Functional Analysis of Amino Acid Residues Constituting the dNTP Binding Pocket of HIV-1 Reverse Transcriptase*

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In order to understand the functional implication of residues constituting the dNTP-binding pocket of human immunodeficiency virus type 1 reverse transcriptase, we performed site-directed mutagenesis at positions 65, 72, 113, 115, 151, 183, 184, and 219, and the resulting mutant enzymes were examined for their biochemical properties and nucleotide selectivity on RNA and DNA templates. Mutations at positions 65, 115, 183, 184, and 219 had negligible to moderate influence on the polymerase activity, while Ala substitution at positions 72 and 151 as well as substitution with Ala or Glu at position 113 severely impaired the polymerase function of the enzyme. The K219A, Y115F, and Q151M mutants had no influence on the fidelity; Y183A, Y183F, K65A, and Q151N mutants exhibited higher fidelity on both RNA and DNA templates, while Y115A was less error-prone selectively on a DNA template. Analysis of the three-dimensional model of the enzyme-template primer-dNTP ternary complex suggests that residues Tyr-183, Lys-65, and Gln-151 may have impact on the flexibility of the dNTP-binding pocket by virtue of their multiple interactions with the dNTP, template, primer, and other neighboring residues constituting the pocket. Recruitment of the correct versus incorrect nucleotides may be a function of the flexibility of this pocket. A relatively rigid pocket would provide greater stringency, resulting in higher fidelity of DNA synthesis in contrast to a flexible pocket. Substitution of a residue having multiple interactions with a residue having reduced interaction capability will alter the internal geometry of the pocket, thus directly influencing the fidelity.

In AIDS patients, genetic variation in the HIV-1 genome is one of the most difficult challenges in the course of searching for inhibitors of certain HIV-specific enzymes. During replication of the HIV-1 genome, approximately 35 mutations per million nucleotides are generated (1). An awesome daily production rate of as many as 10 billion virions per body containing 3–10^10 mutations can rapidly accumulate a significant number of drug-resistant variants that make chemotherapy ineffective. Dynamics of HIV-1 replication in vivo has demonstrated that, within 2–4 weeks of treatment with nucleoside analogs, the wild type virus in the plasma is completely replaced by the drug-resistant variants (2, 3). The single-stranded HIV-1 viral RNA genome is efficiently converted into double-stranded proviral DNA by the virally encoded reverse transcriptase enzyme, which is essential for viral replication and establishing infection. This enzyme is therefore used as a potential target for combating viral infection. However, rapid emergence of drug-resistant viral strains has so far frustrated all efforts in this direction. HIV-1 RT is a highly error-prone enzyme and the most probable source of diversity (4–6). This heterodimeric enzyme consists of the 66- and 51-kDa polypeptides. The smaller subunit is derived from the larger subunit by proteolytic cleavage and removal of the COOH-terminal RNase H domain (7, 8). Various nucleoside drug-resistant phenotypes of HIV have been isolated from patients exposed to prolonged chemotherapy. Two natural mutations, M184V and E89G, which have been shown to confer resistance to didoxynucleoside analogs, also enhance the fidelity of DNA synthesis (9–11). Mutations at codon 219 have been shown to confer resistance to zidovudine (AZT) (12, 13). Structural analysis has revealed that Lys at position 219 may be in contact with the phosphate group of the incoming dNTP (14). A natural mutation at codon 151 (Gln → Met; Q151M) of HIV-1 RT has been shown to confer resistance to all dideoxynucleoside analogs (15–17). Gln-151 is a constituent of the highly conserved LPQG motif found in all reverse transcriptases (18, 19) and is probably a constituent of the dNTP-binding pocket in the enzyme-TP-dNTP ternary complex (20, 21). Earlier mutational studies at position 151 have proposed that besides its direct role in the formation of a dNTP-binding pocket, the side chain of Gln-151 may also help in stabilizing the side chain of Arg-72 (20, 21). Arg-72 has been proposed to be involved in the conformational change of the enzyme-TP-dNTP ternary complex during catalysis (21, 22).

A close examination of the three-dimensional molecular model structure of HIV-1 RT-DNA-dNTP ternary complex, based on the three-dimensional co-crystal structure of HIV-1 RT-DNA complex (22), suggests that a number of amino acid residues may be involved in the formation of the dNTP-binding pocket in the ternary complex. Mutations involving the amino acid residues in the dNTP-binding pocket may, in turn, alter the size and shape of this pocket, thus resulting in an alteration of dNTP selectivity of the correct versus incorrect nucleotide during polymerization. Previous mutagenesis of residue Met-184, a constituent of the dNTP-binding pocket, has shown that changes in this residue have marked effect on the fidelity of DNA synthesis (9, 10). Substitution of Met → Ala at this position makes the enzyme highly error-prone, whereas Met → Val substitution significantly improves the nucleotide selectivity and fidelity of DNA synthesis (9). In our efforts to understand the contribution of other constituents of this pocket to the fidelity of DNA replication, the mutant derivatives of Lys-65, Arg-72, Tyr-115, Gln-151, Tyr-183, Asp-113, and Lys-219 were
studied for their involvement in the process of selection of the correct versus incorrect nucleotide substrates during reverse transcription. Results of these investigations are the subject matter of this report.

