Role of Glutamine 151 of Human Immunodeficiency Virus Type-1 Reverse Transcriptase in Substrate Selection As Assessed by Site-Directed Mutagenesis†

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ABSTRACT: A natural mutation at codon 151 (Gln → Met; Q151M) of HIV-1 RT has been shown to confer resistance to the virus against dideoxy nucleoside analogues [Shirasaka, T., et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92,* 2398], suggesting that Gln 151 may be involved in conferring sensitivity to nucleoside analogues. To understand its functional implication, we generated two mutant derivatives of this residue (Q151M and Q151N) and examined their sensitivities to ddNTPs and their ability to discriminate against rNTPs versus dNTP substrates on natural U5-PBS HIV-1 RNA template. We found that Q151M was highly discriminatory against all four ddNTPs but was able to incorporate rNTPs as efficiently as the wild type enzyme. In contrast, the Q151N mutant was only moderately resistant to ddNTPs but exhibited a higher level of discrimination against rNTPs. The fidelity of misinsertion was found to be highest for the Q151N mutant followed by Q151M and the wild type enzyme. These results point toward the importance of the amino acid side chain at position 151 in influencing the ability of the enzyme in recognition and discrimination against the sugar moieties of nucleotide substrates.

The rapid emergence of human immunodeficiency virus (HIV-1) strains resistant to specific inhibitors has frustrated all efforts to control the spread of acquired immunodeficiency syndrome. Dynamics of HIV-1 replication in vivo have demonstrated that within 2−4 weeks of treatment with nucleoside analogues, the wild type virus in plasma is completely replaced by the drug resistant mutants (*1*, *2*). The single-stranded HIV-1 viral RNA genome is efficiently converted into the double-stranded proviral DNA by the virally encoded reverse transcriptase, which is essential for viral replication and establishing infection. The resistance to nucleoside analogue inhibitors is thought to be due to mutations in HIV-1 reverse transcriptase (*3*). A thorough understanding of the mechanism of drug resistance is hence a prerequisite for a rational design for structure-based drugs. Various nucleoside drug resistant phenotypes of HIV have been isolated from patients exposed to prolonged chemotherapy. A natural mutation at codon 151 (Gln → Met; Q151M) of HIV-1 RT[1] has been shown to confer resistance to all four dideoxynucleoside analogues (*4*). However, in vitro studies with homopolymeric RNA templates have indicated only a moderate increase in the IC_{50} values of ddNTPs for the Q151M mutant enzyme (*5*). Using a single-nucleotide incorporation assay, a marginal increase in K_i values was also observed with the heteropolymeric RNA template (*6*). The sensitivity of this mutant enzyme to ddNTPs in the presence of all four dNTPs with natural heteropolymeric RNA and DNA template has not yet been reported.

Gln151 is a constituent of the highly conserved LPQG motif present in all the reverse transcriptases (*7*, *8*) and is also deduced as a constituent of the dNTP binding pocket in the enzyme−TP−dNTP ternary complex (*9*, *10*). Previous studies have shown that mutations in this motif can severely impair RT functions (*9*−*11*). This motif is located on the β8−αE hairpin in the finger subdomain of the large catalytic cleft in the polymerase domain (*12*). Earlier mutational studies involving substitution of Q → A, and Q → N, at position 151 have proposed that besides its direct role in dNTP pocket formation (*9*), the side chain of Gln151 also helps in stabilizing the side chain of Arg72 which has been proposed to be involved in the conformational change of the E−TP−dNTP ternary complex during catalysis (*10*, *13*). A number of HIV-1 RT strains resistant to drugs have been isolated, and numerous amino acids have been reported to participate in the HIV-1 RT−drug interactions (*14*, *15*). In this paper, we show that on the natural U5-PBS HIV-1 RNA template, the Gln151Met mutant is highly resistant to

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[1] Abbreviations: A, Q, M, N, and R, single-letter codes for Ala, Gln, Met, Asn, and Arg amino acids, respectively; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethanesulfonyl fluoride; IPTG, isopropyl β-thiogalactopyranoside; poly(rA)·$(dT)_{18}$, polyriboadenylic acid annealed with $(oligodeoxythymidylic\ acid)_{18}$; poly(rC)·$(dG)_{18}$, polyribocytidylic acid annealed with $(oligodeoxyguanylic\ acid)_{18}$; poly(dC)·$(dG)_{18}$, polydeoxycytidylic acid annealed with $(oligodeoxyguanylic\ acid)_{18}$; dNTP, deoxyribonucleoside triphosphate; dATP, dGTP, dCTP, and dTTP, nucleoside triphosphates of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine, respectively; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase; IMAC, immobilized metal affinity chromatography; IDA−Sepharose, iminodiacetic acid−Sepharose; MuLV, murine leukemia virus; U5-PBS HIV-1 RNA template, HIV-1 genomic RNA template corresponding to the primer binding sequence region; U5-PBS HIV-1 DNA template, DNA template corresponding to the U5-PBS HIV-1 genomic RNA sequence.

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ddNTPs and exhibits fidelity that is enhanced compared to that of the wild type enzyme but shows no significant discrimination against rNTPs. In contrast, the Glu151Asn mutant displays enhanced fidelity and discrimination against rNTPs substrates but retains near wild type sensitivity to ddNTPs. These results have been discussed in the context of the recently determined three-dimensional crystal structure of the HIV-1 RT−DNA−dNTP ternary complex (*16*).

MATERIALS

Restriction endonucleases and DNA-modifying enzymes were from Promega or Boehringer Mannheim; Sequenase and DNA sequencing reagents were from U.S. Biochemicals. HPLC-purified dNTPs were obtained from Boehringer Mannheim. The 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 available purity grade and were purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad. Fast flow Chelating Sepharose (iminodiacetic-Sepharose) for immobilized metal affinity chromatography (IMAC) was purchased from Pharmacia, and ^{32}P-labeled dNTPs and ATP were the products of Dupont/New England Nuclear Corp. Synthetic template primers, sequencing primers, and mutagenic oligonucleotides were synthesized at the Molecular Resource Facility at the University of Medicine and Dentistry of New Jersey. HIV-RNA expression clone pHIV-PBS was a generous gift from M. A. Wainberg (*17*). All other reagents were purchased from Fisher, Boehringer Mannheim, and Bio-Rad.

