. The symbols are defined in the legend to Figure 2.

(C) The rescue of ddAMP-terminated primer by WT and mutant RTs as a function of ATP concentration. The experiments were performed as described in the legend to Figure 2D but with the ddAMP-terminated 5′-[$^{32}$P]L32 primer/WL50 template.

(D) Dinucleoside polyphosphate synthesis induced by nucleoside di- or triphosphates. The reactions were carried out as in (A) with WT RT (WT) or 67/70/215/219 RT (M) and 6.4 mM of the indicated nucleotide for 2 min at 37°C. The products derived from NDPs are dinucleoside triphosphates (upper bracket). The products derived from NTPs are dinucleoside tetraphosphates (lower bracket).

Table 1. Apparent Second Order Rate Constants for ATP- and GTP-Dependent Dinucleoside Polyphosphate Synthesis and Primer Rescue by HIV-1 WT and Mutant RTs

| | | Dinucleoside Polyphosphate Synthesis[b] $k_{cat}/K_m$, $s^{-1}$ $M^{-1}$ | | Primer Rescue[b] $k_{cat}/K_m$, $s^{-1}M^{-1}$ | |
|---|---|---|---|---|---|
| Primer[a] | RT | ATP | GTP | ATP | GTP |
| AZTMP-L33 | WT | 0.11 +/− 0.01 | 0.22 +/− 0.06 | 0.08 +/− 0.03 | 0.09 +/− 0.01 |
| | 67/70/215/219 | 0.58 +/− 0.06 | 0.81 +/− 0.26 | 0.39 +/− 0.03 | 0.36 +/− 0.01 |
| | 67/70 | 0.44 +/− 0.01 | 0.86 +/− 0.14 | 0.44 +/− 0.02 | 0.56 +/− 0.05 |
| | 215/219 | 0.17 +/− 0.02 | 0.19 +/− 0.03 | 0.12 +/− 0.01 | 0.18 +/− 0.04 |
| ddAMP-L32 | WT | 0.26 +/− 0.02 | 0.46 +/− 0.1 | 0.16 +/− 0.01 | 0.07 +/− 0.01 |
| | 67/70/215/219 | 2.4 +/− 0.3 | 3.1 +/− 0.2 | 1.3 +/− 0.1 | 1.2 +/− 0.1 |
| | 67/70 | 1.2 +/− 0.1 | 3.3 +/− 0.1 | 0.85 +/− 0.15 | 1.6 +/− 0.1 |
| | 215/219 | 0.56 +/− 0.01 | 0.81 +/− 0.0 | 0.42 +/− 0.04 | 0.38 +/− 0.04 |

[a] In each case the template was WL50. Sequences of the primer/template are shown in Figures 2 and 3 and the Experimental Procedures.
[b] Data obtained from experiments performed as described in the legends to Figures 2 and 3 were fitted to theoretical curves. Values represent the average of duplicate or triplicate experiments +/− the average deviation.

### RT Interaction with the Complementary dNTP Is Impaired When the Primer Terminus Is Blocked by AZTMP in Several Sequence Contexts

Formation of DEC was compared at several T incorporation sites for AZTMP- and ddTMP-terminated primer/templates (Figure 4E). WT RT was incubated with the D25/D70 primer/template, all four dNTPs (at the concentrations indicated in the figure), and either AZTTP (left panel) or ddTTP (right panel) at 20% of the concentration of dTTP in the reaction mixture. Chain termination and DEC formation could occur at each T incorporation site. The complexes were separated from free DNA by electrophoresis through a nondenaturing gel and the mixture of DEC species was identified as a low mobility band near the top of the gel (data not shown). DEC and free DNA were eluted from the gel, and the DNA species contained in each fraction were compared by denaturing gel electrophoresis. $K_{d,app}$'s for DEC formation are shown in the middle of the panel for five different T incorporation sites. Much higher concentrations of dNTP (25-fold to >150-fold) were required for DEC formation on the AZTMP-terminated primer/template than on the ddTMP-terminated primer/template, confirming that the presence of a 3′ azido group strongly inhibits formation of DEC in at least five different sequence contexts. DEC formation was less favored at primer positions P41 and P44 than P39, P42, or P46 for both ddTMP- and AZTMP-terminated primers.

### Comparison of Dinucleoside Polyphosphate Synthesis and dNTP Sensitivity in 67/70 RT and 215/219 RT

Synthesis of dinucleoside polyphosphates and primer rescue by 67/70 RT was similar to 67/70/215/219 RT and at least 4-fold higher than WT RT when measured on either the AZTMP-terminated primer/template or the ddAMP-terminated primer/template (Figures 2 and 3 and Table 1). The corresponding rate constants for 215/219 RT were similar to WT RT or slightly increased but not as high as for 67/70 RT or 67/70/215/219 RT. Therefore, we conclude that the increased dinucleoside polyphosphate synthesis activity seen in 67/70/215/219 RT was primarily conferred by the 67/70 mutations.

