Fidelity of Mutant HIV-1 Reverse Transcriptases: Interaction with the Single-Stranded Template Influences the Accuracy of DNA Synthesis†

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Received October 28, 1997; Revised Manuscript Received February 17, 1998

ABSTRACT: We have used random sequence mutagenesis and complementation in a bacterial selection system to establish a large library of immunodeficiency virus type 1 (HIV-1) reverse transcriptase (RT) mutants with amino acid substitutions in the $\beta3-\beta4$ region of the fingers subdomain [Kim, B., Hathaway, T. R., and Loeb, L. A. (1996) *J. Biol. Chem. 271*, 4872−4878]. We show here that one of these mutants, D76V, exhibits increased accuracy of copying both DNA and RNA templates in a primer extension assay with biased dNTP pools. More detailed analysis of DNA-dependent polymerization showed that the D76V mutation conferred an up to 14-fold increase in fidelity of nucleotide insertion and a 9-fold reduced mutation rate in an M13mp2 *lac*Zα forward mutation assay. Substitution at D76 with positively charged (D76R) and nonpolar (D76V and D76I) residues increased replicational accuracy, while substitutions with negatively charged (D76E) and polar residues (D76S and D76C) had little effect on fidelity. We propose that D76 affects replicational accuracy by mediating interaction between the fingers subdomain and the single-stranded template. Our work shows that the *Escherichia coli* complementation system can yield HIV RT mutants with increased fidelity that have not been isolated from the natural host and that are valuable in understanding the molecular bases of replicational accuracy.

Human immunodeficiency virus type 1 reverse transcriptase (HIV RT)[1] is the most error-prone of known DNA polymerases (*1*−*3*). Mutagenic replication by HIV RT is a putative source of the genetic hypervariability which permits the virus to exist as a quasi-species (*4*) and to escape from selective pressures such as host immune responses and antiviral therapies (*5*).

Analysis of mutants is a cornerstone of our current understanding of the mechanisms of replicational accuracy of DNA polymerases (*6*). So far, only two mutant HIV RTs have been shown to exhibit increased fidelity: M184V (*7*−*9*) and E89G (*10*, *11*). M184V resides in the YMDD motif in the conserved catalytic site where aspartate residues D185 and D186 interact with metal ions chelated with the phosphate groups of incoming dNTPs (*6*). It has been suggested that altered interactions with incoming dNTPs reduce the ability of the M184V mutant to incorporate dNTP analogues (*10*), as well as noncomplementary dNTPs (*8*). The mutant E89G mediates resistance to 3TC [(−)-2′,3′-dideoxy-3′-thiacytidine] and several other nucleoside analogues (*12*). It has been proposed that the E89G substitution affects both fidelity and drug resistance by altering interactions with the penultimate nucleotide on the double-stranded region of the template (*10*, *11*).

Structural models have pointed out interactions of specific domains of HIV RT in various steps of the DNA polymerization reaction, such as template−primer binding, dNTP binding, and catalysis (*13*−*16*). Several regions of the p66 monomer have been suggested to interact with DNA or RNA templates along a cleft in the right-hand model. Among these regions is the $\beta3-\beta4$ fingers domain, which has been proposed to directly interact with the single-stranded part of the template (*10*, *17*). Genetic and biochemical evidence indicates that the $\beta3-\beta4$ region plays critical roles in resistance of HIV RT to nucleoside inhibitors. Most of the mutations isolated from HIV-infected individuals that confer resistance to nucleoside analogues are located in this domain. Presumably, interactions between the $\beta3-\beta4$ region and the template affect the ability of HIV RT to discriminate nucleoside analogue drugs from natural dNTP substrates without compromising catalytic activities essential for viral replication (*10*).

We have established a functional complementation system in *Escherichia coli* that permits genetic selection for the DNA-dependent DNA polymerase activity of HIV RT (*18*). In this system, HIV RT allows growth of an *E. coli* mutant harboring temperature-sensitive DNA polymerase I (Pol I^{ts}). By using genetic selection in bacteria, together with random mutagenesis targeting the $\beta3-\beta4$ region, we obtained a large number of active mutants, many of which have not been identified in HIV populations from human cells (*19*). We have screened some of these mutants and determined that D76V, a mutant with wild-type specific activity, exhibited

† This work was supported by grants from the American Foundation for AIDS Research to B.K. (02502-21-RG). The graphic modeling was made possible by UW Center Grant P30 ES07033 from NIEHS.

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[1] Abbreviations: BSA, bovine serum albumin; dNTP, deoxynucleoside triphosphate; EDTA, ethylenediaminetetraacetic acid; HIV, human immunodeficiency virus; MuLV, Moloney murine leukemia virus; Tris-HCl, tris(hydroxymethyl)aminomethane; RT, reverse transcriptase.

S0006-2960(97)02672-X CCC: $15.00
Published on Web 04/09/1998

elevated replicational accuracy. This mutant has not been reported among clinical isolates. Our work shows that mutants selected in *E. coli* can have increased replicational accuracy and concomitant utility in exploring underlying mechanisms.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* NM522 (Stratagene) was used for construction of plasmids and BL21 (Novagen) for overexpression of HIV RT. pBK33 is pHis/*Nde*I (from A. Hizi) encoding full-length wild-type p66 HIV-1 RT, fused at the N terminus to six histidine residues. pBK44 is a pET28a (Novagen) derivative expressing wild-type p51 HIV-1 RT fused at the N terminus to six histidine residues (*19*). To construct pBK33, DNA encoding p66 was PCR-amplified from pHIVRT (*19*) by using the primer SD-RT (5′-GAAGATCTAAGCTTAGGAGGTTGTCCCATATGCC-CATTAGTCC-3′) that hybridizes to the N-terminal sequence of HIV RT and the *Nde*I site and the 3′ primer HSG (5′-TAACGCCAGGGTTTTCCCAG-3′) that hybridizes to a sequence downstream of the mutiple cloning site. Amplified DNA was cloned into pHis/*Nde*I between the *Nde*I and *Sal*I sites. To construct pBK44, DNA encoding p51 was PCR-amplified from DNA encoding p66 by using the SD-RT primer and a p51 primer (5′-TTTTTTGAATTCGTC-GACTTTTAGAAAGTTTCTGCTCC-3′) that hybridizes to the sequence encoding amino acids 436−440 and the *Sal*I site and a termination codon at position 441. Amplified DNA was cloned to pET28a between the *Nde*I and *Sal*I sites. The entire wild-type HIV RT sequences in pBK33 and pBK44 were confirmed by comparing with the original plasmid, pJS-RT (J. B. Sweasy). To construct plasmids expressing mutant p66 and p51 proteins, DNAs were amplified from pHIV RT derivatives containing the indicated mutations.