METHODS

Expression Plasmid Clones and in Vitro Mutagenesis. Two recombinant plasmids, pET-28a-RT66 and pET-28a-RT51 containing p66 and p51 encoding regions, respectively, with metal binding hexahistidine (His-Tag) sequences at the N-terminal region were used for isolating wild type heterodimeric HIV-1 RT (*18*). The *Xba*I and *Sac*I fragment (1.432 kilobases) of pET-3a-RT$_{51}$ encoding the polymerase domain of HIV-1 RT was subcloned in bacteriophage M13 mp18 and used as the template for site-directed mutagenesis (*18*). The mutagenesis protocol in which the dU-containing DNA template was used was essentially as described by Kunkel et al. (*19*). After the mutation in M13 had been ascertained by DNA sequencing, the desired mutation was introduced in both the subunits as follows. The *Nde*I and *Kpn*I fragment from M13 mp18 was cloned into the RT$_{66}$ expression cassette, and the *Nhe*I and *Sac*I fragment was cloned into the RT51 expression cassette (*9*, *18*, *20*).

Preparation of the HIV-1 U5-PBS RNA Template. An HIV-RNA expression clone (pHIV-PBS) was used for the preparation of the U5-PBS HIV-1 genomic RNA template as described previously (*21*). The plasmid pHIV-PBS was linearized with *Acc*I and transcribed using T7 RNA polymerase. The enzyme, buffer, and rNTP solutions were from Boehringer Mannheim, and the transcription reaction was carried out according to the manufacturer's protocol.

Polymerase Activity Assay. Polymerase activity of the wild type and mutant enzymes was assayed on U5-PBS HIV-1 RNA and 49-mer U5-PBS DNA templates primed with 18-mer PBS primer. Assays were carried out in a 50 μL volume containing 50 mM Tris-HCl (pH 8.0), 100 μg/mL bovine serum albumin, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 100 nM TP, 100 μM dNTP (each of the four dNTPs at 25 μM) with one of them being ^{32}P-labeled (0.2 μCi/nmol of dNTP), and 10 nM enzyme. Reactions were carried out at 37 °C for 10 min and were terminated by the addition of ice-cold 5% trichloroacetic acid containing 5 mM inorganic pyrophosphate. The samples were filtered on Whatman GF/B filters and processed for radioactivity counting as described previously (*21*−*23*).

Gel Analysis of Polymerase Reaction Products. For the gel analysis, the 5′-^{32}P-labeled PBS DNA primer annealed with U5-PBS HIV-1 RNA or 49-mer U5-PBS DNA template was used in the polymerase reaction as described above. The primers were end labeled with ^{32}P using T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (3000 Ci/mmol, NEN-DuPont), according to the standard protocol (*24*). The reaction mixture contained 50 mM Tris-HCl (pH 8.0), 100 μg/mL bovine serum albumin, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 mM KCl, 100 nM TP, 100 μM dNTP (each of the four dNTPs at 25 μM) with one of them being ^{32}P-labeled (0.2 μCi/nmol of dNTP), and 10 nM enzyme in a total volume of 6 μL. The reactions were terminated by the addition of 6 μL of Sanger's gel loading dye (*25*) containing 20 mM EDTA. The extension products were resolved on an 8% denaturing polyacrylamide (7 M urea and 1× TBE) sequencing gel.

RNase H Activity Assay. RNase H activity of the wild type and mutant HIV-1 RT was determined using a 5′-^{32}P-labeled 30-mer RNA hybridized to a complementary 30-mer DNA as described previously (*22*). The cleavage products were resolved on a 12% denaturing polyacrylamide urea gel.

Inhibition of Reverse Transcription by Dideoxynucleoside Triphosphates. The wild type HIV-1 RT and mutant derivatives of Gln151 were examined for their ability to incorporate ddNTPs as substrates using the natural U5-PBS HIV-1 RNA template primed with a complementary 19-mer DNA primer. Three independent experiments were carried out using variable concentrations of individual ddNTP inhibitors (0.075−200 μM) while keeping the concentrations of dNTP (total of 20 μM, 5 μM each) and template primer (200 nM) constant at saturating levels. The radioactive labels were [α-^{32}P]dATP and [α-^{32}P]dCTP (0.2 Ci/nmol of dNTP). The enzymes (100 ng) were preincubated with the template primer for 2 min at 25 °C prior to initiation of DNA synthesis by addition of a mixture of Mg·dNTP and ddNTP. The reactions were carried out for 10 min at 37 °C in a standard reaction mixture in 50 μL and were terminated by the addition of cold 5% TCA containing 5 mM PP_i. The reaction mixtures were then filtered on Whatman GF/B filters to wash away all free dNTPs, and the filters were dried and counted in a liquid scintillation counter. RT activity was plotted against individual ddNTP inhibitor concentrations for determination of IC_{50} values using the PROBIT program (*26*).

Primer Extension in the Presence of rNTP Substrate. The ability of the wild type HIV-1 RT and its mutant derivatives to extend the primer by incorporating ribonucleotides was assessed on both U5-PBS RNA and U5-PBS DNA templates primed with 5-^{32}P-labeled PBS DNA primer as described previously (*10*, *21*, *27*). The reactions were initiated by the

Chart 1

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

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

2. 18 mer DNA PBS primer.

5'-GTCCCTGTTCGGGCGCCA-3'

3. 17 mer DNA PBS primer.

5'-GTCCCTGTTCGGGCGCC-3'

4. 19 mer DNA PBS primer.