Interaction of the RT/primer/template complex with dNTP was similar for 67/70 and WT RT as measured both by DEC formation and dNTP inhibition of dinucleoside polyphosphate synthesis, whereas 215/219 RT required higher dNTP concentrations for these interactions (Table 2). These results suggest that the 215/219 mutations are primarily responsible for the decreased sensitivity to dNTP seen for 67/70/215/219 RT.

## Discussion

We have shown that removal of the chain-terminating residue from a blocked primer terminus is enhanced in RT containing AZT resistance mutations and that this

Table 2. The Ability of the Complementary dNTP to Induce DEC Formation and to Inhibit Dinucleoside Polyphosphate Synthesis

| | AZTMP-Terminated Primer/Template | | ddAMP-Terminated Primer/Template | |
|---|---|---|---|---|
| HIV-1RT | DEC Formation $K_{d,app}$, μM[a] | Inhibition of $Ap_4AZT$ Synthesis $IC_{50}$, μM[b] | DEC Formation $K_{d,app}$, μM[a] | Inhibition of $Ap_4ddA$ Synthesis $IC_{50}$, μM[b] |
| WT | 40 +/− 10 | 110 +/− 40 | 3.7 +/− 0.9 | 4.3 +/− 1.3 |
| 67/70/215/219 | 100 +/− 35 | 230 +/− 40 | 20 +/− 3.6 | 12 +/− 1.5 |
| 67/70 | 60 +/− 10 | 120 +/− 20 | 4.0 +/− 0.1 | 8.2 +/− 1.8 |
| 215/219 | 120 +/− 20 | 370 +/− 170 | 12 +/− 3.0 | 21 +/− 4.3 |

[a] Band shift/DEC experiments were performed as described in the legend of Figure 4. Constants were obtained by plotting the amount of DEC formation against dNTP concentration and fitting the data to a hyperbola, as shown for WT and 67/70/215/219RT in Figure 4C. $K_{d,app}$ is the dNTP concentration that induces 50% of maximal amount of DEC.
[b] Reactions were performed as described in the legend of Figure 4D. Constants were obtained by plotting inhibition of dinucleoside polyphosphate synthesis against the dNTP concentration and fitting the data to a hyperbola, as shown for WT and 67/70/215/219RT in Figure 4D. $IC_{50}$ is the concentration of dNTP that inhibits dinucleoside polyphosphate synthesis by 50%.

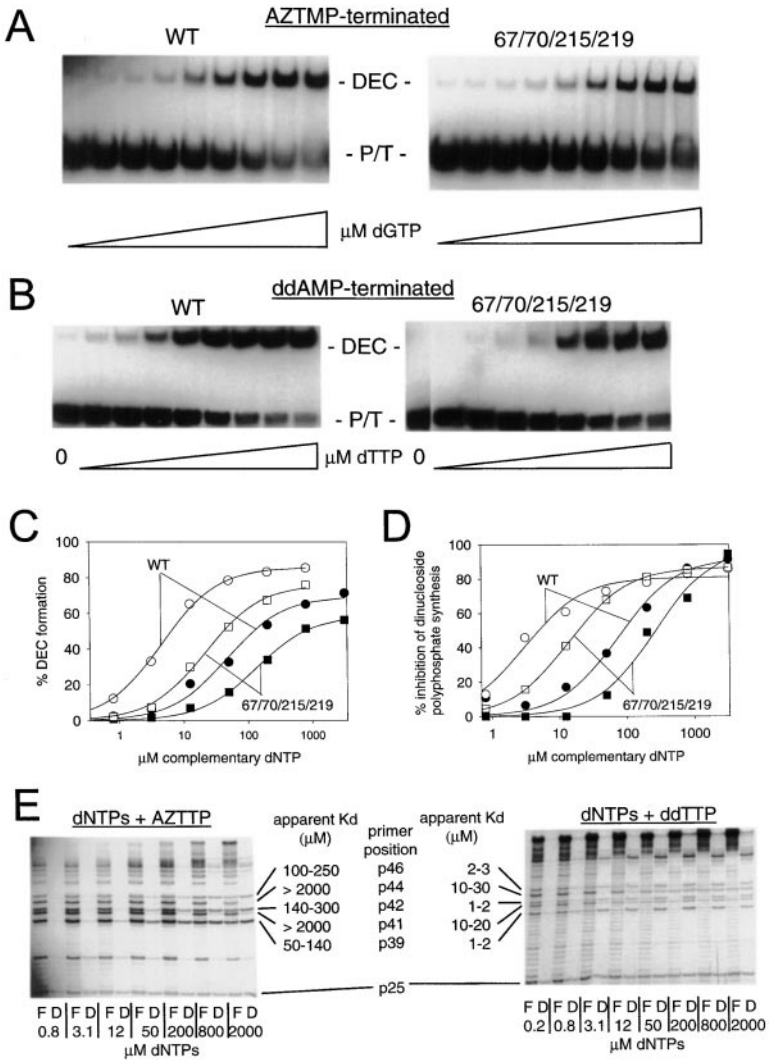


Figure 4. The Ability of the Complementary dNTP to Induce DEC Formation and to Inhibit Dinucleoside Polyphosphate Synthesis by WT and Mutant RTs