**EXPERIMENTAL PROCEDURES**

**Materials**

Restriction endonucleases and DNA-modifying enzymes were from Promega or Boehringer Mannheim; Sequenase and DNA sequencing reagents were from U. S. Biochemicals. High performance liquid chromatography-purified dNTPs were obtained from Boehringer Mannheim. Mutagen-M13 in vitro mutagenesis kit was purchased from Bio-Rad. Expression vector pET-28a and Escherichia coli expression strain BL21 (DE3) were obtained from Novagen. All other reagents were of the highest available purity grade and were purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad. Fast Flow Chelating Sepharose (imino-diactetic-Sepharose) for immobilized metal affinity purification was purchased from Amersham Pharmacia Biotech, and 32P-labeled dNTPs and ATP were the products of NEN Life Science Products. Sequencing primers and oligonucleotides containing the desired mutational changes were synthesized at the Molecular Resource Facility of University of Medicine and Dentistry, New Jersey.

An HIV-1 RNA expression clone pHIV-PBS was a generous gift from Dr. M. A. Wainberg (23). This clone contains a 947-base pair fragment of HIV-1 genome (+473 to +1420), which supplies the RNA corresponding to the PBS region.

**Methods**

Expression Plasmid Clones and in Vitro Mutagenesis—Two recombinant plasmids, pET-28a-Rt66 and pET-3a-Rt51, containing p66 and p51 encoding regions, respectively, with metal binding hexahistidine (His-Tag) sequences at the NH2-terminal region were used for isolating the wild type heterodimeric HIV-1 RT (9). The XbaI and SacI fragment (1.432 kilobase pairs) of pET-3a-Rt51 encoding the polymerase domain (1420) corresponding to the PBS region of the HIV-1 genome (23, 28) was cloned into a 947-base pair fragment of the HIV-1 genome (M13mp18) as the template for site-directed mutagenesis. The mutagenesis protocol using uracil-containing DNA template was essentially as described by Kunkel et al. (24). After ascertaining the mutation in M13 by DNA sequencing, the desired mutation was introduced in both the subunits as follows. The NdeI and KpnI fragment from M13mp18 was cloned into an RT66 expression cassette, and an Nhel and SacI fragment was cloned into an RT51 expression cassette (9).

Expression and Isolation of 66/51 Heterodimeric HIV-1 RT and Its Mutant Derivatives—The growth of E. coli BL21 containing pET-28a-Rt66 and pET-3a-Rt51 clones carrying the WT or mutant subunits, and induction of the enzyme protein was carried out as described before (9, 20, 25). The heterodimers were prepared by mixing the cell pellets of p66 and p51 clones at the appropriate ratios as described by Le Grice et al. (26) with slight modification (9). The protein preparations were stable for months at −20 °C. The protein concentration was determined by using the Bio-Rad calorimetric kit as well as by spectrophotometric measurements using εmax = 2.6 × 104 M−1 cm−1 (27).

DNA Polymerase Assay—Polymerase activity of the WT and mutant enzymes was assayed on three different template-primers, poly(rA)-d(T)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-base pair fragment of the HIV-1 genome (+473 to +1420) corresponding to the PBS region (23, 28). Assays were carried out in a 50-μl volume containing 50 μM Tris-HCl, pH 8.0, 100 μM/ml bovine serum albumin, 5 μM MgCl2, 1 μM dithiothreitol, 50 mM KCl, 100 mM TP, 100 μM dNTP, and 10 mM enzyme. With homopolymeric (rA)9-(dT)18 template primer, the reaction mixture contained 50 μM r3P-labeled TTP (0.4 μCi/nmol) For heteropolymeric template-primers, 25 μM of each of the four dNTPs was included with one of them being 32P-labeled (0.2 μCi/μmol). 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. 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 except that 5′-32P-labeled primer/template (15,000 counts/min/assay) was used instead of 32P-labeled dNTP, and terminated by the addition of 6 μl of Sanger’s gel loading dye (29) 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′-32P-labeled 30-mer RNA hybridized to a complimentary 30-mer DNA to generate the duplex hybrid (30, 31). The sequence of 30-mer RNA corresponds to a small segment of the U5-PBS region of HIV-1 genome (Table I). 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 the addition of 5 μl of Sanger’s gel loading dye. Subsequently, the reactions were heated to 90 °C for 5 min, and the cleavage products were resolved on a denaturing polyacrylamide (7 m urea, 1× TBE) 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 32P-labeled 17-mer DNA primer as well as 49-mer U5-PBS DNA primed with 32P-labeled 19-mer DNA primer was used as the RNA-DNA and DNA-DNA TP, respectively. In each case, the enzyme was pre-incubated with the labeled TP and reactions were initiated by the addition of Mg2+/rNTP (500 μM) or Mg/rNTP (200 μM) complex. The reactions were carried out in a total volume of 8 μl for 10 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.

