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Loss of Polymerase Activity Due to Tyr to Phe Substitution in the YMDD Motif of Human Immunodeficiency Virus Type-1 Reverse Transcriptase Is Compensated by Met to Val Substitution within the Same Motif†

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ABSTRACT: Tyr183 is a constituent of the highly conserved YXDD motif common to all retroviral reverse transcriptases. The two aspartates in this motif are the crucial members of the catalytic carboxylate triad while residue X, which in the case of HIV-1 RT is Met184, is implicated in dNTP substrate recognition and fidelity of DNA synthesis. In an attempt to understand the function of Tyr183 in the catalytic mechanism, we generated mutants of this residue (Y183F and Y183A) and subjected them to in-depth analysis. The efficiency of reverse transcription of natural U5-PBS HIV-1 RNA template was severely impaired by both the conservative and nonconservative substitutions. The major defect identified was at the level of dNTP binding as determined by a 20−80-fold increase in the K_m for the dNTP substrate on both homopolymeric and heteropolymeric RNA and DNA templates. A significant reduction in processivity of DNA synthesis by these mutants was also noted. However, the fidelity of DNA synthesis by the Y183F and Y183A mutants was increased significantly compared to the wild-type enzyme. Interestingly, the reduction in the polymerase activity due to single substitution of Tyr to Phe in the YMDD motif is compensated by a second substitution of Met to Val in the same motif, herein referred to as the FVDD. The loss of dNTP binding as well as decreased processivity of DNA synthesis exhibited by the Y183F mutant was also compensated by mutation at the second site. Curiously, the double mutant did not exhibit any synergistic effect in regard to fidelity of DNA synthesis as might be expected since both the single mutations (Y183F, M184V) exhibited enhanced fidelity compared to the wild-type enzyme. These data implicate Tyr183 and Met184 as important constituents of the dNTP-binding pocket. We propose a model which suggests that subtle structural changes due to mutation in the flexible $\beta9-\beta10$ loop region at the active site of the molecule influence the enzyme activity and substrate recognition.

Reverse transcriptase is an essential enzyme for HIV-1 replication and is currently one of the two major targets against which drugs have been effectively used to reduce viral load in the plasma; the other target being HIV-1 protease. HIV-1 RT[1] is a highly error-prone enzyme and is responsible for the hypermutability seen during HIV-1 replication. One site within RT that has shown a high degree of mutability is the residue 184, corresponding to the X position in the YXDD motif common to all reverse transcriptases (*1*, *2*). Methionine is present at this position in HIV-1 RT and in several variant RTs isolated from AIDS patients and from cell cultures. The M184V mutant that is seen in patients treated with 3TC, and ddI (*3*−*5*) shows a high degree of polymerase activity and increased fidelity over the WT enzyme (*6*, *7*). The neighboring tyrosine residue at position 183 is invariant in all reverse transcriptases, and naturally occurring mutations at this position have so far not been detected in virions. All four residues in the YMDD motif have been shown through X-ray crystallography to be at the active center of the enzyme. Biochemical analysis of the mutant derivatives of D185 and D186 in this motif suggest that they are crucial residues which interact with the 3′OH of the primer terminus and the α-phosphate of dNTP in the ternary complex, respectively (*8*). Met184 and Tyr183 located on the flexible $\beta9-\beta10$ loop in the palm subdomain have been shown to be within interacting distance of the template-primer in the region of the primer terminus (*6*, *9*). Previous studies have shown that Y → S and Y → F substitutions at position 183 resulted in 99 and 70% loss of the polymerase activity, respectively, as compared to the wild-type enzyme (*10*, *11*). In addition, Tyr → Ser substitution reportedly caused a 77-fold increase in the K_m for dNTP

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[1] Abbreviations: A, M, Y, V, and F represent single letter codes for the amino acids, alanine, methionine, tyrosine, valine, and phenylalanine, respectively; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonylfluoride; IPTG, isopropyl-β-thiogalactopyranoside; Poly (rA)·$(dT)_{18}$, polyriboadenylic acid annealed with $(oligodeoxythymidylic\ acid)_{18}$; dNTP, deoxyribonucleoside triphosphate; dATP, dGTP, dCTP, and dTTP represent nucleoside triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine, respectively; HIV-1 RT, human immunodeficiency virus type-1 reverse transcriptase; IMAC, immobilized metal affinity chromatography; IDA-Sepharose, iminodiacetic acid-Sepharose; U5-PBS-RNA template, HIV-1 genomic RNA template corresponding to the primer-binding sequence region; U5-PBS-DNA template, HIV-1 genomic DNA template corresponding to the RNA-PBS sequence.

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substrate implicating this residue in dNTP binding function (*12*). Bakhanashvili et al. (*13*) have recently shown that Tyr → Phe mutation significantly increases the fidelity of HIV-1RT. Taken together, these data suggest that both residues 183 and 184 exert a predominant influence on substrate recognition and binding. With a view to characterize the functions of Tyr183 and Met184 in catalysis, we generated single mutations at position 183 and a double mutation at positions 183 and 184 in the YMDD motif. The results presented in this communication demonstrate that the impaired polymerase activity resulting from a single mutation at position 183 is restored by a second mutation at position 184.

MATERIALS AND METHODS

Materials. Restriction endonucleases, DNA-modifying enzymes, and dNTP solutions were from Promega or Boehringer Mannheim. ^{32}P-Labeled nucleotides were purchased from ICN while Sequenase and other DNA-sequencing reagents were from Amersham Life Sciences. Mutagen-M13 mutagenesis kit was purchased from Bio-Rad Laboratories. Expression vector pET28a and *Escherichia coli* strain BL21 (DE3) were from Novagen. Fast-flow chelating Sepharose (iminodiacetic acid-Sepharose) for immobilized metal affinity chromatography (IMAC) was obtained from Pharmacia. Chemically synthesized HIV-nucleocapsid (1−55 amino acids) was a generous gift from Dr. R. A. Bambara (*14*). Synthetic template-primers, sequencing primers, and mutagenic oligonucleotides were synthesized at the Molecular Resource Facility at UMDNJ. Plasmid pHIV-PBS was a generous gift from Dr. M. A. Wainberg (*15*). All other reagents were purchased from Fisher, Boehringer Mannheim, and Bio-Rad.

Methods. *Site-Directed Mutagenesis of the p66 subunit of HIV-1 RT*. The *Xba*I and *Sac*I fragment of pET-3a-RT51 was subcloned in M13 mp19 and used as a template for site-directed mutagenesis (*6*). The mutagenesis protocol described by Kunkel et al. (*16*) was used to generate mutations in RT66 which were confirmed by DNA sequencing. The *Nde*1 and *Kpn*1 restriction fragment from the RT51 coding region containing the mutation was then subcloned into the pET28a-RT_{66} expression cassette (His-Tag containing expression vector) as described previously (*6, 8, 17*−*19*). The recombinant pET28a-RT_{66} clones carrying the wild-type or mutant RT gene were introduced into *E. coli* BL21 (DE3) strain for induction and expression of enzyme protein.

Expression and Isolation of 66/66 Homodimeric HIV-1 RT and Its Mutant Derivatives. *E. coli* BL21 (DE3) carrying the recombinant clones was grown at 37 °C in Luria Broth containing kanamycin (30 μg/mL). Induction by IPTG and purification of enzyme proteins by IMAC column were carried out as described before (*6*). The purified enzyme preparations were found to be greater than 95% pure as judged by SDS−PAGE (*20*) and were stable at −20° C for several months.