(A) DEC formation on the AZTMP-terminated primer/template. The AZTMP-terminated 5′-[$^{32}$P]L33 primer/WL50 template (2 nM) was incubated with 80 nM WT or 67/70/215/219 RT, as indicated, and dGTP, the complementary nucleotide substrate, increasing in concentration from 0.05 to 3200 μM in 4-fold increments. The free primer/template (P/T) and the dead-end RT/P/T complex (DEC) were separated by electrophoresis through a 6% nondenaturing polyacrylamide gel. The sequence of the primer/template is shown in Figure 2A and the Experimental Procedures.

(B) DEC formation on the ddAMP-terminated primer/template. The reactions were carried out as in (A) with the ddAMP-terminated, 5′-[$^{32}$P]L33 primer/WL50 template and dTTP, the next complementary nucleotide substrate, increasing from 0.05 to 800 μM in 4-fold increments. The sequence of the primer/template is shown in Figure 3A and the Experimental Procedures.

(C) Quantitation of the data in (A) and (B). Circles, WT RT; squares, 67/70/215/219 mutant RT; open symbols, ddAMP-terminated primer/template; closed symbols, AZTMP-terminated primer/template. Each line represents the best fit of the data to a single-ligand binding curve.

(D) The inhibition of dinucleoside polyphosphate synthesis by the complementary dNTPs. The dinucleoside polyphosphate synthesis was performed with either the AZTMP-terminated primer/template, as described in the legend to Figure 2C in the presence of 0.2–3200 μM dGTP (closed symbols), or with the ddAMP-terminated primer/template, as described in the legend to Figure 3B in the presence of 0.2–3200 μM dTTP (open symbols) with WT RT (circles) or 67/70/215/219 mutant RT (squares). The amount of $Ap_4AZT$ or $Ap_4ddA$ synthesized was measured by phosphorimaging, and the percent inhibition was plotted versus dNTP concentration. Each line represents the best fit of the data to a hyperbola.

(E) DEC formation with primers terminated at various positions with AZTMP (left) or ddTMP (right). The 5′-$^{32}$P-labeled D25 primer/D70 template (5 nM) was incubated for 30 min at 37°C with 200 nM WT RT, the indicated concentrations of dNTPs, and AZTTP (AZTTP/dTTP = 1:5) or ddTTP (ddTTP/dTTP = 1:5). DEC (D) and free primer/template (F) were separated on 6% nondenaturing polyacrylamide gels (data not shown), and the DNA species contained in each fraction were eluted from the gel and fractionated on a 10% denaturing polyacrylamide gel. The length of the unextended primer is 25 nucleotides (P25). The amount of primer/template present in the F or D fractions at the indicated primer positions was quantitated by phosphorimaging and the percent DEC [100 × D/(D + F)] was plotted versus the dNTP concentration. The data were fitted to single-ligand binding curves to determine the $K_{d,app}$ for DEC formation at each primer position (range for two experiments).

activity is inhibited by the dNTP that is complementary to the nucleotide at the next position on the template. These results suggest a model (Figure 5A) in which RT bound to a chain-terminated primer/template can either transfer the chain-terminating residue to an acceptor molecule (shown as NTP in the figure) and regenerate an extendible primer (primer rescue, left pathway) or interact with the complementary dNTP and form a stable dead-end complex (DEC formation, right pathway). Formation of DEC prevents the chain-terminated primer/template from entering into the primer rescue pathway. The figure shows a reaction in which the chain-terminating residue (filled box) is transferred to NTP (hatched box) to form dinucleoside tetraphosphate and an unblocked primer/template. At each site where chain termination occurs, either pathway may be selected depending on the intracellular concentrations of the potential acceptor molecules and the concentration of the next complementary dNTP.

