Role of Methionine 184 of Human Immunodeficiency Virus Type-1 Reverse Transcriptase in the Polymerase Function and Fidelity of DNA Synthesis

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ABSTRACT: Methionine 184 of HIV-1 RT is a constituent of the catalytically crucial and highly conserved YXDD motif in the reverse transcriptase class of enzymes. We investigated the role of this residue by substituting it with Ala and Val by site-directed mutagenesis followed by extensive characterization of the two mutant enzymes. The kinetic parameters governing DNA synthesis directed by RNA and DNA templates indicated that both M184A and M184V mutants are catalytically as efficient as the wild type enzyme. Photoaffinity labeling of both the mutant and the wild type enzyme exhibited an identical affinity for RNA−DNA and DNA−DNA template primers. We further demonstrate that M→V substitution at 184 position significantly increases the fidelity of DNA synthesis while M→A substitution results in a highly error-prone enzyme without having compromised its efficiency of DNA synthesis. The M184V mutant exhibited a 25−45-fold increase in mismatch selectivity (ratio of k_{cat}/K_m of correct versus incorrect nucleotides) as compared to the WT enzyme. This pattern of error-prone synthesis is also confirmed by examining the abilities of the enzyme−(template-primer) covalent complexes to incorporate correct *versus* incorrect nucleotide onto the immobilized template-primer. The nature of error-prone synthesis by the M184A mutant shows an increase in both the mismatch synthesis and extension of the mismatched primer termini. Using a three-dimensional molecular model of the ternary complex of HIV-1 RT, template-primer, and dNTP, we observe that the strategic location of M184 may allow it to interact with the sugar moiety of either the primer nucleotide or the dNTP substrate.

Genomic heterogeneity of human immunodeficiency virus (HIV) type-1 resulting from its hyper mutability (Coffin, 1986; Desai et al., 1986) is central to the pathogenesis of this virus which has so far frustrated all efforts to develop effective drugs and vaccines. HIV replication occurs continuously in lymphoreticular tissues (Pantaleo et al., 1991, 1993; Embreston et al., 1993), and viral particles are detectable in the plasma of all patients regardless of the clinical stage (Piatak et al., 1993; Winters et al., 1993; Bagnarelli et al., 1992; Aoki-Sei et al., 1992). Recent studies on the dynamics of HIV-1 replication *in vivo* have shown that the wild type virus in plasma is completely replaced by the drug resistant mutants within 2−4 weeks (Wei et al., 1995; Ho et al., 1995). These observations suggest that continuous viral replication is central to the development of HIV disease. HIV-1 reverse transcriptase, an essential viral enzyme, is responsible for the conversion of the single-stranded RNA viral genome into double-stranded DNA. This enzyme is known to be error prone, induces mutations throughout the viral genome during its replication, and serves as a potential source of diversity (Preston et al., 1988; Roberts et al., 1988; Takeuchi et al., 1988). Various nucleoside drug resistant phenotypes of HIV have been isolated from patients exposed to prolonged chemotherapy. A number of specific mutations in the coding region of reverse transcriptase have been identified displaying resistance to various nucleoside analogs (Tantillo et al., 1994; Gu et al., 1994). One such natural mutation at codon 184 (Met→Val; M184V) of HIV-1 RT[1] has been shown to confer resistance to 2′,3′-dideoxyinosine (ddI), 2′,3′-dideoxycytidine, and 2′,3′-dideoxy-3′-thiacytidine (3TC) (Gu et al., 1992, 1994; Tisdale et al., 1993; Schinazi et al., 1993; Gao et al., 1993; Boucher et al., 1993; Schuurman et al., 1995). M184 is the X residue in the catalytically important YXDD motif found in all of the reverse transcriptases (Johnson et al., 1986; Delarue et al., 1990). Previous studies have shown that mutations in this motif can severely impair RT functions (Larder et al., 1989; LeGrice et al., 1991; Boyer et al., 1992; Wakefield et al., 1992). This motif is located on the $\beta9-\beta10$ hairpin and is part of the dNTP binding site in the 3-D crystal structure of HIV-1 RT (Jacobo-Molina et al., 1993). Earlier mutational

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[1] Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZTTP, 3′-azido-3′-deoxythymidine triphosphate; FTC, (−)-2′-deoxy-5-fluoro-3′-thiacytidine; 3TC, (−)-2′-deoxy-3′-thiacytidine; ddI, 2′,3′-dideoxyinosine; 2′,3′-ddC, dideoxycytidine; dNTP, deoxynucleoside triphosphate; HIV-1 RT, human immunodeficiency virus type-1 reverse transcriptase; D, M, V, A, G, S, L, I, P, and Y represent the single-letter code for amino acids Asp, Met, Val, Ala, Gly, Ser, Leu, Ile, Pro, and Tyr, respectively; IMAC, immobilized metal affinity chromatography; IDA-Sepharose, iminodiacetic acid-Sepharose; MuLV, Murine leukemia virus; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl β-thiogalactopyranoside; poly $(rA)\cdot(dT)_{18}$, polyriboadenylic acid annealed with $(oligodeoxythymidylic\ acid)_{18}$; poly $(rC)\cdot(dG)_{18}$, polyribocytidylic acid annealed with $(oligodeoxycytidylic\ acid)_{18}$; poly $(dC)\cdot(dG)_{18}$, polydeoxycytidylic acid annealed with $(oligodeoxyguanylic\ acid)_{18}$; WT, wild type; M184A and M184V refer to substitution of methionine with valine and alanine, respectively, at position 184 of HIV-1 RT; TP, template-primer.

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studies comprising M→V, M→A, M→S, M→G, M→I, and M→P substitutions at position 184 assessed the polymerase activity of these mutants in the crude enzyme preparations and reported 0%−100% polymerase activity depending upon the nature of substitution and the type of template-primer used (Wakefield et al., 1992; Chao et al., 1995). In our efforts to understand the nature of the contribution by Met 184 of HIV-1 RT in the process of polymerization, we generated two mutations that replaced Met by Val and Ala, and we have extensively characterized the two mutant enzymes. We report here that substitution of Met with Val at codon 184, a naturally occurring 3TC resistant mutation enhances both the fidelity and efficiency of DNA synthesis while Met to Ala substitution (M184A) does not affect the efficiency of DNA synthesis but severely compromises fidelity. We also sought a possible explanation for the somewhat opposite properties conferred by different mutations at codon 184 through analysis of a tentative pre-polymerase ternary complex model of HIV-1 RT, template-primer, and dNTP. The possible interaction of the side chain of residue 184 with the sugar moiety of the primer terminus appears to be responsible for the differential effects of mutation at this codon. Results of these investigations are the subject matter of this communication.

MATERIALS AND METHODS

Materials. Restriction endonucleases, *Taq* DNA polymerase, and DNA-modifying enzymes were from Promega or Boehringer Mannheim, while HPLC-purified dNTPs were obtained from Boehringer Mannheim. Sequenase and other DNA sequencing reagents were from U.S. Biochemicals. Mutagen-M13 *in vitro* mutagenesis kit was purchased from Bio-Rad Laboratories. Expression vector pET-28a and *Escherichia coli* expression strain BL21 (DE3) were obtained from Novagen. All other reagents were of the highest purity grade and were purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad. Fast-flow chelating Sepharose (iminodiacetic acid-Sepharose) for immobilized metal affinity chromatography (IMAC) and synthetic template-primers were purchased from Pharmacia, and ^{32}P-labeled dNTPs and ATP were the products of Dupont/New England Nuclear Corp. Sequencing primers and oligonucleotides containing the desired mutational changes were purchased from Midland Certified Reagent, Dallas, TX.

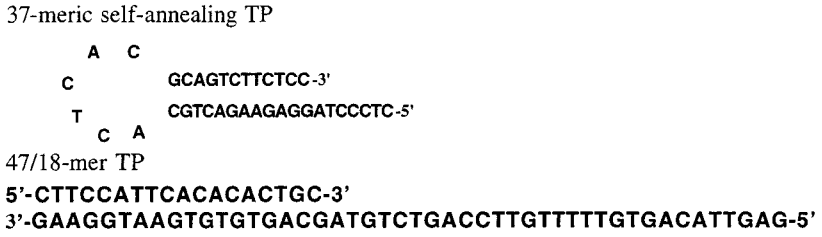
Methods. Construction of Expression Plasmids and in Vitro Mutagenesis. The coding sequence of p66 and p51 was derived from pRC-RT which contains the full coding region for HIV-1 RT [Bacerra et al. (1991), a kind gift from S. Wilson]. The 1.68 kb sequence coding for p66 was amplified from pRC-RT using PCR (Saiki et al., 1985). In the same step, we introduced the *Nde*I restriction site at the 5′-end and *Bam*H1 and a TAA stop codon at the 3′-end of the RT coding sequence. Using these restriction sites, the amplified fragment was cut and ligated with *Nde*I and *Bam*HI digested pET-28a (His-Tag containing expression vector) to construct the pET-28a-RT$_{66}$ plasmid (Sarafianos et al., 1995a,b). This construct contains the T7 promoter and metal-binding hexahistidine (His-Tag) sequences at the N-terminal region of RT$_{66}$. Similarly the 1.32 kb fragment representing the p51 coding region was amplified from pRC-RT by PCR introducing *Nhe*I and *Sac*I sites at the 5′- and 3′-ends, respectively and a TAA stop codon at the 3′-end of the coding sequence. The amplified fragment was restriction digested with *Nhe*I and *Sac*I and ligated with *Nhe*I- and *Sac*I-digested pET-3a to construct the pET-3a-RT$_{51}$. The coding sequence in this plasmid ends with amino acid Phe 440.

