Role of Q190 of MuLV RT in ddNTP resistance and fidelity of DNA synthesis: a molecular model of interactions with substrates

Kamalendra Singh, Neerja Kaushik, Jin Jin, Mohan Madhusudanan and Mukund J. Modak

Department of Biochemistry and Molecular Biology, UMD–New Jersey Medical School and Graduate School of Biomedical Sciences, Newark, NJ 07103, USA

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Introduction

Reverse transcriptase (RT) is a functional component of all retroviruses and belongs to the DNA polymerase class of enzymes (Katz and Skalka, 1994). Although it is not required after integration of the viral genome (via DNA) into host DNA, it is crucial for viral replication. Reverse transcriptases from various retroviruses differ in size and subunit composition. Murine retroviral (MuLV) RT is a monomer, whereas human and avian retroviral RTs are dimers. In spite of varied subunit structures of RTs from different species, strong conservation of many functional domains among them has been noted (Poch et al., 1989; Xiong and Eickbush, 1990; Singh and Modak, 1998). Owing to its clinical relevance, the human retroviral RT from HIV has been the focus of recent studies. Nevertheless, the monomeric structure of MuLV RT with its high structural homology with equivalent segment of HIV-1 RT (Georgiadis et al., 1995) provides a fairly simple model system for understanding the catalytic mechanism of RTs.

In spite of the overall similarities in the catalytic mechanism of DNA synthesis by HIV-1 RT and MuLV RT, the two RTs exhibit some unique properties. For example, sensitivity to dideoxynucleotides (ddNTPs) does not appear to be shared by the two RTs. MuLV RT shows near complete resistance to ddNTPs, while HIV-1 RT is fairly sensitive. Furthermore, definitive differences in the catalytic mechanism of the two enzymes exist as judged by the differential inactivation patterns obtained with the mutation at conserved sites. Thus, Y222F mutant of MuLV RT is fully active, whereas its equivalent Y183F HIV-1 RT is inactive (Harris et al., 1998; Kaushik et al., 1999). Another example is that of Q190, a member of LPQG motif in MuLV RT and its equivalent residue Q151 of HIV-1 RT, which is located in the same motif. The biochemical characterization of Q151 mutants of HIV-1 RT has indicated that Q151N mutant enzyme is as active as WT (Sarafianos et al., 1995; Kaushik et al., 1997; Harris et al., 1998), whereas the substitution of Q190 of MuLV RT by homologous N shows significant loss of the catalytic activity (Jin et al., 1999).

To elucidate the catalytic role of Q190 of MuLV RT, we have previously carried out extensive biochemical characterization of its two mutant derivatives; Q190N and Q190A (Jin et al., 1999). The substitution of Q by N or A significantly reduced the catalytic efficiency of the enzyme (see Table I). Since dideoxynucleotide resistance in HIV-1 RT has been found to be associated with mutation at Q151, we examined the properties of the two Q190 mutants with respect to dideoxynucleotide resistance as well as fidelity of DNA synthesis. The results presented here show that both Q190N and Q190A mutant derivatives of MuLV RT, in spite of being catalytically compromised, show increased fidelity of DNA synthesis as well as acquisition of ddNTP sensitivity.

In order to obtain an insight into the physico-chemical aspects of the interactions related to ddNTP resistance, we resorted to the molecular modeling of binary and ternary complexes of MuLV RT utilizing crystal structures of corresponding complexes of HIV-1 RT and that of a catalytically active fragment of MuLV RT. In addition, a ternary complex crystal structure of T7 DNA polymerase, where ddNTP sensitivity phenomenon has been well established, was also included for comparison. Analysis of these structures has provided (i) a tentative explanation for the general constraints required for ddNTP and ddNTP incorporation by DNA polymerases and (ii) possible interactions of Q190 in the binary and ternary complexes of MuLV RT, which may explain the loss of overall catalytic activity but acquisition of ability to utilize ddNTPs by its mutants.
Materials and methods

Materials

Mutagen-M13 in vitro mutagenesis kit was purchased from Bio-Rad Laboratories. Sequenase and DNA sequencing reagents were obtained from US Biochemicals, restriction endonucleases and DNA-modifying enzymes from Promega or Boehringer Mannheim, HPLC-purified dNTPs from Boehringer Mannheim and 32P-labeled dNTPs from DuPont/New England Nuclear. Fast flow chelating Sepharose (iminodiacetic acid-Sepharose) for immobilized metal affinity chromatography (IMAC) was supplied by Pharmacia. Sequencing primers, synthetic template primers and oligonucleotides containing the desired mutational changes were purchased from the Molecular Biology facility of this school. HIV-PBS RNA was prepared essentially as described before (Chowdhury et al., 1996).

