Biochemical Studies on the Reverse Transcriptase and RNase H Activities from Human Immunodeficiency Virus Strains Resistant to 3'-Azido-3'-deoxythymidine*

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A series of biochemical investigations to compare the DNA polymerase and RNase H functions of the reverse transcriptases (RTs) corresponding to azidothymidine (AZT)-sensitive and -resistant human immunodeficiency virus (HIV) strains are described. Steady-state kinetic studies with purified recombinant enzymes utilizing several templates and three inhibitors, 3'-azido-3'-deoxythymidine triphosphate (AZTTP), 3'-amino-3'-deoxythymidine 5'-triphosphate, and 2',3'-didehydro-2',3'-dideoxythymidine 5'-triphosphate, found consistent 2–4-fold differences between the enzymes from the two strains over a wide pH range. A strong pH dependence for all three inhibitors was found at pH values below 7.4 and suggested an ionizable group on the enzyme with a pK of about 7. The sensitivities of the RNase H activities of the two enzymes to AZTTP and AZTMP were also compared and found to be similar. The nucleotide incorporation fidelities of recombinant RTs corresponding to AZT-sensitive and -resistant clinical isolates were compared and the error specificities determined. No significant differences were found. Both enzymes were equally able to incorporate AZTTP into an elongating M13 DNA strand with concomitant chain termination. Purified wild-type and mutant virions from cell-culture supernatants were compared in "endogenous" DNA synthesis reactions, and the sensitivities of this activity to AZTTP were found to be similar. The contrast between the small differences found in this study and the high level of viral resistance in tissue culture presumably reflects an incomplete understanding of AZT inhibition of HIV in the cell.

The nucleoside analogue 3'-azido-3'-deoxythymidine (zidovudine, AZT, Retrovir) is effective against HIV in vitro and has been shown to extend life expectancy and reduce the incidence of opportunistic infections in patients with AIDS or AIDS-related complex (1, 2). However, after prolonged therapy, HIV isolates from such patients are frequently resistant to AZT in cell culture (3). Partial resistance has also been seen in isolates from asymptomatic individuals following prolonged AZT therapy. So far, no highly resistant isolates have been recovered from patients other than those in late-stage disease (4). The decrease in AZT sensitivity has been shown to correlate with the appearance of multiple nucleotide substitutions in the reverse transcriptase genes of these resistant isolates (5). The construction of infectious molecular clones containing combinations of the four changes commonly seen in resistant strains: Asp-67 to Asn, Lys-70 to Arg, Thr-219 to Phe or Tyr, and Lys-219 to Glu, and measurement of the AZT sensitivity of the recovered virus following transfection of T cells has proved that they are responsible for resistance (5, 6). More recently, a fifth mutation, Met-41 to Leu has been identified by sequence comparison and proven to contribute to AZT resistance (7).

The contribution of each of the changes to AZT resistance has been determined. Clones with single substitutions at codons 67 or 219 showed no decrease in sensitivity, while those with single changes at 70 or 215 were partially resistant. When present in combination with other changes the 67 and 219 mutations increased resistance (5, 6). The recombinant strain, HIV-RTC, with all four changes had an ID50 in a molecular species of the HIV genome in infected MT4 cells that was as sensitive to inhibition by 3'-azido-3'-deoxythymidine triphosphate (AZTTP) as that from sensitive isolates (3). Mutants of feline immunodeficiency virus resistant to AZT have recently been reported (8). These strains, isolated from tissue culture, showed up to a 50-fold decrease in AZT sensitivity. When the RTs were purified from virions and tested for sensitivity to AZTTP, no significant differences in Km values for dTTP and K values for AZTTP were found between the wild-type and mutant enzymes. The nature of

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‡The abbreviations used are: AZT, 3'-azido-3'-deoxythymidine (zidovudine,AZT,Retrovir); AZTTP, 3'-amino-3'-deoxythymidine triphosphate; HIV, human immunodeficiency virus; AIDS, acquired immunodeficiency syndrome; RT, reverse transcriptase; AZTMP, 3'-azido-3'-deoxythymidine 5'-monophosphate; HPLC, high-performance liquid chromatography; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NH4TTP, 3'-amino-3'-deoxythymidine triphosphate; dTTP, 2',3'-didehydro-2',3'-dideoxythymidine triphosphate; kb, kilobase(s).