Site-Directed Mutagenesis of Both RT$_{66}$ and RT$_{51}$ Subunits. The *Xba*I and *Sac*I fragment (1.37 kb) of pET-3a-RT$_{51}$ encoding the polymerase domain of HIV-1 RT was subcloned in bacteriophage M13 mp18 and used as a template for site-directed mutagenesis. The mutagenesis protocol using uracil-containing template was essentially as described by Kunkel et al. (1987). After the mutation in M13 was ascertained by DNA sequencing, the desired mutation was introduced by subcloning the appropriate restriction fragment in the RT$_{66}$ and RT$_{51}$ expression cassettes. Subsequently, it was introduced into *E. coli* BL21 (DE3) for expression. Induction by IPTG was performed as described before (Pandey et al., 1994a; Desai et al., 1994).

Expression and Isolation of 66/51 Heterodimeric HIV-1 RT and Its Mutant Derivatives. The growth of *E. coli* BL-21 containing pET-28a-RT$_{66}$ and pET-3a-RT$_{51}$ clones carrying the WT or mutant subunits, and induction of the enzyme protein was carried out as described before (Sarafianos et al., 1995a,b). The heterodimers were prepared by mixing the cell pellets of p66 and p51 clones at the appropriate ratios (Le Grice et al., 1991). In brief, the cell paste harvested from the 400 mL batch was suspended in 8 mL of lysis buffer (40 mM Tris-HCl, pH 8.0, 1 mM PMSF, and 2 mg of lysozyme/mL) and stirred at 4 °C for 1 h. The suspension was briefly sonicated, adjusted to 0.5 M NaCl, and centrifuged at 28 000*g* for 45 min. The cell-free extract was applied to the pre-equilibrated (2.5 mL) Ni^{2+}−iminodiacetate-Sepharose (IDA-Sepharose) column at a flow rate of 1 mL/min. The column was washed extensively with buffer A (40 mM Tris-HCl, pH 8.0, 5 mM imidazole, and 0.5 M NaCl) until the A_{280} was reduced to a negligible level. A second wash (5 mL) with 60 mM imidazole in buffer A removed most of the weakly bound contaminating proteins. Next, the HIV-1 RT was eluted from the column with a gradient of 60−300 mM imidazole (30 mL) in buffer A, and 1 mL fractions were collected. Every alternate fraction was analyzed on SDS−PAGE, and only those fractions containing equal amounts of both the p66 and p51 subunits were pooled and dialyzed against buffer B (50 mM Tris HCl, pH 7.0, 100 mM NaCl, and 1 mM DTT) and finally concentrated by dialyzing in the same buffer containing 50% glycerol. The isolated proteins were more than 98% pure, as judged by SDS−PAGE (Laemmli, 1970). The protein preparations were stable for months at −20 °C. The protein concentrations were determined by using the Bio-Rad calorimetric kit.

Enzyme Assay. The composition of the reverse transcriptase reaction mixture is described below; any variation used in a specific experiment is as described in the appropriate figure legends. Unless otherwise indicated all reactions were carried out at 25 °C. Reactions were quenched either by the addition of 20 mM EDTA and 0.1% SDS or by the addition of ice-cold 5% trichloroacetic acid. The quenched reaction products were analyzed in some cases by urea−polyacrylamide denaturing gel or quantitated by collecting the TCA-precipitable materials on Whatman GF/B filters and counting for radioactivity in a liquid scintillation counter, as described before (Pandey et al., 1988). The reactions were carried out in a final volume of 100 μL containing 50 mM Tris-HCl, pH 8.0, 1 mM dithiothrietol, 0.1 mg of bovine serum albumin/mL, 5 mM $MgCl_2$, 200 nM of the desired

Chart 1. Oligomeric DNA Template-Primers Used



template-primer, and ^{3}H-labeled 20 μM dNTP substrate corresponding to the homopolymeric template-primers. With heteromeric template-primers 20 μM each of the four dNTPs were present with one of them being radiolabeled. The molar ratio of the primer to template was 1:1 unless indicated otherwise. The concentrations are expressed in terms of the 3′-hydroxyl primer termini. RNAse-H activity assays were performed essentially as described elsewhere (Basu et al., 1989).

Steady-State Kinetics of Polymerization. Kinetic studies were carried out as described previously (Majumdar et al., 1988; Bryant et al., 1983; Pandey et al., 1994a) using homopolymeric poly (rA)·(dT)$_{15}$, poly (rC)·(dG)$_{15}$, or poly (dC)·(dG)$_{15}$ as template-primers and complementary dTTP or dGTP as the nucleotide substrates. K_m and V_{max} were calculated graphically from Eadie−Hofstee plots of steady-state kinetic data; k_{cat} was calculated from the equation $V_{max} = k_{cat}[E]$. Kinetic experiments were performed twice, and values were averages of at least duplicate samples.

Kinetic Parameter for Incorporation of Correct and Incorrect Nucleotide. The rate of correct nucleotide (dTMP) incorporation on 47/18-mer template-primer was determined by incubating 4 nM heterodimeric (66/51) WT or mutant enzyme in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg of BSA/mL, 5 mM $MgCl_2$, 100 nM 47/18-mer TP, and 2−50 μM [α-^{32}P]-dTTP in a final volume of 100 μL. The reaction was initiated by the addition of Mg^{2+} ion and terminated by addition of ice-cold 5% TCA after incubation for 90 s at 25 °C. The acid insoluble radioactive material was quantitated, and kinetic constants were calculated graphically from Eadie−Hofstee plots as described before (Pandey et al., 1994a). The rate for incorrect nucleotide incorporation was also determined as above except that dTTP was replaced with dATP, dGTP, or dCTP and the incubation time was extended to 5 min.

Preparation of Template-Primer with Single-Base Misinsertion. Exonuclease deficient Klenow fragment and 47/[5′-^{32}P]-18-mer template-primer were used to prepare template-primer containing the mispaired terminal nucleotide. In this template primer [47/18(AT)], T and A represent the correct nucleotide substrates at the first and second positions, respectively (see Chart 1). The reaction mixture (8 μL) contained 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg of BSA/mL, 5 mM $MgCl_2$, 200 nM 47/[5′-^{32}P]-18-mer, 50 nM of the exonuclease deficient Klenow fragment, and 50 μM of dGTP, dCTP, or dATP for misinsertion at the first position (to reach a primer length of 19-mer). The reaction mixture containing 50 μM dTTP results in the misinsertion product of 20-mer with T:T misinsertion at the terminal nucleotide. The reaction mixtures were incubated at room temperature for 30 min and terminated by the addition of 25 mM EDTA and 0.1% SDS. The labeled products corresponding to 19-mer bands of A, G, or C termination and 20-mer band of T termination products were purified on 16% polyacrylamide−urea gel and quantitated by Cerenkov counting of the excised gel piece after locating them by autoradiography. The gel-purified primers were annealed individually with an excess of 47-mer template and used in the mismatch primer extension assay described below.

Extension of Primers in the Presence of Three dNTPs. [5′-^{32}P]-labeled 18-mer primer annealed with a 3-fold molar excess of 47-mer template (see Chart 1) was used for determining the extent of misincorporation in the presence of only three dNTPs (Preston et al., 1988). The labeled 47/18-mer template primer at 2 nM concentration was incubated with 25 nM WT or mutant enzymes at 25 °C for 30 min in a total volume of 5 μL containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 0.1 mg of BSA/mL, 5 mM $MgCl_2$, and only three dNTPs at a concentration of 50 μM each (−A = dCTP, dGTP, dTTP; −T = dATP, dGTP, dCTP; −G = dATP, dCTP, dTTP; −C = dGTP, dATP, dTTP). Each of the dNTPs used was of the highest purity grade (HPLC purified) supplied as 0.1 M solution (Boehringer Mannheim). At the end of the incubation period the reaction was quenched by the addition of 5 μL of the stop solution containing 40 mM EDTA, 0.33% SDS, 0.014% each of bromophenol blue and xylene cyanole and 85% formamide. The reaction products were analyzed on a denaturing 16% polyacrylamide−8 M urea gel.