We have shown that the 67/70/215/219 mutant RT has elevated nucleotide-dependent primer unblocking activity and reduced sensitivity to inhibition of this activity by the complementary dNTP. These properties map to different regions of the protein, with the 67/70 mutations largely accounting for the elevated primer unblocking activity, and the 215/219 mutations, for the reduced inhibition by the next complementary dNTP (Figure 5B). Canard et al. (1998) have suggested that enhanced binding of AZT-resistant RT to an AZTMP-terminated primer increases the likelihood that the terminating residue will be removed by transfer to pyrophosphate. We have shown that transfer to a nucleoside

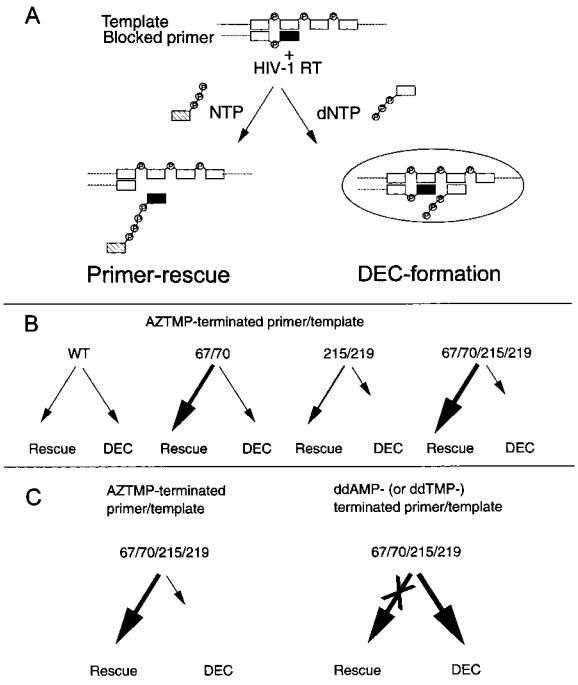


Figure 5. A Model for Potential Interactions between HIV-1 RT and the Chain-Terminated Primer/Template

(A) Alternative pathways for RT/primer/template. Open boxes represent natural deoxynucleosides; closed boxes, chain-terminating nucleosides; hatched boxes, ribonucleosides.

(B) Alternative interactions between WT or mutant RTs and the AZTMP-terminated primer/template. The relative ability of each RT to catalyze the nucleotide-dependent unblocking reaction (rescue) or undergo DEC formation (DEC) are indicated by the thickness and lengths of the arrows. A decreased ability to form DEC by 215/219 and 67/70/215/219 mutant RTs indicates that a higher concentration of the complementary dNTP is required for DEC formation (increased $K_{d,app}$), which translates into a decreased sensitivity of the rescue reaction to the inhibition by the complementary dNTP.

(C) Interaction between 67/70/215/219 RT and the primer/template terminated with ddAMP, ddTMP, or AZTMP. The thickness and lengths of the arrows represent the relative ability of 67/70/215/219 RT to catalyze the nucleotide-dependent unblocking reaction (rescue) or undergo DEC formation (DEC) on the AZTMP-terminated primer/template versus the ddAMP- (or ddTMP-) terminated primer/template. An "X" indicates the inhibition of the rescue reaction under conditions that favor DEC formation.

triphosphate acceptor is elevated with the mutant enzyme whereas transfer to PPi or nucleoside diphosphate acceptors are not, suggesting that, in addition to enhanced binding to AZTMP at the primer terminus, the mutant RT forms unique interactions with the NTP acceptor that are not formed with PPi or NDP. Our results do not agree with those of Arion et al. (1998) who reported an increased rate of pyrophosphorolysis in the mutant enzyme. We do not understand the reason for this difference.

Our results provide an explanation for the lack of cross-resistance of the 67/70/215/219 mutant virus to other nucleoside inhibitors in in vivo assays (Larder et al., 1989, 1990). As shown in Figure 5C, the removal of the chain-terminating residue (rescue) by the mutant enzyme occurs to similar extents for the AZTMP- and ddAMP-terminated primers; however, the enzyme is much more easily captured as a dead-end complex (DEC) when the primer is terminated with ddAMP than when it is terminated with AZTMP. As a result, the removal of ddAMP is strongly inhibited by the next complementary dNTP ($IC_{50}$ = 12 $\mu$M), while the removal of AZTMP is relatively insensitive to this inhibition ($IC_{50}$ > 200 $\mu$M). DEC formation on AZTMP-terminated primers was impaired in all sequence contexts examined (Figure 4E), suggesting that the 3′ azido group was responsible for the reduced dNTP binding. These results indicate that the nucleotide structure at the primer terminus plays an important role in the inhibition of DNA synthesis by chain-terminating inhibitors.

Huang et al. (1998) have determined the crystal structure of a covalently trapped catalytic complex containing HIV-1 RT, chain-terminated primer/template, and dTTP in a complex similar to the DEC we have described (Tong et al., 1997). Residues 67 and 70 lie near the $\gamma$-phosphate of dTTP in this structure, but not close enough to make contact. The covalently trapped catalytic complex can be considered equivalent to the complex formed immediately after the synthesis of dTTP by pyrophosphorolysis. By analogy, the replacement of dTTP with dinucleoside tetraphosphate in this structure would give the complex formed immediately after the removal of the primer terminus by the transfer to nucleoside triphosphate but prior to the dissociation of the product. The K70R mutation could stabilize the binding of the dinucleoside tetraphosphate in place of dTTP by repositioning the positive charge to allow the interaction with the additional phosphate residue. The D67N mutation could also enhance the dinucleoside tetraphosphate binding by removing a negative charge from the environment. This would explain the increased transfer of nucleotide from the primer terminus to nucleoside triphosphate acceptors, but not to PPi or nucleoside diphosphate acceptors, by the mutant enzyme, since residues 67 and 70 are not likely to make contact with the products of the latter transfer reactions.