In vitro site-directed mutagenesis

Uracil containing bacteriophage M13 mp19 carrying a KpnI and SalI fragment (847 bp) of MuLV RT coding sequence was used as the template for the site-directed mutagenesis. Q190N and Q190A were created using appropriate mutagenic primers and the Mutagen-M13 in vitro mutagenesis kit. This method is based on the procedure described by Kunkel et al. (1987). After ascertaining the mutation in M13 by DNA sequencing, we subcloned the KpnI and SalI fragment into the MuLV RT expression plasmid, pET-28a-MRT (Chowdhury et al., 1996).

Expression and purification of the wild-type (WT) and mutant proteins

The WT and mutant proteins were purified to homogeneity by metal affinity chromatography (Jin et al., 1999). Protein concentrations were determined by the Bradford colorimetric assay (Bradford, 1976) and also by the staining intensity of the protein on SDS–polyacrylamide gels.

ddNTP sensitivity assays

The ddNTP incorporation into product was assessed by using HIV-1 PBS RNA and PBS 19-mer DNA as template primer. The 5'-end of PBS 19-mer was labeled with [32P]ATP, 40 nM wild-type enzyme or 400 nM mutant enzyme was incubated in a reaction mixture containing 2 nM template primer, 50 mM Tris–HCl (pH 7.8), 60 mM KCl, 5 mM MgCl2, 40 nM wild-type enzyme or 400 nM mutant enzyme in a final volume of 5 μl. To assess the fidelity characteristics of the WT and mutant enzymes in the context of template nucleotide, four primers of varying lengths were used. These primers were annealed with the U5-PBS RNA or 49-mer DNA containing PBS sequence (see Chart 1). The length of the primer was varied such that an individual template primer requires one of the four correct incoming dNTPs. The synthesis on a given template primer in the presence of each of the three incorrect nucleotides individually, to monitor the misincorporation and its extension by the WT and mutant enzymes, represents the fidelity of the enzyme employing a single dNTP. The reactions were incubated at 25°C for 30 min and the products were analyzed on a 16% polyacrylamide–urea gel. The gels were scanned on a PhosphorImager and each band was quantitated. The percentage of misincorporation and mispair extension in the case of each enzyme was calculated and plotted for each mismatch.

Molecular modeling of the ternary complex

Since a high structural homology exists between the catalytic floor of HIV-1 RT and MuLV RT, the ternary complex crystal structure of HIV-1 RT (Huang et al., 1998; PDB file 1rtd) and the crystal structure of the catalytically active fragment of MuLV RT (Georgiadis et al., 1995; PDB file 1mml) were used to model the ternary complex of MuLV RT. The ternary complex crystal structure of HIV-1 RT contains p66 and p51 subunits, a template primer, dNTP and two metal ions bound at the active site. The Cα atoms of the palm domain of HIV-1 RT composed of β-strands 6, 9 and 10 and α-helices E and F were superimposed on the Cα atoms of the palm domain of MuLV RT which contains β-strands 7, 10 and 11 and α-helices H and I. As a result, the Cα atoms of the catalytic carboxylates of HIV-1 RT (e.g. D110, D185 and D186) superpose very well on the equivalent carboxylates of MuLV RT (D150, D224 and

Table I. Kinetic parameters of WT, Q190N and Q190A mutant MuLV RT

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Poly (rC-dG)/dGTP</th>
<th>Poly (dC-dG)/dGTP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Km</td>
<td>kcat</td>
</tr>
<tr>
<td>WT</td>
<td>30.9</td>
<td>0.45</td>
</tr>
<tr>
<td>Q190N</td>
<td>141.9</td>
<td>0.048</td>
</tr>
<tr>
<td>Q190A</td>
<td>212.8</td>
<td>0.083</td>
</tr>
</tbody>
</table>

*These kinetic constants were taken from Jin et al. (1999). Reprinted with permission from the Journal of Biological Chemistry.
Table II. Utilization ratios of dNTP/ddNTP by MuLV RT and its Q190 mutant

<table>
<thead>
<tr>
<th>Nucleotide substrate</th>
<th>Ratio of utilization</th>
<th>WT</th>
<th>Q190N</th>
<th>Fold decrease</th>
</tr>
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<tbody>
<tr>
<td>dA/ddA</td>
<td>400</td>
<td>80</td>
<td>5.0</td>
<td></td>
</tr>
<tr>
<td>dC/ddC</td>
<td>180</td>
<td>50</td>
<td>3.6</td>
<td></td>
</tr>
<tr>
<td>dG/ddG</td>
<td>520</td>
<td>80</td>
<td>6.5</td>
<td></td>
</tr>
<tr>
<td>dT/ddT</td>
<td>890</td>
<td>100</td>
<td>8.9</td>
<td></td>
</tr>
<tr>
<td>Average (dNTP/ddNTP)</td>
<td>497</td>
<td>77</td>
<td>6.4</td>
<td></td>
</tr>
</tbody>
</table>

The incorporation ratio of dNTP to ddNTP was determined by gel analysis of the products synthesized in the presence of ddNTP by the wild-type or the Q190N mutant enzyme (Figure 2). Quantitative analysis of the ddNTP-terminated bands was carried out by fitting the band intensities to an exponential decay curve.