Extension of Primer Containing Mispaired Termini. 47/[5′-^{32}P]-19-mer DNA template-primers containing an A:A, A:G, or A:C mismatch and 47/[5′-^{32}P]-20-mer with a T:T mispaired terminus were enzymatically synthesized as described above. The extension reactions were initiated with the WT and mutant HIV-1 RTs in the presence of all four dNTPs at 20 μM concentration. The reaction mixture contained 5 nM of the labeled template-primer containing one of the four mispaired termini, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 100 μg of BSA/mL, 20 μM each of the four dNTPs, 5 mM $MgCl_2$, and 4 nM of the WT or its mutant derivatives in a final volume of 15 μL. Incubations were carried out at room temperature, and 3 μL samples were withdrawn at desired time intervals and mixed with equal volumes of 88% formamide containing 40 mM EDTA, 0.1% SDS, and 0.014% each of bromophenol blue and xylene cyanole. The products of the extension reaction were analyzed on a denaturing polyacrylamide−urea gel and quantitated by measuring the amount of radioactivity present in the extended products. The ratio of radioactivity in the original substrate to that in the extended product provided an estimate of the extension reaction by the individual enzyme.

Cross-Linking of the Enzyme to Template-Primer. The cross-linking of the enzyme to DNA was carried out as described previously (Pandey et al., 1994a,b). Primarily, the 47/18 template-primer was used (see Chart 1) in this study;

however, the results were confirmed with a self-annealing DNA template-primer (37-mer TP, Chart 1). The [5′-^{32}P]-labeled 18-mer primer was appropriately diluted with nonradioactive 18-mer and annealed with equimolar amounts of the unlabeled 47-mer template.

Nucleotidyltransferase Activity of E−TP Covalent Complex. Nucleotidyltransferase activity of the enzyme containing covalently cross-linked template-primer was carried out as described previously (Pandey et al., 1994b). A 15 pmol amount of the enzyme was cross-linked with 25 pmol of the unlabeled TP in a standard irradiation mixture containing 50 mM Tris-HCl, pH 8.0, 1 mM DTT, and 5 mM $MgCl_2$ in a final volume of 50 μL. The nucleotidyltransferase reactions were initiated by the addition of 10 μCi of complementary [α-^{32}P]-dNTP at 0.5 μM concentration. The reaction mixture was incubated for 30 min at room temperature and terminated by the addition of 1% SDS and 20 mM EDTA. An aliquot of the reaction mixture was subjected to SDS−polyacrylamide gel electrophoresis followed by autoradiography. The radioactivity associated with the E−TP covalent complex was determined by Cerenkov counting after excising the radioactive band from the gel. In order to show that the nucleotide addition to the cross-linked DNA was catalyzed by the enzyme species in the covalent complex and not by the free enzyme, enzyme−TP covalent complexes were purified as follows: Scaled-up (20×) irradiated samples were loaded on DEAE-Sephadex column (0.5 mL) pre-equilibrated with 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 200 mM NaCl, and 5% glycerol. Following extensive washing of the column with the same buffer to remove the uncross-linked free enzyme, the enzyme−TP covalent complexes were recovered from the column by elution with 1.0 M NaCl in the same buffer. The eluate was desalted and concentrated using Centriprep-30. The final preparation was free of the uncross-linked enzyme, as judged by the lack of activity on the externally added synthetic template-primer. When the non-cross-linked template-primer−enzyme complexes were processed as per the same procedure, the entire enzyme activity was recovered in the 200 mM salt wash while no detectable activity was associated with the 1.0 M eluent.

Processivity Measurements. We used the 18/47-mer primer-template as well as poly (rA)·$(dT)_{18}$ for a qualitative measurement of the processivity of DNA synthesis by the mutant and the wild type enzymes. A 5 pmol amount of the 18-mer primer was 5′-end-labeled with [γ-^{32}P]-ATP (Ausubel et al., 1987) and annealed with 5 pmol of 47-mer template. The appropriate enzyme was first incubated with the labeled TP in a reaction mixture containing 40 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 2 nM primer termini, and 100 nM of the wild type or mutant enzyme in a total volume of 3 μL. After incubation for one min at room temperature the polymerase reaction was initiated by the addition of 3 μL solution containing 200 μM dNTPs (50 μM each) and calf thymus DNA trap (Joyce, 1989) equivalent to approximately 50 μM primer termini, 40 mM Tris-HCl, pH 7.5, and 5 mM $MgCl_2$. The reaction was allowed to continue at 25 °C, and 2.5 μL samples were withdrawn after 1 and 10 min and subsequently mixed with 4 μL of a stop solution containing 40 mM EDTA, 0.33% SDS, 0.014% each of bromophenol blue and xylene cyanole, and 85% formamide. Products formed with the individual enzyme were analyzed on a denaturing 16% polyacrylamide gel containing 7 M urea followed by autoradiography of the gel.

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

enzyme	template-primer	units/mg of protein ($\times 10^4$) average	units/mg of protein ($\times 10^4$) range	percent of wild type activity
WT	poly (rA)·$(dT)_{18}$	1.1	0.98−1.45	100
	poly (rC)·$(dG)_{18}$	0.2	0.19−0.22	100
	poly (dC)·$(dG)_{18}$	0.8	0.75−0.86	100
M184V	poly (rA)·$(dT)_{18}$	2.5	2.4 −2.6	227
	poly (rC)·$(dG)_{18}$	0.3	0.36−0.25	150
	poly (dC)·$(dG)_{18}$	2.2	1.9−2.5	275
M184A	poly (rA)·$(dT)_{18}$	2.6	2.5−2.7	236
	poly (rC)·$(dG)_{18}$	0.28	0.26−0.3	140
	poly (dC)·$(dG)_{18}$	2.4	2.2−2.6	300

[a] The specific polymerase activity of WT HIV-1 RT and its mutant derivatives was determined with the indicated template-primers at saturated dNTP concentrations as described in Materials and Method. One unit is defined as the amount of enzyme activity necessary to incorporate 1 nmol of dNMP into acid insoluble form in 10 min at 37 °C.

RESULTS

Construction and Purification of the Mutant Enzymes. The M184A and M184V mutants of HIV-1 RT were constructed and expressed in *E. coli* as described before (Sarafianos et al., 1995a,b; Pandey et al., 1994a; Desai et al., 1994). The rationale for these mutations is that M→V mutation is known to occur naturally in response to 3TC monotherapy (Tisdale et al., 1993; Schinazi et al., 1993; Schuurman et al., 1995) whereas M→A mutation represents a mutant derivative without a functional side chain at position 184. The purified heterodimeric (p66/p51) enzyme preparations were found to be more than 95% pure. The level of their expression, solubility, and yield, and their chromatographic characteristics were identical with those of the wild type enzyme, suggesting that substitution at these positions did not cause any perturbation in the enzyme structure. All the mutants were RNase-H positive and showed a heat inactivation pattern identical to the WT HIV-1 RT (results not shown), thus providing additional evidence that these mutations had not altered the folding pattern of the protein.

Specific DNA Polymerase Activities and Kinetic Parameters of the Mutant Enzyme. Earlier reports have shown that M→Y, M→L, or M→G substitution at position 184 of HIV-1 RT results in drastic reduction in the polymerase activity as compared to the wild type (Larder et al., 1989; Boyer et al., 1992; Wakefield et al., 1992), whereas 45%−100% of WT activity was reported for M→V and M→A substitutions depending upon the template-primer used (Chao et al., 1995; Boyer & Hughes, 1995). To assess the effect of the different substitution that we had introduced, we evaluated the polymerase activity of M184A and M184V mutants with different template-primers. Table 1 shows the specific activities of the WT enzyme and its mutant derivatives with the poly (rA)·(dT)18, poly (rC)·$(dG)_{18}$, and poly (dC)·$(dG)_{18}$ template-primers. Both M184A and M184V mutants exhibited 1.5−3-fold higher specific polymerase activity compared to the WT enzyme (Table 1).