Residues 215 and 219 do not make direct contact with either the dNTP or the primer terminus in the covalently trapped catalytic complex (Huang et al., 1998); however, the T215F and K219Q mutations give rise to indirect structural changes at the polymerase active site (Ren et al., 1998) and may indirectly alter protein interactions with the primer terminus and the incoming nucleotide. Altered binding to either the dNTP or the primer terminus could explain our observations that DEC formation by the 215/219 mutant RT requires higher concentrations of dNTP in comparison with the WT and 67/70 enzymes and that elevated dNTP concentrations are required to inhibit the removal of chain-terminating nucleotides by the 215/219 mutant enzyme.

We have demonstrated that the nucleotide-dependent primer unblocking activity is elevated in an AZT-resistant form of RT that predominates under selective conditions in vivo. In addition, the $K_m$ for this activity (Meyer et al., 1998) is in the range of the ribonucleotide concentrations found in vivo (Hauschka, 1973), strongly supporting our conclusion that this reaction is likely to play an important role in AZT-treated cells. The ability of HIV-1 RT to remove chain-terminating nucleotides under physiological conditions must be taken into consideration in evaluating HIV sensitivity to chain-terminating inhibitors as well as the effects of drug resistance mutations. The inhibitory effects depend not only on the

efficiency of phosphorylation of the compound to its active triphosphate derivative and its incorporation into the DNA product, but also on the efficiency of removal of the blocked primer terminus and the sensitivity of the removal reaction to inhibition by the next complementary dNTPs. The discovery that this novel enzymatic activity is elevated in the AZT-resistant RT offers a new target for the design of anti-HIV chemotherapeutic agents that may help prevent and/or reverse the resistance to AZT, an important component of the current treatment regimens for HIV infection.

## Experimental Procedures

### The Expression and Purification of HIV-1 RT and Herpes Simplex Virus Thymidine Kinase

His-tagged HIV-1 RT was prepared as previously described (Meyer et al., 1998). Mutations were introduced using the megaprimer PCR method (Sarkar and Sommer, 1990) with Pwo polymerase (Boehringer Mannheim), and the mutant clones were sequenced by the dideoxy method. The specific RNA-dependent DNA polymerase activities (assayed as described by Tan et al., 1991) of the wild-type and mutant enzymes were: WT RT, 20,000 U/mg; 67/70 RT, 8,000 U/mg; 215/219 RT, 12,000 U/mg; 67/70/215/219 RT, 10,000 U/mg; where one unit (U) is the amount of enzyme required for the incorporation of 1.0 nmol [$^3$H]dTMP in 10 min at 37°C using poly(rA)/oligo(dT) as the substrate. Herpes simplex virus thymidine kinase (HSV-TK) was expressed in BL21(DE3) tk$^-$ *E. coli* containing the expression plasmid pET23d:HSVTK, a generous gift from Dr. Margaret Black at Washington State University (Pullman, WA), and purified by affinity chromatography on aminothymidine cross-linked to sepharose beads (M. Black, personal communication).

### The Synthesis and Purification of [$\alpha$-$^{32}$P]AZTTP

HSV-TK (400 nM) was incubated with 750 $\mu$Ci [$\gamma$-$^{32}$P]ATP and 3 mM AZT in 1 ml TK activity buffer (50 mM Tris-HCl [pH 7.5], 4 mM $MgCl_2$, 2.5 mM DTT, 12 mM KCl, 2.5% glycerol [Munir et al., 1992]) for 2 hr at 37°C, and the HSV-TK was inactivated at 90°C for 5 min. To convert [$^{32}$P]AZTMP to AZTTP, 180 $\mu$l of BL21 cell extract (M. Black, personal communication) and 20 $\mu$l 350 mM ATP were added, and the mixture was incubated for 16 hr at 37°C. The cell debris was removed by addition of 1/10 volume ice-cold 11 N formic acid, incubation on ice for 30 min, and centrifugation at maximal speed for 20 min at 4°C in a Microspin 12 tabletop centrifuge (Sorvall). The supernatant solution was neutralized by addition of 1/8 volume triethanolamine and dried to a viscous residue (Bochner and Ames, 1982). The residue was resuspended in 50 $\mu$l 100 mM triethylammonium bicarbonate (pH 7.0) and fractionated by reverse phase HPLC using 0.1 M triethylammonium bicarbonate buffer (pH 7.0), containing 8% acetonitrile, with a 250 $\times$ 4.6 mm sephasil peptide C18 5u ST column (Pharmacia) and a flow rate of 1 ml/min. The fractions (1 ml) were collected, vacuum dried, and resuspended in 100 $\mu$l double-distilled water. The [$\alpha$-$^{32}$P]AZTTP was identified by comigration with unlabeled AZTTP (Amersham) during thin layer chromatography (Bochner and Ames, 1982) and incorporation by HIV-1 RT into a primer/template resulting in 3′ labeling of the primer, formation of a chain-terminated primer, and extension of the primer by exactly one base (data not shown).