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the mechanism by which the changes in the RT allow the resistant HIV strains to evade the action of AZT is clearly an important question. In the present work we describe a series of biochemical investigations intended to address this problem.

**EXPERIMENTAL PROCEDURES**

**Materials**—AZTTP and AZTMP were supplied by Wayne Miller of Burroughs Wellcome Co. (Research Triangle Park, NC). 3-Aminothymidine 5’-triphosphate and 2’,3’-dideoxy-2’,3’-dideoxythymidine 5’-triphosphate were synthesized from AZTTP by reduction with dithiothreitol as described (9) and purified by HPLC. Ultrapure deoxynucleoside 5’-triphosphates, poly(rA)1000, and oligo(dT)16 were purchased from Pharmacia Molecular Biology Products. The 44- and 21-mer oligonucleotides were prepared by Midland Certified Reagent Company (Midland, TX) (10). Preparation and annealing of the DNA and RNA templates for the kinetic studies has been described previously (10). The Escherichia coli strains, bacteriophage M13 mp2 and other materials required for the fidelity assay, have been described (11, 12).

**Viruses**—The virus strains HXB-2 (AZT-sensitive) and HIVRTMC (RTM-C) were obtained on a 30-ml 5-40% Nycodenz gradient in TNE and layered onto a 30-ml 5-40% Nycodenz gradient in TNE and centrifuged at 160 min at 4°C. The virus pellet was resuspended in 1 ml of TNE and used for infection at a multiplicity of infection of 1 MOI.

**Reverse Transcriptase Assays**—Reaction mixtures for steady-state kinetic studies on recombinant reverse transcriptase contained 5 mM Tris-HCl, pH 7.72 (37°C), 5 mM MgCl2, 0.025% Triton X-100 in a total volume of 100 μl of poly(rA)-poly(dT)24 as template-primer at 2 μM and 50 mM KCl. The reaction mixtures were incubated at 37°C and initiated by the addition of enzyme. Five 15-μl samples were removed during the course of the assay and spotted onto DE81 paper. The paper was washed with 125 mM Na2HPO4, water, washed briefly with ethanol, dried, and counted.

**RNase H Hydrolysis/Primer Extension Assays**—These were done essentially as described previously (12). Briefly, a 211-nucleotide single-stranded RNA template was prepared by SP6 polymerase in vitro transcription of PvuI-linearized pGEM3zf. The RNA was purified by agarose gel electrophoresis and electroelution of the RNA from an excised gel slice. The RNA was dephosphorylated using calf intestinal alkaline phosphatase and then radiolabeled with [α-32P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase. The radioabeled RNA was annealed with a 5% polyacrylamide gel spotted from sia-urea gel and annealed to a 40-nucleotide DNA primer complementary to the RNA template from the 5’th through the 96th nucleotide. The enzyme assays contained 12 nm HIV RT, 0.15 μg/ml bovine serum albumin, 2 units/μl RNasin, the nucleoside triphosphates (when present, these were at 1 μM and the ddNTPs at 45 μM), 70 mM Tris-HCl (pH 8.8), 0.1% Triton X-100, 50 mM MgCl2, 2 mM dithiothreitol, and 5 mM MgCl2 in a total volume of 5 μl. The reactions, which were performed at 37°C, were initiated by the addition of the template-primer to a mixture of enzyme, bovine serum albumin, and RNasin with or without the nucleotides. The reactions were quenched at 20 min by addition of 5 μl of Maxim-Gilbert loading dye (0.1% bromophenol blue and xylene cyanol, 50 mM Tris-Clate (pH 8.3), 1 mM EDTA at 80% formamide). The quenched reaction mixtures were heated to 90°C for 2 min and then cooled on ice and electrophoresed in a 7% acrylamide/8% urea gel at 85 constant watts until the bromophenol blue eluted from the gel. The gel was exposed to film at −70°C overnight with intensifying screens. The sensitivity of RNase H to AZTTP and AZTMP was also assessed as described by Tan et al. (18) using the homopolymeric template-primer, [3H]-poly(rA)-poly(dT)16.

**Fidelity Assay**—The mutational target for polymerase errors was the 258-nucleotide lacZ α-complementation sequence present as a single-stranded DNA or as a double-stranded M13 mp2 DNA. The DNA from error-free gap-filling transcription, when transfected into competent host cells, yields blue M13 plaques. Errors introduced by the reverse transcriptase that result in a decrease in α-complementation of β-galactosidase activity were selected by growth in liquid cultures containing 5-bromo-4-chloro-3-indolyl β-D-galactoside and isopropyl-1-thio-β-D-galactopyranoside. This assay detects 221 different single-base substitution errors at 114 template positions, 150 single-base frameshift errors, and a variety of more complex errors.