The kinetic parameters of M184A and M184V mutants determined using homopolymeric poly (rA)·$(dT)_{18}$, poly (rC)·$(dG)_{18}$, and poly (dC)·$(dG)_{18}$ template-primers also indicated a 2−5-fold increase in k_{cat} (Table 2). However, the ratio of efficiency of the polymerization reaction

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

template-primer and dNTP substrate	enzyme	$K_{m_{dNTP}}$ (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	ratio, mutant/WT
poly (rA)·$(dT)_{18}$ (dTTP)	WT	2.16 ± 1.5	0.17 ± 0.09	7.8×10^4	
	M184A	13.40 ± 3.6	0.64 ± 0.15	4.7×10^4	0.6
	M184V	2.40 ± 0.5	0.62 ± 0.12	2.5×10^5	3.2
poly (rC)·$(dG)_{18}$ (dGTP)	WT	2.60 ± 1.2	0.27 ± 0.11	1×10^5	
	M184A	5.90 ± 2.4	0.56 ± 0.20	9.3×10^4	0.93
	M184V	8.30 ± 2.1	0.62 ± 0.14	7.5×10^4	0.75
poly (dC)·$(dG)_{18}$ (dGTP)	WT	1.90 ± 1.2	0.30 ± 0.07	1.6×10^5	
	M184A	3.10 ± 1.7	1.25 ± 0.06	4.0×10^5	2.53
	M184V	2.68 ± 0.6	1.48 ± 0.20	5.4×10^5	3.41

[a] The steady-state kinetic parameters for the WT HIV-1 RT and its mutant derivatives were measured with indicated template-primers and corresponding dNTP substrate as described in Materials and Methods. The purified mutant and WT enzymes were used in heterodimeric form (p66/p51). Please note that these determinations were carried out at subsaturating concentration of the respective dNTP substrates.

catalyzed by the mutants versus the WT enzyme varied from 0.6 to 3.4 with poly (rA)·$(dT)_{15}$ and from 2.5 to 3.4 with poly (dC)·$(dG)_{18}$. The highest efficiency of polymerization (k_{cat}/K_m) was seen by the M184V mutant, the increase being 3−4-fold on both poly (rA)·$(dT)_{18}$ and poly (dC)·$(dG)_{18}$ template-primers. With poly (rC)·$(dG)_{18}$ the efficiency of M184V was found to be slightly reduced due to a marginal increase in the K_m for dGTP substrate. Similar results were also obtained with M184A mutant except with poly (rA)·$(dT)_{18}$, where its efficiency of polymerization was significantly compromised due to reduced affinity for dTTP substrate (6-fold increase in K_m), in spite of higher k_{cat} of polymerase reaction. This preference for only DNA−DNA template-primer may well explain the lower infectivity observed for M184A virus as compared to M184V or WT virus (Wakefield et al., 1992).

Effect of M184A and M184V Mutations on the Formation of E−TP Binary Complexes. The increased efficiency of polymerization seen with M184A and M184V prompted us to ascertain whether these mutant enzymes have greater affinity for the template-primers. Direct photochemical cross-linking of RNA−DNA template primer [poly (rA)·[5′-^{32}P]-$(dT)_{18}$] and DNA−DNA template primer (47/18-mer) to the mutant and wild type enzymes was performed by UV irradiation of the E−TP complex under standard conditions (Pandey et al., 1987, 1994b), and the extent of E−TP covalent complex formed was analyzed by SDS polyacrylamide gel electrophoresis. Results shown in Figure 1 indicate no change in the E−TP binary complex formation with both the mutants.

Fidelity of DNA Synthesis. Mutation at position 184 (M184V) has been shown to confer 3TC, FTC, ddC, and ddI resistance phenotype (Gu et al., 1992; Schinazi et al., 1993; Tisdale et al., 1993; Boucher et al., 1993; Schuurman et al., 1995). We further observed that resistance of M184A to ddITP is 5 times greater than that of M184V (results not shown). The degree of discrimination against ddNTP analogs by HIV-1 RT mutants may be determined largely by the residues surrounding the dNTP binding pocket. Since M184 resides very close to the catalytic aspartate residues in the palm subdomain, it may be in a position to directly or indirectly affect dNTP binding. Alternatively it may participate in the positioning of the primer terminus, thereby influencing the fidelity of DNA synthesis by discriminating against the mismatched nucleotide. To assess these possibilities, we carried out experiments to measure the rates of synthesis and extension of the various mispairs by these

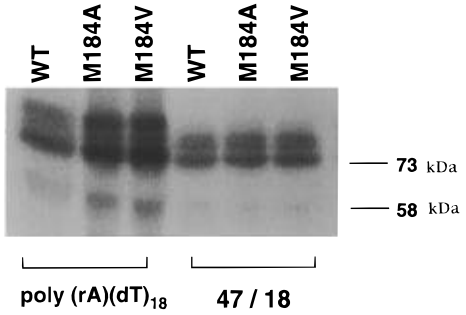


FIGURE 1: Photochemical cross-linking of RNA−DNA and DNA−DNA template primers to M184A and M184V mutants and to the wild type HIV-1 RT: 512 nM of WT HIV-1 RT and its mutant derivatives were incubated on ice with poly (rA)·$(dT)_{18}$, or 47/18-mer at a concentration of 200 nM with respect to the [5′-^{32}P]-labeled primer termini. The incubation buffer contained 20 mM Tris-HCl, pH 7.5, 1 mM DTT, and 2 mM $MgCl_2$ in a reaction volume of 50 μL. The mixtures were irradiated in the UV-Spectrolinker and subjected to SDS−PAGE and autoradiography as described before (Pandey et al., 1994b; Desai et al., 1994). The extent of E−TP covalent complex formation was estimated either by determining the Cerenkov CPM of the excised radioactive gel piece or by densitometric scanning of the autoradiogram.

mutants and compared them with those of the wild type enzyme. Synthesis catalyzed by the WT and mutant HIV-1 RT was carried out on 47/[5′-^{32}P]-18-mer template-primer in the presence of only three dNTPs so as to examine the pattern of misincorporation at the template position complementary to the missing dNTP. Four separate reactions were carried out, and in each of which one of the dNTPs was omitted. The results are as shown in Figure 2, Lanes 1−4 in each panel represent the reaction conditions in which dATP, dTTP, dGTP, and dCTP, respectively, were omitted in order to assess the extent of misincorporation against T, A, C, and G template nucleotides. In all reactions with the wild type and mutant enzymes, a substantial accumulation of the DNA product at the site corresponding to the missing nucleotide was clearly seen. Further extension of a part of the product into longer product under these conditions was also evident. However, the extent of conversion into longer products under these conditions was clearly different with different mutant enzymes. These results gave some indication of the extent of errors committed by the WT and its mutant derivatives. As can be seen from Figure 2, mismatched products that accumulate opposite T and A template bases are poorly extended by the WT and M184V mutant in the presence of the three dNTPs, whereas M184A mutant could further extend the mispaired products up to 12−20

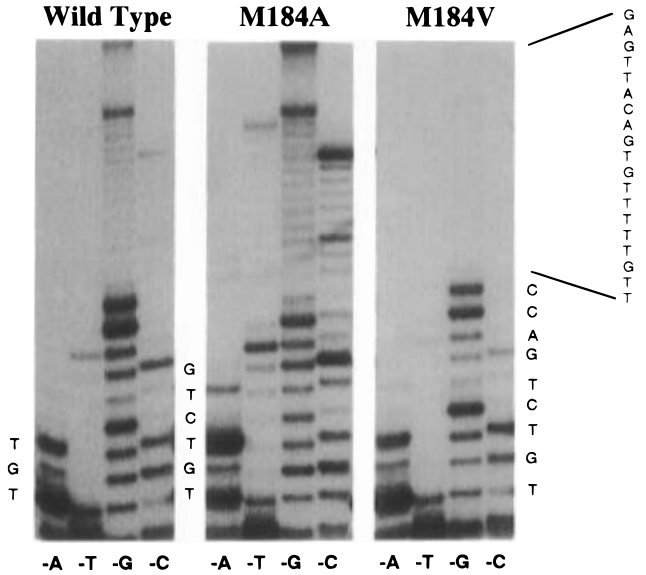


FIGURE 2: Misinsertion and subsequent extension of the mismatched primer in the presence of 3 dNTPs. The [5′-^{32}P]-labeled 18-mer primer annealed with 47-mer template was used to assess the extent of misinsertion and extension. 25 nM WT HIV-1 RT and the mutant enzymes was incubated with 2 nM labeled template-primer in the presence of only three dNTPs as described in Materials and Methods. At the end of the incubation the reaction products were analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. The sequence of the single-stranded template is as shown to the right. The lanes marked as −A, −T, −G, and −C represent the missing fourth nucleotide from the dNTP mix in the respective set of experiment.

nucleotides (Figure 2, lane −A and −T in each set). A high rate of extension of the mispaired products opposite C and G template nucleotides was also seen with both M184A and the WT enzyme. In fact both of these enzyme species extended the mispaired terminus to full-length products, even under the condition in which one of the four nucleotides was missing from the reaction (Figure 2, lanes labeled −G and −C). An approximate quantitation of the misincorporation and their subsequent extension as determined by densitometric scanning of the autoradiogram is shown in Table 3. As seen from the table, M184A mutant seems to be more error prone than the WT enzyme as judged by the extent of misincorporation as well as subsequent extension of mismatched termini. Most interestingly, the M184V mutant is not as efficient as the M184A and the WT enzyme in mismatch insertion and extension. Most of the products synthesized by M184V accumulate opposite C template (4th , 8th, and 9th positions) in the absence of dGTP and opposite G template (Figure 2, lane −G, 3rd and 6th positions) in the absence of dCTP (lane −C). These results indicate that fidelity of DNA synthesis by HIV-1 RT may be influenced by the residue at position 184. Interestingly, Murine leukemia virus reverse transcriptase (MuLV-RT) having Val in place of Met in the YXDD motif exhibited a similar misincorporation and extension pattern as that shown by the M184V mutant of HIV-1 RT (Figure 2).