**Single Nucleotide Misincorporation Assays**—In these assays, the U5-PBS RNA and 49-mer U5-PBS DNA templates primed with 5′-32P-labeled 17-mer DNA were used as template primers. The gel-purified 5′-32P-labeled 17-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/min. The [dNTP] was kept at 200 μM for the correct nucleotide and 500 μM for the incorrect nucleotides. The enzymes were pre-incubated with the labeled TP, and the reactions were initiated by the addition of a single Mg2+/rNTP. 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

Construction and Purification of Mutant Enzymes—Fifteen site-directed mutants of amino acid residues at positions 65, 72, 113, 115, 151, 183, 184, and 219 in the polymerase domain of HIV-1 RT were constructed and expressed in E. coli as described previously (9, 21, 22). In the three-dimensional co-crystal structure of HIV-1 RT-DNA complex, residues Asp-113, Tyr-115, Tyr-183, Met-184, and Lys-219 are located on the palm subdomain, Lys-65 and Arg-72 on the finger subdomain, and Gln-151 at the junction of finger and palm subdomain in the polymerase cleft; all these residues are constituents of the putative dNTP-binding pocket. The functional side chains of these residues were replaced with either alanine or substituted by a residue with a similar geometry, hydrophobicity, or charge. The homodimeric form (p66/66) of the enzyme was used throughout these studies since the kinetic parameters of both the heterodimeric (p66/p51) and homodimeric enzymes have been shown to be similar (32).

The purified enzyme preparations were found to be homogeneous with purity greater than 95%. The levels of their expressions, solubility, and yield, as well as their chromatographic characteristics, were identical with that of the wild type enzyme, suggesting that substitutions at the site of mutation did not cause any significant perturbation in the enzyme structure. All the mutants were RNase H-positive and showed a heat inactivation pattern identical to the wild type HIV-1 RT (results not shown), thus providing additional evidence that these mutations did not significantly alter the folded structure of the enzyme protein.

DNA Polymerase Activity of the dNTP Pocket Mutants on Different Template Primers—The RNA-dependent DNA polymerase activity of the mutant enzymes was assessed using the homopolymeric poly(rA)\(\cdot(dT)_{18}\) and the heteropolymeric U5-PBS RNA templates primed with complementary 17-mer PBS-DNA primer. The DNA template-dependent primer extension reaction was monitored by using the 49-mer U5-PBS DNA template primed with the PBS primer (Table I). As shown in Fig. 1A, polymerase activity of Y115F, Q151M, Q151N, M184A, M184V, K219A, and K219R mutants remained more or less unaffected on both RNA and DNA templates while drastic to moderate reduction was observed with K65A, R72A, D113E, D113A, Q151A, Y183A, and Y183F mutants. Similar results were obtained on a DNA template with all mutants except Y183A, which exhibited approximately 28% of the wild type activity on a DNA template but was inactive on RNA template (Table II; Fig. 1A). The primer extension reaction catalyzed by the mutant derivatives of Asp-113 (D113E, D113A) was severely impaired on both RNA and DNA templates. The results obtained with Asp-113 are at great variance with earlier reports, where conservative and nonconservative substitutions at position 113 were shown to retain 60–80% of the wild type activity (33, 34). These authors, however, assayed the mutant enzymes in the crude bacterial extracts using poly(rA)\(\cdot(dT)_{18}\) as the template primer, which is also efficiently utilized by E. coli DNA polymerase I, a possible
The polymerase activities of the WT HIV-1 RT and mutant derivatives were determined using three template primers in the presence of Mg$^{2+}$ or Mn$^{2+}$ as the divalent metal ions. The values represent a percentage of the wild type enzyme activity. Values shown in the parentheses are total picomoles of acid-insoluble dNMP incorporated into the primer DNA by WT HIV-1 RT in 10 min at 37 °C.

| Enzyme | Poly(rA)·(dT)$_{18}$ | U5-PBS RNA/18-mer | U5-PBS DNA/18-mer |
|--------|---------------------|-------------------|-------------------|
|        | Mg$^{2+}$ | Mn$^{2+}$ | Mg$^{2+}$ | Mn$^{2+}$ | Mg$^{2+}$ | Mn$^{2+}$ |
| WT     | 100 (222) | 100 (34) | 100 (19) | 100 (11) | 100 (24) | 100 (9) |
| M184V  | 147       | 143       | 98       | 127       | 105       | 88       |
| M184A  | 165       | 110       | 107      | 125       | 112       | 90       |
| Y183F  | 22        | 31        | 23       | 68        | 21        | 50       |
| Y183A  | 2         | 24        | 3        | 32        | 28        | 42       |
| Q151M  | 105       | 51        | 88       | 110       | 65        | 74       |
| Q151N  | 58        | 163       | 86       | 232       | 32        | 120      |
| Q151A  | 2         | 6         | 3        | 9         | 2         | 14       |
| D113A  | 4         | 8         | 5        | 11        | 6         | 19       |
| D113E  | 4         | 7.6       | 6        | 13        | 6         | 21       |
| Y115F  | 106       | 230       | 103      | 256       | 148       | 205      |
| Y115A  | 7         | 173       | 8        | 31        | 24        | 82       |
| K219A  | 72        | 80        | 66       | 107       | 73        | 65       |
| K219R  | 173       | 90        | 92       | 95        | 101       | 69       |
| R72A   | 3         | 5.6       | 4        | 7         | 3         | 12       |
| K65A   | 21        | 59        | 38       | 65        | 22        | 91       |

TABLE II
Polymerase activity of wild type HIV-1 RT and its mutant derivatives

The polymerase activity of the assembly of wild type HIV-1 RT and mutant derivatives was determined using three template primers in the presence of Mg$^{2+}$ or Mn$^{2+}$ as the divalent metal ions. The values shown are picomoles of acid-insoluble dNMP incorporated into the primer DNA by WT HIV-1 RT in 10 min at 37 °C.