Polymerase Activity Assay. Polymerase activity of the WT and mutant enzymes were assayed on three different template-primers, the poly (rA)•$(dT)_{18}$, U5-PBS HIV-1 RNA and 49-mer U5-PBS-DNA templates primed with 17-mer or 18-mer PBS primer. The U5-PBS HIV-1 RNA template was transcribed from the plasmid pHIV-PBS which contains a 947 bp fragment of the HIV-1 genome (+473 to +1420) corresponding to the PBS region (*15*, *21*). Assays were carried out in a 50 μL volume containing 50 mM Tris HCl, pH 8.0, 100 μg/mL bovine serum albumin, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 100 nM TP, 100 μM dNTP, and 10 nM enzyme. With homopolymeric (rA)•$(dT)_{18}$ template primer, the reaction mixture contained 50 μM ^{32}P-labeled dTTP (0.4 μCi/nmol). For heteropolymeric template-primers, 25 μM of each of the four dNTPs was included with one of them being ^{32}P-labeled (0.2 μCi/nmol dNTP). Reactions were incubated at 37 °C for 10 min and were terminated by addition of ice-cold 5% trichloroacetic acid containing 5 mM inorganic pyrophosphate. The samples were filtered on Whatman GF/B filters. The filters were dried and counted for radioactivity in a liquid scintillation counter. For the gel analysis of products, reactions were carried out in a total volume of 6 μL under similar conditions and terminated by the addition of 6 μL of Sanger's gel loading dye (*22*) containing 20 mM EDTA. The terminated reactions were heated to 90 °C for 5 min and the products were resolved on an 8% denaturing polyacrylamide (7 M urea, 1× TBE) sequencing gel.

RNase H Activity Assay. RNase H activity of the WT and mutant HIV-1 RT was determined using a 5′-^{32}P-labeled 30-mer RNA hybridized to a complimentary 30-mer DNA to generate the duplex hybrid. The sequence of 30-mer RNA corresponds to a small segment of the U5-PBS region of HIV-1 genome (Chart 1). The labeled duplex hybrid was incubated with 50 ng of the wild-type HIV-1 RT or its mutant derivative for 30 s at 25 °C in a volume of 5 μL and terminated by addition of 5 μL of Sanger's gel loading dye. Subsequently, the reactions were heated to 90 °C for 5 min, and cleavage products were resolved on a denaturing polyacrylamide urea gel.

Steady-State Kinetics of Polymerization. The initial velocity of the reaction was determined by measuring the rate of incorporation of α-^{32}P-labeled dNTP into the template-primer as a function of Mg-dNTP concentration (*23*−*25*). The enzyme was incubated with the template primer at 25 °C for 2 min, and then reactions were initiated by the addition of a solution containing all other components such that the final concentrations were 100 nM TP, 10 nM enzyme, 100 μg/mL BSA, 2 mM $MgCl_2$, 1 mM DTT, 50 mM Tris HCl, pH 8.0, 50 mM KCl, and variable dNTP concentration with one of the four dNTPs being ^{32}P-labeled in a final volume of 50 μL. Reactions were carried out for 2−5 min and were terminated by the addition of ice-cold 5% TCA containing 5 mM inorganic pyrophosphate. Samples were filtered on Whatman GF/B filters, the filters were dried and counted in a liquid scintillation counter. The K_m values for dNTP substrate and V_{max} for the polymerase reaction catalyzed by the wild-type HIV-1 RT and its mutant derivatives were calculated graphically by Eadie-Hofstee plots of the initial velocity data using the program Enzyme Kinetics v1.1. The k_{cat}s were calculated from the equation $V_{max} = k_{cat}[E]$.

Cross-Linking of Enzyme to Template-Primer. We have used poly(rA)5′-^{32}P-$(dT)_{18}$ as the RNA−DNA template-primer and 49-mer U5-PBS-DNA/5′-^{32}P-18-mer as the DNA−DNA template primer for binding studies (see Chart 1). The primers were 5′-labeled using [γ-^{32}P]ATP and T_4 polynucleotide kinase according to the standard protocol (*26*). The labeled oligomers were purified on a NAP-10 column

Chart 1

1. U5-PBS HIV-1 RNA containing the primer binding site.

<----------PBS---------->

3'- CAG GGA CAA GCC CGC GGU GAC GAU CUC UAA AAG GUG UGA CUG AUU UUC CCA GAC UCC CUA GAG AUC AAU GGU CUC AGU GUG UUG UCU GCC CGU GUG UGA UGA ACU UCC UGA GUU CCG UUC GAA AUA ACU CCG AAU UCG UCA CCC AAG GGA UCA UCG GUC UCU CGA GGG UCC GAG UCU AGA-5'

2. 17 mer DNA PBS primer

5'-GTCCCTGTTCGGGCGCC-3'

3. 18 mer DNA PBS primer.

5'-GTCCCTGTTCGGGCGCCA-3'

4. 19 mer PBS DNA primer

5'-**GTCCCTGTTCGGGCGCCAC-3'**

5. 20 mer DNA PBS primer.

5'-GTCCCTGTTCGGGCGCCACT-3

6. 21 mer DNA PBS primer

5'-GTCCCTGTTCGGGCGCCACTG-3

7. Synthetic 30 mer RNA corresponding to U5-PBS RNA sequence

3'-CAG GGACAAGCCCGCGGUGACGAUCUCUAA-5'

8. 30 mer DNA complementary to 30 mer U5-PBS RNA

5'-**GTCCCTGTTCGGGCGCCACT GCTAGAGATT-3'**

9. 49 mer U5-PBS DNA template corresponding to U5-PBS sequences.

3'-CAGGGACAAGCCCGCGGTGACGATCTCTAAAAGGTGTGACTG ATTTTCC-5'

(Pharmacia) and adjusted to the required specific activity with the unlabeled oligomer. The TP was prepared by mixing equimolar aliquots of primer and template in an annealing mixture containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 100 mM NaCl followed by heating the mixture at 65 °C for 10 min and then slow cooling to room temperature. For cross-linking, 512 nM enzyme and 10 nM labeled TP (3×10^5 Cerenkov cpm/pmol) were incubated on ice for 10 min in a reaction mixture containing 50 mM Tris HCl, pH 7.5, 1 mM DTT, 2 mM $MgCl_2$, and 5% glycerol in a final volume of 50 μL (*6*). The mixture was exposed to 254 nm UV in a Spectrolinker (300 mJ/cm^2) and the TP cross-linked enzyme species were resolved by electrophoresis on SDS−polyacrylamide gel and detected by autoradiography.

Processivity Assay. The processivity of the wild-type and mutant enzymes was assayed using the U5-PBS HIV-1 RNA template primed with ^{32}P-labeled 18-mer DNA primer. The 30/21-mer was used as the DNA trap at a final concentration of 50 μM of primer termini. In reactions where processivity was assessed, the enzymes were preincubated with the labeled TP for 2 min and then synthesis was initiated by the addition of a solution containing Mg-dNTP and DNA trap. A control reaction was included for each 1 and 10 min time point in which synthesis was not restricted by the presence of a trap. The efficiency of the DNA−DNA trap was examined by addition of the trap at the TP-binding step. The molar ratio of the labeled TP to trap was approximately 1:10000 so that the probability of an enzyme rebinding a labeled TP after one round of processive synthesis and dissociation was negligible. The final [dNTP] was 200 μM and [Mg^{2+}] was 2 mM. The reaction was carried out at 25 °C for 1 and 10 min and terminated by addition of Sanger's gel loading dye. The samples were then heated at 90 °C for 5 min, and the products were resolved by denaturing 8% polyacrylamide 7 M urea gel electrophoresis.