5'-GTCCCTGTTCGGGCGCCAC-3

5. 20 mer DNA PBS primer

5'-GTCCCTGTTCGGGCGCCACT-3

6. 49 mer U5-PBS DNA template corresponding to U5-PBS sequence

3'-CAG GGA CAA GCC CGC GGT GAC GAT CTC TAA AAG GTG TGA CTG ATT TTC C -5'

addition of 500 μM Mg·rNTPs in a final reaction volume of 5 μL. For comparison, control reactions were also carried out in the presence of dNTP substrates (200 μM). The reaction mixtures were incubated at 25 °C for 10−30 min and then the reactions terminated by the addition of an equal volume of Sanger's gel loading dye. The reaction products were resolved by denaturing 12% polyacrylamide−7 M urea gel electrophoresis.

Sensitivity to ddNTP and Gel Analyses of the Reaction Products. The template primers used in this assay were the same as those used in the rNTP incorporation assay. One hundred nanograms of wild type and mutant derivatives of Q151 was incubated with U5-PBS RNA or U5-PBS DNA template primed with 5′-^{32}P-labeled PBS DNA primer as described above. Reactions were initiated by the addition of dNTP and ddNTP at a ratio of 2:1 or 1:1 in a final reaction volume of 5 μL. After incubation for 30 min at 25 °C, the reactions were terminated by the addition of an equal volume of Sanger's gel loading dye, and the reaction products were then resolved by denaturing 12% polyacrylamide−7 M urea gel electrophoresis.

Extension of Primers in the Presence of Three dNTPs. 5′-^{32}P-labeled 17-mer primer annealed with a 2-fold molar excess of U5-PBS HIV-1 RNA and DNA templates (see Chart 1) was used for determining the extent of misincorporation in the presence of only three dNTPs (*18*). The labeled template primer at a concentration of 2 nM was incubated with the wild type or mutant enzymes (25 nM) 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/mL BSA, 5 mM $MgCl_2$, and only three dNTPs each at a concentration of 10 μM. Each of the dNTPs that was used was of the highest purity grade (HPLC purified) supplied as a 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% bromophenol blue, 0.014% xylene cyanol, and 85% formamide. The reaction products were analyzed on a denaturing 12% polyacrylamide−8 M urea gel.

Determination of the Kinetics of a Single Correct and Incorrect Nucleotide Incorporation. Misinsertion efficiency (f_{ins}) for all mispairs was determined on U5-PBS HIV-1 RNA template using a gel-based steady-state kinetic assay for nucleotide misinsertion (*28*−*31*). We used a 19-mer and 20-mer PBS primer to determine misinsertions against a purine and pyrimidine template base, respectively. Twenty-five picomoles of RNA template was annealed with 20 pmol of the desired ^{32}P-labeled DNA primer, in a final volume of 50 μL in an annealing buffer containing 50 mM Tris-HCl (pH 7.8) and 100 mM KCl. The misinsertion reactions were carried out at varying enzyme concentrations in a buffer containing 100 nM TP (20K Cerenkov counts per lane), 50 mM Tris-HCl (pH 7.8), 1 mM DTT, 0.1 mg/mL BSA, 60 mM KCl, 5 mM $MgCl_2$, and the respective single dNTP in a final volume of 5 μL. The amount of enzyme was 3 or 11 ng, depending on whether the match or mismatch was to be determined. The mismatch reactions were carried out at 22 °C at increasing concentrations of dNTP (from 0.01 to 10 mM) for an empirically determined reaction time to allow the conversion of about 10−25% of the primer to extended products. Reactions were terminated by the addition of an equal volume of Sanger's gel loading solution (*25*). The reaction mixture was heated at 90 °C for 3 min and loaded on a denaturing 12% polyacrylamide−urea gel and electrophoresed for 2.5 h at 45 W. The products were quantitated by PhosphorImager analysis using the Image Quant software as described by Drosopoulos and Prasad (*29*). The initial velocities of product formation were determined from the percent extension of the primer and plotted as a function of the substrate dNTP concentrations. The relative V_{max} ($V_{max,rel}$) and K_m values were determined using the Enzyme kinetic program. V_{max} values were normalized on the basis of the enzyme concentration factor and the reaction time. Misinsertion efficiency (f_{ins}) was then determined as the $V_{max,rel}/K_m$ ratio of the respective incorrect and correct base pairs as described previously (*29*).

RESULTS AND DISCUSSION

In the three-dimensional cocrystal structure of the HIV-1 RT−DNA complex, Gln151 is located at the junction of the finger and palm subdomain in the polymerase cleft (*12*, *16*) and is a constituent of the putative dNTP binding pocket (*9*, *27*). Two site-directed mutants of Gln151 of HIV-1 RT were constructed and expressed in *E. coli* as described previously (*10*, *13*, *18*). The functional side chain of Gln151 was replaced with asparagine having similar geometry or with methionine, a naturally occurring substituent in the nucleoside drug resistant HIV-1 strain. The enzyme preparations were greater than 95% pure. The polymerase activity of both Q151M and Q151N mutants, as assessed by primer extension assay, was found to be almost similar to that of the wild type enzyme on both RNA (Figure 1A) and DNA templates (Figure 1B).

The emergence of a natural mutation at Gln151 (Gln151Met) of HIV-1 RT confers drug resistance to the virus against all four dideoxynucleoside analogues (*4*), suggesting the involvement of this residue in discrimination and recognition of the sugar moiety of the incoming nucleotides. It was therefore of interest to examine if the mutant derivatives of Gln151 could discriminate between