Reaction mixtures (50 μl) contained 20 mM Hepes (pH 7.8), 2 μM dithiothreitol, 10 mM MgCl2, 300 ng of gapped M13 mp2 DNA, each of the four dNTPs at 1 mM, and 1.2 μg of A012#2 RT or 2.4 μg of A012#25 RT. Incubation was for 1 h at 37°C, and reactions were terminated by adding EDTA to a final concentration of 15 mM. Aliquots were analyzed by gel electrophoresis as described (11) to ensure complete gap filling. All polymerase reactions reported here generated products that migrated coincident with the fully double-stranded, replicative form II DNA. Preparation of DNA substrates, transfections, and plating of competent cells and RNA sequence analysis of mutants were performed as described (11, 12).

**Preparation of Virions for Endogenous DNA Synthesis Assays**—MT2 cells were infected using HXB-2 and HIVRTMC virus stocks generated by transfection of MT2 cells with molecular clones. At a stage of advanced cytopathic effect, 5 × 106 cells were sedimented at 3000 rpm for 15 min. The cell pellet was resuspended in 1 ml of TNE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA). The cell debris was sedimented at 3000 rpm for 15 min at 4°C. The supernatant was removed to a new tube, and the virus was pelleted by centrifugation at 20,000 rpm in a Sorvall SS34 rotor for 180 min at 4°C. The virus pellet was resuspended in 1 ml of TNE and centrifuged for 180 min at 20,000 rpm in a SS34 Sorvall rotor at 4°C. The gradient was unloaded into fractions which were assayed for RT activity in a standard poly(rA)-oligo(dT) template-exogenous assay. The peak fractions were pooled and the purified virions pelleted by centrifugation at 50,000 rpm in a Beckman TL100 centrifuge for 15 min and resuspended in 1 ml of TNE.

**Endogenous DNA Synthesis Reactions**—Reactions (100 μl) contained 50 mM Tris-HCl (pH 8.0), 50 mM NaCl, 10 mM MgCl2, 15 mM dithiothreitol, 1 mM each dCTP, dATP, and dGTP, 5 μCi of [α-32P]dATP (3000 Ci/mmol), 0.05% Triton X-100, 20 μl of virion preparation, and varying concentrations of AZTTP. The reactions were incubated at 41°C for 14 h, boiled, and extracted with phenol/chloroform. The reaction products were electrophoresed on 1% alkaline agarose gels and visualized by autoradiography.

**Purification of HIV Reverse Transcriptase**—The HXB-2, HIVRTMC, A012#2, and A012#25 reverse transcriptases produced in E. coli were purified by means of immunofinity chromatography as M, 66,000/51,000 heterodimers (A012#2, A012#25) or as M, 66,000 homodimer (HXB-2, HIVRTMC-C) (15, 16). The RTMC enzyme contains four amino acid substitutions relative to the HXB-2 enzyme (5). The #2 and #25 designations, respectively, denote DNA clones from the clinical isolates A012D (AZT-resistant) and A012B (sensitive). The RTM-C enzyme contains four amino acid substitutions relative to the HXB-2 enzyme (5). The A012D enzyme contains four changes of RTM-C and A012B (sensitive). The A012D enzyme contains four changes of RTM-C and A012B (sensitive).

**Kinetic Studies on Recombinant Reverse Transcriptases**—The HXB-2 and RTMC RTs, obtained by expression in E. coli and purified as homodimers of 66-kDa subunits, were studied in a series of DNA polymerase assays employing each of three synthetic template-primers: poly(rA)-oligo(dT)16, r44d21-mer, and d44d21-mer. The Michaelis constants for dTTP were determined for the two enzymes and found to be similar, independent of the template-primer used. The ability of AZTTP to inhibit the polymerization of dTTP in each
of the template-primers by each enzyme was measured. The
$K_i$ values of the two enzymes for AZTTP with all three
templates differed by no more than 2-fold, although interest-
ingly, RTMC consistently had the higher value (Table I). The
reverse experiment; the ability of dTTP to inhibit the incor-
poration of $[^3H]AZTMP$ was also done with a similar result
(data not shown).