D225, respectively) with a root mean square (r.m.s.) deviation of ~0.3 Å. The side chain conformation of the catalytic aspartates and the position of metal ions in the active site were modeled such that the metals formed the octahedral coordination sphere. For both HIV-1 RT and MuLV RT, modeling of mutant enzymes was performed by LOOK (version 3.0) molecular modeling software (Molecular Application Group, Palo Alto, CA). In order to project the specific interactions between the desired side chains of amino acids and TP and/or dNTP, a conformational search was performed using Kollman United charges on the protein and TP (Weiner et al., 1984). In this analysis, a binary complex consisting of HIV-1 RT and DNA (Ding et al., 1998) and change in the position of the desired residue at the active site, upon binding to dNTP (ternary complex formation), served as the guiding factor. Net atomic charges obtained from MOPAC calculations (QCPE, Indiana University, Bloomington, IN) were used on ddNTP with a +2e charge on individual metal ions. The active site geometry of the ternary complex is shown in Figure 1.

Results

General catalytic properties of Q190 mutants

We have previously carried out an extensive biochemical characterization of Q190N and A mutants of MuLV RT (Jin et al., 1999). The properties of these mutants were as follows. The substitution of Q by N or A resulted in a significant reduction in the catalytic efficiency of the enzyme (see Table I). The kinetic analyses of mutant proteins indicated a 3–10-fold increase in $K_m$ (dNTP) and a ~20-fold reduction in $k_{cat}$. Measurement of rates for the first and second nucleotide addition by mutants and the WT enzyme suggested that Q190 might also be required at the conformational change step before the chemical bond formation. Since Q190 of MuLV RT is topologically equivalent to Q151 of HIV-1 RT, some functional similarities between the two residues were expected. Q151 of HIV-1 RT has been reported to be associated with deoxynucleotide (ddNTP) drug resistance. We therefore assessed the ddNTP sensitivity patterns of Q190 mutants of MuLV RT.

Q190 mutant enzymes show increased sensitivity to ddNTPs compared with the wild-type MuLV RT

Detailed investigation of the effect of Q190 mutations on dNTP/ddNTP selectivity was carried out on a U5-PBS HIV-1 RNA annealed with a 5'-32P-labeled PBS primer in the presence of a single ddNTP and four dNTPs. Sensitivity to individual ddNTP was determined at three different ratios of dNTPs to ddNTP, i.e. 2:1, 1:1 and 1:2. The results are shown in Figure 2A, B and C. Examination of primer extension products shows that the ddNTP terminations, as indicated by the strong stops at the corresponding sequence, are few for the wild-type enzyme. In contrast, significantly increased number of pauses and also enhanced intensity at the pause sites were consistently observed for both Q190N and Q190A mutant enzymes with all four ddNTPs. The difference in ddNTP discrimination between the wild-type and the mutant enzyme was more pronounced at increased ratios of ddNTP to dNTP.

In order to analyze quantitatively ddNTP incorporation by the wild-type MuLV RT and its mutant derivatives, we quantitated each radioactive band in Figure 2 on a Phosphor-Imager and calculated the incorporation ratio of dNTP/ddNTP as described in Materials and methods. A similar method has been employed by Tabor and Richardson (1995) in their analysis of ddNTP selectivity of ddNTPs by a mutant of T7 DNA polymerase. Table II shows the results of this determination. It is evident that the wild-type enzyme incorporated dNTPs on average 500 times better than ddNTPs (represented by the incorporation ratio of dNTP/ddNTP). On the other hand, this ratio was around 77 for the Q190N mutant, approximately 6-fold lower than that observed for the wild-type enzyme. Although small differences were observed for
Fig. 2. Sensitivity of MuLV RT and its Q190N and A mutant enzymes to ddNTPs. The sensitivity of WT and mutant MuLV RTs to the indicated ddNTP was assessed using PBS RNA and 32P-labeled 19-mer DNA primer. The products of the reaction were resolved on a 16% polyacrylamide-urea gel. Lanes marked A, C, G and T in each panel represent the type of dideoxynucleotide present in the reaction (e.g. lane A contained ddATP), in addition to all four dNTP substrates. The lane marked +4 represents the presence of all four dNTPs in the absence of ddNTP. (A) Four dNTPs at 1 mM, while the indicated ddNTP at 500 µM were used; (B) four dNTPs and desired ddNTP were at 500 µM each; (C) dNTPs were at 500 µM and the indicated ddNTP was at 1mM. A portion of the template sequence is shown on the right. Number 19 indicates the position of 19-mer primer.

individual ddNTP incorporations for both the wild-type and the mutant MuLV RTs, the overall decrease in dNTP/ddNTP ratio in the case of Q190N mutant enzyme consistently persisted.