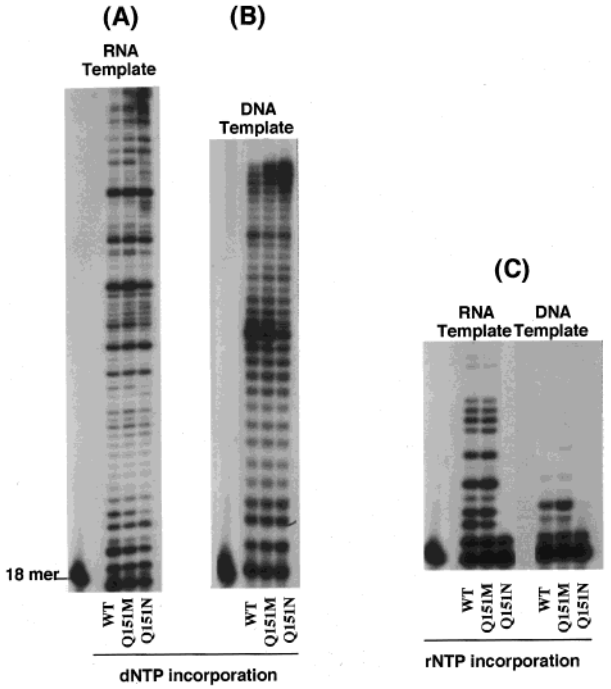

FIGURE 1: DNA polymerase and RNA polymerase (RNA replicase) activities of wild type HIV-1 RT and its mutant derivatives. DNA polymerase activity of wild type HIV-1 RT and mutant derivatives of Q151 was assessed on the heteropolymeric U5-PBS RNA template (A) and the 49-mer U5−PBS DNA template primed with the ^{32}P-labeled 18-mer PBS DNA primer (B). The extension reactions were carried out in the presence of Mg·dNTP as described in Methods. The RNA-dependent RNA replicase and DNA-dependent RNA polymerase activities were monitored using RNA−DNA and DNA−DNA template primers having the same sequence in the presence of 0.5 mM Mg·rNTP substrate (C).

rNTPs and dNTPs on RNA and DNA templates. A mutation at a single site (F155V) in MuLV reverse transcriptase has been implicated in conferring RNA polymerase activity to the enzyme. The K_m values for rNTP substrates with this MuLV RT mutant is comparable to that for dNTPs (*32*). Our studies indicate that both the wild type HIV-1 RT and the Q151M mutant enzymes are able to catalyze RNA-dependent incorporation of several ribonucleotides at a stretch, unlike the Q151N mutant which incorporates only a single ribonucleotide but is unable to extend it further. The ability to discriminate against rNTPs is exhibited by the Q151N mutant for both RNA and DNA templates (Figure 1C). The wild type and the Q151M mutant on the other hand exhibit relatively higher efficiencies of rNTP incorporation on an RNA template, as compared to on a DNA template.

In AIDS patients receiving combinational therapy as well as an HIV-1-infected cell culture treated with dideoxynucleoside analogues, early emergence of the Q151M mutation was noted in the drug resistant viral strains (*4*). Since in the single-nucleotide incorporation assay, response of the HIV-1 RT carrying Q151M mutation to ddNTP has been shown to be only marginally increased (*6*), we examined the sensitivities of the mutant enzymes to ddNTPs in the presence of all four dNTPs using natural U5-PBS HIV-1 RNA and DNA templates and compared these with those of the wild type enzyme. The results are shown in Table 1. The Q151M mutant exhibited a markedly higher resistance to all four ddNTPs on the RNA template than the Q151N mutant. The IC_{50} values of these analogues for inhibiting the reverse

Table 1: Inhibition of the Reverse Transcription Reaction of the Wild Type HIV-1 RT and the Mutant Derivatives of Q151 by ddNTP Inhibitors[a]

	IC_{50} (μM)			
enzyme	ddATP	ddCTP	ddGTP	ddTTP
wild type	6.0 ± 1.2	2.0 ± 0.6	3.0 ± 0.7	5.0 ± 1.1
Q151N	11.0 ± 2.0	9.0 ± 2.4	15.0 ± 3.6	12.0 ± 2.8
Q151M	47.0 ± 5.0	29.0 ± 4.6	24.0 ± 5.0	76.0 ± 6.5

[a] The inhibition assays for determining the sensitivity of the wild type enzyme and its mutant derivatives to each of the dideoxynucleoside triphosphates were performed as described in Methods. Reactions were carried out at varying concentrations of the inhibitor ranging from 0.075 to 200 μM, while the concentrations of the normal dNTPs were kept constant at 20 μM. The IC_{50} values were determined from the graph of percent inhibition as a function of inhibitor concentration.

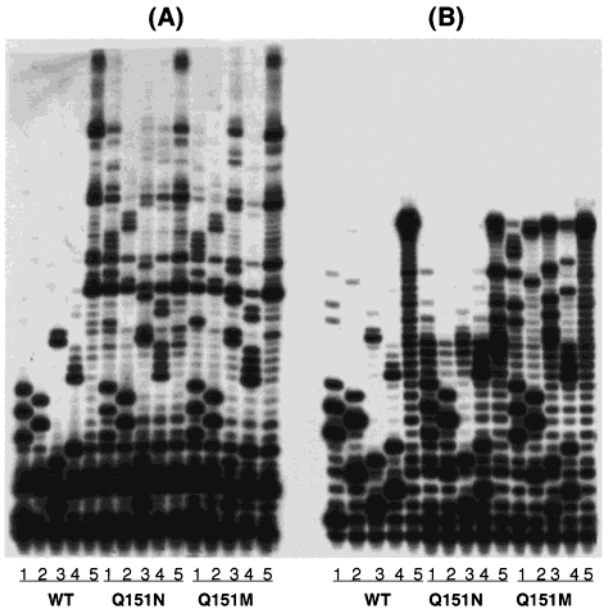

FIGURE 2: Sensitivity of wild type HIV-1 RT and Gln151 mutant derivatives to dideoxynucleoside triphosphates. The U5-PBS RNA template (A) and U5-PBS DNA template (B) were primed with 5′-^{32}P-labeled 18-mer PBS primer and used in these extension reactions in the presence of dNTP or ddNTP as described in Methods. The reaction proceeded for 30 min at 25 °C. The control reaction for each enzyme is represented in lane 5, in which synthesis was carried out in the absence of ddNTPs. Lanes 1−4 represent the reaction carried out in the presence of ddATP, ddCTP, ddGTP, and ddTTP, respectively.

transcription reaction of the Q151M mutant were 8−15-fold higher than for the wild type enzyme, whereas a moderate 2−5-fold increase was observed with the Q151N mutant. This observation was further confirmed by gel analysis of products of reverse transcription in the presence and absence of the individual inhibitors (Figure 2A).