Construction of D76 Mutants. Amino acid substitutions at D76 were generated by using PCR-based site-directed mutagenesis with D76 primers (5′-CTTATTGAGCTCTCT-GAANNNTACTAATTTTCTCCATTTAG-3′) that contained 100% random nucleotides (designated "N") at the positions encoding residue 76. The products of PCR amplification with primers D76 and SD-RT were inserted into pBK33 after digestion with *Bsr*GII and *Sac*I. D76 mutations were introduced into pBK44 as described above. The 126 bp region between *Bsr*GI and *Sac*I of the plasmids expressing D76 mutants was sequenced to confirm mutatuions at position 76.

Purification of p66/p51 Heterodimers. Procedures for purification of the hexahistidine-tagged p66 and p51 monomers by Ni^{2+} chelation chromatography have been described; equimolar amounts of separately purified p66 and p51 were mixed prior to dialysis to form p66/p51 heterodimers (*19*, *20*). In the case of mutant HIV RT heterodimers, the same mutations were present in both subunits. Reconstructed p66/p51 heterodimers of wild-type and D76 mutant proteins were used for the fidelity assays described below. Amounts of purified monomers were estimated in the Bradford assay (Biorad) with a bovine serum albumin standard; all preparations were of >95% purity, estimated by visual inspection of Coomassie Blue-stained polyacrylamide gels. DNA- and RNA- dependent DNA polymerase activities were determined as described (*19*); the assays employed a gapped salmon sperm DNA template−primer, poly(rA)/oligo(dT) (Pharmacia), or an RNA template encoding the HIV RT gene. Preparation of the RNA template is described in the next paragraph. The p66/p51 heterodimers of HIV proteins used in this study contain the His tag at the N termini. We previously reported that the presence of a His tag in purified HIV RT did not significantly alter the DNA polymerase activity or sensitivity to AZT (*19*). We directly compared the fidelity of wild-type HIV RT with and without the His tag. HIV RT proteins (p66 and p51) overexpressed and purified from pET28a constructs were treated with thrombin as described (Novagen). Using the misinsertion assay, both HIV RT proteins with and without the His tag showed the same level of misinsertion in four different types of primer extension reactions with biased dNTP pools as described in Figure 1.

Primer Extension Assay with Deoxyoligonucleotide or RNA Templates. Procedures were modified from those of Preston et al. (*1*). The deoxyoligonucleotide template−primer was prepared by annealing a 63-mer (5′-TAATAC-GACTCACTATAGGGAGGAAGCTTGGCTGCAGAATAT-TGCTAGCGGGAATTCGGCGCG-3′) to a 14-mer (5′-CGCGCCGAATTCCC3′) ^{32}P-labeled at the 5′ end with T4 polynucleotide kinase (2.5:1 template−primer). Assay mixtures (20 μL) contained 100 nM template−primer, 5−50 nM RT as specified in the figure legends, three or four dNTPs (250 μM each), 25 mM Tris-HCl (pH 8.0), 40 mM KCl, 2 mM DTT, 5 mM $MgCl_2$, and 0.1 mg/mL bovine serum albumin. Reaction mixtures were incubated at 37 °C for 5 min and reactions terminated by addition of 5 μL of 40 mM EDTA and 90% formamide. Reaction products were denatured by incubating at 95 °C for 5 min and analyzed by electrophoresis in 14% polyacrylamide−urea gels. The 1600-nucleotide RNA template used in this assay contains the coding sequence of the HIV RT gene and was synthesiszed by T7 RNA polymerase-catalyzed transcription of pBK8 (*19*) which encodes the HIV RT gene between the *Hin*dIII and *Eco*RI sites of pBluescript (Stratagene). The transcribed RNA was annealed to a ^{32}P-labeled 21-mer [3305 RT primer (*19*)] complementary to nucleotides encoding amino acids 239−246. Reaction conditions were otherwise the same as described above. Extension products were resolved in 10% denaturing gels.

M13mp2 lacZ Forward Mutation Assay. The mutation frequencies for wild-type and mutant HIV RT were measured essentially as previously described (*21*, *22*). M13mp2 DNA containing a 361-nucleotide single-stranded gap was prepared as specified (*23*). Gapped DNA (1 μg) was incubated with RT (10 nM) or the exonuclease-deficient Klenow fragment of *E. coli* DNA polymerase I (0.1 unit, Amersham Lifescience) at 37 °C for 5 min under the conditions described below for the misinsertion assays, except that the dATP was radiolabeled. Incorporation of the label from [α-^{32}P]dATP was linear with incubation time for up to 20 min. The extent of gap filling was assessed by monitoring production of a 268-nucleotide fragment upon *Pvu*II digestion of the copied DNA (*23*) and was comparable for the three polymerases utilized. The mutation frequency was determined as the ratio of mutant (pale blue or white) plaques to mutant and wild-type (dark blue) plaques as described (*2*, *24*).