Polymerase Activity in the Presence of Nucleocapsid Protein. The wild-type enzyme and its mutant derivatives were incubated for 2 min with either U5-PBS-RNA or 49-mer U5-PBS-DNA templates primed with 5′-[^{32}P]18-mer PBS primer in the absence or presence of 1 μM nucleocapsid protein in a standard reaction mixture. The reaction mixture (5 μL) contained 50 mM Tris HCl, pH 8.0, 100 μg/mL bovine serum albumin, 1 mM dithiothreitol, 50 mM KCl, and 100 nM TP. Reactions were initiated by the addition of Mg-dNTP (100 μM dNTP and 5 mM $MgCl_2$) and

terminated by the addition of equal volume (5 μL) of Sanger's gel loading dye following incubation for 15 min at 25 °C. The samples were then heated at 90 °C for 5 min and the products were resolved by denaturing 8% polyacrylamide 7 M urea gel electrophoresis.

rNTP Incorporation Assays. The ability of the wild-type HIV-1 RT and its mutant derivatives to incorporate rNTPs as substrate was assessed on both RNA and DNA templates. The U5-PBS-RNA template primed with ^{32}P-labeled 17-mer DNA primer as well as 49-mer U5-PBS-DNA primed with ^{32}P-labeled 19-mer DNA primer was used as the RNA−DNA and DNA−DNA TP, respectively. In each case, the enzyme was preincubated with the labeled TP and reactions were initiated by the addition of Mg-dNTP (200 μM) or Mg-rNTP (500 μM) complex. The reactions were carried out in a total volume of 8 μL for 30 min at 25 °C and terminated by the addition of equal volume of Sanger's gel loading dye. The samples were then heated at 90 °C for 5 min and the products were resolved by denaturing 8% polyacrylamide 7 M urea gel electrophoresis.

ddNTP Sensitivity Assays. The template-primers used in this assay were the same as those used in the rNTP assay. Samples were incubated at 25 °C for 30 min for both DNA−DNA and RNA−DNA TPs. Forty nanograms of the wild-type HIV-1 RT and M184V mutant enzyme, 50 ng of the FVDD double mutant, and 160 ng of the Y183F and Y183A mutant enzymes were used for each reaction. Final concentrations were [dNTP] = 200 μM, [ddNTP] = 100 μM, and [Mg^{2+}] = 2 mM. All other conditions were identical to the rNTP incorporation assay.

Single Nucleotide Misincorporation Assays. In these assays, the U5-PBS-RNA and 49-mer U5-PBS-DNA templates primed with 5′-^{32}P-labeled 20-mer DNA were used as template primers. The gel-purified 5′-^{32}P-labeled 20-mer PBS primer was annealed with a 2-fold excess of RNA or DNA template by heating the TP mixture to 65 °C and cooling to 30 °C at the rate of 1 °C/minute. The [dNTP] was kept at 200 μM for the correct nucleotide and 500 μM for the incorrect nucleotides. The enzymes were preincubated with the labeled TP and the reactions were initiated by addition of a single Mg-dNTP. The reactions were carried out in a total volume of 8 μL for 30 min at 30 °C and terminated by the addition of equal volume of Sanger's gel loading dye The reaction products were analyzed by denaturing polyacrylamide gel electrophoresis.

RESULTS

Differential Polymerase Activity with RNA and DNA Templates. We compared the polymerase activities of the mutant enzymes using poly(rA)·$(dT)_{18}$ as well as natural RNA and DNA templates corresponding to the U5-PBS region of HIV-1 genome primed with the PBS primer. The results depicted in Figure 1 and Table 1 indicate that the Y183F mutant exhibited 25−30% of the wild-type activity on all template primers displaying no preference for RNA or DNA template. In contrast, the Y183A mutant showed a significant preference for DNA template as it exhibited 30% of the wild-type activity with the DNA template and only 3% activity with the RNA templates. The selective preference for DNA template displayed by the Y183A mutant suggests that tyrosine at position 183 is indispensable for

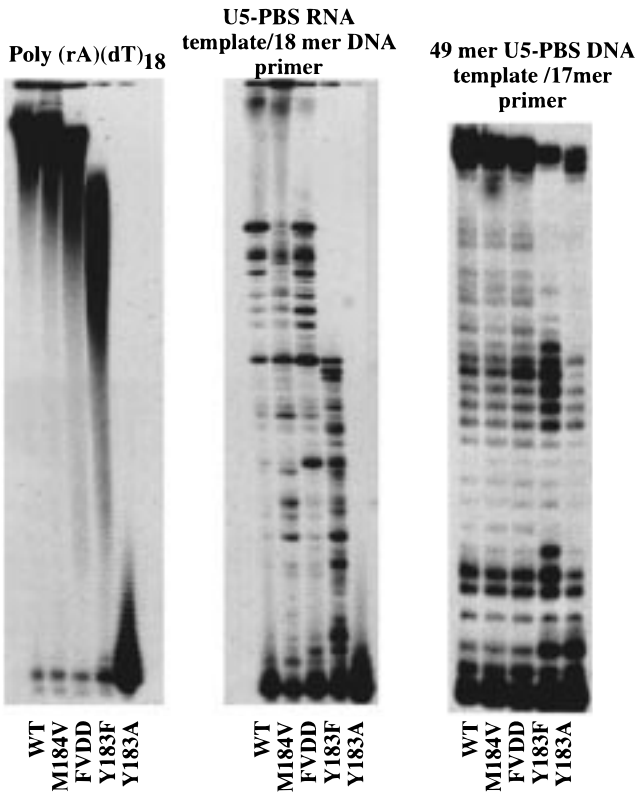

FIGURE 1: RNA- and DNA-directed DNA polymerase activity of wild-type HIV-1 RT and its mutant derivatives. Three template primers, poly (rA)·$(dT)_{18}$, U5-PBS-RNA/18-mer DNA, and 49-mer U5-PBS-DNA/17-mer DNA primer were used to assess the extension reaction catalyzed by wild-type HIV-1 RT and its mutant derivative. The DNA primers labeled with ^{32}P at the 5′-terminus were annealed with the respective templates and used in the reaction as described in the Materials and Methods. The reactions were carried out in a total volume of 6 μL for 10 min at 37 °C and terminated by the addition of equal volume of Sanger's gel loading dye. The samples were then heated at 75 °C for 5 min and the products were resolved by denaturing 8% polyacrylamide 7 M urea gel electrophoresis.

reverse transcription while it may have a relatively minor role in second strand DNA synthesis. This contention is supported by the fact that Tyr in the YMDD motif is an invariant residue among all retrovrial reverse transcriptases. Surprisingly, the double mutation at positions 183 and 184 (FVDD) restored the polymerase activity of the enzyme suggesting a possible communication between these two vicinal residues in the YMDD motif (Table 1 and Figure 1). The RNase-H activity of these mutants was not altered as judged by gel analysis of the RNase-H cleavage products (data not shown).

Steady-State Kinetics Analysis. The kinetic parameters of the mutant derivatives of Y183 determined using both poly(rA)·$(dT)_{18}$ as well as natural U5-PBS-RNA and DNA templates primed with complementary PBS primer indicated a drastic reduction in the affinity for dNTP substrate by both the Y183A and Y183F mutants. An 18-fold increase in K_m for dTTP for the Y183F mutant and an 80-fold increase by the Y183A mutant on the homopolymeric RNA template was noted with corresponding 20- and 800-fold decrease in the catalytic efficiency, respectively (Table 2). Similarly, high K_m values for dNTP with the homopolymeric TP have also been reported for the Y183S mutant of HIV-1 RT (*12*). In contrast, the FVDD double mutant showed no change in the

Table 1: Polymerase Activity of Wild-Type HIV-1 RT and Its Mutant Derivatives[a]

enzyme	template-primer	picomoles of dNMP incorporated/10 min/ 50 ng enzyme	% of WT activity
WT	poly (rA)·$(dT)_{18}$	104	100
	U5-PBS-RNA/18-mer	16	100
	49-mer U5-PBS DNA/17-mer	18	100
M184V	poly (rA)·$(dT)_{18}$	195	187
	U5-PBS-RNA/18-mer	18	109
	49-mer U5-PBS DNA/17-mer	22	124
Y183F	poly (rA)·$(dT)_{18}$	26	25
	U5-PBS-RNA/18-mer	4.6	29
	49-mer U5-PBS DNA/17-mer	4.6	26
Y183A	poly (rA)·$(dT)_{18}$	2.1	2
	U5-PBS-RNA/18-mer	0.6	3.6
	49-mer U5-PBS DNA/17-mer	5.4	30
FVDD	poly (rA)·$(dT)_{18}$	62	60
	U5-PBS-RNA/18-mer	14	86
	49-mer U5-PBS-DNA/ 17-mer	7	40

[a] The polymerase activity of the wild-type HIV-1 RT and its mutant derivatives was determined with the indicated template-primers at saturated dNTP concentrations. Reactions were carried out at 37 °C for 10 min and the total pmole dNMP incorporated was determined as described in Materials and Method.