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A notable observation was with the Asp-113 mutants, which significantly recovered their polymerase activity on DNA template in the presence of Mn$^{2+}$. A moderate increase with Mn$^{2+}$ on the RNA-dependent DNA polymerase activity was also seen with the Y183F and Y183A mutants, but the most significant increase was found with the Y115A mutant (Table II, Fig. 1B). These results suggest that the nature of the dNTP-binding pocket differs depending on the metal ion employed in the catalytic mechanism. Tyrosine 183 is the only residue of HIV-1 RT that is capable of hydrogen bonding with nucleic acid bases, as observed in a recently solved crystal structure (39), and this observation highlights the importance of this residue which is conserved among all retroviral reverse transcriptases.

Interestingly, we find that, similar to the Y183A mutant, the Y115A mutant also exhibited increased DNA polymerase activity on the DNA template as compared with the RNA template (Fig. 1, A and B). This, in conjunction with other observations discussed below, suggests that the nature of the dNTP-binding pocket differs depending on RNA or DNA templates.

Utilization of rNTP Substrates by Wild Type HIV-1 RT and Its Mutant Derivatives—Emergence of natural mutations conferring resistance to dideoxynucleoside analogs are known to occur in the dNTP-binding pocket residues of HIV-1 RT. These mutations may confer greater discrimination in the recognition of ribose moiety of the incoming nucleotides. It was therefore interesting to examine whether mutants of the dNTP-binding pocket residues could discriminate between rNTPs and dNTPs. A recent report states that a single mutation in MuLV reverse transcriptase is responsible for conferring RNA polymerase activity to the enzyme (40). The $K_m$ for rNTP substrates with this MuLV RT mutant was found to be comparable to that for dNTPs. We thus examined the ability of these mutants to incorporate rNTP substrates using both RNA and DNA templates in an attempt to better understand the role of each of these residues in substrate recognition. The wild type HIV-1 RT is able to catalyze DNA-dependent incorporation of 12–15 ribonucleotides before termination of synthesis under our assay conditions, and several of the mutants including M184V, Q151M, K219A, and K219R exhibited a pattern similar to the WT enzyme (Fig. 2A). Two of the mutants, M184A and Y115F, were able to synthesize RNA with efficiency greater than the wild type HIV-1 RT. However, the Y183F, Y183A, Q151N, Y115A, and K65A mutants were able to incorporate only 1 or 2 ribonucleotides before terminating synthesis. Some interesting differences in rNTP incorporation were noted for the RNA-directed reactions (Fig. 2B). Both the M184V and M184A mutants catalyzed RNA polymerization with less efficiency than...
The ability of WT and its mutant derivatives to catalyze incorporation of rNTPs and dNTPs was assessed on both DNA and RNA templates primed with 5'-32P-labeled 17-mer DNA primer. Reactions were carried out as described under "Experimental Procedures." The ability of the Lys-219 mutants remained unchanged (Fig. 2B). Likewise, mutant derivatives of Tyr-183 exhibit contrasting pyrophosphorolytic activity with respect to the template primer used in the reaction. The Y183A mutant, consistent with its recovery of polymerase activity displayed greater pyrophosphorolytic activity on DNA template. In contrast, pyrophosphorolytic activity of Y183F mutant was severely impaired on the DNA template, although its polymerase activity was more or less identical on both DNA and RNA templates (Fig. 3A). The most interesting results obtained were with the K65A mutant, which is significantly active in catalyzing the polymerase reaction but was found to be greatly impaired in pyrophosphorolytic activity on DNA template and substantially decreased on RNA template. This observation provides a subtle clue that this residue may be involved in PPi binding/release or may interact with the primer terminus nucleotide during the reverse reaction. Furthermore, the dissimilarities observed between the RNA- and DNA-directed reactions emphasize the template-dependent variation in the dNTP substrate-binding pocket. This contention is also supported by the differential fidelity of DNA synthesis observed, as described below, on RNA and DNA templates.

The fidelity of DNA Synthesis—HIV-1 and retroviruses in general show a high degree of variability in their genomes, which gives rise to "quasi species" (43). It is now accepted that the high frequency of mutations seen in retroviruses is due to the low fidelity of the reverse transcriptase, which lacks a proofreading exonuclease activity (5). The HIV-1 RT mutants with reduced misinsertion fidelity such as the M184V, Y115F mutant with higher pyrophosphorolytic activity selectively on DNA template (Fig. 3A). The most interesting results obtained were with the K65A mutant, which is significantly active in catalyzing the polymerase reaction but was found to be greatly impaired in pyrophosphorolytic activity on DNA template and substantially decreased on RNA template. This observation provides a subtle clue that this residue may be involved in PPi binding/release or may interact with the primer terminus nucleotide during the reverse reaction. Further, the dissimilarities observed between the RNA- and DNA-directed reactions emphasize the template-dependent variation in the dNTP substrate-binding pocket. This contention is also supported by the differential fidelity of DNA synthesis observed, as described below, on RNA and DNA templates.

Pyrophosphorolysis—The pyrophosphorolysis activity is a reversal of the polymerase reaction and therefore would require the participation of the same residues. In the overall pyrophosphorolysis reaction, the DNA primer is sequentially cleaved from the 3' terminus in the presence of PPi resulting in the generation of dNTP. We have previously shown that mutant derivatives of the carboxylate triad (Asp-110, Asp-185, Asp-186), as well as R72A and K151A mutants of HIV-1 RT, are deficient in the polymerase reaction and severely impaired in their ability to catalyze the pyrophosphorolysis reaction (20–22, 25). In the present study, we have shown that several mutant derivatives of the dNTP-binding pocket, namely M184V, M184A, K219A, K219R, Q151M, and Q151N, exhibit near identical wild type pyrophosphorolysis. In contrast, the Y115F mutant exhibited higher pyrophosphorolytic activity selectively on DNA template (Fig. 3, A and B). Likewise, mutant derivatives of Tyr-183 exhibit contrasting pyrophosphorolytic activity with respect to the template primer used in the reaction.