Kinetic Parameters for Incorporation of Correct and Incorrect Nucleotide(s). The above results showing extension of [5′-^{32}P]-18/47-mer primer-template in the presence of only three dNTPs (Figure 2 and Table 3) have suggested that M184V mutant enzyme may catalyze less error-prone DNA synthesis than the WT enzyme and M184A mutant. In order to confirm this we have determined the kinetic

Table 3: Misinsertion and Subsequent Extension of Mispaired Termini[a]

template base	missing dNTP substrate	primer sequence and position of the first misinsertion	% of primer termini misinserted and subsequently extended by: wild type	M184A	M184V
T	dATP	**TGC**TX(CAGACTGG)-3′	22	34	15
A	dTTP	**TGC**X(ACAGACTGG)-3′	25	40	10
C	dGTP	**TGC**TACAX(ACTGG)-3′	63	69	39
G	dCTP	**TGC**TAX(AGACTGG)-3′	36	51	10

[a] The percent of the primer termini misinserted and subsequently extended by the WT HIV-1 RT and its mutant derivatives was determined by densitometric scanning of the autoradiogram from the experiment shown in Figure 2. The sequence of the primer strand in bold is part of the duplex region preceding the primer terminus. X indicates the position of the first misinsertion in the absence of the corresponding dNTP in the reaction mixture. The sequences beyond the mismatch position are shown in parentheses. The total amount of product accumulated at and beyond the first misinsertion position was quantitated to determine the percent of misinserted primer molecules.

parameters for correct and incorrect nucleotide incorporation opposite dA template base using 18/47-mer as the primer template. The results are shown in Table 4. The M184V mutant exhibited the highest affinity for the correct nucleotide (dTTP) followed by the WT enzyme and M184A mutant. In contrast, the K_m values for incorrect nucleotide (dATP, dGTP, and dCTP) were highest for M184V and lowest for M184A mutant with intermediate values for the WT enyme. As expected, the highest catalytic efficiency (k_{cat}/K_m) for correct nucleotide and lowest for incorrect nucleotides observed with M184V mutant indicates the highest mismatch selectivity (ratio of k_{cat}/K_m for correct versus incorrect) by this mutant.

Extension of Purine−Purine, Purine−Pyrimidine, and Pyrimidine−Pyrimidine Mispair. In order to confirm the patterns of misinsertion and subsequent extensions of the mispaired primer termini shown in Figure 2, we examined the extension reactions of the defined mispairs with the WT and the mutant enzymes. Using template-primer containing pur−pur, pyr−pyr, or pur−pyr mispaired termini, we determined the rates of extension of the mismatched base pairs. Reaction conditions adapted for the extension reactions were similar to those used in the insertion reactions. As seen in Figure 3, A:C and A:A mispairs were least utilized as compared to T:T or A:G mispairs by all the three enzymes. However, the magnitude of mispair extension reaction catalyzed by M184V mutant is significantly lower than the WT and M184A mutant. The maximal misinsertion occurred with M184A mutant followed by the WT enzyme. The rate constants determined from these data clearly suggest that M184V has the lowest rate for all the mispair extension reaction (Table 5). The maximal rate of misinsertion occurred with M184A mutant followed by the WT enzyme.

We suspected that the M184V mutant enzyme, not being able to extend the mispaired primer terminus as effectively as the WT and M184A mutant enzymes, may have comparatively less binding affinity for such mispaired template-primers. In order to examine this possibility, we enzymatically synthesized different combinations of the mispaired template-primer and compared their binding affinity by the mutant and the WT enzymes by photochemical cross-linking and found it to be true (Figure 4). As shown in the figure, the WT and M184A show similar affinity for all the

Table 4: Kinetic Parameters for Correct (dTTP) and Incorrect Nucleotide (dATP, dGTP, and dCTP) Incorporations on 47/18-mer Template-Primer (Opposite dA Template Base) by WT HIV-1 RT, M184A, and M184V Mutant Enzymes[a]

correct and incorrect dNTP	enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	mismatch selectivity (ratio of k_{cat}/K_m of correct versus incorrect nucleotide)
dTTP	WT	10.5 ± 2.5	0.10 ± 0.04	9.5×10^3	
(correct)	M 184A	20 ± 5.0	0.17 ± 0.025	8.5×10^3	
	M 184V	1.4 ± 0.6	0.15 ± 0.03	1.0×10^5	
dATP	WT	32 ± 6.0	0.09 ± 0.05	2.8×10^3	3.4
(incorrect)	M184A	25 ± 4.5	0.08 ± 0.02	3.2×10^3	2.6
	M184V	78 ± 12.0	0.05 ± 0.02	0.64×10^3	156.0
dGTP	WT	45 ± 9.0	0.11 ± 0.07	2.4×10^3	4.0
(incorrect)	M184A	30 ± 3.0	0.04 ± 0.01	1.3×10^3	6.5
	M184V	51 ± 5.0	0.05 ± 0.015	0.98×10^3	102.0
dCTP	WT	67 ± 11.0	0.05 ± 0.01	0.78×10^3	12.0
(incorrect)	M184A	51 ± 15.0	0.04 ± 0.02	0.84×10^3	10.1
	M184V	208 ± 30.0	0.05 ± 0.01	0.24×10^3	416.0

[a] The steady-state kinetic parameters for correct and incorrect nucleotide incorporation on heteromeric 47/18 template-primer by the WT HIV-1 RT and its mutant derivatives were determined as described in the Materials and Methods. The purified mutant and WT enzymes were used in heterodimeric form (p66/p51).

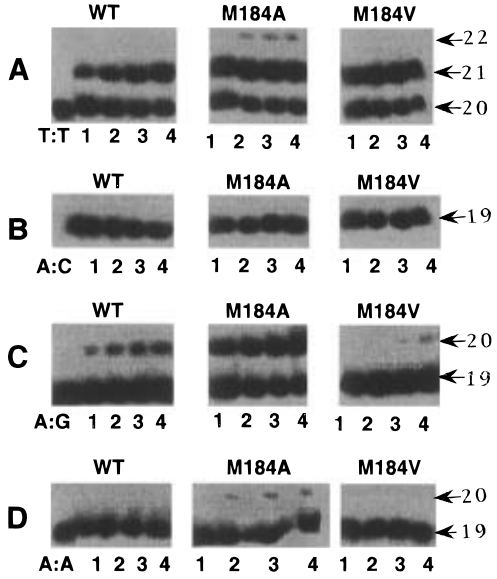


FIGURE 3: Extension of mispaired primer termini. The 47/19-mer template primer containing an A:A, A:G, or A:C mispaired terminus and 47/20-mer with a T:T mismatched terminus were prepared and used to assess the extension of each mispairs as described in Materials and Methods. The template-primer containing the mispair terminus was incubated with WT and the mutant enzymes in the presence of all four dNTPs. Samples were removed at different time intervals and analyzed on denaturing polyacrylamide urea gel. Lanes 1−4 of each panel represent the reaction products at 5, 10, 15, and 20 min of incubation, respectively. The lanes marked at T:T, A:C, A:G, and A:A to the left of each panel represent the mispaired template-primer as the starting substrate.

mispaired TPs, while M184V shows selective affinity for pyr−pyr mismatches and very poor affinity for pur−pur mispaired termini. In the autoradiogram we have consistently seen a minor band appearing below the 63 kDa band. This may be due to the presence of trace amounts of proteolytic fragment of the enzyme, smaller than 51 kDa which has retained the ability to bind/cross-link DNA template-primer. Nevertheless, the cross-linking results are consistent with the observation that the M184V mutant enzyme can extend pyr−pyr (T:T) mispair better than pur−pur (A:A, A:G) mismatches.