### The Labeling of Oligonucleotides and Dinucleoside Tetraphosphates with $^{32}$P

The oligonucleotide primer L32 (5′-CTACTAGTTTTCTCCATCTAGACGATACCAGA-3′) was 3′ labeled with [$^{32}$P]ddAMP by annealing to the template WL50 (5′-GAGTGCTGAGGTCTTCATTCTGGTATCGTCTAGATGGAGAAAACTAGTAG-3′) and extending the primer with [$\alpha$-$^{32}$P]ddATP as previously reported (Meyer et al., 1998). The oligonucleotide primer L33 (5′-CTACTAGTTTTCTCCATCTAGACGATACCAGAA-3′) was 3′ labeled with [$^{32}$P]AZTMP by annealing to the template WL50 and extending the primer with [$\alpha$-$^{32}$P]AZTTP. The oligonucleotides were 5′ labeled with $T_4$ polynucleotide kinase. Firefly luciferase and D-luciferin were employed to prepare the $^{32}$P-labeled dinucleoside polyphosphates (Sillero et al., 1997; Meyer et al. 1998). AZTTP and [$\alpha$-$^{32}$P]ATP were used to make [$^{32}$P]$Ap_4$AZT. AZTTP and [$\alpha$-$^{32}$P]GTP were used to make [$^{32}$P]$Gp_4$AZT. An alkaline phosphatase-resistant labeled product was formed in each of these reactions with an electrophoretic mobility expected for the dinucleoside tetraphosphate (Figure 2A). In addition, a phosphatase-sensitive labeled product was formed that may arise by hydrolysis of an unstable $^{32}$P-labeled luciferin intermediate.

### Primer Extension in the Presence of a Chain-Terminating Nucleotide, Dinucleoside Polyphosphate Synthesis, and Detection of Unblocked Primer

The primer extension experiments were carried out with 5′-$^{32}$P-labeled D25 primer (5′-GTTTCTGATCTGGTGTGAAAAGTCC-3′) (2.5 nM) annealed to excess D70 template (5′-CATAGAACAAAAATAGAGGAGCTGAGACAACATCTGTTGAGGTGGGGACTTTTCACACCAGATCAGAAAC-3′) and 100 nM WT or mutant RT in 20 $\mu$l RB buffer containing all four dNTPs and AZTTP, ddTTP, or ddATP (concentrations given in the legend to Figure 1) in the presence or absence of 3.2 mM ATP or 50 $\mu$M PPi. After incubation for 30 min at 37°C, the reaction mixture was heated for 5 min at 90°C, an equal volume of 2$\times$ urea/TTE loading buffer (16 M urea, 180 mM Tris, 58 mM taurine, 1 mM EDTA, 0.5% bromphenol blue, 0.5% xylene cyanol) was added, and the products were fractionated on a 10% denaturing polyacrylamide gel.

The synthesis of dinucleoside polyphosphate was measured by the transfer of a $^{32}$P-labeled ddAMP or AZTMP residue from a 3′-labeled primer to a nucleotide acceptor to form a $^{32}$P-labeled dinucleoside polyphosphate (Meyer et al., 1998). The primer rescue assays, in which a 5′-labeled chain-terminated primer/template was incubated with WT or mutant RT and then extended with the exonuclease-free Klenow fragment of *E. coli* DNA polymerase I to measure the formation of unblocked primer, were performed as previously described (Meyer et al., 1998), and the products were quantitated by phosphorimaging. The rates of dinucleoside polyphosphate synthesis and primer rescue were determined with saturating amounts of RT so that the reactions were limited by the concentration of the RT/primer/template complex, which was assumed to be equal to the primer concentration. The apparent $k_{cat}$ and $K_m$ were obtained by fitting the data to the Michaelis-Menten equation using Sigmaplot 3.0.