Polymerases have evolved with a high degree of substrate specificity, and it has recently been shown that minor structural changes can alter this specificity. Tabor and Richardson (41) have demonstrated that a single point mutation in DNA polymerase I (F762Y) renders the enzyme capable of incorporating dNTPs with high efficiency, whereas the wild type enzyme is highly discriminatory against deoxy analogs. The change in specificity involving recognition at the 3' position of the ribose moiety of the incoming dNTP facilitates the use of ddNTPs lacking the 3' hydroxyl group. Also, in contrast to the findings by Gao et al. (40), wherein a point mutation allows the use of rNTPs by MuLV RT, mutations in T7 RNA polymerase renders it capable of incorporating dNTPs (42). A single residue in HIV-1 RT, which influences ribose selection in a similar manner, has yet to be identified.
this assay as a measure of fidelity (45–47). The wild type enzyme is able to generate all possible mispairs on both DNA and RNA templates, as well as extend these mispairs on RNA template. The G:T mispair, however, could not be extended on the DNA templated reactions, whereas all other mispairs could be extended in a manner similar to RNA-directed reactions. Our findings that HIV-1 RT displays less accuracy when copying natural RNA versus DNA templates of identical sequence are in general agreement with those of Huber et al. (48). A recent report by Kerr and Anderson showing higher fidelity on synthetic RNA template may be attributed to either the sequence context or secondary structure of the template (49). The RNA and DNA templates we have used correspond to the U5-PBS (primer binding site) region of the HIV-1 genome (23); to our knowledge, this is the first fidelity study performed on a retroviral RNA template. The M184A mutant, which had been shown to be more error-prone than the wild type when copying a DNA template (9), is less error-prone than the wild type enzyme when copying an RNA template (Fig. 4A). This mutant (M184A) displays greater efficiency than the WT enzyme in incorporating rNTPs on a DNA template and substantially lower efficiency on an RNA template (Fig. 2, A and B). This observation suggests that the side chain of Met-184 may be dispensable in terms of enzymatic activity, but is essential for nucleotide selection during DNA-directed second strand (+ stand) DNA synthesis. The M184V mutant has greater fidelity than the wild type on both RNA and DNA templates. Other mutants with enhanced nucleotide selectivity are Y183F, Y183A, Q151N, and K65A. The Y115A mutant also appears to have greater fidelity than the wild type enzyme, as it is unable to generate any of the mispairs on the U5-PBS DNA template (Fig. 4B, panel a) although it is able to generate mispairs on U5-PBS RNA template (Fig. 4A) albeit with efficiency lower than the wild type enzyme. This observation is in contrast to earlier reports, where the Y115A mutant was shown to be more error-prone than the wild type enzyme (44, 50). The fidelity patterns exhibited by the K219A, K219R, Q151M, and Y115F mutants were similar to the wild type enzyme. The enhanced fidelity of the Y183F mutant observed on a DNA template is in agreement with an earlier report (51). We have further demonstrated that the Y183A mutant displaying its polymerase activity similar to the Y183F mutant also exhibits higher fidelity on DNA template (Fig. 4B, panel a).

It is worth noting that mutant derivatives of Gln-151, Tyr-183, and Met-184 show an increase in fidelity of DNA synthesis and all of these residues are within interacting distance of the nucleic acid substrate in the region of the primer terminus where the dNTP-binding pocket is formed (39). The K65A mutant of HIV-1 RT also displays an increased fidelity with only marginal reduction in the polymerase activity and affinity for dNTP. The K65R mutant shows an increase in processivity during DNA synthesis (52), and this effect may be mediated through alterations in dNTP substrate binding, although this segment of the “fingers” subdomain of RT has previously been proposed to interact with the template primer (53). The K65R mutant also confers cross-resistance to ddI and 3TC but not AZT (36). The mechanism whereby removal of the positively charged side chain of Lys-65 enhances nucleotide selectivity is not yet clear, and this residue may have multiple contacts including interaction with the single-stranded region of the template, but there are as yet no crystal structures available with a template overhang long enough to make this determination.

Fidelity of DNA Synthesis in the Presence of Mn2+—All polymerases require divalent metal cation for catalysis, and it has been shown that substitution of magnesium with manganese in the polymerase reaction profoundly influences the polymerase activity, processivity, and fidelity of DNA synthesis (54). It has recently been shown that several mutants of the Klenow fragment of DNA polymerase I which suffer a substantial decrease in Mg2+-dependent polymerase activity experience a recovery of activity when Mn2+ is substituted as the divalent metal ion (37). Similar results were obtained with some of the HIV-1 RT mutants, where a partial recovery of the polymerase activity on DNA template was noted in the presence of Mn2+ (Table II). In order to examine the effects of Mn2+ on the fidelity of DNA synthesis, we have carried out misinsertion assay with high fidelity mutants (Y183A, Y183F, Q151N, K65A) in the presence of Mn2+ (Fig. 4B, panel b) and compared with the pattern obtained with Mg2+ (Fig. 4B, panel a). The high fidelity mutants are unable to catalyze misinsertion in the presence of Mn2+ (lanes 3–5, panel a), while they are able to extend the primer with the respective Mn-dNTP substrates (lanes 3–5, panel b). This observation confirms previous reports stating that fidelity is compromised when Mg2+-dependent polymerase activity is replaced with Mn2+-dependent polymerase activity (54).