**Fidelity of DNA synthesis by Q190N and Q190A MuLV RT**

The low catalytic activity of both Q190N and Q190A raised the possibility that the activity of mutant enzymes to incorporate ddNTPs may be an aberration of the overall catalytic process. In order to rule out such a possibility, we examined the fidelity of DNA synthesis. Furthermore, previous studies on HIV-1 RT nucleoside drug-resistant mutants have shown some correlation between sensitivity to nucleoside analogs and increase in the fidelity of DNA synthesis (Bakhanashvili et al., 1996; Drosopoulos and Prasad, 1996; Pandey et al., 1996; Wainberg et al., 1996; Rezende et al., 1998).

**The extent of mismatch nucleotide incorporation with MuLV and HIV-1 RT and their Q mutants**

To obtain a qualitative indication of the relative degree of mismatch nucleotide synthesis between MuLV RT and HIV-1 RT and their Q mutants, we examined the incorporation pattern in the presence of a single dNTP with PBS-RNA as a template. Thus, the extension products resulting from the incorporation of the correct dNTP (dTTP) and the incorrect dNTPs (dATP, dGTP, dTTP) by MuLV RT, HIV-1 RT and their mutant derivatives (Figure 3A and B) were generated. The results shown in Figure 3A indicate that the wild-type MuLV RT preferentially formed G:A, G:G and G:T mismatches and was able to extend the mismatches. However, Q190N mutant enzyme could not form G:G mismatch at all and very little misinsertion of dATP was noted. The dTTP incorporation and its extension by Q190N enzyme also appeared to be lower than the wild-type MuLV RT (Figure 3A, lane 4). These results indicate that mutations at Q190 of MuLV RT afford higher fidelity of DNA synthesis. The results for the misincorporation and extension pattern for HIV-1 RT are shown in Figure 3B. A significantly higher extent of misinsertion and extension was seen for HIV-1 RT compared with the MuLV RT. These observations are consistent with the previous report by Bakhanshivili and Hizil (1996). However, decreased mismatch formation and extension were also observed for the Q151N mutant of HIV-1 RT, indicating that the Q residue in both MuLV RT and HIV-1 RT may have some role in the fidelity of DNA synthesis.

**Relative misincorporation fidelity against different template nucleotides**

In order to clarify if there is preference for mismatch with certain template nucleotides, primers providing four different template nucleotides (thereby four different correct incoming dNTPs) were used (Chart 1). In addition to PBS RNA template, we also included the PBS 49-mer DNA template annealed with the same four primers shown in Chart 1. Fidelity assays in the presence of a single dNTP using these template primers were carried out and the products were resolved on a 16% acrylamide gel similar to that shown in Figure 3. The radiolabeled bands were quantitated on a PhosphorImager and the percentage of misincorporation was calculated as described in Materials and methods and is shown in Figure 4. Similarly to the results shown in Figure 3, Q to N mutation in both MuLV RT and HIV-1 RT significantly reduced the percentage of misinsertion compared with the wild-type RTs. Similar differences in the percentage misincorporation between the wild-
Interactions of Q190 of MuLV RT in nucleotide recognition

Fig. 3. Increased fidelity exhibited by Q190N of MuLV RT and Q151N of HIV RT. Fidelity of the WT and mutant RTs on RNA template in the presence of a single dNTP substrate (A and B) was determined using the PBS RNA template primed with $^{32}$P-labeled 18-mer primer. In panels A and B, lanes 1, 2, 3 and 4 represent reactions carried out in the presence of dATP, dCTP, dGTP and dTTP, respectively. Lane 5 represents the reaction carried out in the presence of all four dNTP substrates. A portion of the template sequence is shown on the right. Number 18 indicates the position of 18-mer primer.

Fig. 4. Graphic representation of relative degree of misincorporation on RNA and DNA templates by the wild-type and the mutant MuLV and HIV-1 RTs. Fidelity assays were carried out in the presence of single dNTP, PBS RNA or PBS 49-mer DNA using four different primers which provided A, C, G or T as the template nucleotide complementary to the incoming dNTP (Chart 1). The gels were scanned on a PhosphoImager and each band was quantitated. The percentage of misincorporation was calculated and plotted for each mismatch and for each enzyme.