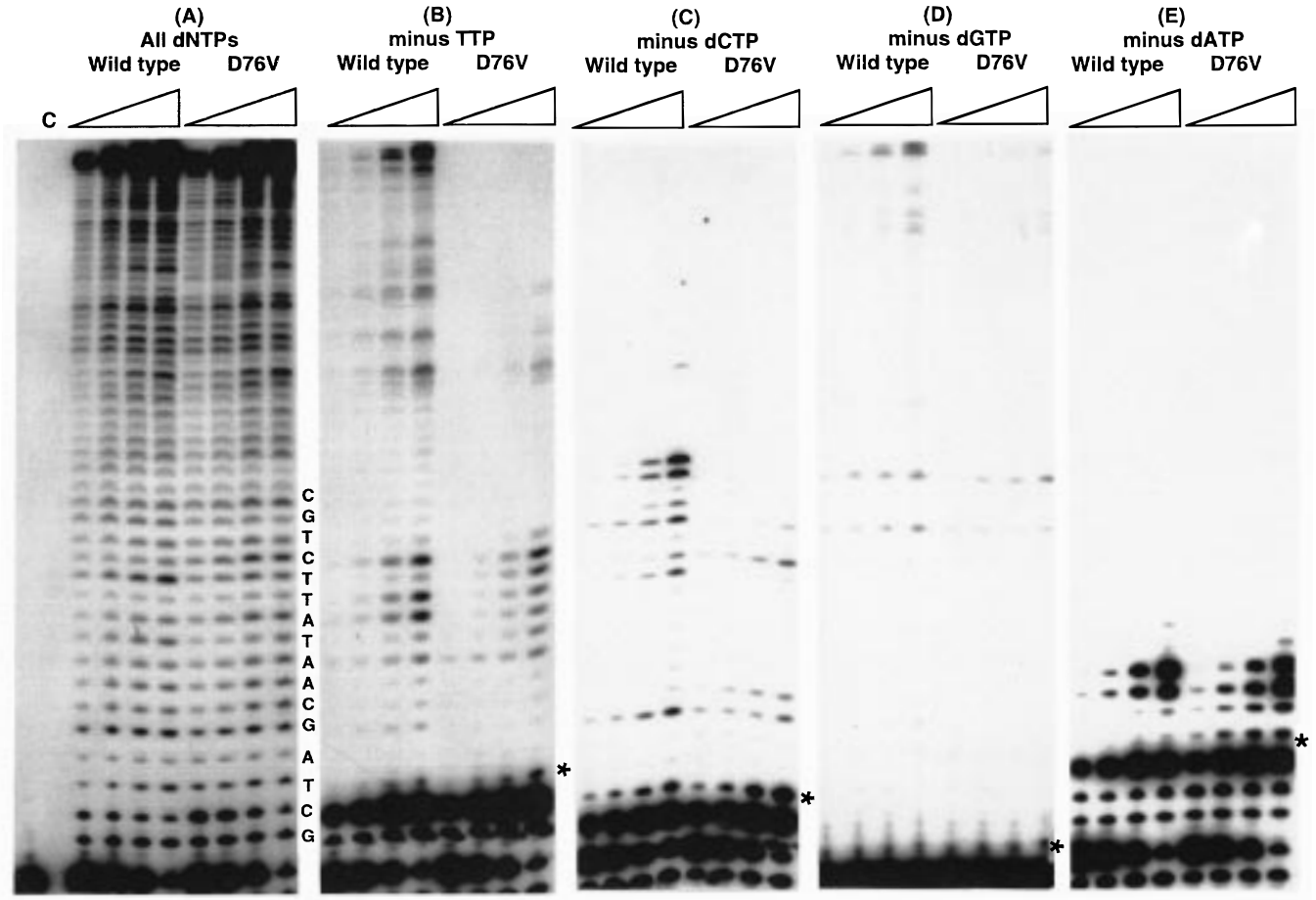

FIGURE 1: Primer extension by wild-type and D76V HIV RTs in assays containing a deoxyoligonucleotide template and lacking a complementary dNTP. A 14-mer primer annealed to a 63-mer deoxynucleotide template was extended in reaction mixtures to which all four dNTPs were added at 250 μM each (A) or reaction mixtures from which TTP (B), dCTP (C), dGTP (D), or dATP (E) was omitted. The first complementary nucleotide to be added to the primer terminus is dGMP. The sequence given is the expected sequence in the newly synthesized strand. Each set of four reactions contained 5, 10, 20, and 40 nM WT RT or 7.5, 15, 25, and 50 nM D76V RT, representing equal amounts of DNA-dependent DNA polymerizing activity on a gapped DNA template (19). The asterisk (*) indicates the unextended mismatched primer produced by misinsertions. The first stop sites of the reactions with only three nucleotides are one nucleotide shorter than the mismatched primers.

Nucleotide Misinsertion Efficiencies. A modification of the steady-state kinetic assay of Boosalis et al. (*27*) was used to determine misinsertion frequencies. Reaction conditions were the same as those described in the primer extension assay except for the primers, the concentrations of dNTPs and RT, and the incubation time. The following four primers, 5′ end-labeled with ^{32}P, were annealed individually to the 63-mer deoxynucleotide template: a 14-mer "G primer" (5′-CGCGCCGAATTCCC-3′) for measurement of correct insertion of dGMP, a 15-mer "C primer" (5′-CGCGCCGAATTCCCG-3′), a 16-mer "T primer" (5′-CGCGCCGAATTCCCGC-3′), and a 17-mer "A primer" (5′-CGCGCCGAATTCCCGCT-3′). The rate of product formation was measured for seven different concentrations of each correct and incorrect dNTP. For each dNTP, the amount of RT and the incubation time were adjusted to yield extension of approximately 10−35% of the labeled primers at the highest triphosphate concentration. Products were resolved in 14% polyacrylamide−urea gels and quantitated by phosphor imaging analysis. k_{cat} and K_m values were determined from Hanes−Woolf plots as described (*25*). The misinsertion frequency, f_{ins}, was calculated from the k_{cat} and K_m values as specified by Goodman et al. (*26*, *27*).