Effect of M→A and M→V Substitution on the Processivity of DNA Synthesis. The highest specific polymerase activity as well as fidelity and efficiency of catalysis observed with the M184V mutant suggested that this mutant enzyme may

Table 5: Rate (min^{-1}) of Extension of A:A, A:G, A:C, and T:T Mismatched Template-Primer by Wild Type and Mutant HIV-1 RT[a]

enzyme	A:A	A:G	A:C	T:T
WT HIV-1 RT	$<1 \times 10^{-4}$	3×10^{-2}	$<1 \times 10^{-4}$	0.35
M184A	2×10^{-3}	0.45	$<1 \times 10^{-4}$	0.55
M184V	$<1 \times 10^{-4}$	1×10^{-2}	$<1 \times 10^{-4}$	0.25

[a] The first-order rate constants were determined from an experiment of the type shown in Figure 3. A plot of the log of mismatched primer remaining unutilized versus time gave the slope equal to the apparent first order rate constant for the extension of the individual mismatched base pairs.

be more processive than M184A and the WT enzyme. Processivity is the probability of translocation of the polymerase enzyme along the template and represents the average number of cycles of nucleotide addition during a single enzyme DNA encounter. The DNA substrates were prepared by annealing [5′-^{32}P]-labeled $(dT)_{18}$ and 18-mer heteromeric oligo annealed with poly rA and 47-mer DNA, respectively. Wild type HIV-1 RT and its mutant derivatives were first incubated with the labeled template primer and then supplemented with dNTPs and DNA trap to initiate the polymerization reaction. DNA trap present in the reaction would prevent the enzyme from binding to the same labeled TP molecule from which it has dissociated after completing one cycle of processive synthesis (Figure 5A,B, lanes 1 and 2). In the absence of DNA trap, the labeled products ranging from 19-mer and longer, resulting from both processive and nonprocessive synthesis, could be seen (Figure 5A,B, lanes 3 and 4). Both the mutant enzymes as well as the WT enzyme were found to be highly processive with both template-primers as judged by equal extension of the 18-mer primer within 60 s of incubation (Figure 5A,B, lanes 1 and 2). The intensity of each labeled product remained same even upon 10 min incubation, suggesting that most of the enzyme molecules, after completing synthesis ranging from one to hundreds of processive cycles, dissociated from the labeled TP and remained effectively tied to the DNA trap. Our results are at variance from those reported by Boyer and Hughes (1995) in which slightly lower processivity of DNA synthesis by M184V enzyme was observed. Somewhat unusual conditions used by the authors for the processivity determination may be responsible for the different results. For example, unrestricted synthesis was allowed for 5 min at 25 °C followed by addition of DNA trap and then

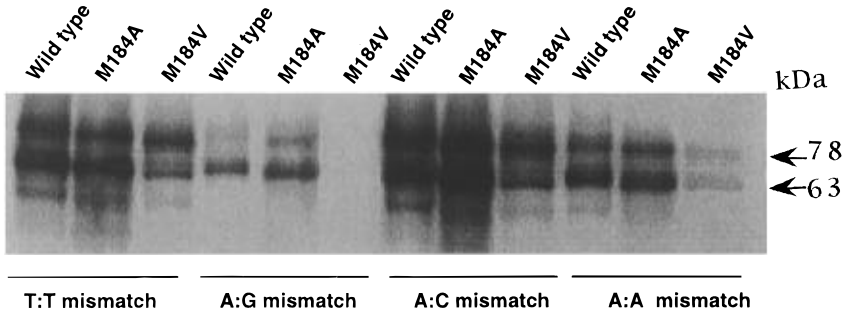


FIGURE 4: Qualitative assessment of the binding affinity of WT HIV-1 RT and its mutant enzymes (M184A, M184V) to various mispaired template-primers. The mismatched template primers were prepared by converting a [5′-^{32}P]-labeled self-annealing 37-meric template-primer (see Chart 1) into 38-meric product containing an A:G, A:C, A:A mismatched terminus and 39-mer product with a T:T terminus using Klenow fragment and respective single-dNTP substrate as described in Materials and Methods. Individual enzyme protein (500 nM) incubated with the labeled template-primer (100 nM) containing the indicated mispaired terminus was irradiated in the Spectrolinker, and the extent of E−TP covalent complexes formed was analyzed by SDS−PAGE as described before.

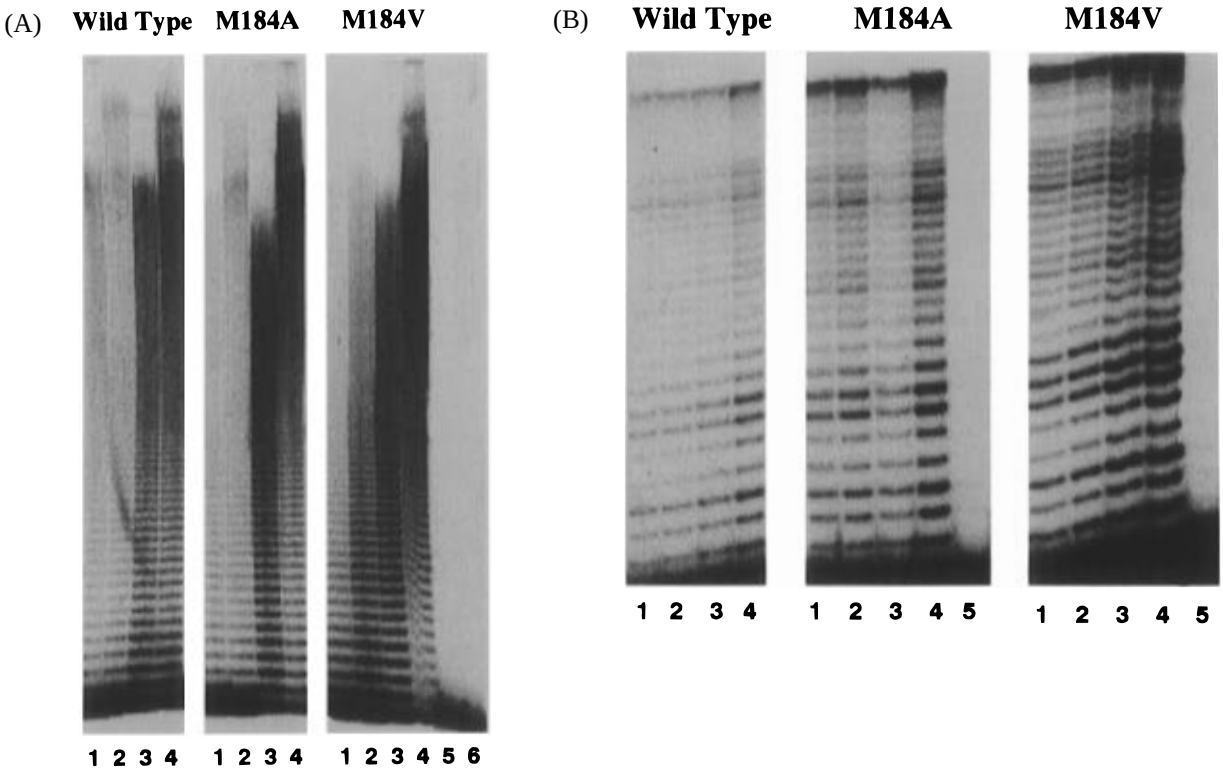


FIGURE 5: Effect of M184A and M184V mutations on the mode of DNA synthesis on RNA−DNA and DNA−DNA template primers. The [5′-^{32}P]-labeled dT_{18} annealed with poly rA and heteromeric 18-mer primer annealed with 47-mer template were used as RNA−DNA (A) and DNA−DNA (B) template primers, respectively. Following incubation of the individual enzyme with the labeled TP, the reaction was initiated by the addition of 50 μM each of the four dNTPs (or 200 μM TTP with poly rA directed reaction) and calf thymus DNA trap (see Materials and Methods). Each set represents an experiment with the wild type, M184A, or M184V mutant enzyme. In lanes 1 and 2 of each set the 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 3 and 4 represent the DNA synthesis occurring on the labeled template-primer in the absence of DNA trap for 1 and 10 min, respectively. In lane 5, the DNA trap was added to the labeled template primer before the binding step.

incubation was continued for additional 5 min at 37 °C. In addition, the homodimeric form of mutant enzyme and a high salt concentration (150 mM KCl) were used in these investigations.

Incorporation of Correct and/or Incorrect Nucleotide on the Template-Primer Covalently Immobilized in the Polymerase Domain. We have earlier shown that DNA template-primer covalently cross-linked in the polymerase domain of the Klenow fragment (Pandey et al., 1994b) as well as that of HIV-1 RT (Sarafianos et al., 1995a,b) can be effectively used as the substrate for the addition of a single nucleotide on the immobilized primer terminus. It was therefore, interesting to examine whether the dNTP binding pocket in the E−TP covalent binary complexes of the mutants and the WT enzyme are rigid and stringent to accommodate only the correct nucleotide in the ternary complexes or they are flexible enough to take up either the correct or the incorrect nucleotides in the pocket. We therefore, prepared the covalent E−TP complexes and assessed their nucleotidyltransferase activity in the presence of [α-^{32}P]-labeled dTTP (correct nucleotide), or [α-^{32}P]-labeled incorrect dNTP (dGTP, dATP, or dCTP). Binding and subsequent incorporation of the labeled dNTP (correct or incorrect) onto the immobilized template-primer (E−37-mer TP) result in the labeling of E−(template-primer) complex, which migrates at the 78 kDa position upon SDS polyacrylamide gel