Molecular modeling of binary and ternary complexes of MuLV RT

In order to clarify the structural basis for the ddNTP response, we resorted to the modeling of binary and ternary complexes of MuLV RT. The crystal structure of MuLV RT bound with DNA bound in the active center or DNA with dNTP has not yet been solved. However, the corresponding structures of HIV-1 RT are readily available (Ding et al., 1998; Huang et al., 1998). The only available structure of MuLV RT apo-enzyme is the one consisting of the N-terminal 265 amino acid fragment which contains the catalytic core (Georgiadis et al., 1995) and LPQG motif where Q190 resides. Recently, the structure of this fragment with DNA bound outside the
active site has also been reported (Najmudin et al., 2000). For our model building, we chose 76 Ca atoms within the palm subdomain containing an αβα motif from the previously reported structure (Georgiadis et al., 1995), which includes LPQG sequence and the catalytic carboxylate triad, and superposed these on to the corresponding Ca atoms of HIV-1 RT. The individual binary and the ternary complexes of HIV-1 RT were used to model corresponding complexes of MuLV RT. The choice of the core region that we selected for the superposition purpose is justified by the fact that in HIV-1 RT crystal structure, no significant change in these regions of apo- and DNA-bound enzymes has been seen (r.m.s. deviation 0.7 Å). Therefore, the DNA present in the binary complex of HIV-1 RT was docked ‘as is’ in the MuLV RT apo-enzyme to model MuLV RT binary complex. In this binary complex model, Q190 appears to form a hydrogen bond with the base of the template. Analysis of the DNA-bound crystal structure of HIV-1 RT (PDB file 2hmi) shows that a similar contact does not exist in HIV-1 RT. This is one of the several interactions of Q190 of MuLV RT that differs from those of Q151 in the DNA-bound HIV-1 RT crystal structure. Since Q151 of HIV-1 RT and Q190 of MuLV RT are known to be at topologically equivalent positions and they are essential for the activity, we constructed a ternary complex model of MuLV RT (Figure 1) based on the ternary complex model of HIV-1 RT (see Materials and methods section). The examination of the two model structures showed a differential interaction of Q151 and Q190 with substrate dNTP in the respective model structures (data for HIV-1 RT not shown). Of particular interest was the exclusive interaction of Q151 of HIV-1 RT with dNTP in the ternary complex structure (Huang et al., 1998), whereas Q190, in the corresponding MuLV RT model, displayed an interaction with base moieties of both template and dNTP (see Figure 1). However, differential dideoxynucleotide resistance properties of WT and Q191N mutant MuLV RT suggested the interaction of Q190 side chain with 3’OH of incoming dNTP. We therefore performed a conformational search of the Q190 side chain to find an appropriate conformer that can display a contact with 3’OH of bound dNTP. This search failed to identify an appropriate conformer. We then carried out a loop search for the LPQG motif and identified 50 loops with <1 Å r.m.s. deviation for the Ca position of L188 and G191 residues. This was followed by a rotamer search for Q190 side chain in individual loops in order to find the one with its side chain orientation within 3.8 Å of the 3’OH of bound dNTP. The conformer with no steric hindrance was finally induced in the ternary complex structure. This structure differs from the initially modeled ternary complex in that the contact of Q190 with the base of template nucleotide was interchanged with a new contact at 3’OH of dNTP, while the contact with the base moiety of dNTP is retained. Thus, the position of Q190 in the first complex is within the interacting distance (<3.8 Å) from the base moieties of both the template and dNTP (Figure 1), while in the second complex (represented by Q190; see Figure 1), the shift from the template base contact to 3’OH of dNTP contact is apparent. Since the modeling of the second complex requires a slight conformational change of LPQG loop structure, this structure may be considered to represent a distinct step in the multi-step process of DNA synthesis. The two ternary complex structures with different interactions of its Q190 are consistent with the observed biochemical properties revealed by the characterization of Q190 mutants of MuLV RT (Jin et al., 1999).

Since HIV-1 RT and MuLV RT display differential ddNTP sensitivity and Q in the LPQG motif appears to be one of the residues involved, we examined the interactive environment around Q151 HIV-1 RT and Q190 of MuLV RT in their respective ternary complexes. In MuLV RT, Q190 appears to make a total of seven contacts including two with dNTP, while Q151 of HIV-1 RT has eight contacts in the ternary complex. The intramolecular contacts of Q190 and Q151 of HIV-1 RT are listed in Table III. Many interactions seen in the crystal structure of HIV-1 RT are retained in MuLV RT.

Discussion

The results presented here demonstrate that both the homologous (Q190N) and non-homologous (Q190A) mutants of Q190 have acquired sensitivity to all four dideoxynucleotides in contrast to near complete resistance displayed by the wild-type MuLV RT. The wild-type MuLV RT incorporates deoxynucleotides 500 times better than dideoxynucleotides (Table II). This ratio is reduced to ~77 when Q190N is used as a test enzyme. A similar decrease in dNTP/ddNTP utilization ratio was seen for Q190A (Figure 2). Therefore, Q190 may be considered as one of the key residues that permit MuLV RT to be relatively refractory to dideoxynucleotide inhibition. Since the major difference between the deoxynucleotide and its dideoxy analog is in the sugar moiety, it seemed plausible that Q190 may interact directly with the 3’OH of dNTP at some stage during catalysis. Earlier studies have also indicated a role for Q190 in the catalytic process including dNTP binding (Jin et al., 1999).