One possibility considered was that a metabolite of AZT
other than AZTTP was important in inhibition of the viral
RT in vivo and that the resistance of the RTMC strain was
due to an alteration in the kinetic properties of the RT with
respect to this metabolite. It has been shown that AZT can be
reduced to 3' -aminothymidine by thiols such as glutathione,
dithiobitol, or mercaptoethanol under physiologically
relevant conditions (9). The treatment of AZTTP with dithi-
othreitol yields 3' -aminothymidine 5'-triphosphate and 2',3'-
didehydro-2',3'-dideoxythymidine 5'-triphosphate.1 The rel-
ative sensitivities of the two enzymes to these two inhibitors
were compared, using poly(rA)-oligo(dT)$_{20}$ as the substrate.
Both were potent inhibitors of both enzymes with $K_i$
values of the same order as that of AZTTP. Again, it was found that
RTM-C had a slightly higher $K_i$ value for both inhibitors, the
largest difference, 4-fold, being seen with NH$_2$TTP. Table I
summarizes all of the kinetic data presented so far.

Since three of the four amino acid substitutions in RTM-C
confering AZT resistance involve a charged group (Asp-67 to
Asn, Lys-70 to Arg, and Lys-219 to Gln), it is conceivable
that an alteration in local charge might be important in
resistance, and that this effect is manifested only under
relevant conditions of pH. Most of the kinetic studies in the
literature have been conducted at pH values in excess of 7.6.
We therefore examined the variation in $K_i$ of AZTTP and
NH$_2$TTP for both enzymes as a function of pH over the range
5.8-8.7 using poly(rA)-oligo(dT)$_{20}$ as the template. Fig. 1
shows a plot of $K_i$ versus pH for the RTMC and HXB-2
enzymes. The $K_i$ of both enzymes for AZTTP was found to
be highly sensitive to pH below the pH of 7.4, while the $K_i$
for dTTP was not greatly changed (data not shown). This pH
dependence was also seen for the inhibitors NH$_2$TTP and
d$_4$TTP and can be fit to an ionizable group on the enzyme
with a pK of approximately 7. A second ionizable group with a
pK of >8.5 may also be involved, but this cannot be
confirmed with the present data. Both the enzymes showed a
similar variation in kinetic properties with pH, indicating

\[ \text{Table I} \]

| Substrate    | Inhibitor  | HXB-2  | RTMC  |
|--------------|------------|--------|-------|
| Poly(rA)-oligo(dT)$_{20}$ dTTP | AZTTP     | $1.6 \pm 0.1$ | $3.3 \pm 0.3$ |
|             | AZTTP     | $0.59 \pm 0.05$ | $0.41 \pm 0.09$ |
| dTTP         | NH$_2$TTP  | $0.010 \pm 0.001$ | $0.046 \pm 0.008$ |
|             | d$_4$TTP   | $0.0020 \pm 0.0002$ | $0.0047 \pm 0.0006$ |
| r$_{4}$d$_{21}$-mer dTTP | AZTTP     | $0.051 \pm 0.006$ | $0.043 \pm 0.005$ |
|             | AZTTP     | $0.056 \pm 0.004$ | $0.064 \pm 0.006$ |
|             | AZTTP     | $0.059 \pm 0.008$ | $0.071 \pm 0.009$ |
| d$_{44}$d$_{21}$-mer dTTP | AZTTP     | $2.1 \pm 0.3$ | $4.8 \pm 0.6$ |

\[ \text{Fig. 1. Plot of pK, of AZTTP for HXB-2 (open circles) and}
\text{RTMC (filled circles) reverse transcriptases against pH. Two}
\text{buffers were used: Bis-Tris-HCl for pH 6.35-7.15; Tris-HCl for pH 6.88-8.63.} \]

that the charged groups affected by the mutations conferring
AZT resistance do not correspond with those affecting $K_i$ as
a function of pH.

Another possibility considered was that the mutations
might influence the binding of AZTMP. AZTMP is a very
weak inhibitor of HIV RT but may compete with the triphos-
phate for the binding site on the enzyme and has been
reported to be able to accumulate in cells to almost millimolar
levels (18). It is conceivable that, should the mutant enzyme
have an increased affinity for the monophosphate, this might
reduce the potency of AZTMP inhibition. The inhibitory effect
of AZTP on wild-type and mutant enzymes was examined in
the presence of various levels of AZTMP. Very high con-
centrations (>1 mM) of AZTMP had a weak additive effect
with AZTTP, but no significant differences were seen between
the two enzymes (Fig. 2).