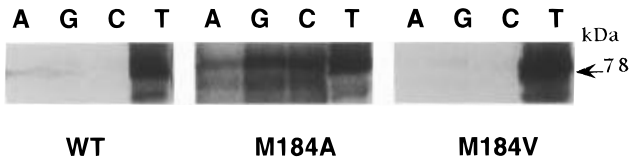


FIGURE 6: Nucleotidyltransferase activity of E−TP covalent complexes of wild type HIV-1 RT and its M184A and M184V mutants and their ability to incorporate correct and incorrect nucleotide corresponding to first template base. The individual enzyme protein was cross-linked with unlabeled 37-meric self-annealing template-primer as described in the Materials and Methods. The resultant E−TP covalent complexes were incubated in the presence of either 2 μM of [α-^{32}P]-dTTP corresponding to the first template base A (Lane T) or 2 μM of incorrect nucleotide dATP, dCTP, or dGTP (lanes A, C, and G). The reactions were carried out at 37 °C for 30 min. Additions of correct or incorrect nucleotide corresponding to the first template base were analyzed by SDS−PAGE and autoradiography as described in Materials and Methods.

electrophoresis (Figure 6). A minor band migrating at approximately the 63 kDa position was also seen in the autoradigram. This band may represent trace amounts of the catalytically active E−TP covalent complexes of the 51/51 homodimeric form of the enzyme present in the enzyme preparations. As shown in the figure, selection of dNTP by E−(template-primer) complex of M184V mutant is highly stringent in catalyzing the nucleotide addition as only the correct nucleotide (dTTP) was added (lane T) while none of the incorrect dNTPs could be utilized in the *in situ* nucleotidyltransferase reaction. In contrast, the E−TP covalent complexes of M184A did not display high stringency as they bound and catalyzed the incorporation of both the correct (lane T) and incorrect dNTPs (lanes A, C, and G) on to the immobilized template-primer. However, incorporation of dG and dC nucleotides opposite the T template was found to be prominent of all the three incorrect dNTPs by M184A mutant. Incorporation of the incorrect nucleotide by the WT enzyme was also seen albeit with less efficiency as compared to the correct nucleotide.

DISCUSSION

Our data indicate that one of the major roles of Met 184 of HIV-1 RT is to help copy the viral genome with moderate fidelity, thereby ensuring the relatively high mutation rates which results in the constantly changing virus progeny. Met 184 is the second residue of the highly conserved YXDD motif, present in the RNA dependent DNA polymerase class of enzymes (Johnson et al., 1986). Two of the four residues in this motif following Met 184 are Asp 185 and Asp 186, which have been shown to be crucial for polymerase function of the enzyme (Larder et al., 1989; Le Grice et al., 1991). The residue preceding Met 184 in this motif is Tyr 183 which has also been found to be indispensable for the catalytic function as both conservative (Y→F) and nonconservative (Y→S) mutations are deleterious for the polymerase function of the enzyme (Larder et al., 1987, 1989). Substitution of Met→Tyr, Met→Leu, Met→Ile, Met→Pro, or Met→Gly at position 184 has been reported to inactivate 80%−100% of the polymerase activity, whereas Met→Val and Met→Ala mutants had WT activity (Larder et al., 1989; Boyer et al., 1992; Wakefield et al.,1992, Chao et al., 1995). In the 3-D crystal structure of HIV-1 RT Met 184 is located on β9−β10 hairpin in the palm subdomain of the large catalytic cleft in the polymerase domain (Kohlstaedt et al., 1992; Jacobo-Molina et al., 1993). The structural data from the RT−DNA binary complex suggest that M184 interacts with the sugar moiety of the 3′-terminal primer nucleotide, and therefore the effects of mutation on the dNTP binding site may be indirect (Tantillo et al., 1994). Among the naturally occurring mutants at codon 184 as well as those generated by site-directed mutagenesis, none of them has been characterized in detail and most of the data available on these are limited to examination of polymerase activity in crude enzyme preparations. Further, the polymerase activity reported for these mutant enzymes differs from different laboratories. This may probably be due to different levels of enzyme present in the crude preparations or *in situ* degradation of RNA−DNA template primers in the crude extracts.

In order to probe the role of M184 in the catalytic mechanism of RT, we generated two point mutants at this position (M184A, M184V) and extensively characterized their properties. Steady-state kinetic analyses of these enzymes, show that these mutants are more efficient catalyst than the WT enzyme on poly (dC)·(dG)$_{15}$ template primer (Table 2). This was rather surprising since near-complete lack of activity was reported with other M184 mutants, such as M184Y (Boyer et al., 1992), M184G and M184P (Wakefield et al., 1992), and the conservative M184L mutant (Larder et al., 1989). The above results suggest that the size and bulk of the 184 side chain may be a major factor in determining the catalytic activity of the enzyme. The long and bulky side chains, as in the case of tyrosine and leucine, seem to be detrimental to the polymerase activity, whereas the presence of valine, alanine, or methionine with smaller side chains exhibits no inhibitory effect on the activity. The deleterious effect of M→P and M→G substitution on the polymerase activity may have been due to alteration in the β9−β10 hairpin structure. Most interestingly, the difference in the efficiency with which M184A and M184V mutant catalyze certain mismatch syntheses gives some insight into the importance of this residue in the fidelity of DNA synthesis by HIV-1 RT. The mutant M184A having the shortest side chain at this position is a highly error-prone enzyme without having compromised its efficiency of carrying out polymerization. It exhibited a lowest mismatch selection (Table 4) resulting in high rate of misincorporation and subsequent extension of the mismatched primer terminus. In contrast, M184V with a side chain, smaller than Met but larger than Ala, was found to exhibit the highest mismatch selection and therefore, lowest rate of misincorporation and extension of the mismatched termini (Figures 2 and 3, and Tables 3−5).

The residue at this position could conceivably affect the activity by steric effects. For example, the side chain of a substituent amino acid may prevent the correct alignment of the 3′-O of the primer terminus with the α-phosphate moiety of dNTP. This in turn would disturb the positioning of the complementary template strand that is known to form part of the dNTP binding pocket. This type of "template effect" is proposed to be responsible for the ddNTP inhibitor resistance of certain HIV-1 RT mutants (Boyer et al., 1994; Tantillo et al., 1994), even though the actual mutation sites are distant from the actual dNTP binding pocket. As stated before in the cocrystal structure of DNA and HIV-1 RT, the side chain of Met 184 has been reported to be in the vicinity of the deoxyribose moiety of the terminal nucleotide of the primer strand. In order to assess the contribution of met