RESULTS

Primer Extension Assay with a Deoxynucleotide Template and Biased dNTP Pools. All experiments were carried out with essentially homogeneous p66/p51 heterodimers of wild-type (WT) and mutant HIV RTs. For initial assessment of the fidelity of D76V RT, we employed an assay that monitors primer extension in the presence of only three of four dNTPs complementary to template nucleotides. Elongation of the primer past a template nucleotide complementary to the deleted dNTP requires that the enzyme insert an incorrect nucleotide (misinsertion) and then extend the mismatched primer generated by misinsertion. Figure 1A displays the products of primer extension by increasing concentrations of wild-type or D76V RT in the presence of four dNTPs; equivalent amounts of DNA-dependent DNA polymerizing activity of each RT were utilized, as measured on a gapped DNA template. The amounts of fully extended, 63 bp product increased with increasing enzyme concentrations and were similar for the WT and D76V RTs. The patterns of partially extended products were also alike, though not identical. Panels B−E of Figure 1 show the products formed in the absence of a single dNTP. In every reaction, both WT and D76V elongated the 14-mer primer beyond the first

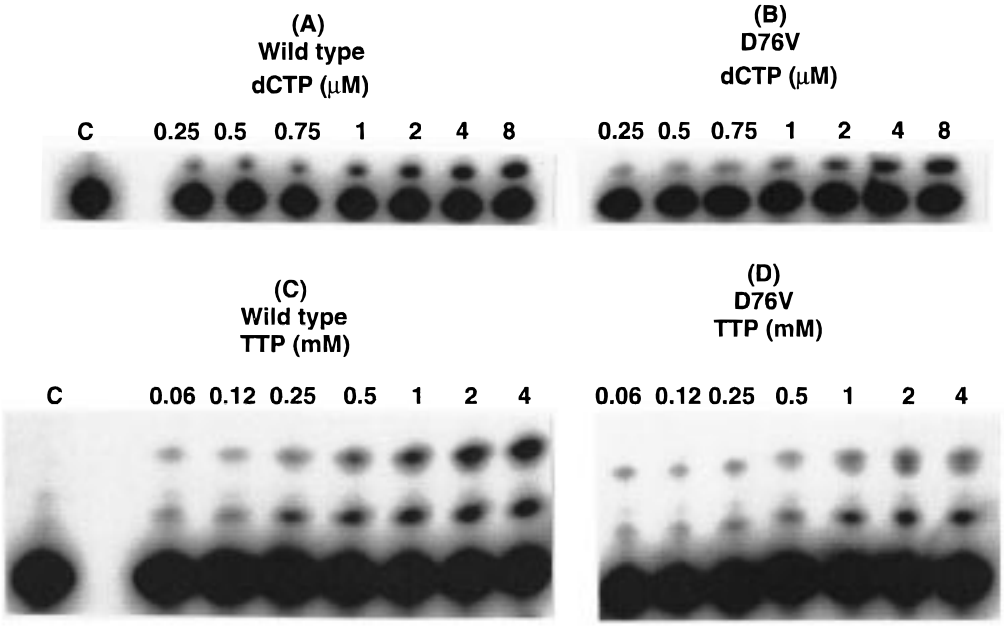

FIGURE 2: Incorporation of correct dCMP and incorrect TMP by wild-type and D76V HIV RTs. A 15-mer primer (C primer) annealed to a 63-mer deoxynucleotide template (75 nM) was extended by wild-type (A, 4 nM; C, 8 nM) or D76V (B, 2 nM; D, 6 nM) HIV RT. Reaction products were resolved by electrophoresis through 14% polyacrylamide−urea gels. The products formed at seven different concentrations of correct dCTP (A and B) and incorrect TTP (C and D) are shown. Lane C contains the unextended 15-mer C primer. Two molecules of TMP are incorporated per primer, the second resulting from extension of the G·T mismatch generated by misinsertion of the first TMP. Incorporation of the only first TMP was used in determining kinetic values for misinsertion (see Table 2, particularly footnote c).

nucleotide for which the correct dNTP was lacking. However, the mutant enzyme did not extend the primer as far and formed smaller amounts of extended products, in reactions lacking TTP (Figure 1B), dCTP (Figure 1C), and dGTP (Figure 1D); phosphor image analysis revealed that 6, 5, and 3 times less primer was elongated beyond the first stop site, respectively. The discrepancy was less marked in reaction mixtures lacking dATP (Figure 1E), where a 1.6-fold difference was observed. Notably, D76V RT left more unextended mismatched primer (see sites marked with an asterisk) directly following the first stop sites in reaction mixtures lacking each of the NTPs than wild-type RT, providing further evidence that D76V has a diminished ability to extend mismatches. Taken together, the data of Figure 1 indicate that the D76V mutation confers elevated replicational accuracy by increasing both misinsertion and misextension fidelities.

M13mp2 lacZ Forward Mutation Assay. To confirm the increased fidelity of DNA synthesis by D76V RT, we employed the M13 *lac*Z forward mutation assay (*2*, *24*). This assay scores errors made during in vitro copying of the *lacZ*α gene encoded within a single-stranded gap in M13mp2 DNA. Error-free polymerization produces DNA that yields dark blue plaques by α-complementation on transfection and plating of an appropriate *E. coli* strain. Errors that reduce or abolish complementation are detected as light blue or white plaques. The mutant frequency is calculated as the ratio of mutant to total plaques. As shown in Table 1, the mutant frequency observed for wild-type HIV RT was 2.3×10^{-2}, similar to that reported previously (*21*, *22*). In contrast, the mutant frequency for D76V RT was 2.6×10^{-3}, about 9 times lower than that for WT RT and comparable to that observed for the exonuclease-deficient Klenow fragment of *E. coli* DNA polymerase I.

Table 1: Accuracy of Wild-Type and D76V HIV RTs in a *lac*Zα Forward Mutation Assay

polymerase	total plaques	mutant plaques	mutant frequency ($\times 10^{-4}$)	-fold increase
wild type	11250	261	232	−
D76V	10020	26	26	8.8
Exo$^-$ KF[a]	8150	31	38	−

[a] The Klenow fragment of *E. coli* DNA polymerase I lacking 3′ → 5′ exonuclease activity.