RNase H Activity of Recombinant RTs—The purified recom-
binant HXB-2 and RTMC reverse transcriptases used in
the above kinetic studies were also compared in a series of
RNase H hydrolysis/primer extension assays (data not pre-
icted). The cleavage/extension patterns seen were qualita-
tively similar to those seen with other purified HIV RTs in
previous studies (13). The RNase H activities of the two
enzymes were equally sensitive to AZTTP. Furthermore, no
difference in the inhibition of the enzymes by AZTMP was observed (17).

Fidelity of A0122#2 and A0122#25 RTs in Forward Mutation
This paper describes a series of studies designed to elucidate a central problem in HIV resistance to azidothymidine; the biochemical mechanism by which multiple amino acid substitutions in the reverse transcriptase gene can enable the virus to replicate in the presence of AZT. The construction of molecular clones with defined mutations and recovery of the virus (5).

Endogenous Synthesis Assays—The similarity of the purified wild-type and mutant RTs in kinetic studies may imply that a difference in AZTTP sensitivities of the polymerase activities may be mediated by factors relating to the environment in which RT exists in the virion DNA synthesis complex. To address this possibility the DNA polymerase activities associated with virions of the HXB-2 and HIVRTMC strains were examined in endogenous synthesis reactions employing [α-32P]dTTP as the label and Triton X-100 as the permeabilizing agent. Fig. 4 shows the products of these reactions in the presence of a range of AZTTP concentrations. In the absence of inhibitor a smear representing a range of products from <100 to approximately 9 kb was seen. No discrete bands representing full-length proviral DNA or replication intermediates were seen. When AZTTP was present at a concentration of 0.05 micromolar, formation of this product was almost completely inhibited. The sensitivities of virus (5).

DISCUSSION

AZTTP Incorporation/Chain Termination Assay—We compared the A012#2 and A012#25 enzymes in an assay which provided a visual measure of the ability of reverse transcriptases corresponding to AZT-sensitive and -resistant HIV clinical isolates to incorporate AZTTP into an elongating M13 DNA strand with concomitant chain termination. As shown in Fig. 3, AZTTP was well able to substitute for dDTTP in the reaction. Both enzymes behaved similarly in this assay. It should be noted that in a previous study we observed a 3-fold increase in K_i for AZTTP with RT A012#2 from mutant virus (5).

To check this possibility two recombinant RTs purified from E. coli and corresponding to two clinical isolates from the same patient were tested in a forward mutation assay.

Reactions catalyzed by the A012#2 (mutant) and A012#25 (wild-type) reverse transcriptases produced α-complementation mutants during DNA-dependent DNA synthesis with a similar frequency of 4 and 3.2%, respectively (Table II). These values were approximately 50-fold higher than the background mutant frequency of uncopied DNA and are similar to the mutant frequencies reported for natural and recombinant preparations of wild-type HIV-1 RT (19). To determine the error specificities of the two enzymes, 50 randomly chosen, independent mutants produced by each reverse transcriptase were analyzed by DNA sequence analysis. The calculated error rates per detectable nucleotide polymerized are summarized in Table III for the various classes of mutations.

Single-base substitution mutations were the predominant class of polymerase-mediated errors in both spectra. The base substitution error rates were 1/2700 and 1/4000, respectively, for the A012#2 and A012#25 RTs (Table III). A mutational "hotspot" for T to C transition errors occurred at position -36 in both collections; the calculated error rate per nucleotide for this specific change was 1/54 for the A012#2 enzyme and 1/110 for the A012#25 enzyme.

In addition, both enzymes produced a substantial number of single base frameshift mutations during DNA-dependent DNA synthesis in vitro. The frequency of single-base frameshift errors was approximately 1/10,000 for the A012#2 enzyme, while the A012#25 polymerase produced frameshifs at a slightly higher rate of 1/8,600. For both polymerases the majority of frameshift errors occurred within reiterated DNA sequences (data not shown).