The catalytic role of Q190 of MuLV RT appears similar to that of Q151 of HIV-1 RT, also a component of the conserved LPQG motif and located at a topologically identical position, since their mutation to alanine produces a catalytically compromised phenotype (Sarafianos et al., 1995; Kaushik et al., 1997; Jin et al., 1999). However, the homologous substitution (Q→N) in two enzymes gave different results. Q190N of MuLV RT had severely reduced activity, while the same substitution in HIV-1 RT (Q151N) showed no change in the catalytic activity. Nevertheless, during the development of dideoxy nucleoside drug resistance, the mutation of Q151 (to Q151M) has been consistently detected in HIV-infected patients undergoing nucleoside drug treatment (Shirasaka et al., 1995). Thus, Q of the LPQG motif in RTs seems to be associated with dideoxynucleotide sensitivity/resistance. In HIV-1 RT, drug-resistant mutant species were also reported to be less error prone (i.e. the frequency of mismatch incorporation or extension of mismatched termini was reduced from the levels seen in WT HIV-1 RT). We therefore examined the fidelity of DNA synthesis by Q190N and Q190A MuLV RT. The results presented in Figure 3 clearly show that Q190NA MuLV RT has increased fidelity as judged by the decreased mismatch insertion/extension efficiency and extension of mismatched termini corresponds to WT MuLV RT. Thus, MuLV RT appears to differ from HIV-1 RT in that its increased fidelity of DNA synthesis is not associated with resistance to ddiNTPs. In order to understand the mechanism of participation of Q190 of MuLV RT and its mutants in the recognition of dNTPs and ddNTPs, we carried out a comparative analysis of the crystal structures of Pol I family DNA polymerases, HIV-1 RT and model structures of MuLV RT. The analysis suggested the possible mode of participation of Q190 in the catalytic reaction and that of Q190N in the ddNTP recognition. We present below the various steps of our analysis and their implications.
comes from the fact that the mental support for the increased binding of dNTP mediated by a T-shaped arrangement and Richardson, 1995. Therefore, the presence of phenyl-

MuLV RT and its Q190N mutant
To understand the participation of Q190 in the recognition of dNTP and ddNTP, we constructed a molecular model of the ternary complex of MuLV RT (Figure 1). In the modeled structure, the interactions of Q190 with dNTP substrate are slightly different than those seen for HIV-1 RT in its ternary complex (Huang et al., 1998). The interaction of Q with 3′OH of dNTP and the intramolecular contact between 3′OH and β-phosphate exist in both structures. However, Q190 of MuLV RT does not seem to interact with β-phosphate (as does Q151 of HIV-1 RT). Therefore, the intramolecular interaction between 3′OH and β-phosphate appears to be sufficient for the catalytic activity. The loss of catalytic activity of Q190N may therefore be related to the inability of N190 to interact with 3′OH, which, in turn, does not promote the 3′OH-β-phosphate interaction. It is interesting that the side chain of Q190 also has a van der Waals interaction with the side chain of F155, which seems to maintain its phenyl ring parallel to the sugar moiety. However, upon substitution of Q190 by N190, subtle changes in the interacting environment are noted. The mutant molecular modeling of Q→N using Look (version 3.0) program (Molecular Application) shows that in addition to the loss of 3′OH contact, the position and conformation of F155 is also altered (data not shown). Upon mutation of Q→N, the conformation of F155 changes such that the arrangement between sugar moiety and phenyl ring of F155 acquires a T-shape. Based on the analysis of high-resolution crystal structures, Singh and Thornton (1990) have shown that the aromatic residues prefer to pack against the hydrophobic core of protein interior, in T-shape, suggesting stronger hydrophobic interactions in T-arrangement. Therefore, Q190N, in spite of being unable to establish 3′OH-β-phosphate contact, allows stable binding of ddNTP mediated by a T-shaped arrangement between the rings of F155 and the sugar moiety. The experimental support for the increased binding affinity and turnover comes from the fact that the $K_m$ (ddNTP) for Q190N enzyme (~50 μM) is much smaller that WT MuLV RT (>1 mM) (data not shown).

Clearly, there are other residues that participate in the process of ddNTP discrimination in RTs, as judged by the observation that the development of ddNTP resistance in HIV requires simultaneous mutations at few other sites (for details see Sarafianos et al., 1999). Nevertheless, Q190 is the first residue in MuLV RT that has been found to be at least partly responsible for the discrimination between ddNTP and dNTP.

HIV-1 RT and ddNTP recognition
In contrast to the Pol I type of DNA polymerases, where a single residue alters the ddNTP recognition by ~1000 fold, the ddNTP recognition properties of RTs appear to be more complex. For example, in HIV-1 RT, multiple mutations have been observed to emerge as the result of dideoxynucleotide inhibitor treatments. Of these, Q151M mutation has consistently been noted to occur, suggesting the participation of Q151 in dideoxynucleotide incorporation. Unlike Pol I enzymes, the structural information in RTs is only available for HIV-1 RT. In the ternary complex crystal structure of HIV-1 RT, Q151 has similar interactions as seen for Y526 in T7 DNA polymerase. It interacts with both the 3′OH and the β-phosphate of incoming dNTP. An additional interaction of 3′OH of dNTP with its own β-phosphate is also noted (Huang et al., 1998). Therefore, it appears that HIV-1 RT shares the same mechanism as seen for Pol I family of DNA polymerases described below.