Table 2: Steady-State Kinetic Parameters of WT HIV-1 RT and Its Mutant Derivatives[a]

template-primer and dNTP substrate	enzyme	K_{mdNTP} (μM)	k_{cat} (s^{-1})	K_{cat}/K_m ($M^{-1}s^{-1}$) × 10^4
poly (rA)·$(dT)_{18}$ (dTTP)	WT	6	0.26	4.4
	Y183F	108	0.24	0.22
	Y183A	480	0.026	0.0054
	FVDD	6	0.09	1.4
U5-PBS-RNA/ 18-mer TP (4 dNTPs)	WT	8	0.053	0.66
	Y183F	150	0.052	0.034
	Y183A	313	0.028	0.0089
	FVDD	28	0.043	0.154
49-mer U5-PBS-DNA/ 17-mer TP (4 dNTPs)	WT	17	0.043	0.24
	Y183F	152	0.055	0.036
	Y183A	87	0.038	0.044
	FVDD	33	0.044	0.13

[a] The steady-state kinetic parameters for wild-type HIV-1 RT and its mutant derivatives were measured with indicated template-primers and corresponding dNTP substrate as described in the Materials and Methods. The purified mutant and WT enzymes were used in homodimeric form (p66/p66). These determinations were carried out at sub saturating concentrations of the respective dNTP substrates.

affinity for dTTP indicating that the decrease in dTTP-binding affinity due to Tyr to Phe mutation at position 183 was compensated by Met → Val substitution at position 184 within the same motif. Steady-state kinetic parameters were also obtained using the natural U5-PBS HIV-1 RNA template corresponding to the primer-binding site (PBS) of the HIV-1 genome. The average K_m for all four dNTPs was determined by varying the concentration of dNTP substrate at fixed concentration of the TP. The Y183F mutant exhibited an 18-fold increase in K_{mdNTP} with the natural U5-PBS−RNA TP as compared to the wild-type enzyme, while a 40-fold increase in the K_{mdNTP} was noted with the Y183A mutant. Interestingly, the FVDD mutant exhibited a marginal increase of 3-fold in the average K_{mdNTP} on this TP and no change in the affinity for dTTP on rA·dT. These results are consistent with the earlier reports suggesting possible interactions of Tyr183 and Met184 with the template-primer and/or dNTP substrate (*6*, *13*, *9*). In contrast, both the mutant derivatives

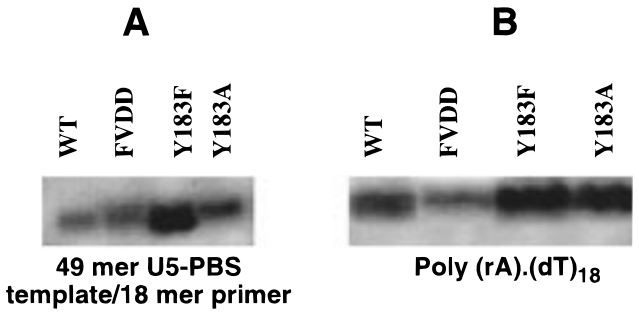

FIGURE 2: Photochemical cross-linking of RNA-DNA and DNA-DNA template primers to the wild-type HIV-1 RT and its mutant derivatives. Wild-type HIV-1 RT and its mutant derivatives were covalently cross-linked with (A) 49-mer U5-PBS/5′-[^{32}P]18-mer or (B) poly(rA)·5′-[^{32}P](dT)18 by UV irradiation as described in the Materials and Methods. The irradiated mixtures were subjected to SDS−PAGE and the labeled enzyme-TP covalent complexes were detected by autoradiography.

of Tyr183 exhibited an increase in the affinity for template primers. As shown in Figure 2, the extent of photoaffinity binding/cross-linking of both Y183A and Y183F mutants with both heteropolymeric and homopolymeric template primers was significantly higher than the wild-type enzyme and the FVDD mutant enzyme.

Mode of DNA Synthesis. As described above, both Y183F and Y183A mutants displayed higher affinity for DNA and decreased affinity for dNTP substrate with significant reduction in their polymerase function. Since reduction in the polymerase activity and accumulation of smaller reaction products were more pronounced on RNA template, it was presumed that in addition to dNTP binding, other steps such as translocation of the enzyme along the RNA template may have also been affected. To ascertain this possibility, the processivity of the polymerase reaction catalyzed by the wild-type enzyme and its mutant derivatives was evaluated (Figure 3). Processivity is a measure of the number of nucleotides incorporated into a primer strand during a single binding event before the enzyme dissociates from the template-primer. Some mutations in HIV-1 RT have been shown to compromise the processivity of the polymerase reaction (*17*, *19*), and our analysis of the Y183F mutant has revealed that removal of the OH group at position 183 significantly decreased processive DNA synthesis (Y183F, lanes 3 and 4). However, processivity of the Y183A mutant could not be estimated due to the very low level of the polymerase activity of this mutant on the RNA template. Interestingly, a second mutation at position 184 (FVDD) restored the processivity of DNA synthesis to levels similar to the wild-type enzyme (FVDD, lanes 3 and 4). The single M184V mutation had been shown earlier to have little or no effect on processivity (*6*). Although, the affinity for TP is increased by Y→ F substitution at position 183, the low processivity resulting in accumulation of shorter products was surprising. It is possible that the Y183F mutant with its side chain devoid of the hydroxyl group may compromise the ability of RT to move through the secondary structure that exists within the U5-PBS-RNA template. These observations suggest that, in addition to dNTP binding, the hydroxyl group of Tyr183 may also be required for the processive synthesis of the full-length viral cDNA. However, a second mutation at the neighboring 184 residue (FVDD) resulting in complete restoration of the dNTP-binding affinity and processive DNA

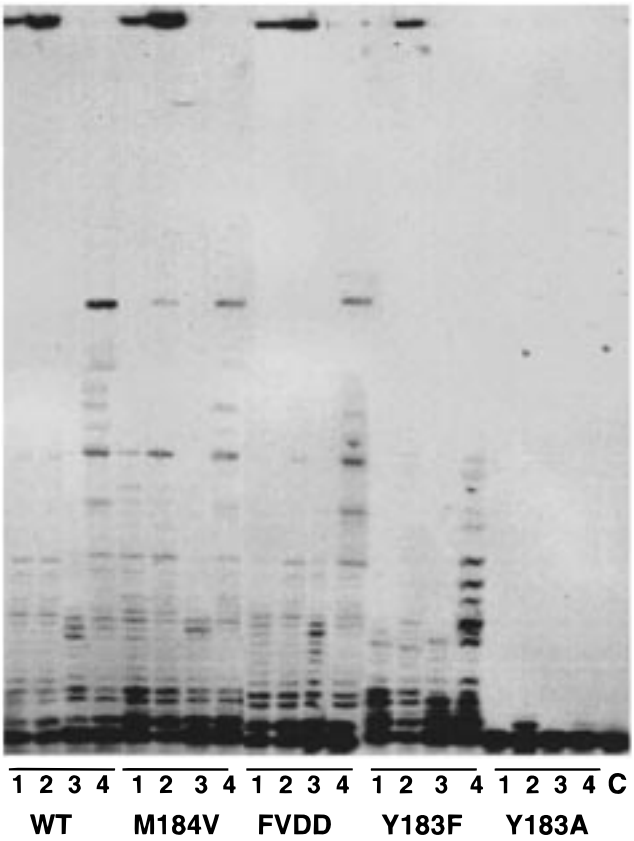

FIGURE 3: Mode of DNA synthesis catalyzed by wild-type HIV-1 RT and its mutant derivatives. The 5′-^{32}P-labeled 18-mer primer annealed with U5-PBS-RNA template was used to assess the mode of DNA synthesis. Following incubation of the individual enzymes with the labeled TP, the reaction was initiated by the addition of 200 μM of dNTPs and 50 μM of 30/21-mer as the DNA trap. Each set represents experiments with the wild-type, M184V, FVDD, Y183F, and Y183A mutant enzymes. In lanes 3 and 4 of each set, DNA trap was added along with the dNTP substrates and the product length generated represents a single processive synthesis event during 1 and 10 min of incubation, respectively. Lanes 1 and 2 of each set represent the DNA synthesis occurring on the labeled template-primer in the absence of DNA trap for 1 and 10 min, respectively. In lane C, both DNA trap and labeled TP were premixed and incubated with the enzyme prior to initiation of DNA synthesis.