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DISCUSSION

This paper describes a series of studies designed to elucidate a central problem in HIV resistance to azidothymidine; the biochemical mechanism by which multiple amino acid substitutions in the reverse transcriptase gene can enable the virus to replicate in the presence of AZT. The construction of molecular clones with defined mutations and recovery of resistant virus following transfection of T cells with the DNA (5) has not only eliminated the possibility of changes in other genes being involved in AZT resistance but has allowed work with clonal resistant virus stocks compared to previous studies employing heterogenous clinical isolates (19). Studies on the RT activities associated with these clinically derived viruses may be complicated not only by background mutations in the RT gene but also by changes in other virus genes. In the in vitro AZTTP incorporation/chain termination experiment described here the purified, recombinant RTs corresponding
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Fig. 3. Autoradiograph of a 6% polyacrylamide DNA sequencing gel. Chain termination reactions were performed as described under “Experimental Procedures” with HIVRT A012#2 (R) or A012#25 (S) as indicated. dTTP lanes contained 1 μM dTTP. AZTTP lanes contained 1 μM, 0.1 μM, or 0.01 μM AZTTP, respectively. The size markers on the right of the figure are in base pairs.

to AZT-sensitive and -resistant clinical isolates showed no significant difference in their ability to incorporate AZTTP into and terminate an elongating M13 DNA chain (Fig. 3). The apparent paradox may be due to the in vitro experiment not adequately reflecting the in vivo situation due to the differences in the template or the absence of an accessory factor(s) or set of reaction conditions.

Similarly, no large difference was found between purified recombinant HXB-2 and RTMC RT 66K homodimers in detailed kinetics on DNA primed RNA and DNA templates. This agrees with our previous results on virion-associated RT activities (5), results from other workers on HIV RT (20), and on feline immunodeficiency virus RT (9). However, there was a slight elevation in the Ki values for the mutant enzyme with most of the inhibitor/template combinations investigated, the largest difference (4.6-fold) relative to the wild-type enzyme being found when NH2TTP was used to inhibit dTTP incorporation into poly(rA)-oligo(dT)20. We also found no major differences in the specificities or rates of the RNase H activities of the recombinant RTs in the presence or absence of AZTTP or AZTMP. In any case, the location of the mutations in the polymerase domain of the enzyme makes it unlikely that AZT resistance is mediated by an alteration in the RNase H activity which is localized to a different domain (21).

Our observations reported here demonstrate that the fidelities of the A012#2 and A012#25 reverse transcriptases corresponding to an AZT-sensitive and -resistant clinical isolate are not significantly different from each other. This suggests that the amino acid substitutions present in the RT derived from the AZT-resistant clone do not strongly influence fidelity during DNA-dependent DNA synthesis in vitro. The overall accuracy of both forms of RT was very similar to that observed in an earlier study using a different HIV RT isolate (19). Furthermore, in the error spectra for the A012#2 and A012#25 enzymes presented in Fig. 2, both base substitution and frameshift errors were clustered at several mutational hotspots which are characteristic of those observed before with HIV RT (22).

The specificity and position of these mutations suggest that the majority of errors produced by the HIV-1 RT during in vitro DNA synthesis are initiated by template-primer misalignments. Most frameshifts occur at iterated template nucleotides, where, as proposed by Streisinger (23), template-primer slipping may allow the formation of a misaligned frameshift intermediate. A common feature of the base substitution hotspots is that they occur at the end of homopolymeric runs. For example, the T to C transitions at positions -36 and the G to A transitions seen at position 90 are consistent with a mechanism whereby the base substitution is templated by an adjacent base via a transient misalignment mechanism (24).

Finally, the endogenous DNA synthesis assay with purified HIV virions indicated that the reverse transcriptase activity on the endogenous template/primer was sensitive to AZTTP for both wild-type and AZT-resistant virus. It is possible that the conditions used for this endogenous assay did not adequately reflect the in vivo reaction as full-length DNA synthesis was not observed. On the other hand, the AZTTP sensitivities of the RT activities in this assay agreed well with
the data from the studies on the purified recombinant enzymes described in this work.

The resolution of the paradox described in this paper may lie in a better understanding of the role of HIV reverse transcriptase within the infected cell. It may be that one or more cellular and/or viral factors interact with the HIV replicative complex and influence differential sensitivity to AZTTP due to the resistance-conferring mutations. It may also be that the nature of the RT-catalyzed DNA synthesis reaction varies according to the intracellular compartment in which it occurs. Another possibility is that the consistent small differences we have observed between the wild-type and the mutant enzymes are sufficient to cause the observed resistance phenotype. A small change in the rate of incorporation of AZTMP into virus DNA might be amplified in vivo to allow escape of mutant virus from inhibition by AZT. We are currently engaged in studies to investigate these hypotheses.

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