### Electrophoretic Mobility Shift Assays for Detection of DEC and Comparison of DEC Formation at Multiple Termination Sites

The 5′-$^{32}$P-labeled, chain-terminated primer/template was incubated with HIV-1 RT and dNTPs, and electrophoretic mobility shift assays were carried out as previously described (Tong et al., 1997) except as follows. The primer/template was chain terminated with WT HIV-1 RT and reisolated prior to incubation with WT or mutant RT for 15 min at 37°C with various concentrations of the next complementary dNTP in 10 $\mu$l RB buffer (40 mM HEPES [pH 7.5], 20 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 2.5% glycerol, and 80 mg/ml BSA). The reaction mixture was placed on ice for 5 min, followed by the addition of 3 $\mu$l heparin loading buffer (0.01 U/ml of heparin, 30% glycerol, 0.25% bromphenol blue, and 0.25% xylene cyanol). The electrophoresis was carried out in Tris-taurine buffer (90 mM Tris, 30 mM taurine). AZTTP (10 $\mu$M) was added to the reactions containing AZTMP-terminated primer/templates to replace the terminal AZTMP residues removed during the incubation. The radioactivity in DEC and free DNA was determined by phosphorimaging. The apparent dissociation constant ($K_{d,app}$) for DEC formation was obtained by fitting the data to a single-ligand binding curve using Sigmaplot 3.0.

DEC formation on primer/template terminated at multiple sites was carried out using 5 nM 5′-$^{32}$P-labeled D25 primer annealed to excess D70 template. The labeled primer/template was incubated for 30 min at 37°C in 20 $\mu$l RB buffer with 200 nM WT RT, dNTPs, and AZTTP or ddTTP (concentrations given in Figure 4E). After incubation on ice for 5 min, 6 $\mu$l of heparin loading buffer was added and the mixture was fractionated as described for the electrophoretic mobility shift assay. The radioactive bands corresponding to DEC and free DNA were visualized by autoradiography, cut out of the gel, and eluted in 500 $\mu$l DNA elution buffer (0.5 M $NH_4$ acetate, 10

mM Mg acetate, 1 mM EDTA [pH 8.0], 0.1% SDS) for 16 hr at room temperature with constant shaking. The eluate (200 μl) was ethanol precipitated and fractionated on a 10% denaturing polyacrylamide gel. The radioactivity recovered as DEC or free DNA corresponding to each primer termination site was determined and $K_{d,app}$'s were calculated as described above.

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**References**

Arion, D., Kaushik, N., McCormick, S., Borkow, G., and Parniak, M.A. (1998). Phenotypic mechanism of HIV-1 resistance to 3′-azido-3′-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase. Biochemistry *37*, 15908–15917.

Battula, N., and Loeb, L.A. (1976). On the fidelity of DNA replication. Lack of exodeoxyribonuclease activity and error-correcting function in avian myeloblastosis virus DNA polymerase. J. Biol. Chem. *251*, 982–986.

Bochner, B.R., and Ames, B.N. (1982). Complete analysis of cellular nucleotides by two-dimensional thin layer chromatography. J. Biol. Chem. *257*, 9759–9769.

Canard, B., Sarfati, S.R., and Richardson, C.C. (1998). Enhanced binding of azidothymidine-resistant human immunodeficiency virus 1 reverse transcriptase to the 3′-azido-3′-deoxythymidine 5′-monophosphate-terminated primer. J. Biol. Chem. *273*, 14596–14604.

Carroll, S.S., Geib, J., Olsen, D.B., Stahlhut, M., Shafer, J.A., and Kuo, L.C. (1994). Sensitivity of HIV-1 reverse transcriptase and its mutants to inhibition by azidothymidine triphosphate. Biochemistry *33*, 2113–2120.

Cheng, Y.-C., Dutschman, G.E., Bastow, K.F., Sarngadharan, M.G., and Ting, R.Y.C. (1987). Human immunodeficiency virus reverse transcriptase. General properties and its interactions with nucleoside triphosphate analogs. J. Biol. Chem. *262*, 2187–2189.

Emini, E.A., and Fan, H.Y. (1997). Immunological and pharmacological approaches to the control of retroviral infections. In Retroviruses, J.M. Coffin, S.H. Hughes, and H.E. Varmus, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 637–706.

Furman, P.A., Fyfe, J.A., St. Clair, M.H., Weinhold, K., Rideout, J.L., Freeman, G.A., Nusinoff-Lehrman, S., Bolognesi, D.P., Broder, S., Mitsuya, H., and Barry, D.W. (1986). Phosphorylation of 3′-azido-3′-deoxythymidine and selective interaction of the 5′-triphosphate with human immunodeficiency virus reverse transcriptase. Proc. Natl. Acad. Sci. USA *83*, 8333–8337.

Hauschka, P.V. (1973). Analysis of nucleotide pools in animal cells. Methods Cell Biol. *7*, 361–462.

Hsieh, J.-C., Zinnen, S., and Modrich, P. (1993). Kinetic mechanism of the DNA-dependent DNA polymerase activity of human immunodeficiency virus reverse transcriptase. J. Biol. Chem. *268*, 24607–24613.

Huang, P., Farquhar, D., and Plunkett, W. (1990). Selective action of 3′-azido-3′-deoxythymidine 5′-triphosphate on viral reverse transcriptases and human DNA polymerases. J. Biol. Chem. *265*, 11914–11918.