Misinsertion Fidelity. The primer extension assays illustrated in Figure 1 indicate that the D76V mutation diminishes the ability of HIV RT to carry out the first step of mutation synthesis, i.e., insertion of a noncomplementary nucleotide. To further characterize the decrease in misinsertion efficiency, we utilized primer extension in the presence of a single dNTP to determine K_m and k_{cat} values for incorporation of correct dNTPs and incorrect dNTPs by the wild-type and D76V RTs. The deoxynucleotide template was the 63-mer used in the extension reactions shown in Figure 1, and the four primers were the same 14-mer G primer seen in Figure 1, correctly extended by insertion of dGMP, a 15-mer C primer, correctly extended by insertion of dCMP, a 16-mer T primer, and a 17-mer A primer. Figure 2 shows data for incorporation of correct dCMP and incorrect TMP onto the C primer. Whereas the wild-type (A) and D76V (B) RTs yielded equivalent incorporation of dCMP, the mutant (D) catalyzed less incorporation of TMP than the wild-type enzyme (C) at both the first and second positions downstream of the primer terminus. In reactions with the C primer and TTP, two nucleotides can be incorporated per primer because the second nucleotide is opposite a template dAMP. Only misincorporation of the first nucleotide was

Table 2: Misinsertion Frequencies for Wild-Type and D76V HIV RTs

	k_{cat} (min^{-1})		K_m (μM)		f_{ins} ($\times 10^{-3}$)[a]		
T·P	WT	D76V	WT	D76V	WT	D76V	ratio[b]
A·T	2.0	1.5	5.1	3.6	1000	1000	
A·C	0.62	0.16	1050	2590	1.5	0.15	10
A·G	0.05	0.0224	460	910	0.30	0.06	5
A·A	0.29	0.38	760	1400	1.0	0.6	1.7
G·C	0.14	0.26	1.4	0.9	1000	1000	
G·A	0.11	0.20	990	2520	1.1	0.27	4.1
G·T[c]	1.00	0.29	186	260	54	3.9	14
G·G	0.08	0.11	560	592	1.4	0.60	2.3
T·A	2.2	1.5	1.0	1.3	1000	1000	
T·G	1.9	1.5	320	695	3.0	1.9	1.6
T·C	0.31	0.23	582	1110	0.3	0.18	1.7
T·T	0.06	0.14	770	755	0.04	0.16	0.25
C·G	1.2	1.4	0.55	0.45	1000	1000	
C·A	0.11	0.03	760	840	0.07	0.01	7
C·T	0.06	0.06	830	920	0.03	0.02	1.5
C·C	ND[d]	ND	ND	ND	−	−	−

[a] f_{ins} is the ratio of k_{cat}/K_m for correct dNTP versus k_{cat}/K_m for incorrect dNTP, as described (*27*). [b] Ratio of f_{ins} for WT versus $f_{ins}$for D76V. [c] Determined for incorporation of the first, incorrect TMP only (see Figure 2). k_{cat} and K_m values determined for incorporation of both the first, incorrect TMP and the second, correct TMP (see Figure 2C,D) were 1.9 min^{-1} and 165 μM for WT and 0.55 min^{-1} and 310 μM for D76V, respectively. The ratio of f_{ins} for WT versus D76V was 18.8. [d] Not determined because incorporation of dCTP was too low to obtain kinetic values.

utilized for determination of kinetic parameters. Notably, however, K_m and k_{cat} values determined for incorporation of two TMP residues were similar to those for incorporation of a single TMP, indicating that, as expected, incorporation of the second residue is not rate-limiting (see the footnote of Table 2). Table 2 records K_m and k_{cat} values for incorporation of the 4 correct and 12 incorrect nucleotides. These values were used to determine the misinsertion frequencies, f_{ins}, for wild-type and D76 HIV RTs (*27*, *28*). The data (Table 2) show that the D76V mutation confers a specific pattern of enhanced insertional fidelity, rather than an overall increase in stringency. The mutant protein shows misinsertion fidelity 4−14 times higher than that of the wild type in the G·T (Figure 2), G·A, A·C, A·G, and C·A reactions and a less than 2-fold increase in the remaining reactions. In general, misincorporation frequency opposite template purines was reduced more than the frequency opposite pyrimidines, in accord with the results shown in Figure 1. Interestingly, however, the D76V protein displayed a misinsertion frequency 4 times lower in the T·T reaction.

Primer Extension Assay with an RNA Template and Biased dNTP Pools. To test whether the D76V mutation also confers increased fidelity in copying RNA, we used a 1600-nucleotide RNA template containing the coding sequence of the HIV RT gene and a deoxynucleotide primer encoding the sequence of HIV RT from amino acids 239 to 246. Reaction mixtures contained either all four dNTPs or biased pools lacking a single dNTP and equivalent amounts of RNA-dependent DNA polymerizing activity of wild-type or D76V RT. As shown in Figure 3A, the wild-type and D76V proteins yielded about the same level of extension with all four dNTPs. In contrast, the D76V mutant showed a lower level of extension than wild-type protein in the minus TTP reactions (Figure 3B); thus, at the two highest protein concentrations, the D76V mutant extended the primer to 60