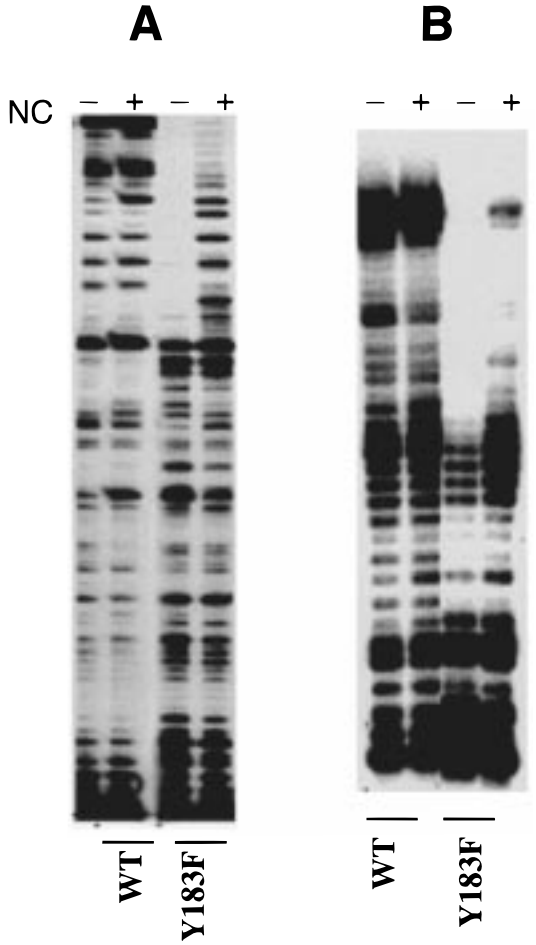

FIGURE 4: Effect of nucleocapsid (NC) protein on the DNA synthesis catalyzed by WT enzyme and its mutant derivatives. 5′-^{32}P-labeled DNA primer annealed with either U5-PBS-RNA template (panel A) or 49-mer U5-PBS-DNA template (panel B) was used to assess the effect of nucleocapsid protein on DNA synthesis by the mutant and wild-type enzymes. Lanes (−) and (+) of each set represent the extension reaction carried out in the absence and presence of 1 μM nucleocapsid protein, respectively, as described in the Materials and Methods. Extension reactions were performed for 15 min at 25 °C and the products were resolved by denaturing polyacrylamide 7 M urea gel electrophoresis.

synthesis suggests that distortion in the dNTP-binding pocket caused by the single mutation (Y183F) may be successfully reversed by the second mutation (FVDD).

Effects of NC Protein on DNA Synthesis. Since the Y183F mutant exhibited low processivity of DNA synthesis on U5-PBS-RNA template, it was thought that the observed effect could be due to the inability of the mutant enzyme to destabilize the secondary hairpin structure of the RNA template. To explore this possibility, we examined the effect of NC on DNA synthesis catalyzed by the WT and mutant enzyme. The nucleocapsid protein of HIV-1 RT is found in virions and is associated with both RT and the single-stranded RNA genome. This protein is proposed to be important in the process of reverse transcription and in production of proviral DNA. Since NC has been shown to enhance reverse transcription of HIV-1 RT presumably by promoting unwinding of the secondary structure of the viral RNA template and tRNA primer (*27*, *28*), it was of interest to examine whether the nucleocapsid showed a similar effect with the Y183F mutant. The results shown in Figure 4 indicate that the polymerase activity of the Y183F mutant is stimulated with nucleocapsid protein on both RNA template (Figure 4A) and DNA template (Figure 4B). The stimulation on the DNA template was moderate for the wild-type HIV-1 RT but was dramatic with the Y183F mutant (Figure 4B).

Utilization of rNTP versus dNTP Substrates. Tyrosine in the YXDD motif is highly conserved among RNA-dependent RNA polymerases as well as reverse transcriptases (*29*). We, therefore, examined whether HIV-1 RT, with the YXDD motif common in RNA polymerases, is also able to utilize rNTP as substrate in the polymerase reaction. In this analysis, the M184V mutant was also included for comparison. The ability of the wild-type enzyme and its mutant derivatives to catalyze the incorporation of rNTPs was tested using primed heteromeric DNA and RNA templates (Figure 5). As shown in the figure, the order of efficient rNTP incorporation on the RNA template was as follows: wild-type > M184V > FVDD > Y183F = Y183A (Figure 5A). This order essentially remained the same on DNA except that the double mutant (FVDD) was also able to incorporate

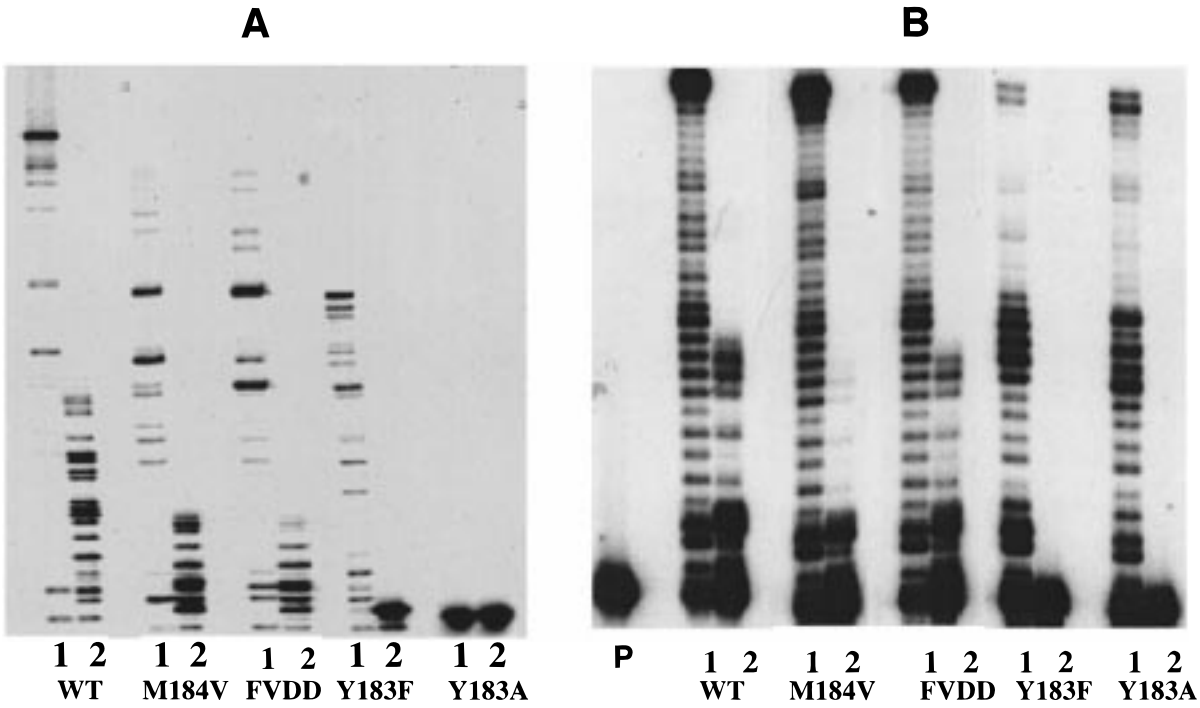

FIGURE 5: Utilization of rNTP in the extension reaction by wild-type HIV-1 RT and its mutant derivatives. The ability of WT HIV-1RT and its mutant derivatives to catalyze incorporation of rNTP substrates was examined either using U5- PBS-RNA template (panel A) or 49-mer U5-PBS-DNA template (panel B) annealed with 5′-^{32}P-labeled 18-mer PBS primer. Lanes 1 and 2 represent extension reactions carried out in the presence of 200 μM dNTPs and 500 μM rNTP, respectively. All reactions were carried out at 25 °C for 20 min.

rNTPs more efficiently on the RNA template as compared to the DNA template (Figure 5B). The loss of the functional side chain of Y183 (Y183A) as well as the mere loss of the OH group (Y183F) renders the enzyme largely incapable of recognizing rNTPs as substrates for polymerization. However, mutation at a second site (FVDD) reduces the discrimination against rNTPs and allows the enzyme to incorporate them, albeit not as efficiently as the wild-type enzyme. These findings suggest that residues 183 and 184 operate in conjunction with each other to influence the shape and size of the dNTP-binding pocket. The nature of the binding pocket apparently differs depending on the type of the template being read as seen with the FVDD mutant, which is able to catalyze the addition of rNTPs with high efficiency on an RNA template but with low efficiency on a DNA template.