Similar results were also obtained with DNA-directed DNA synthesis catalyzed by the wild type enzyme and its mutant derivatives (Figure 2B). As is evident from the IC_{50} values, the Q151M mutants displayed slightly reduced resistance to the ddGTP analogue when compared to ddATP, ddCTP, and ddTTP. In these studies, the IC_{50} values that were obtained were higher than the reported values (*5*). This may be due to differences in the templates that were used. We have used natural U5-PBS HIV-1 RNA for these studies, whereas the previously reported values were obtained using a homopolymeric RNA template. The secondary structure

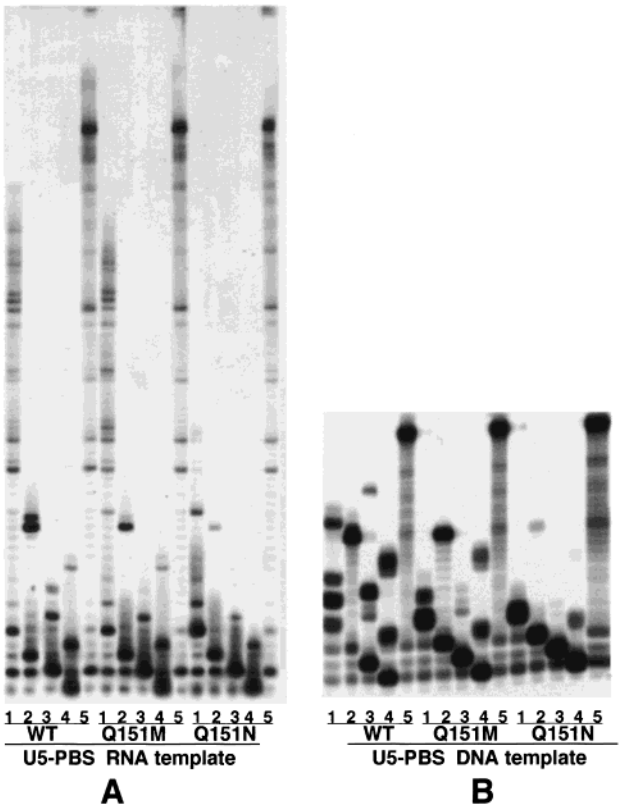

FIGURE 3: Misincorporation and mispair primer extension catalyzed by mutant derivatives of Q151. The U5-PBS HIV-1 RNA and DNA templates were primed with 5′-^{32}P-labeled 19-mer PBS primer and used to assess the extent of misinsertion and mispair extension. Wild type HIV-1 RT (25 nM) or the mutant enzymes (30 nM) were incubated with the labeled template primer in the presence of only three dNTPs as described in Methods. The reaction products were analyzed by denaturing polyacrylamide gel electrophoresis followed by autoradiography. Lanes 1−4 represent reactions carried out in the absence of the fourth nucleotide, dATP, dCTP, dGTP, and dTTP, respectively, from the dNTP mix in the respective set of experiments. Lane 5 represents the reaction in the presence of all four dNTPs.

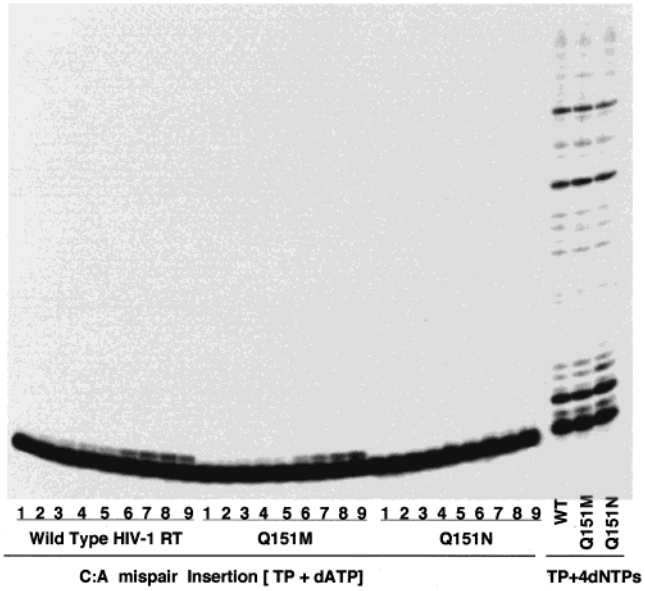

FIGURE 4: C·A mispair insertion by wild type HIV-1 RT and its mutant derivatives. U5-PBS HIV-1 RNA template (100 nM) primed with 100 nM 5′-^{32}P-labeled 20-mer PBS DNA primer was used for C·A mispair insertion in the presence of varying concentrations of dATP ranging from 10 μM to 10 mM. The reactions were carried out for 1 min for the wild type and Q151M and for 3 min for Q151N at 25 °C. The reaction products were resolved on a 16% polyacrylamide−urea gel and quantitated by phosphorimager analysis using Image Quant software. Lanes 1−6 correspond 10, 25, 50, 100, 250, and 500 μM dATP, respectively, while lanes 7−9 correspond 2, 5, and 10 mM dATP, respectively. The lanes on the extreme right show the extension in the presence of all four dNTPs.

of the natural heteropolymeric RNA template used in our studies may be an important factor for this discrepancy.