Pol I family of DNA polymerases and ddNTP recognition
It has been shown that Escherichia coli DNA polymerase I (Atkinson et al., 1969; Tabor and Richardson, 1987, 1989, 1995; Brandis et al., 1996; Astatke et al., 1998a,b) and Taq DNA polymerase (Innis et al., 1988) preferentially select dNTPs over ddNTPs by several hundred-fold, in spite of their similar binding affinity for dNTP and ddNTP. F762 in E.coli DNA polymerase I and its equivalent residue F667 in Taq DNA polymerase were subsequently identified to be responsible for the discrimination between deoxy and dideoxynucleotides (Tabor and Richardson, 1995). T7 DNA polymerase, which shows no discrimination against ddNTPs, has a tyrosine (Y526) at the equivalent position. Replacement of phenylalanine with tyrosine in KF and Taq severely decreases the discrimination against all four dideoxynucleotides, whereas the replacement of tyrosine with phenylalanine in T7 DNA polymerase increases the discrimination by several thousand-fold (Tabor and Richardson, 1995). Therefore, the presence of phenylalanine or tyrosine at a specific location in the Pol I type of enzymes appears to be entirely responsible for the dideoxynucleotide recognition property of individual polymerase.

Different models have been proposed to explain the biochemical data on dNTP and ddNTP selection by the Pol I type of enzymes (Tabor and Richardson, 1995; Astatke et al., 1998b). Using the ternary complex crystal structure of T7 DNA polymerase, Doublie et al. (1998) have provided an explanation for ddNTP selection by this polymerase. They noted that Y526 (an equivalent of F762 of KF) forms a hydrogen bond with the β-phosphate as well as 3′OH of incoming dNTP. In addition, 3′OH of dNTP was also noted to interact with β-phosphate oxygen. Based on this interaction pattern involving Y526, 3′OH and β-phosphate of dNTP, they proposed that the loss of one or more of these contacts may

### Table III. The interactions of Q190 of MuLV RT compared with those of Q151 of HIV-1 RT

<table>
<thead>
<tr>
<th>Interaction (HIV-1 RT)</th>
<th>Type</th>
<th>Comment</th>
<th>Interaction (MuLV RT)</th>
<th>Type</th>
<th>Comment</th>
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</thead>
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<td>SC–SC</td>
<td>Q190–V112</td>
<td>Hydrophobic</td>
<td>SC–SC</td>
</tr>
<tr>
<td>Q151–P116</td>
<td>Hydrophobic</td>
<td>SC–SC</td>
<td>Q190–F155</td>
<td>Hydrophobic</td>
<td>SC–SC</td>
</tr>
</tbody>
</table>

*The interactions of Q151 of HIV-1 RT noted in this table are derived from the analysis of the ternary complex crystal structure (Huang et al., 1998; PDB file 1rdt). The interactions for Q190 are from the model built ternary complex of MuLV RT (see text for details). Note that most of the interactions seen in the crystal structure of HIV-1 RT are retained in MuLV RT. SC represents side chain and MC main chain.
be involved in the discrimination between dNTP and ddNTP in T7 and related polymerases. In a later report, Astatke et al. (1998b) argued that the interaction between F762 and 3′OH is chemically implausible and therefore, suggested that F762 interacts hydrophobically with the ribose moiety of dNTP, which in turn positions the 3′OH to interact elsewhere in the ternary complex. They inferred that the phenolic-OH of tyrosine (in the case of F762Y mutation) is able to compensate for the lack of 3′OH in the dideoxynucleotide, most likely by interacting with an ‘X’ group which would also bind to the 3′OH (Astatke et al., 1998b). The possibility that β-phosphate may serve as that ‘X’ group has not been ruled out.

β-Phosphate interaction may be the key to the catalytic activity

One of the indirect but important outcomes from our structural analysis described above and amplified below suggests that the interaction with β-phosphate oxygen may be a crucial factor for the efficient incorporation of dNTPs and ddNTPs. The observation that non-homologous mutation (F/Y→A in Pol I and Q→A in HIV-1 RT) results in nearly inactive phenotypes (Astatke et al., 1995, 1998; Kaushik et al., 1997; Harris et al., 1998) implies the catalytic importance of Y/F and Q/N in their representative systems. The fact that homologous substitution generally provides catalytically competent phenotypes with altered ddNTP sensitivity suggests that the loss of interaction(s) at specific site(s), without compromising the overall chemical environment, necessary for catalysis, may have occurred. In this context, an analysis of various interactions that sugar moiety of the dNTP/ddNTP exhibits, in the available structures of the ternary complexes of T7 DNA polymerase, KlenTaq and HIV-1 RT, provides useful indication of their functional contributions.