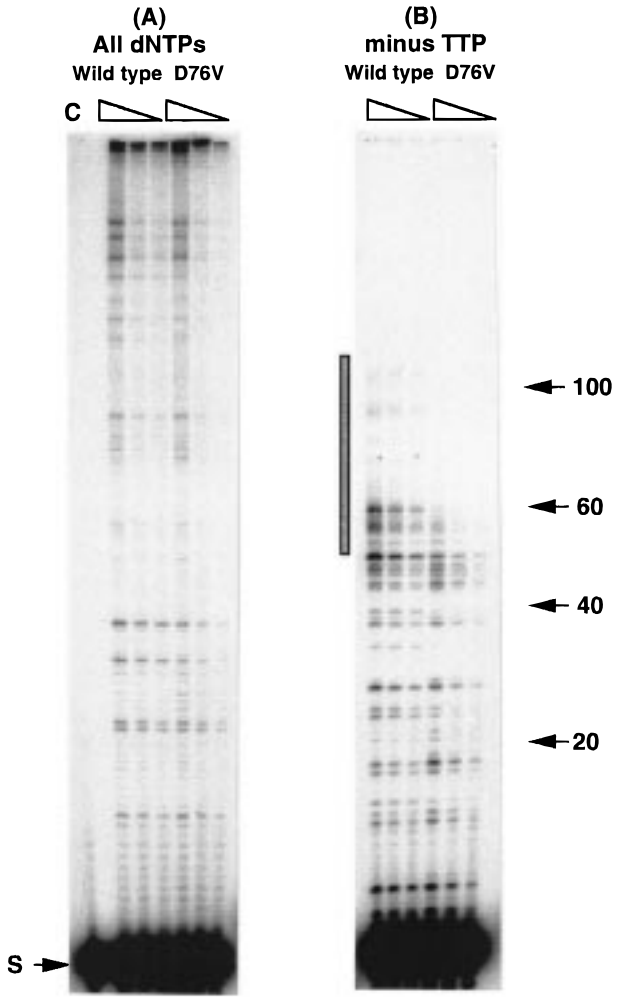

FIGURE 3: Primer extension by wild-type and D76V HIV RTs in assays containing an RNA template and lacking a complementary dNTP. The 21-mer 3305 RT primer was annealed to a 1600-nucleotide RNA template encoding HIV RT. Reaction mixtures contained 100 nM template−primer, either wild-type RT (10, 5, or 2.5 nM) or D76V RT (3, 1.5, or 0.75 nM), and either all four dNTPs at 250 μM each (A) or a biased pool from which TTP was deleted (B). The specific activity of D76V RT is 2.5 times that of wild type in an RNA-dependent DNA polymerase assay that utilizes the same template−primer and monitors incorporation of the label from [α-^{32}P]TTP (our unpublished data). The length of extended products is indicated at the right.

base pairs while wild-type RT extended the primer up to 200 base pairs. Less difference was observed in the minus dGTP and minus dATP reactions (not shown), indicative of a specific pattern of enhanced fidelity akin to that found for a deoxynucleotide template (Figure 1). These data indicate that the D76V mutation increases the accuracy of RNA-dependent DNA polymerization.

Generation of Additional D76 Mutants with Altered Fidelity. To determine whether other mutations at D76 affect replicational fidelity, we used PCR-based site-directed mutagenesis to generate additional amino acid substitutions. Of the 10 mutants that we purified (substitutions to E, R, L, I, S, C, W, F, P, and K), six exhibited DNA polymerase activity that was greater than 50% of the wild-type value. The fidelity of these six was examined in primer extension assays with a 63-mer deoxynucleotide template and biased dNTP pools, as illustrated in Figure 1 for the D76V mutant. Figure

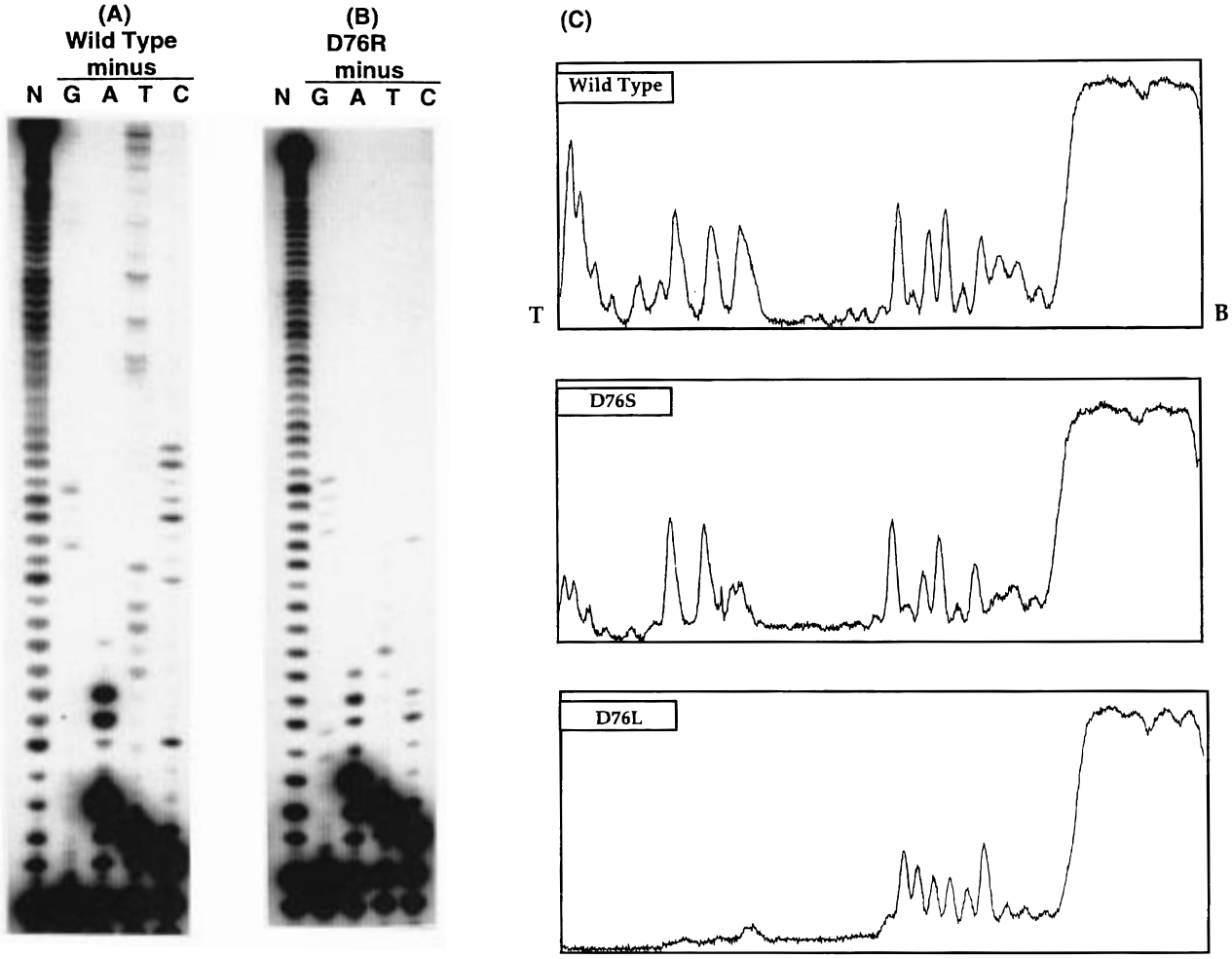

FIGURE 4: Primer extension by the wild type and D76 mutants in assays containing a deoxyoligonucleotide template and lacking a complementary dNTP. A 14-mer primer annealed to a 63-mer template was extended in reaction mixtures to which all four dNTPs were added at 250 μM each or reaction mixtures from which a single dNTP was omitted, as in the experiments of Figure 1. The first complementary nucleotide to be added to the primer terminus is dGMP. The concentrations of wild-type (A and C), D76R (B), D76S (C), and D76L (C) proteins were 10, 20, 15, and 15 nM, respectively, representing equivalent amounts of DNA-dependent DNA polymerizing activity on a gapped DNA template (*19*, *20*). At these protein concentrations, approximately 55% of the 14-mer primer was extended. The products formed by the wild type, D76S, and D76L in reactions lacking TTP were quantitated by phosphor image analysis (C). B and T indicate the bottom and top of the gel.