The degree of discrimination against ddNTP analogues by HIV-1 RT mutants may be determined largely by the residues constituting the dNTP binding pocket. Previously, we have proposed that Q151 participates in the dNTP binding function with the DNA template while its involvement in the conformational change step of the ternary complex may be mediated via its interaction with R72 in the presence of the RNA template (*10*). Since the discrimination against the incorrect versus the correct nucleotide is influenced at both the initial dNTP binding step and the subsequent conformational change step (*10*, *33*), it was of interest to ascertain if the mutant derivatives of Q151 exhibit any change in the fidelity of DNA synthesis. Experiments were therefore carried out to assess the synthesis and extension of the various mispairs by these mutants and compare them with those of the wild type enzyme. Synthesis catalyzed by the wild type HIV-1 RT and its mutant derivatives was carried out on natural U5-PBS RNA and DNA templates primed with 17-mer PBS primer in the presence of only three dNTPs to determine the patterns of misincorporation at the template position complementary to the missing dNTP. Four separate reactions were carried out; in each, one of the dNTPs was omitted. The results depicted in Figure 3 indicate a substantial accumulation of the DNA products at the sites preceding the missing nucleotide in all the reactions with the wild type and mutant enzymes. A significant portion of these accumulated products were further extended to longer products due to misinsertion and subsequent extension, the magnitude of which varied depending on the enzyme and the nature of the template. As seen in Figure 3, the wild type and Q151M mutant enzymes were able to extend the mismatched products to near similar extents on both RNA and DNA templates. In contrast, the Q151N mutant was found to be highly discriminatory against the incorrect versus correct nucleotides on both the RNA and DNA templates, as evidenced by its poor ability to execute misinsertion and subsequent extension of the mismatched products.

To quantitatively estimate the misinsertion efficiency (f_{ins}) of Q151M and Q151N mutants against various mispairs, we carried out a steady-state kinetic analysis using U5-PBS HIV-1 RNA as a template primed with a 5′-^{32}P-labeled PBS primer. The PBS primers 19- and 20-mer providing dTTP and dGTP as the first incoming correct nucleotide, respectively, were used to examine various misinsertions against dA and dC template nucleotides. Figure 4 shows a representative pattern of this kinetic experiment for the C·A mispair insertion with the wild type as well as the mutant enzymes. As seen in the figure, the wild type enzyme and its two mutants displayed different levels of C·A mispair insertion, although all three enzymes exhibited near equivalent activity in the presence of four dNTP substrates. The individual product bands on the gel were quantitated by phosphorimager analysis using ImageQuant (Molecular Dynamics). The kinetic parameters for misinsertion on the

Table 2: Kinetic Parameters for Single Correct and Incorrect Nucleotide Incorporation on the U5-PBS HIV-1 RNA Template Primed with 19-mer or 20-mer by Wild Type HIV-1 RT and Its Mutant Derivatives, Q151M and Q151N[a]

	$V_{max,rel}$ (%/min)[c]			K_m (μM) (for correct and incorrect nucleotide)[c]			f_{ins} (for correct and incorrect nucleotide)[d]		
base pair[b]	WT	Q151M	Q151N	WT	Q151M	Q151N	WT	Q151M	Q151N
A·T	**5.8**	**5.1**	**4.7**	**1.0 ± 0.4**	**0.5 ± 0.2**	**0.6 ± 0.2**	**1.0**	**1.0**	**1.0**
A·A	2.5	2.3	0.5	35 ± 5	55 ± 7	86 ± 11	1.2×10^{-2}	4.5×10^{-3}	7.6×10^{-4}
A·C	0.7	1.2	0.4	7.9 ± 1.6	25 ± 5	37 ± 9	1.6×10^{-2}	4.9×10^{-3}	1.3×10^{-3}
A·G	1.4	1.3	0.3	85 ± 4	101 ± 26	125 ± 15	3.0×10^{-3}	1.4×10^{-3}	3.6×10^{-4}
C·G	**1.4**	**1.7**	**1.4**	**0.3 ± 0.05**	**0.1 ± 0.03**	**0.3 ± 0.07**	**1.0**	**1.0**	**1.0**
C·A	0.6	0.6	0.1	30 ± 13	60 ± 17	127 ± 42	5.3×10^{-3}	8.18×10^{-4}	2×10^{-4}
C·C	0.2	0.2	0.07	20 ± 8	35 ± 8	69 ± 23	2.6×10^{-3}	4.3×10^{-4}	2×10^{-4}
C·T	0.6	0.4	0.1	81 ± 21	79 ± 30	223 ± 65	1.7×10^{-3}	3.8×10^{-4}	1.5×10^{-4}

[a] The steady-state kinetic parameters for correct and incorrect nucleotide incorporation on the U5-PBS HIV-1 RNA template primed with either the 19-mer or 20-mer PBS primer (see Chart 1) were determined as described in Methods. [b] Base pairs are shown with template base first. [c] Both $V_{max,rel}$ and K_m are relative values determined by using the Enzyme Kinetics program. [d] Fidelity of insertion (f_{ins}) was evaluated from the $V_{max,rel}/K_m$ ratio.

U5-PBS RNA template by the wild type and mutant enzymes and the misinsertion efficiency are summarized in Table 2. As is evident from the f_{ins} values, the Q151N mutant displayed the best fidelity of insertion followed by Q151M and the wild type enzyme. The range of misinsertion efficiencies for the Q151N varied from 1.5×10^{-4} to 1.3×10^{-3}. In the case of the wild type and Q151M, this range varied from 1.7×10^{-3} to 1.6×10^{-2} and from 3.8×10^{-4} to 4.9×10^{3}, respectively. The Q151N mutant displayed enhanced insertion fidelity for all the mispairs tested and exhibited relatively lower V_{max} values in the presence of individual incorrect dNTP substrates with a large increase in their K_m values. Interestingly, the Q151M mutant also exhibited enhanced fidelity than the wild type enzyme, although in the M13 lacZ forward mutation assay (with DNA template) it has been shown that its overall error rate is similar to that of the wild type enzyme (*34*).