Huang, H., Chopra, R., Verdine, G.L., and Harrison, S.C. (1998). Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance. Science *282*, 1669–1675.

Kerr, S.G., and Anderson, K.S. (1997). Pre-steady-state kinetic characterization of wild type and 3′-azido-3′-deoxythymidine (AZT) resistant human immunodeficiency virus type 1 reverse transcriptase: implication of RNA directed DNA polymerization in the mechanism of AZT resistance. Biochemistry *36*, 14064–14070.

Krebs, R., Immendörfer, U., Thrall, S.H., Wöhrl, B.M., and Goody, R.S. (1997). Single-step kinetics of HIV-1 reverse transcriptase mutants responsible for virus resistance to nucleoside inhibitors zidovudine and 3-TC. Biochemistry *36*, 10292–10300.

Lacey, S.F., Reardon, J.E., Furfine, E.S., Kunkel, T.A., Bebenek, K., Eckert, K.A., Kemp, S.D., and Larder, B.A. (1992). Biochemical studies on the reverse transcriptase and RNase H activities from human immunodeficiency virus strains resistant to 3′-azido-3′-deoxythymidine. J. Biol. Chem. *267*, 15789–15794.

Larder, B.A., Darby, G., and Richman, D.D. (1989). HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy. Science *243*, 1731–1734.

Larder, B.A., Chesebro, B., and Richman, D.D. (1990). Susceptibilities of zidovudine-susceptible and -resistant human immunodeficiency virus isolates to antiviral agents determined by using a quantitative plaque reduction assay. Antimicrob. Agents Chemother. *34*, 436–441.

Meyer, P.R., Matsuura, S.E., So, A.G., and Scott, W.A. (1998). Unblocking of chain-terminated primer by HIV-1 reverse transcriptase through a nucleotide-dependent mechanism. Proc. Natl. Acad. Sci. USA *95*, 13471–13476.

Mitsuya, H., Yarchoan, R., and Broder, S. (1990). Molecular targets for AIDS therapy. Science *249*, 1533–1544.

Munir, K.M., French, D.C., Dube, D.K., and Loeb, L.A. (1992). Permissible amino acid substitutions within the putative nucleoside-binding site of herpes simplex virus type 1 established by random sequence mutagenesis. J. Biol. Chem. *267*, 6584–6589.

Reardon, J.E. (1993). Human immunodeficiency virus reverse transcriptase. A kinetic analysis of RNA-dependent and DNA-dependent DNA polymerization. J. Biol. Chem. *268*, 8743–8751.

Ren, J., Esnouf, R.M., Hopkins, A.L., Jones, E.Y., Kirby, I., Keeling, J., Ross, C.K., Larder, B.A., Stuart, D.I., and Stammers, D.K. (1998). 3′-Azido-3′-deoxythymidine drug-resistance mutations in HIV-1 reverse transcriptase can induce long range conformational changes. Proc. Natl. Acad. Sci. USA *95*, 9518–9523.

Roberts, J.D., Bebenek, K., and Kunkel, T.A. (1988). The accuracy of reverse transcriptase from HIV-1. Science *242*, 1171–1173.

Sarkar, G., and Sommer, S.S. (1990). The "megaprimer" method of site-directed mutagenesis. BioTechniques *8*, 404–407.

Sillero, M.A.G., Madrid, O., Zaera, E., and Sillero, A. (1997). 2′,3′-dideoxynucleoside triphosphates (ddNTP) and di-2′,3′-dideoxynucleoside tetraphosphates ($ddNp_4ddN$) behave differently to the corresponding NTP and $Np_4N$ counterparts as substrates of firefly luciferase, dinucleoside tetraphosphatase and phosphodiesterases. Biochim. Biophys. Acta *1334*, 191–199.

St. Clair, M.H., Richards, C.A., Spector, T., Weinhold, K.J., Miller, W.H., Langlois, A.J., and Furman, P.A. (1987). 3′-azido-3′-deoxythymidine triphosphate as an inhibitor and substrate of purified human immunodeficiency virus reverse transcriptase. Antimicrob. Agents Chemother. *31*, 1972–1977.

Tan, C.-K., Zhang, J., Li, Z.-Y., Tarpley, W.G., Downey, K.M., and So, A.G. (1991). Functional characterization of RNA-dependent DNA polymerase and RNase H activities of a recombinant HIV reverse transcriptase. Biochemistry *30*, 2651–2655.

Tong, W., Lu, C.-D., Sharma, S.K., Matsuura, S., So, A.G., and Scott, W.A. (1997). Nucleotide-induced stable complex formation by HIV-1 reverse transcriptase. Biochemistry *36*, 5749–5757.

Ueno, T., and Mitsuya, H. (1997). Comparative enzymatic study of HIV-1 reverse transcriptase resistant to 2'-3′-dideoxynucleotide analogs using the single-nucleotide incorporation assay. Biochemistry *36*, 1092–1099.