dNTP Binding Pocket—Based on site-directed mutagenesis, biochemical results, co-crystal structure of RT-DNA complex, and three-dimensional molecular model, the amino acid residues involved in the formation of the putative dNTP-binding pocket along with the template nucleotide and dNTP are schematically shown in Fig. 5. Although some of these residues do not have direct interaction with dNTP, they are in a position to influence the interaction with other constituents of the pocket. To analyze the geometry and flexibility of the dNTP-binding pocket, we performed a systematic conformational search of the side chains of these residues along the Cα-Cβ bond. We noted that, within structural constraint, Phe-160 and Tyr-183 adopt only a single conformation while Lys-65, Arg-72, Gln-151, and
Arg-219 have multiple conformational states and Asp-113, Tyr-115, Phe-116, and Met-184 are stabilized at two possible orientations (Table III).

**DISCUSSION**

With the availability of the crystal structures, mutational analysis has become a powerful tool for studying the structure-function relationships in HIV-1 RT, as meaningful inferences can be drawn regarding the interactions and possible roles of a particular amino acid residue in catalysis. Although crystal structures of the ternary complex of RT-DNA-dNTP are not yet available, several residues have been identified through mutational and modeling studies that are implicated in the substrate dNTP binding function (9, 21, 22).

The amino acid residues involved in the formation of the putative dNTP-binding pocket are schematically shown in Fig. 5. A highly conserved YXDD motif found in all reverse transcriptases (18, 19) forms the catalytic center of the enzyme where metal complexed dNTP and template primer are aligned to catalyze the nucleotidyltransferase reaction (22). In HIV-1 RT, Tyr-183, Met-184, Asp-185, and Asp-186 constitute this motif and are part of the dNTP-binding pocket of the enzyme. The other constituents of the dNTP-binding pocket are Lys-65, Arg-72, Gln-151, Asp-110, Tyr-115, Phe-116, and Phe-160. Asp-113 of HIV-1 RT has been proposed to be equivalent to Glu-710 of the Klenow fragment and may also be involved in the coordination of dNTP (55). Substitution of Glu to Ala at position 710 of the Klenow fragment results in complete loss of enzyme activity (35). Our results with the Asp-113 mutants demonstrate that both conservative (D113E) and non-conservative (D113A) mutations cause greater than 90% loss of polymerase activity, thus sup-
porting the contention of its functional equivalence with Glu-710 of the Klenow fragment. The side chain of Asp-113 is suitably positioned to influence the binding of dNTP in the catalytic site.

Larder et al. (56) first showed that a Y183S mutation resulted in a 99% reduction in polymerase activity, and a subsequent report demonstrated that this same mutation caused a 77-fold decrease in the affinity for dNTP substrates (57). Recent data from our laboratory as well as other laboratories have shown that Y183F mutation increases the fidelity of HIV-1 RT (51, 58) and that the Y183A mutant is active on DNA templates but not on RNA templates. The Met-184 mutant has been studied extensively, and a naturally occurring M184V mutation displaying cross-resistance to several of the dNTP analogs (59–61) has been shown to confer greater fidelity to HIV-1 RT (9, 10). Given that the fact that both of these residues are within interacting distance of the DNA in the region of the primer terminus (14, 39), it is feasible that their influence on dNTP substrate binding may be mediated via stabilization/stabilization of the dNTP-binding pocket. The other two residues in the YMDD motif are Asp-185 and Asp-186, which, along with Asp-110, form the crucial metal-coordinated catalytic triad that is indispensable for catalysis as any conservative or nonconservative changes in these positions abolishes the enzyme activity (22, 26, 57, 61, 62). We have proposed that Asp-185 and Asp-110 interact with the 3'-O of the primer terminus and β,γ-phosphate of dNTP through metal coordination, respectively, while Asp-186 coordinates with the α-phosphate of dNTP and catalyzes the phosphodiester bond formation (22).