The Gln151Met mutant displayed poor discrimination against rNTPs while exhibiting higher selectivity against ddNTP inhibitor substrates. The Q151N mutant, on the other hand, displayed enhanced discrimination against incorrect dNTPs, rNTPs, and ddNTPs, resulting in enhanced fidelity of DNA synthesis without compromising its catalytic efficiency. These observations would imply that the length of side chain at position 151 may be a crucial determinant in the selection of nucleotide with ribose, deoxyribose, and dideoxyribose moieties. The wild type enzyme having the side chain of glutamine at position 151 exhibited moderate discrimination against rNTP versus dNTP but showed a similar preference for dNTP versus ddNTP. While 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 displayed a greatly enhanced fidelity compared to that of the wild type enzyme, indicating that the reduction in the length of the side chain at position 151 by one methylene group (Q → N) had drastically enhanced the stringency toward dNTP selection. Although the Q151N mutant was moderately resistant against ddNTPs, it displayed a reduced ability, compared to the wild type enzyme, to incorporate rNTPs which have hydroxyls at both the 2′ and 3′ positions. This observation is consistent with the idea that the Q151N mutation results in a more stringent, less flexible dNTP binding pocket. Interestingly, substitution of Gln with a hydrophobic side chain of similar length profoundly influenced discrimination against ddNTP versus dNTP, but the resulting mutant exhibited moderate discrimination against rNTP versus dNTP.

We have previously proposed that the Q151 side chain interacts with dNTP substrate in a DNA-templated reaction, while with the RNA template, it predominantly interacts with the template nucleotide (*10*). An examination of the three-dimensional crystal structure of the RT−DNA−dNTP ternary complex (*16*) reveals that the side chain of Q151 is indeed within interacting distance of the 3′-OH of the sugar moiety of the dNTP substrate. The results obtained with the mutant derivative of Q151 may be due to their ability to influence the overall flexibility of the dNTP-binding pocket via direct interaction with the sugar moiety of the dNTP substrate. To analyze the mechanism of discrimination of the sugar moiety of nucleotide substrates by the wild type and its mutant derivatives in the ternary complex, the crystal structures of rATP, dATP, and ddATP were superimposed by aligning them along their base moiety in a Watson−Crick hydrogen bonding fashion with the template nucleotide. It was observed that sugar puckering of these nucleotides greatly differs from one to the other, leading to significant differences in their positioning and orientations (Figure 5A). Due to differences in the sugar puckering, incorporation of ddNTP or rNTP may also influence the dissociation of the enzyme from the template primer. It has been shown that in vitro inhibition of the wild type HIV-1 RT results from slow dissociation of the enzyme from the chain-terminated template primer (*35*). It is possible that mutant derivatives of Q151 (Q151 and Q151N) exhibiting enhanced resistance to ddNTP inhibitors may form a rapidly dissociating complex from the chain-terminated template primer.

An interesting feature emerged on examination of the orientation of the sugar moieties in the context of the mutation of Gln151 to Met151 and Asn151 in the three-dimensional model structure of HIV-1 RT (Figure 5A,B). The hydrophobic side chain of Phe116 together with the side chain of Gln151 seems to form an entry gate for incoming nucleotides. The hydrophobic side chains of residues in the active site of DNA polymerases have been proposed to be involved in exclusion of water molecules from the active site, resulting in a large free energy difference between the correct and incorrect nucleotides (*36*). Substitution of the polar side chain with a hydrophobic side chain has been shown to confer enhanced fidelity to HIV-1 RT (*27*). The Q151M mutant enzyme with the hydrophobic side chain of