In T7 and KlenTaq DNA polymerase ternary complex crystal structures (Doublie et al., 1998; Li et al., 1998, 1999), the sugar moiety of ddNTP is wedged between the aromatic ring of tyrosine/phenylalanine (Y526 in T7 and F667 in KlenTaq) and the aliphatic carbons of glutamate (E480 in T7 and E615 in KlenTaq). In addition, Y526 in T7 DNA polymerase also interacts with 3′OH and β-phosphate of dNTP. In KlenTaq ternary complex crystal structures, the direct interaction between its F667 and 3′OH and β-phosphate of dNTP is not possible. However, there is a strong hydrophobic interaction between F667 and the sugar that may place 3′OH to interact with β-phosphate.

In the HIV-1 RT ternary complex, the stabilization of dNTP is provided by the interaction of the Q151 side chain with 3′OH and hydrophobic interaction between the sugar of dNTP and Y115. Yet in MuLV RT, its Q190 interacts with 3′OH which, in turn, may interact with β-phosphate. A schematic representation of these interactions is presented in Scheme 1. Panel A represents dNTP incorporation and panel B dNTP. The comparison of the interactions in panels A and B clearly suggests that an interaction with β-phosphate is an important factor for activity with both dNTP and ddNTP and this interaction may be effected directly by an external ligand or by an external ligand via 3′OH. An example of the direct contacts with β-phosphate is that of Y526 of T7 DNA polymerase or Q151 of HIV-1 RT, while the F762 of KF and Q190 of MuLV RT appear to induce the intramolecular contact of 3′OH with β-phosphate.

Participation of Q190 of MuLV RT in catalytic reaction

The requirement of Q190 in the catalytic reaction of MuLV RT (Jin et al., 1999) may be explained as follows: Q190 of MuLV RT does not appear to interact directly with β-phosphate of bound dNTP. However, it can interact with 3′OH of dNTP, which in turn may position it to interact with β-phosphate, permitting full catalytic activity. The mutant Q190N or A, on the other hand, cannot interact with 3′OH of dNTP and thereby may not position it for interaction with β-phosphate resulting in the catalytically deficient phenotype. Nevertheless, the substitution of the Q side chain with shorter (N or A) residues may permit the stable binding of ddNTP via a strong hydrophobic interaction with F155, as discussed earlier. In spite of stable binding of ddNTPs, its turnover is rather compromised, since catalytically important β-phosphate interaction is lacking. A similar explanation can also be attributed to the F762A mutant of KF, where the mutant enzyme exhibits significantly decreased catalytic activity but a moderate increase in ddNTP incorporation (Astatke et al., 1998b).

In our efforts to understand the structural basis for ddNTP/dNTP discrimination and the role of Q190 in this process, we also noted that a conformational change in the loop structure of LPQG motif was necessary to bring the Q190 within interacting distance of the 3′OH of dNTP. In the absence of the above conformational change, catalytic reaction may be severely affected. The characterization of the properties of Q190N and Q190A of MuLV RT has indeed shown that an essential conformational change step prior to and after the bond formation was effected in the mutant enzymes (Jin et al., 1999). Therefore, the two model structures of MuLV RT

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**Scheme 1.** The relationship among 3′OH of dNTP, its β-phosphate and aromatic amino acid residues (Pol I type) or amido-containing amino acid residues (RTs). Schematic representation of the interactions seen among 3′OH and β-phosphate of dNTP with an external functional group (side chain) in the crystal structures of T7 DNA polymerase I (Doublie et al., 1998) and HIV-1 RT (Huang et al., 1998) is shown. The interactions for MuLV RT shown in this scheme are derived from the modeled ternary complex (Figure 1). The dotted lines represent the direct interactions and the dashed lines indicate hydrophobic interactions. In those cases where interactions are not seen or are lost owing to the substitution of dNTP by ddNTP or that by substitution of the amino acid side chain, no lines are drawn. Only the amino acid residue and nucleotide positions are indicated. The interaction of β-phosphate by intra- and intermolecular contacts in Pol I type (T7 and KF) or RT group of enzymes (HIV and MuLV RT) are shown in triangular fashion. This scheme depicts that (i) for the activity of a DNA polymerase, the interaction of β-phosphate with 3′OH of incoming dNTP or a vicinal side chain is required and (ii) for the DNA polymerases that have higher sensitivity for dideoxynucleotides, a functional group side chain that has an interaction with β-phosphate is necessary. Furthermore, it appears that the alignment of β-phosphate (and thereby that of τ-phosphate) by either intra- or intermolecular interaction may be required for the incorporation of ddNTP and dNTP. In the case of ddNTP, this alignment is provided by intramolecular interaction and for dNTP by intermolecular interaction.
ternary complexes that we have presented (Figure 1) are likely to represent the structures before and after conformational change steps. In summary, our analysis of dNTP sensitivity in the mutants of MuLV RT has provided a model for physical interaction of Q190 with substrates.

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References


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