Other residues in HIV-1 RT have also been proposed to exert their effects on dNTP binding/drug resistance through repositioning of the template primer (11, 15). Guanine 151 has been observed to be within interacting distance of the first template nucleotide ahead of the primer terminus (39) and has been shown to be an important residue in catalysis (20). The Q151M mutation confers high level resistance to several nucleoside analogs (15–17). We suggest that the altered substrate binding properties of Met-151 can be attributed to its varied interaction with the template. Although the conservative Q151N mutation did not change the dNTP binding properties of the enzyme with regard to recognition of the 3'-position of the ribose moiety, there was a significant change in nucleotide base selection. The Q151N mutant showed increased fidelity over the wild type enzyme, indicating that reduction in length of the side chain at position 151 by one methylene group (Gln → Asn) significantly influences the stringency of dNTP selection (Fig. 4, A and B). The Q151M mutant displayed no significant alteration in its fidelity characteristics as compared with the WT HIV-1 RT and this is in agreement with a recent report (63). The Q151N mutant exhibiting higher discrimination not only against the correct versus incorrect dNTPs but also versus rNTP substrates (Fig. 2, A and B). This observation is consistent with the idea that the Q151N mutation results in a more stringent, less flexible dNTP-binding pocket. The Q151A mutant is severely impaired in its polymerase function on RNA template, and its behavior is much like that of the R72A mutant. Arginine 72 has been proposed to be involved in the pyrophosphate binding/removal function of HIV-1 RT (25), and, as neither the Q151A mutant or the R72A mutant shows appreciable pyrophosphorolytic activity, these residues may be stabilizing each other (21). Interestingly, the side chain of lysine 65 is also in close proximity to that of Arg-72. Lysine 65 is a member of the highly conserved IIKK motif situated on the flexible β3-β4 loop of the finger subdomain. Since the K65A mutant is defective in pyrophosphorylase as well (Fig. 3, A and B), it may also be implicated in the binding or removal of PPi. Arg-72 has been proposed to be involved in the PPi binding/release and conformational change step before and after the chemical step of the reaction (22, 25). Another residue that has been thought to bind to the phosphate portion of the incoming dNTP is Lys-219 (14), but our results show that the K219A mutant has wild type levels of polymerase activity, arguing against earlier proposals. In fact, it is not clear from our studies how Lys-219 exerts its influence on dNTP substrate recognition. As shown in the three-dimensional molecular model (Fig. 6A), Lys-65, Arg-72, and Lys-219 are the only three basic residues in the vicinity of PPi moiety of dNTP that provide a complementary positive electrostatic charge cloud, thus influencing the binding and orientation of dNTP in the pocket. The positive charge clouds generated by these basic residues compensate the complementary negative charge cloud of the triphosphate moiety, thus stabilizing the dNTP substrate in the ternary complex. Interestingly, in one of the conformations when distance between Arg-72 and Lys-65 is minimal, Lys-219 is stabilized closer to Asp-113 (Fig. 6B). Based on this scenario, we postulate that the role of Asp-113 may be to minimize the positive charge cloud effect of Lys-219 transiently, thus facilitating the transit of the PPi moiety toward the overall positive charge cloud of Lys-65 and Arg-72. This postulation offers a putative role for Asp-113 residue in facilitating the release of PPi, moiety of dNTP after the phosphodiester bond formation, which is essential for efficient catalysis.

The side chain of methionine 184 seems to be dispensable, since no significant reduction in polymerase activity of the M184A mutant could be seen on either RNA or DNA templates. The importance of Tyr at position 183 is clearly evident in reverse transcription, since Tyr → Ala and Tyr → Phesubstitutions at this positions drastically reduced the enzyme activity on the RNA template (58). In contrast, the OH group of Tyr at position 115 is not essential for viral replication, as a Tyr → Phesubstitution has no influence on either RNA- or DNA-directed DNA synthesis, although a Tyr → Ala substitution at this position drastically reduced the catalytic activity of the enzyme selectively with RNA templates. However, their mutant derivatives have significant and contrasting influence on the fidelity of DNA synthesis. For instance, substitution of Met → Ala and Tyr → Ala at position 184 and 115, respectively, results in an error-prone enzyme (9, 50) while Lys → Ala and Tyr → Ala substitutions at positions 65 and 183, respectively,

### Table III

| Amino acid residues | Conformational search along the Cα-Cβ bond from 0 to 360° at 30° intervals | Angle |
|---------------------|---------------------------------------------------------------|-------|
| Lys-65              | 3                                                             | 150, 180, 210 |
| Arg-72              | 6                                                             | 150, 120, 90, 60, 30, 300 |
| Asp-113             | 2                                                             | 150, 120 |
| Tyr-115             | 2                                                             | 150, 180 |
| Phe-116             | 2                                                             | 150, 180 |
| Glu-151             | 6                                                             | 30, 60, 90, 120, 150, 270 |
| Phe-180             | 2                                                             | 180, 210 |
| Tyr-183             | 0 (original)                                                  | No change |
| Met-184             | 2                                                             | 120, 150, 180 |
| Arg-219             | 4                                                             | 90, 120, 150, 180 |
significantly improve the fidelity of the enzyme.

As shown in the ternary complex model (Fig. 7), Tyr-115 is probably involved in dNTP binding through base stacking with the incoming dNTP and extensive analysis of mutations at position 115 supports this contention (44). When the aromatic character of position 115 is preserved by a Y115F mutation, neither polymerase activity nor substrate binding is significantly affected. However, elimination of the aromatic ring by other mutations such as Y115A or Y115S results in a severe loss of polymerase activity as well as greatly increased Km for dNTP substrates. Our data are in good agreement with these earlier findings (44). Furthermore, we find that Y115A shows a substantial recovery of activity on DNA versus RNA templates, suggesting that the aromatic ring of Tyr-115 is relatively crucial for the RNA-directed reactions as compared with the DNA-directed reactions. We have recently reported a similar behavior for the Y183A mutant of HIV-1 RT, in which the aromatic ring seems essential for RNA-dependent polymerase activity but not for DNA-dependent activity (58). Interestingly, substitution of Mg2+ with Mn2+ significantly recovered the RNA-dependent DNA polymerase activity of both the Y183A and Y115A mutants noticeably (Fig. 1, A and B). These data strongly suggest that the structure and composition of the dNTP-binding pocket is very different, depending not only on the template being read but also on the divalent ion used and the nature of coordinating residues in the polymerase reaction.