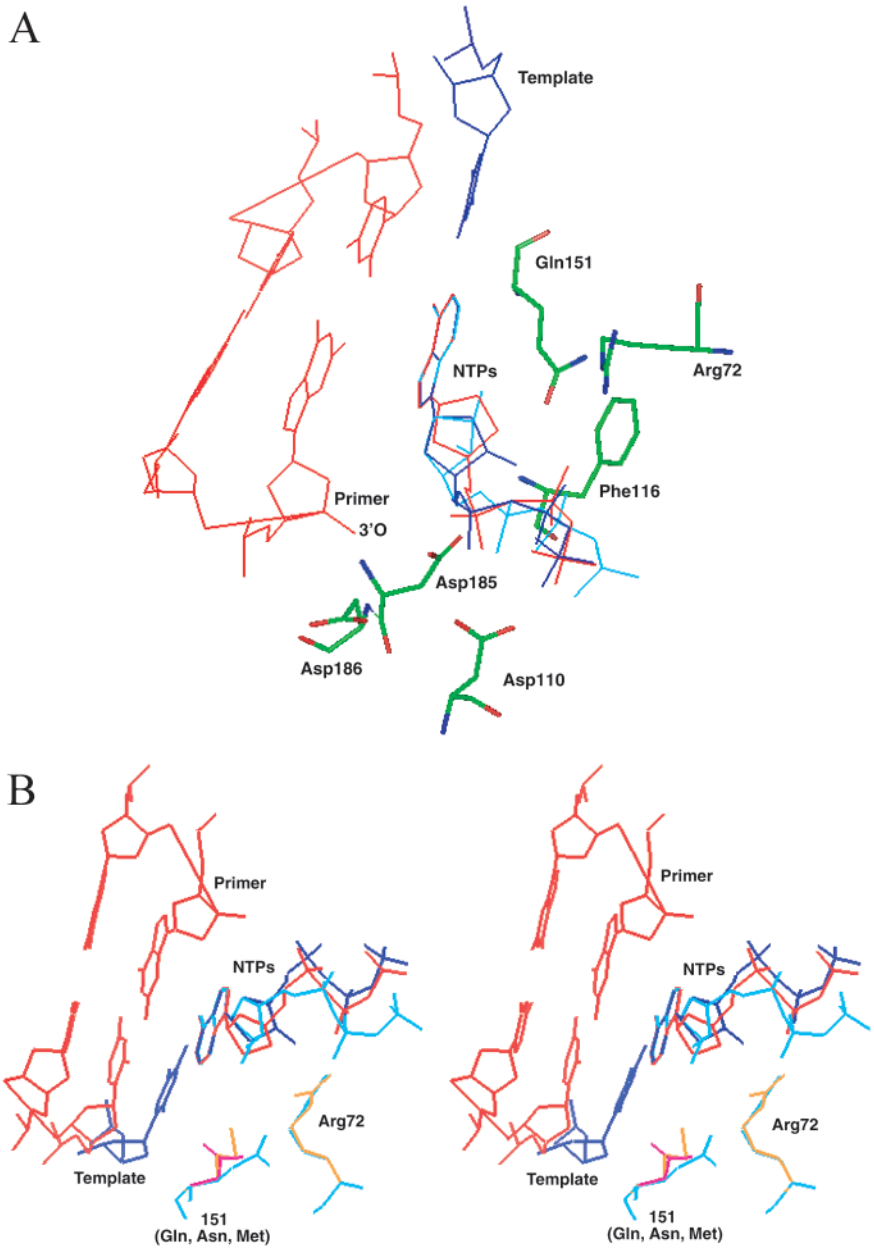

FIGURE 5: Three-dimensional molecular model of the ternary complex of the wild type and mutant HIV-1 RT complexed with DNA and NTP substrates. We have used the three-dimensional crystal structure of the ternary HIV-1 RT−DNA−dNTP complex. Modeling of the mutants (Q151M and Q151N) was performed on the crystal structure of HIV-1 RT extracted from the ternary complex using the mutant modeling module of the Look Modeling package. The complete geometry refinement and energy minimization of the modeled structure were carried out using the Kollman United Atom approach of the SYBYL Modeling package. The structures of various NTPs used in this modeling were either taken or extracted from the three-dimensional crystal structures of Protein Data Bank files (165d, 1d88, 216d, and 219d). The mutant ternary complex was generated by replacing the wild type RT structure of the ternary complex with the mutant model structure. The base moiety of dNTP was used as a site for superimposition of various NTPs into the crystal structure as well as into the mutant model structure ternary complex. The glycosyl bond conformation and the orientation of the triphosphate moiety were adjusted to mimic the crystal structure dNTP conformation and orientation. (A) Variations in the sugar puckering and positioning of the α-phosphate of various nucleotide analogues in the HIV-1 RT−DNA−dNTP ternary complex. The base moiety of rATP and ddATP are superimposed along the base moiety of dATP. Residues shown in the bold line along with the 3′ primer and template nucleotide represent the dNTP binding pocket. Due to slight differences in the sugar puckering, the position of the α-P atom of rATP (blue), dATP (red), and ddATP (cyan) significantly changed with respect to 3′-O of the primer terminus. The hydrophobic side chain of Phe116 together with polar side chains of Gln151 and Arg72 seems to provide the access route of NTPs to the catalytic center. (B) Stereomodel of the ternary complex of the wild type HIV-1 RT and its mutant derivatives complexed with DNA and with nucleotide derivatives. Various nucleotides shown in the figure such as rNTP (blue), dNTP (red), and ddNTP (cyan) are superimposed along their base moiety. The relative orientation and interaction of the side chain at positions 72 and 151 in the wild type and in the mutant enzymes (Met151 and Asn151) with respect to sugar moieties of nucleotides are shown. The interaction of the side chain of Gln151 and Arg72 is more favorable than that of the Asn151 and Met151 mutants. We propose that the amino acid residue at position 151 directly influences the orientation of Arg72 which, in turn, has direct interaction with the α-phosphate of dNTPs.

Met is also able to exhibit enhanced discrimination against ddNTP analogues while retaining the wild type activity with the normal dNTP substrates. Substitution of Gln to Met may also have a significant influence on the stabilization of the side chain of Arg72, an important residue proposed to be involved in the conformational change step before and after

phosphodiester bond formation (*10*, *13*). In the case of the wild type enzyme, the side chain of Q151 is within interacting distance of the side chain of Arg72 and thus may participate in facilitating the conformational change required before and after phosphodiester bond formation (*10*, *13*). In the three-dimensional ternary complex model structure of wild type HIV-1 RT, the α-P of ddNTP is farther from the 3′-O of the primer terminus as compared to the normal dNTP as well as rNTP and yet it is able to utilize ddNTP as the normal substrate. This may be possible due to its ability to influence conformational change in ddNTP via the interaction of its Gln151 side chain with Arg72 which, in turn, interacts with the α-P of ddNTP. This interaction is lost when the amide side chain of Gln is replaced with Met, resulting in insensitivity toward ddNTPs. However, with the shorter amide side chain of Asn, the Q151N mutant may still be able to interact electrostatically with Arg72 through the water molecule. A similar interaction via a water bridge has been proposed by Wang and Ben-Naim (*37*). Thus, the possibility of the interaction of *HØI* of Asn151 and *HØI* of Arg72 may be able to affect the conformational change step, albeit less efficiently as compared to the wild type enzyme, resulting in the manifestation of a marginal resistance against ddNTP analogues. With the DNA−DNA template primer, its side chain is within the interacting distance of the base moiety of the dNTP substrate, while with the RNA−DNA template primer, it is in the interacting vicinity of the template nucleotide base. Multiple interactions of Gln151 may influence the flexibility of the dNTP binding pocket so that it accommodates dNTP, ddNTP, and rNTP substrates. Such a flexibility can be partially affected by an inability to undergo multiple interactions due to a shorter side chain (Asn151) or a side chain lacking the amide group (Met151) conferring enhanced discriminatory ability to Q151N and Q151M mutant enzymes against rNTP versus dNTP and ddNTP versus dNTP, respectively.

Minor structural changes in the side chain of DNA and RNA polymerases have been shown to drastically alter the specificity for nucleotide substrates. For example, a single substitution of Phe → Tyr at position 762 of *E. coli* DNA polymerase I (F762Y) drastically inhibited the ability of the enzyme to discriminate against ddNTP versus dNTP substrates (*38*). Similarly, a single substitution of Phe → Val at position 155 in Murine leukemia virus reverse transcriptase (*32*) and Tyr → Phe at position 639 in T7 RNA polymerase (*39*) abolishes the ability of these enzymes to discriminate against rNTP versus dNTP substrates. Q151 is one such residue in HIV-1 RT which may influence the ribose selection as Gln → Asn and Gln → Met substitutions at this position confer the enzyme with wild type polymerase activity but endow the mutant enzymes with attributes of enhanced discrimination against rNTP and ddNTP substrates, respectively, while retaining their polymerase activity at the wild type level.

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