Sensitivity to ddNTPs. Dideoxynucleotides have been shown to be competitive inhibitors of HIV-1 RT and have been used in the treatment of HIV infection by targeting the function of this enzyme. Since mutant derivatives of Tyr183 were found to be highly discriminatory against rNTP substrate, we examined whether these mutants also exhibit any such discrimination against ddNTPs. It is known that the wild-type HIV-1 RT is sensitive to dideoxy chain terminators and that the M184V mutant is less sensitive to some of these dNTP analogues such as ddI, 3TC, and ddC (*4*, *30*, *31*). In the present study, both Y183F and Y183A mutants were found to be highly resistant to ddNTPs as no significant change in the DNA synthesis pattern was noted in the presence or absence of ddNTPs on both RNA and DNA templates (Figure 6). These results are in agreement with a previous report by Bakhanashvili et al. (*13*). In contrast, the FVDD double mutant was found to be more sensitive to the dideoxynucleotides compared to Y183F and Y183A mutants. This is surprising for two reasons: first, the Y183F mutation confers a high degree of resistance to ddNTPs, and second, the M184V mutation alone also confers resistance against some of the dideoxy analogues. We expected these two mutations to act synergistically in conferring higher resistance, but paradoxically, the FVDD mutant displays greater sensitivity than the mutant derivatives of Tyr183. These observations seem to suggest a correlation between fidelity and resistance to nucleoside analogues.

Fidelity of DNA Synthesis. To investigate the effects of mutations at positions 183 and 184 on the fidelity of HIV-1 RT, we employed an assay in which addition of a single nucleotide was examined to assess the ability of RT to incorporate and extend correct versus the incorrect nucleotide on RNA and DNA templates. This assay revealed that the mutant derivatives of Y183 exhibit higher fidelity on RNA template compared to M184V, FVDD, and the wild-type enzymes (Figure 7A). The Y183F mutant was unable to catalyze the incorporation of any of the incorrect nucleotides when provided with a single nucleotide at concentrations of 500 μM. However, both Y183F and Y183A were able to generate mispairs (C:C, C:T) and extend them on the DNA template (Figure 7B). Although FVDD mutant exhibited lower fidelity as compared to Y183F and Y183A mutants, its overall fidelity was higher than M184V and the wild-type enzyme. The order of increasing fidelity was as follows: WT < M184V < FVDD < Y183F = Y183A. Although Bakhanashvili et al. (*13*) observed no difference in misincorporation between the wild-type and the Y183F mutant, we clearly demonstrate here that the Y183F mutant is unable to generate and extend mispairs on RNA template while the wild-type enzyme is able to generate and extend all mispairs in the presence of a single nucleotide under our assay conditions.

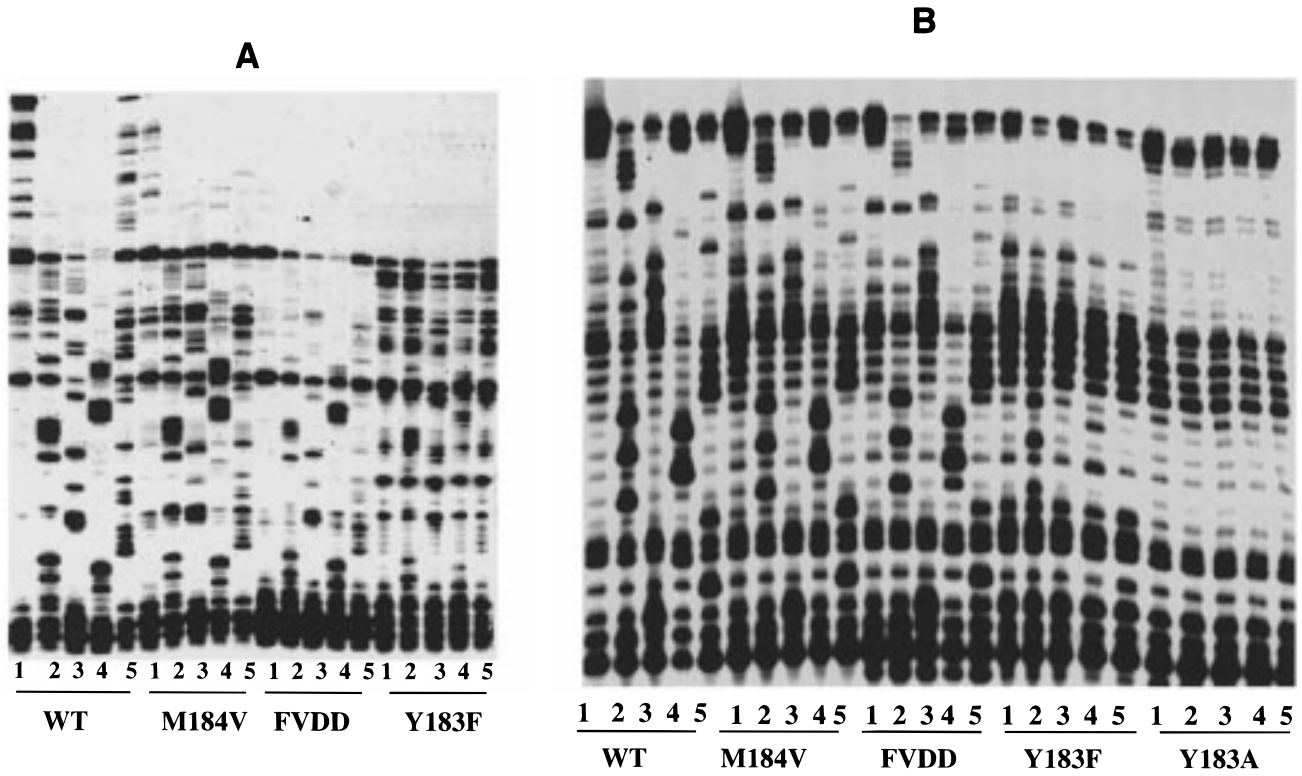

FIGURE 6: Sensitivity of wild-type HIV-1 RT and its mutant derivatives to dideoxynucleoside triphosphates. Effect of ddNTPs on the DNA synthesis catalyzed by the wild-type and mutant enzymes was assessed on U5-PBS-RNA template (panel A) and on 49-mer U5-PBS-DNA template (panel B) primed with 5′-^{32}P-labeled 18-mer PBS primer. The concentration of dNTP was 200 μM and the concentration of each of the ddNTPs was 100 μM. Lane 1 in each set of experiments represents the extension reaction in the absence of ddNTPs. Lanes 2−5 represent extension reaction in the presence of ddATP, ddCTP, ddGTP, and ddTTP, respectively.