Elucidation of the mechanisms used by DNA polymerases to achieve high fidelity during replication has been a long-standing investigation. Although the predicted error frequency based on free energy differences between correct and incorrect base pairs can provide only a 5–150-fold discrimination against insertion of a wrong nucleotide (64), there should be specific mechanisms employed by polymerases that provide additional criteria for discrimination in nucleotide selection and ensure high fidelity during replication. It has been suggested that exclusion of water molecules from the polymerase active site magnifies the free energy differences between the correct and incorrect base pairs and this may in itself account for high polymerase fidelity (65). The ability to perform pre-steady state kinetics analysis has facilitated detailed depiction of the polymerase reaction and identified several kinetically distinct steps. Evidence that suggested a rate-limiting conformational change step prior to the phosphodiester bond formation was first proposed in the kinetic scheme for DNA polymerase I (66, 67). This rate-limiting step was later identified in the kinetic mechanism of HIV-1 RT using pre-steady-state methods (27, 68) and may be a major factor in achieving fidelity during DNA synthesis. While dNTPs may be bound indiscriminately during the initial binding step k2, binding of the correct nucleotides may facilitate the conformational change k after which nucleotide addition is essentially irreversible. It is not yet clear which residues in HIV-1 RT are involved in triggering this conformational change, which, in the case of an enzyme lacking proofreading activity, is the ultimate deciding factor in nucleotide selection. Once incorporated, a mismatched nucleotide is not excised, and perhaps this explains the ability of RT to extend mispaired DNA with greater efficiency than other DNA polymerases (69). Instead of terminating synthesis, HIV-1 RT can continue replication after a mutation is generated, and this can certainly be seen as beneficial to the virus. Although genetic variation may be beneficial to the virus, this variation is extremely deleterious to the host and has presented major problems in terms of treating HIV-1 infection, as has been seen from the emergence of genetic variants that are resistant to all drugs so far used in the treatment of AIDS.

We propose that among the amino acids residues involved in constituting the dNTP-binding pocket, those having more that one allowed conformations and/or multiple center of interactions with dNTP, template, and primer as well as with other components of the pocket may provide greater flexibility to the size and shape of the pocket (Figs. 5 and 7). Such residues may also be the center for greater error-prone synthesis due to their ability to change the topology of the pocket to accommodate the nonspecific nucleotide. Removal or alteration of the side chains
of these residues may endow greater fidelity due to loss of flexibility of the pocket, conferring higher specificity for correct dNTP.

Analysis of the three-dimensional crystal structure of RT-DNA complex (70) as well as the three-dimensional molecular model of the RT-DNA-dNTP ternary complex (Fig. 7) revealed that Lys-65, Tyr-183, and Gln-151 exhibit multiple interaction with the components of the dNTP-binding pocket. For example, Tyr-183 has been shown to be within interacting distance of bases of the template and primer (39) as well as Met-184, Met-230, and Gln-91 (58). A systematic conformational search of the side chain of the dNTP-binding pocket residues along the Ca-Cβ bonds revealed more than one allowed conformation for most of the residues, suggesting their dynamic interaction with the components of the dNTP-binding pocket during the polymerase reaction (Table III). The only exception is Tyr-183, which does not have flexibility to move within the structural constraint. The ability of Tyr-183 to contribute to the flexibility of the dNTP-binding pocket may be due to multiple centers of interactions residing within its seemingly inflexible side chain. Removal of the hydrophobic phenyl ring by alanine substitution (Y183A) or removal of the OH group by phenylalanine substitution (Y183F) yields an enzyme with higher fidelity. Similarly, abolition of the side chain of Lys-65 by the K65A mutation increased the fidelity of HIV-1 RT drastically.

Further, Lys-65 is suitably positioned to interact with the template nucleotide influencing the proper positioning of the template strand, an important component of the dNTP-binding pocket. Template length-dependent sensitivity to ddNTP analogs reported earlier suggests that the β3-β4 loop region may be important in positioning the template strand (53). Recently, an insertion of 15 amino acids into the β3-β4 loop of HIV-1 RT has been shown to enhance the processive DNA synthesis probably by increased contact between the finger subdomain and the single-stranded portion of the template (71). Thus, the multiple interactions of Lys-65 may be lost as a result of the K65A mutation effecting a more rigid dNTP-binding pocket. Gln-151 interacts with the first template nucleotide ahead of the 3'-OH of the primer and also may interact with the dNTP. The shortening of this residue by introducing a Q151N mutation evidently results in a loss of one or more of these interactions, which normally affords the dNTP-binding pocket a good degree of flexibility. In addition, Gln-151 has also been proposed to interact with the side chain of Arg-72, as both residues seem to be functionally interdependent (21).

Recently, it has been established that the size and shape complementarity, rather than the ability of dNTP to hydrogen bond to the template nucleotide, is responsible for the high fidelity during DNA replication (72). The recruitment of correct versus incorrect nucleotides may be a function of the flexibility in the dNTP-binding pocket. A more rigid pocket is expected to be responsible for high fidelity of DNA synthesis, whereas a flexible pocket may result in error-prone DNA synthesis. Among the amino acid residues that constitute the binding pocket, one that has multiple interactions with the substrates (dNTP, PPi, and TP) and other constituents of the pocket may greatly influence this flexibility and may cause a less stringent selection of the incoming dNTPs. Replacement of such a residue with one that has reduced capability for multiple interactions will alter the geometry and flexibility of the pocket and thus may have direct influence on accommodating the correct versus incorrect nucleotides in the pocket.

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