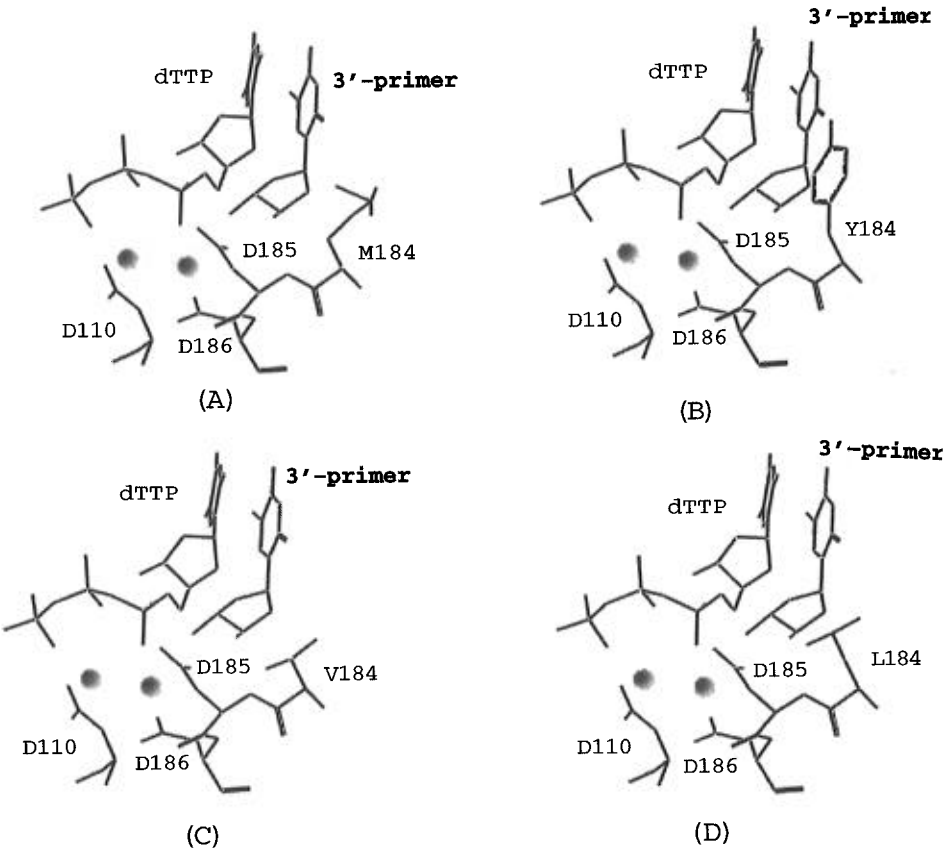


FIGURE 7: Molecular model of the catalytic region of the ternary complex of HIV-1 reverse transcriptase showing the suggested interaction of Met 184 (panel A), Tyr 184 (panel B), Val 184 (panel C), and Leu 184 (panel D) with the 3′-primer nucleotide. The entire backbone structure of HIV-1 RT including the side chains was completed using Cα coordinates of DNA-bound HIV-1 RT [PDB file 1HMI, Jacobo Molina et al. (1993)] and SYBYL modeling package as described before (Yadav et al., 1992, 1994). A duplex DNA in the A form comprising the AAA/TT sequence was modeled from the P atom positions as seen in the crystal structure using the algorithm of Arnott and Hukins (1972). The ternary complex was generated by orienting the base moiety of dTTP substrate in a Watson−Crick base-pairing mode and placing the Pα at approximately 3 Å distance from the 3′-O of the primer terminus and β and γ phosphates at an interacting distance (through Mg^{2+}) from the oxygens of the catalytic carboxylate triad, D110, D185, and D186 (details of the modeling protocol by Yadav et al. are to be published elsewhere). The Mg^{2+} ions are shown as two closed circles. The resulting pre-polymerization ternary complex was used to examine the position and possible interaction of the side chain of Met 184 in the catalytic center. The sugar moiety of the primer nucleotide is seen as the most proximal entity, suggesting a hydrophobic interaction with the methionine side chain. Methionine monomer in the model was then replaced by Tyr, Val, and Leu followed by a systematic conformational search in the absence of the substrates. The most favorable conformation of the side chains was selected in each case, and the substrates were then inserted in the same position and orientation as present in the prepolymerase ternary complex. Panel A shows the position of Met 184 which is in the close vicinity of the 3′-primer nucleotide but there is no specific interaction with the primer nucleotide. In panels B and D, Tyr and Leu side chains (M184Y, M184L) create a steric hindrance with the sugar moiety of 3′-primer nucleotide which is in accordance with the observation that the polymerase activity of these two mutants is reduced by 80%−90% (Larder et al., 1989; Boyer et al., 1992). However, in the case of Met 184 substitution to Val 184 (panel C), the isopropyl group of valine is within 3 Å distance from the sugar moiety of the primer nucleotide, and this position is most appropriate for hydrophobic interaction. Differential stabilization effects of Met and Val on the position of primer terminus may therefore be responsible for the differences in the observed catalytic activity. However, in the absence of hydrophobic side chain at position 184 (as in case of M184A), polymerase reaction appears to proceed normally, albeit the fidelity of synthesis (loss of primer stabilization) is severely compromised. The hydrophobhic interaction of Val side chain with the sugar moiety may help stabilize the 3′-O of the primer strands in a proper position for the polymerization reaction. Since the 3′-primer nucleotide and the dNTP substrate/inhibitor binding positions are close to one another, the methionine mutation may have a role in discriminating between the incorporation of normal and modified dNTP substrate (inhibitor) by displacing the 3′-O of the primer strand alignment, which is crucial with respect to the Pα of dNTP for the polymerization reaction.

184 and other substituents in the polymerase reaction we used a model built ternary complex of HIV-1 RT−DNA−dNTP (Yadav et al., submitted) based on the Cα coordinates of the above refined binary complex. In the ternary complex, D110, D185, and D186 are shown to bind both dNTP and 3′-O of the primer terminus through metal coordination (Figure 7). This region also includes Y183 and M184, which appear to be involved in positioning of the primer terminus, as in the binary complex. In our modeling studies, we sought to explain the lack of activity of the M184Y and M184L mutants. A systematic computer search of all possible side chain conformations of the mutants in the absence of DNA revealed that the most stable structures for M184Y and M184L bring the Y184 or L184 side chains to a position that sterically hinders with the primer terminus (Figure 7). This hindrance may, in turn, disturb the side chain orientation of the catalytic carboxylates. Interestingly, substitution of M184 by Val and Ala results in mutants that are not sterically impeded. Furthermore, substitution of Met by Val places the isopropyl group of the latter at a favorable distance of 2.2−3 Å from the sugar moiety of the primer terminus. An optimal hydrophobic interaction between Val and the sugar moiety may stabilize the template-primer and the interrelated dNTP binding site, resulting in a higher fidelity. The increase in mismatch selection by 25−45-fold exhibited by M184V mutant with decreased affinity (high K_m values) for

incorrect nucleotide also supports this presumption (Table 4). This is not the case with WT and M184A mutants, providing a possible explanation for their decreased fidelity. The position and chemistry of the primer terminus nucleotide have been reported previously to be implicated in conferring fidelity during polymerization (Kuchta et al., 1988; Carrol et al., 1991). The "thumb" region of HIV-1 RT is known to be involved in the template-primer binding, and it has also been reported to influence the fidelity of DNA synthesis (Beard et al., 1994). The reverse transcriptase of another virus, MuLV, exhibits much higher fidelity of DNA synthesis than HIV-1 RT (Preston et al., 1988). Interestingly, MuLV RT contains Val at the X position of the YXDD motif (Johnson et al., 1986). The possibility that other residues in the vicinity of primer terminus and dNTP substrate have a similar function is currently being investigated.

An alternative scenario in which Met 184 interacts with the sugar moiety of substrate dNTP has not been ruled out. In fact a number of experimental observations support a direct role for Met 184 in the substrate binding function. A natural mutation at this position (M184V) has been shown to confer high levels of resistance to 3TC and FTC and relatively low levels of resistance to ddI and ddC (Schinazi et al., 1993; Tisdale et al., 1993; Boucher et al., 1993; Schuurman et al., 1995; Gu et al., 1992). These observations suggest a direct interaction of the 184 side chain with the sugar moiety of the dNTP substrate. Furthermore, the selective resistance to 3TC, FTC, ddI, and ddC inhibitors suggests that the 184 side chain may not interact uniformly with all dNTPs and dNTP-analog inhibitors. A systematic computer search of the possible M184 side chain conformations, in the presence of DNA, revealed that the methyl group of M184 could be placed as close as 3.0−3.5 Å from the deoxyribose ring of the dNTP substrate in the ternary prepolymerase complex, without any significant compromise in the stability of the complex (data not shown). Such a direct dNTP−M184 interaction does not preclude the primer terminus−M184 interactions, as the Cα and Cβ carbons of M184 remain proximal to the primer terminus ribose ring. Such dNTP−M184 interaction is absent in the case of M184A and M184V mutants and could explain the drug resistance of these mutants. Further support for the interaction of Met 184 with dNTP binding comes from the comparison of the 3-D structure of HIV-1 RT and Klenow fragment (Yadav et al., 1994). The Met 184, Asp 185, and Asp 186 of HIV-1 RT have been found to be spatially homologous to the His 881, Asp 882, and Glu 883 residues, respectively, of the Klenow fragment (Kohlstaedt et al., 1992; Yadav et al., 1994). Since direct photoaffinity labeling of the Klenow fragment with dTTP substrate identified His 881 as the residue that is in close vicinity to the dNTP binding pocket (Pandey et al., 1987), it is likely that Met 184 of HIV-1 RT may also be in a position to interact with the dNTP substrate.

In any event, the relatively high degree of fidelity exhibited by M184V, which also occurs as a natural mutation upon prolonged treatment with 3TC and FTC (Gu et al., 1992; Schinazi et al., 1993; Tisdale et al., 1993; Schuurman et al., 1995), may have potential applications in the treatment of AIDS. We could visualize an alternative treatment regime, where a patient is treated with 3TC to the point where he/she carries only the 3TC resistant phenotype. At this stage, the RT of the virus is presumably of the M184V genotype and therefore has high fidelity. Subsequent treatment with a series of other non-nucleoside and/or nucleoside inhibitors would, thus, stand a better chance for decreasing the viral load. While this manuscript was being revised, Larder et al. (1995) reported results of combination therapy of 3TC−AZT that showed a markedly greater decrease in the serum viral load than treatment with AZT alone. The success of combination therapy was attributed to the rapid emergence of M184V mutants. This mutation has been thought to induce the suppression of AZT resistant phenotype. The continued susceptibility of M184V virus to AZT−3TC combination therapy without the emergence of any new resistant phenotype for several generations may be a direct effect of the increased fidelity of DNA synthesis, which, in turn, may prevent the emergence of new resistance-conferring phenotypes. Alternatively, M184V mutant may have a higher binding affinity for AZTTP due to favorable interaction between the isopropyl group of Val 184 and the 3′-azidodeoxyribose moiety of the analog resulting in its efficient incorporation. In summary, our studies have shown that Met 184 of HIV-1 RT plays a role in the fidelity of DNA synthesis and that its possible interaction with the sugar moiety of the primer terminus and substrate dNTP may be responsible for this property.

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