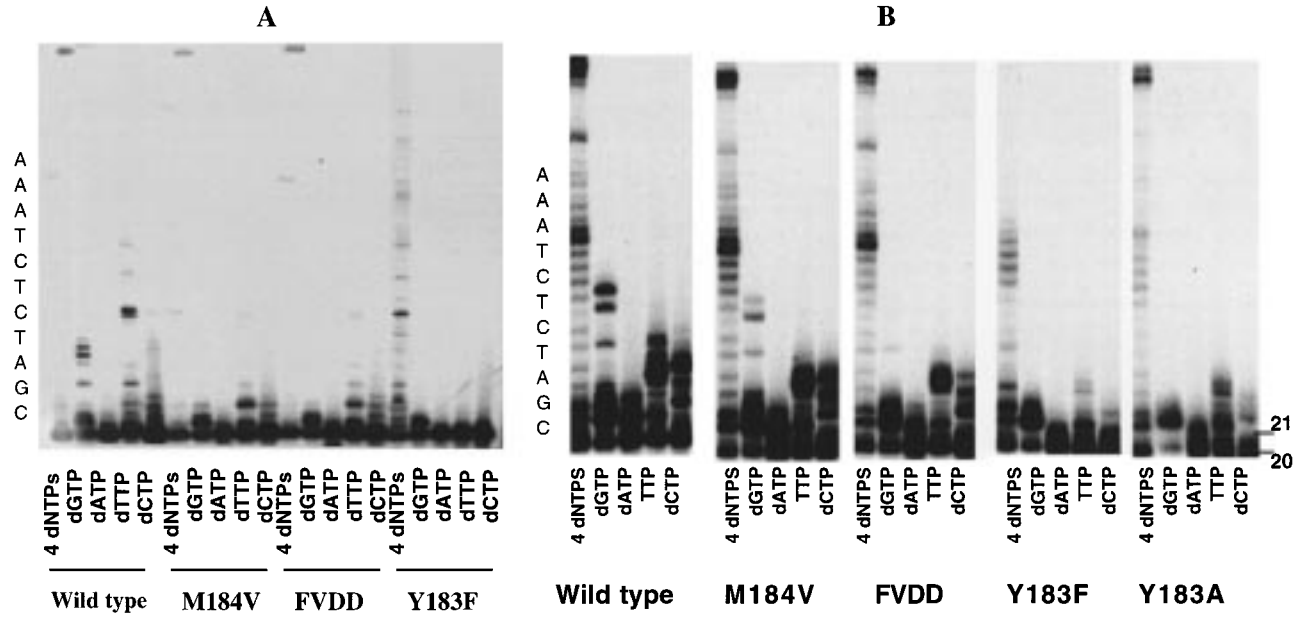

FIGURE 7: Misinsertion and mispair extension catalyzed by wild-type enzyme and its mutant derivatives in the presence of a single dNTP. The ability of the enzymes to generate and extend mispairs in the presence of only a single dNTP was assessed on both U5-PBS-RNA (panel A) and U5-PBS-DNA (panel B) templates primed with 5′-[^{32}P]20-mer PBS primer. Each set of experiments represents extension of primer in the presence of all four dNTPs as well as in the presence of a single indicated dNTP. Reactions were carried out for 30 min and the products were analyzed by denaturing polyacrylamide gel electrophoresis.

DISCUSSION

These studies show that residues at position 183 and 184 in the YMDD motif seem to communicate with each other through their functions during catalysis. Partial loss of polymerase activity due to single mutation at position 183 (Y183F) within the YMDD motif is compensated by a second mutation at position 184 (FVDD) within the same motif. In the 3-D crystal structure of HIV-1 RT, both Tyr183 and M184 are located on the β9 and β10 hairpin loop, respectively, in the palm subdomain of the large catalytic

cleft (*32*, *33*). Mutant derivatives of Y183 (Y183A, Y183F) exhibit differential polymerase activity on RNA versus DNA templates. Y183F, a conservative mutant, retained 25−30% polymerase activity on both RNA and DNA templates. Interestingly, polymerase activity of the Y183A mutant on the RNA template is less than 4% of the WT enzyme, while on a DNA template, it is equal to that of the Y183F mutant. In vivo, RT plays a dual role in synthesizing the proviral DNA. In the first step, it copies the genomic viral RNA template into the (−)strand cDNA. In the second step, the negative strand cDNA serves as template for the synthesis of a complementary plus strand DNA. Our results suggest that both the OH group and the phenyl ring at position 183 may be crucial for the synthesis of the negative strand cDNA while neither of these may have a significant role in the plus strand DNA synthesis. However, the role of the OH group for efficient reverse transcription is completely redundant when valine is present at position 184 since the FVDD is nearly as active as the wild-type enzyme on both RNA and DNA templates. This indicates that proper positioning of the catalytic center is reinforced by the combined effect of both Phe and Val side chains. Furthermore, single mutation at position 183 (Y183A, Y183F) caused substantial decrease in the affinity for dNTP as judged by the severalfold increase in the K_{mdNTP} exhibited by these mutants. A second mutation at position 184 substituting Met → Val (FVDD) fully restored the affinity for dNTP, suggesting that direct involvement of the side chain of Y183 in dNTP binding is unlikely. Our earlier 3-D model of the RT-DNA-dNTP ternary complex suggested that Tyr183 is within interacting distance from the penultimate primer terminus nucleotide while Met184 is closer to the sugar moiety of the 3′-terminal nucleotide (*6*, *8*). Therefore, the effect of mutant derivatives of these residues on the affinity for dNTP substrate may be via their interaction with the primer nucleotides in the dNTP-binding pocket.

The low efficiency with which Y183A and Y183F mutants catalyze certain mismatch synthesis gives some insight into the importance of this residue in the fidelity of DNA synthesis. The Y183F mutant exhibits highest fidelity on the RNA templates. However, with DNA template, both Y183F and Y183A mutants are able to catalyze C:C and C:T mismatches and could also extend these mispairs with low efficiency. The FVDD mutant exhibited lower fidelity than both the individual mutant derivatives of Tyr183 (Y183F, Y183A). This result seemed paradoxical as it was speculated that the double mutant would show a synergistic effect on fidelity. Nonetheless, the fidelity of the FVDD mutant was still higher than that of the wild-type enzyme and M184V mutant. These results suggest that elimination of either the OH group or the aromatic ring at position 183 may result in greater rigidity in an otherwise flexible dNTP-binding pocket. The rigid pocket thus enhances the fidelity by accommodating only the correct nucleotide for catalysis either during the initial binding step (k_2) or by inhibition of the conformational change step (k_3) after binding of the incorrect nucleotide (*34*, *8*). Kinetic analyses have shown that mutation at a second site (M184V) restores the affinity for the dNTP substrate but simultaneously compromises the fidelity of the enzyme. This restoration of the affinity for dNTP as well as the polymerase activity in the FVDD double mutant may be partly accounted for by a more stable interaction of the TP with the side chain of valine as compared to methionine. It has been proposed that there is a correlation between an increase in K_m for dNTP substrate and a corresponding increase in the fidelity of the mutants of HIV-1 RT (*35*). Analysis of the Y183 mutants are in agreement with this correlation. A higher affinity for the dNTP substrates dictated by the residues of the dNTP-binding pocket may result in a higher error rate during polymerization, whereas a decreased binding affinity may provide an additional constraint for discrimination against the incorrect nucleotides. In *E. coli*, DNA polymerase I mutation at position 850 (Gly850Arg) results in enhanced fidelity of the enzyme which is attributed to reduced processivity of DNA synthesis (*36*). Our present study also supports this phenomenon. A correlation between decrease in the processivity of DNA synthesis with a corresponding increase in fidelity was observed with the mutant derivatives of Tyr183.

To assess the contribution of Tyr183 and M184 in the polymerase reaction, we have analyzed several crystal structures of HIV-1 RT, in liganded and unliganded forms, for the possible interaction of Y183 with its neighboring residues. We found that the interatomic distance between the side chains of Q91 and Y183 in different crystal structures greatly varied over the range 3.5−9.6 Å (*33*, *37*), thus making it difficult to predict any specific interaction between these two residues. This large variation has prompted us to perform a systematic conformational search of the side chain of Y183 along the Cα−Cβ bond. For this purpose, we used a 3-D molecular model of HIV-1 RT/DNA/dNTP ternary complex (*6*, *8*, *17*) based on the C-α coordinates of the refined RT-DNA binary complex (*33*). We found that in one of the seven allowed conformations, the side chains between O(H) of Y183 and CO of Q91 are positioned within a distance of 3.2 Å with an angle O−H···O of 145°, which is appropriate for interaction via hydrogen bonding (Figure 8A). This interaction between Q91 and Y183 is strongly supported by the fact that substitution of Gln → His at position 91 resulted in a 70% loss of the polymerase activity (*38*); a value similar to that observed with Y→ F substitution at position 183.