4 shows the products of reactions with the wild type (A) and D76R (B), together with phosphor image scanning analysis of the minus TTP reactions carried out with D76S and D76L. Among the mutants examined, D76R (B), D76L (C), and D76I (data not shown) showed reduced extension of the G primer, relative to that observed for the wild-type protein (A and C). In contrast, D76S (C), D76E, and D76C (data not shown) showed a level of extension higher than that observed for wild-type HIV RT. Notably, these six mutants displayed >50% of the wild-type specific activity on a gapped DNA template. These data are consistent with the premise that the presence of negatively charged or polar amino acids at D76 is associated with a wild-type level of fidelity, whereas the presence of positively charged or nonpolar amino acids increases fidelity. Taken together, our findings demonstrate that the amino acid at position 76 is a determinant of the replicational accuracy of HIV RT.

DISCUSSION

The HIV RT mutant D76V is one of many created by random mutagenesis of the $\beta3-\beta4$ fingers subdomain and recovered by positive genetic selection in an *E. coli* complementation system (*19*). The D76V mutant substitutes for temperature-sensitive DNA polymerase I in the *E. coli* host to the same extent as wild-type HIV RT, suggesting a normal level of DNA-dependent DNA polymerase activity. In fact, the purified D76V heterodimer has 80% of the wild-type activity, measured on a gapped DNA template. Most importantly, it exhibits a higher fidelity than wild-type HIV RT in copying both DNA and RNA templates. In the primer extension assays shown in Figure 1, D76V displayed a reduced ability to extend primers in reactions lacking TTP, dCTP, or dGTP, indicative of increased replicational accuracy. To confirm this result, we employed two additional assays. The first, an M13mp2 *lac*Zα forward mutation assay, measures the mutation rate of the enzyme during copying of DNA encoding the *lac*Zα gene. In accord with the primer extension results, D76V showed a mutation rate that was approximately 9-fold lower than that of wild-type HIV RT. The second, a gel-based steady-state kinetic assay, measures the ability to incorporate correct and incorrect dNTPs. The misinsertion frequencies (f_{ins}) in Table 2 show that D76V

has a decreased capacity, overall, to incorporate incorrect dNTPs. However, the reductions in misinsertion frequencies were much greater opposite template purines, affecting in particular the template−primer pairs A·C, A·G, G·A, and G·T. With the exception of the C·A pair, little difference between wild-type and D76V proteins was observed opposite template pyrimidines. These data are consistent with the results of the primer extension assays illustrated in Figure 1 where a major difference between the wild-type and D76 proteins was observed only in the minus TTP and dCTP reactions (Figure 1B,C), in which the template bases are A and G, respectively. Importantly, the primer extension assays also show that, relative to the wild-type enzyme, D76V leaves behind an increased level of unextended mismatched primers at the first stop sites, indicating that D76V is less able to extend the mismatched primers generated by misinsertion. For this reason, the overall fidelity of DNA-dependent DNA synthesis by D76V may be even higher than that indicated by the misinsertion frequencies in Table 2. Finally, primer extension assays indicate that the D76V mutation also confers increased accuracy of RNA-dependent DNA synthesis (Figure 3), which is the first step in viral replication.

Two drug-resistant HIV RT mutants with increased fidelity, M184V and E89G, have been characterized to date. In contrast to D76V, which shows the greatest decreases in misinsertion rates at template purines, the largest decreases observed for M184V were in T·T and C·T template−primer pairs (*29*). For E89G, the biggest differences were in T·T, T·G, and G·A pairs (*11*). Thus, these three mutations that cause increased fidelity confer different spectra of misinsertion mutagenesis. Interestingly, the misinsertion fidelity of D76V is as great or greater than that of M184V in the primer extension assay exemplified in Figure 1, judging by the length and relative amounts of extended products (data not shown). Moreover, D76V leaves behind more unextended primers, M184V being indistinguishable from the wild type in this regard. Thus, our data indicate that D76V is more accurate than M184V in this assay, due to insertion fidelity that is at least as high and extension fidelity that is higher.

Mechanisms controlling the replicational accuracy of HIV RT have been suggested on the basis of study of the drug-resistant mutants M184V and E89G that display increased fidelity. M184 is located near the catalytic site in the conserved YXDD motif. According to a proposed model (*6*), metals (Mg^{2+} or Mn^{2+}) chelated to the incoming dNTP interact with the two negatively charged D185 and D186 residues in the YXDD sequence. Presumably, the adjacent M184 may affect fidelity by mediating interaction between the catalytic site and the incoming dNTP. A second mechanism is suggested by studies with E89G. E89 interacts with the penultimate nucleotide of a double-stranded template, suggesting that interaction with the double-stranded region of the template affects replicational accuracy (*11*, *13*). Additionally, certain mutations in the thumb and fingers domains of both HIV RT (*21*, *30*) and the Klenow fragment (*28*, *31*), affect the level of frame-shift mutations, apparently by a template−primer slippage mechanism. These HIV RT mutants (*21*) and other mutants such as M184V (*32*) also display altered processivity, indicating that processivity is related to the accuracy of the DNA synthesis by at least some DNA polymerases. Another mechanism affecting replicational accuracy, suggested by studies with mutants of the Klenow fragment of *E. coli* DNA polymerase I (*33*) and thermostable *Taq* DNA polymerase I (*22*), may be interactions between the dNTP binding site (the O helix of these polymerases) and the incoming dNTP. In addition, substitutions at a distance from the dNTP binding site should alter the conformation of the enzyme and affect the rate-limiting step involved in fidelity. As a result, there may be many alterations that can enhance the accuracy of DNA synthesis.