Analysis of the mutation of single amino acid in the 3-D model structure of HIV-1 RT using the LOOK program has revealed that substitution of Tyr to Phe caused a notable perturbation in the local structure of the enzyme. A major shift in the orientation of both Phe183 and Gln91 is observed possibly due to lack of the hydrogen-bonding interaction between their side chains. Interestingly, a significant shift in the position of the side chain of Asp110, Tyr181, and Met184 is also observed. Since Asp110 is a member of the catalytic triad and has been proposed to be involved in coordinating with the γ,β-phosphate of Mg-dNTP substrate (*8*), a slight shift in its position may adversely influence the catalytic coordination in the dNTP-binding pocket. A shift in the position of Met184 observed as a result of mutation of the amino acid from Tyr to Phe at position 183 may have resulted in the misalignment of the template primer since Met184 has been proposed to be interacting with the sugar moiety of the primer terminus nucleotide (*6*). Modeling of a second mutation (changing the FMDD motif to FVDD) did not reveal any significant change other than restoration of the original position of Asp110. This seems relevant since

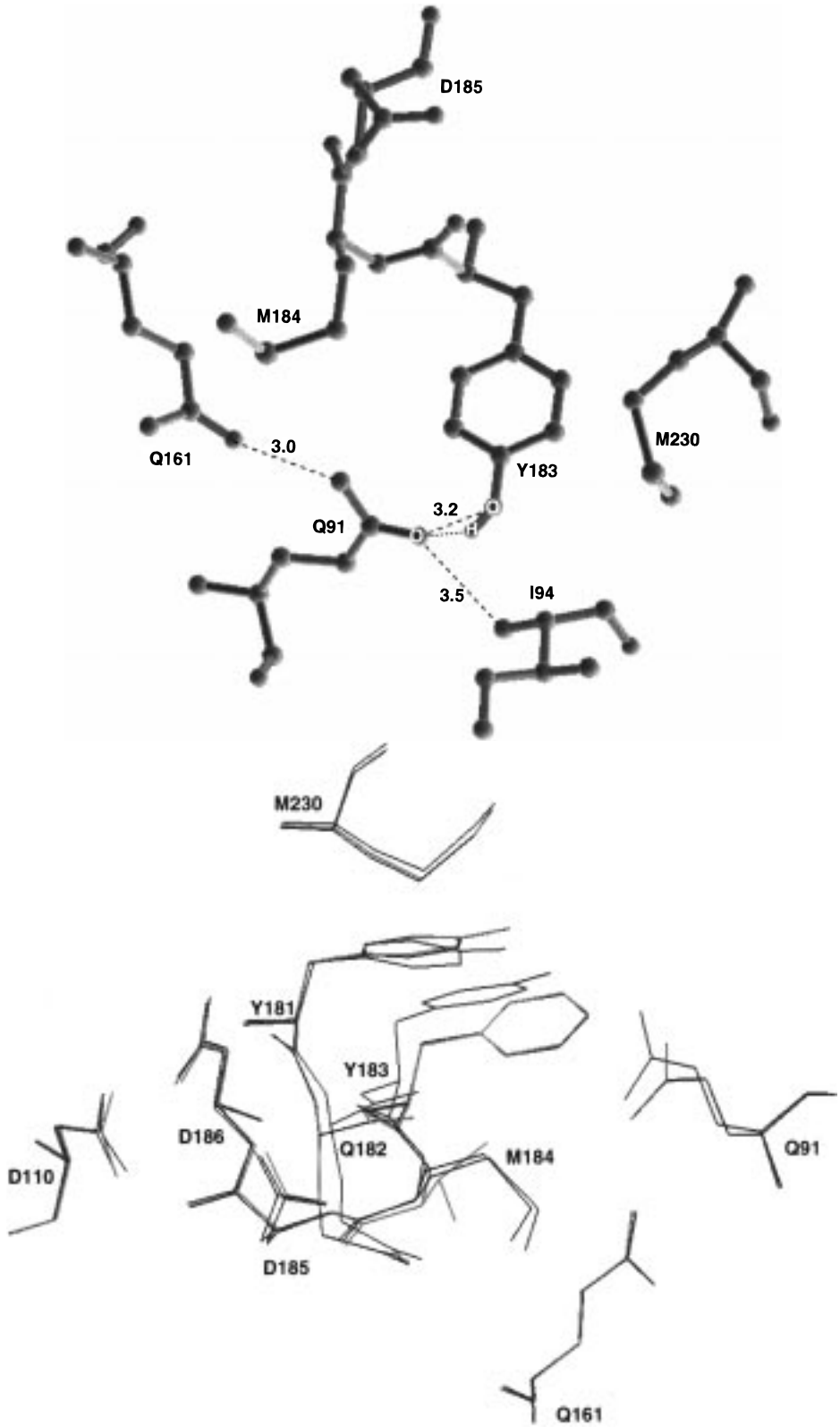

FIGURE 8: (A) Interaction of Tyr183 with surrounding residues and (B) superimposition of 3-D model structure of wild-type, Y183F, and FVDD double mutant of HIV-1 RT. The prepolymerase ternary complex model of HIV-1 RT (*6*, *8*, *17*) based on the 3-D crystal structure of HIV-1 RT−DNA complex (*33*) was used to analyze the structural changes in and around the amino acid residue at position 183 and 184. The systematic conformational search of the side chain of Y183 was performed to explore the possibility of its interaction with the surrounding residues. In one of the seven allowed conformations obtained, the interatomic distance between O(H) of Y183 and CO of Gln91 is found to be within 3.2 Å and angle O−H···O is 145°. This geometry is appropriate for hydrogen bonding interaction between O(H) of Tyr183 and C═O of Gln91 (top). To analyze the effect of mutation(s) at position 183 and 184, we mutated these residues in the modeled structure of HIV-1 RT−DNA−dNTP ternary complex using the LOOK homology and mutant modeling protocol. An important change noticed in the model structure of the mutant was with respect to the orientation of the side chain of Tyr183 and Gln91 suggesting a possible interaction between the side chains of Tyr183 and Gln91 (bottom). The fact that Y183F and Q91H mutants of HIV-1 RT display a similar reduction in their polymerase activity (*38*) also suggests a likely interaction between the side chains of these two residues. The ring structure of Phe183 is stabilized at a different position from that of Tyr183. The green line represents the position of WT while blue and red lines represent Y183F mutant and FVDD double mutant, respectively. The deviation in the side chains at positions 110, 183, 91, 184, and 181 can be seen.

the second mutation (FVDD) restored the affinity for dNTP substrate as well as catalytic activity of the enzyme which was substantially decreased by the Y183F mutation.

In the 3-D crystal structure of HIV-1 RT-DNA complex, Tyr183 is seen intercalating between the first and second nucleotide bases of the primer 3′-terminus. In addition, a likely interaction also exists between Tyr183 and Gln91 since mutation of either residue has a similar effect on the polymerase activity of the enzyme. The proximity of Tyr183 to the catalytic center of HIV-1 RT allows it to establish multiple contacts with the template primer, dNTP, and the surrounding amino acid residues constituting the dNTP-binding pocket. These multiple contacts may influence the flexibility of the dNTP-binding pocket resulting in more error prone DNA synthesis. By substituting Tyr → Phe at position 183, some of these interactions may be lost creating a more rigid dNTP-binding pocket that severely compromises the polymerase efficiency with significant increase in the fidelity. The second mutation may have restored the flexibility of the pocket by reestablishing the multiple contacts essential for efficient reverse transcription.

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