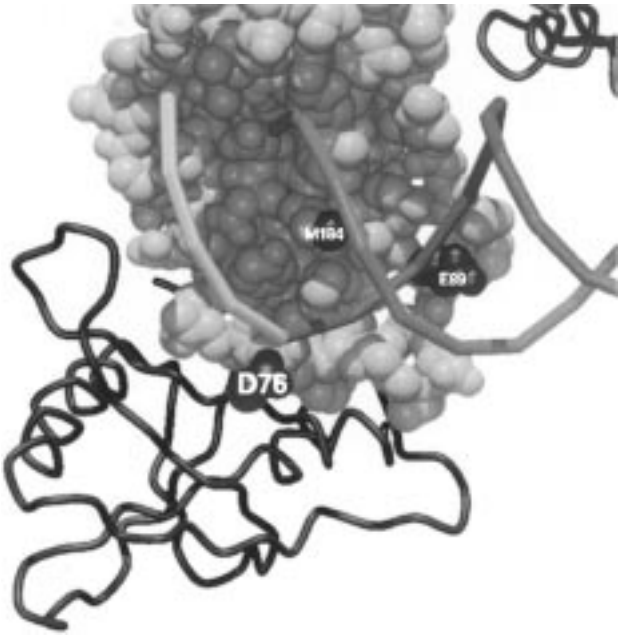

FIGURE 5: Modeled interaction of Asp-76 of wild-type HIV RT with an extended single-stranded template. Cα coordinates of HIV RT and phosphorus coordinates of a double-stranded deoxynucleotide template−primer were obtained from the Protein Data Bank (PDB) [file 1HMI (*13*)]. Since no side chain positions were provided in PDB file 1HMI, coordinates of HIV RT complexed with an inhibitor were obtained from the PDB [file 1HNI (*35*)], rotated and translated to best fit the 1HMI structure, and used for locating the side chain atoms. The position of the extended template strand was generated from the template phosphorus coordinates in 1HMI by doing a rigid body rotation and translation of the template strand so that residues T9−T17 of the new template position overlapped positions T3−T11 of the old; the graphics program O (*36*) was used for this. The drawing was made by E. Adman using MOLSCRIPT (*37*) and Raster3D (*38*). Only residues from the p66 domain are shown, with the fingers in red, the palm in green, and connection residues 323−437 in green. The thumb region is not included in this view. The phosphate backbone of the primer is colored in magenta, the template paired with the primer in blue, and the translated and rotated single-stranded template phosphate backbone in yellow. Residues D76, E89, and M184 are in green.

Structural models of HIV RT indicate that the $\beta3-\beta4$ fingers subdomain, where many drug-resistant mutations have been found, interacts with the single-stranded part of the template. Since D76 is located in this subdomain, we attempted to observe interaction between D76 and the template by building a structural model for HIV RT complexed with template−primer (Figure 5). We modeled the single-stranded region of the template by extending four nucleotides (yellow) from the 5′ end of the template. For the extension, we copied the structure of the first four nucleotides at the 3′ end of the template (purple) that anneals to the primer (blue). As seen in the model, D76 is located very close to the first nucleotide of the single-stranded part

of the template (yellow) that will base pair to the incoming dNTP. Possibly, in the wild type, interaction between the carboxyl group of D76 and the first nucleotide of the single-stranded region of the template promotes mispairing between the template nucleotide and the incoming dNTP, resulting in mutation. Alternatively, interaction between D76 and the template might affect translocation of the tertiary complex (RT−DNA−dNTP), facilitating incorporation of a non-complementary nucleotide. Since our kinetic measurements were made under steady-state conditions, it is not clear which steps of the DNA polymerization reaction—dNTP binding, conformational change, or catalysis—are affected by the D76 mutations. Pre-steady-state kinetic measurements employing, for example, rapid quenching techniques will afford further understanding of the mechanisms of the D76 mutations.

Abolishing the negative charge at D76 by substitution of positively charged (D76R) or nonpolar amino acids (D76V, D76L, and D76I) might antagonize the mutagenic effect of D76, while substitution with negatively charged (D76E) or polar amino acids (D76S and D76C) would preserve the wild-type level of replicational accuracy. One D76 mutation, D76N, has been identified in natural viral populations. Possibly, the polar asparagine residue at D76 does not affect fidelity, permitting D76N HIV to maintain a wild-type rate of evolution. Taken together, the available observations and the model suggest that polarity and charge at D76 play significant roles in fidelity. As seen in Figure 5, we also visualized the locations of M184 and E89, which affect replicational accuracy. As discussed above, M184 is located in the palm domain near the 3′ end of the primer (blue), whereas E89 is located near the double-stranded part of the template (purple).

Why has the D76V mutation not been identified in clinical isolates of HIV? One possibility is that the slight reduction in DNA-dependent DNA polymerase activity precludes efficient viral replication. However, the L74M mutation, which reduces DNA-dependent polymerase activity to 37% of the wild-type value, is able to replicate in the natural host (*34*). Therefore, it is unlikely that the D76V mutant, with 80% of the wild-type DNA-dependent and 250% of the wild-type RNA-dependent DNA polymerase activity, cannot replicate. The D76V mutation possibly affects other catalytic properties essential to viral replication, such as strand transfer activity. However, a more interesting possibility is that the increased replicational accuracy is detrimental to viral fitness. During the course of HIV infection, the viral mutation frequency must be maintained at a level which is not so high that too few infectious virus are made, but not so low that insufficient variants are produced to permit escape from selective pressures. For this reason, the low level of mutagenesis conferred by the D76V mutation may not be favored. Although M184 was identified as a drug-resistant variant in human cells, our preliminary results (see above) indicate that its overall accuracy may be less than that of D76V and closer to that of wild-type HIV RT. In fact, it is possible that mutants with greatly increased fidelity may be difficult to recover from clinical isolates. Such mutants can clearly be selected in *E. coli* however, and could become an important component of the mutational database required for producing a coherent molecular analysis of polymerase accuracy (*6*).

ACKNOWLEDGMENT

We especially thank Ann Blank for critical discussions and E. Adman for modeling HIV RT complexed with primer−template.

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BI972672G