Articles

Tyrosine 222, a Member of the YXDD Motif of MuLV RT, Is Catalytically Essential and Is a Major Component of the Fidelity Center†

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ABSTRACT: Tyrosine 222 of MuLV RT is an invariant residue of the highly conserved YXDD motif in the reverse transcriptase class of enzymes. The residue X is Met 184 in HIV-1 RT and Val 223 in MuLV RT. This residue has been implicated in the fidelity of DNA synthesis, whereas the role of the preceding tyrosine in this aspect, as well as in the catalytic mechanism of MuLV RT, remains to be elucidated. We have substituted Tyr 222 with Phe, Ser, and Ala by site-directed mutagenesis and have characterized the properties of the individual mutant enzymes. The results show that Tyr→Phe substitution did not affect the polymerase activity of the enzyme, while Tyr→Ser and Tyr→Ala substitutions significantly reduced the polymerase activity. The pyrophosphorolysis activities of these mutants showed the same trend as the polymerase activities, suggesting an essential role for Y222 in the catalytic mechanism of MuLV RT. One of the most interesting observations of Y→F substitution was the significantly increased fidelity of DNA synthesis on RNA templates. In addition, a limited extent of ribonucleotide incorporation on RNA template that was consistently noted with the wild-type enzyme was reduced with the Y222F mutant. The resistance to all four ddNTPs, however, persisted in the wild type and Y222 mutants on the RNA template. A ternary complex model of MuLV RT shows that (a) the aromatic ring of Tyr/Phe is positioned between the terminal and penultimate primer bases and (b) the phenolic OH group is seen within hydrogen bonding distance with the base moieties of two template and penultimate primer nucleotides. We propose that the base stacking interaction of Tyr 222 stabilizes the primer terminus position which is essential for the catalytic reaction. However, the weaker stacking interaction of Y compared to F, due to polarization of the π-charge toward the phenoxyl-OH as well as the resonating character of its H-bond center, may provide slight flexibility to the position of the template base which may be responsible for the error-proneness of MuLV RT.

Reverse transcriptase (RT),[1] a member of the DNA polymerase group, is encoded by all retroviruses and plays a defining role in retroviral replication (*1*−*3*). This enzyme has both RNA- and DNA-dependent DNA polymerase activities and an RNase H activity which functions in both an exo- and an endonucleolytic manner (*4*−*7*). Reverse transcriptase is responsible for the synthesis of a double-stranded linear proviral DNA copy of the RNA genome, which is then integrated into the host chromosome. The retroviral reverse transcriptase, specifically HIV-1 RT, has been the most extensively characterized enzyme both biochemically and structurally for obvious reasons. Murine leukemia virus reverse transcriptase (MuLV RT) is another member of the reverse transcriptase family, and investigations of its properties and mechanism are expected to reveal unique properties of the individual RT. It has been purified from virions, and its catalytic properties have been well characterized *(8, 9)*. In contrast to the heterodimeric HIV-1 RT, MuLV RT exists as a monomer in solution (*10, 11*). Several studies have documented RTs as highly hyper-mutable and error-prone enzymes, and it is this property that permits rapid emergence of drug-resistant phenotypes (*12*−*19*).

The two aspartates of the highly conserved YXDD motif (D185 and D186) in HIV-1 RT were the first to be recognized as constituents of the catalytically essential

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[1] Abbreviations: A, F, S, and Y, single-letter codes for the amino acids alanine, phenylalanine, serine, and tyrosine, respectively; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; IPTG, isopropyl-β-thiogalactopyranoside; PP_i, pyrophosphate; RT, reverse transcriptase; IMAC, immobilized metal affinity chromatography; IDA−Sepharose, iminodiacetic acid−Sepharose; MuLV, murine leukemia virus; pol I, *Escherichia coli* DNA polymerase I; PSM, protein solubilizing medium; poly(rA)·$(dT)_{18}$, polyriboadenylic acid annealed with $(oligodeoxythymidylic\ acid)_{18}$; 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; U5-PBS RNA template, HIV-1 genomic RNA template corresponding to the primer binding sequence region; U5-PBS-DNA template, HIV-1 genomic DNA template corresponding to the RNA PBS sequence; PBS, primer binding site.

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carboxylate triad since their mutation resulted in complete inactivation of the enzyme (*20*−*26*). The equivalent carboxylate residues in MuLV RT are Asp 224 and Asp 225, respectively. Mutation of these acidic residues has also been found to result in polymerase-deficient phenotypes (Chowdhury et al., unpublished data). The X position of this motif is occupied by valine, alanine, or methionine depending on the retrovirus or retroelement (*27*−*31*). In MuLV RT, the residue at the X position is valine while in HIV RT it is methionine. An M184V mutation in HIV-1 RT has been encountered in AIDS patients treated with nucleotide analogue drugs (*13, 14, 32*−*35*). In addition to the drug-resistance phenotype, M184V has also been found to exhibit increased fidelity of DNA synthesis (*13*−*15, 36*−*38*). In MuLV RT, mutation of V223 to M223 exhibits reciprocal characteristics with respect to fidelity properties (Chowdhury et al., submitted for publication).

Compared to other members of the YXDD motif, the role of the Y residue is not well documented. Mutations of this residue in HIV-1 RT seem to cause substantial reduction in enzyme activity (*23, 25, 26, 3*9). Biochemical studies of Y183F, Y183S, and Y183A mutants of HIV-1 RT have exhibited an increase in the K_m for the dNTP substrate as well as altered processivity of DNA synthesis (*25, 39*). Despite reduced catalytic activity, the Y183F mutant has been reported to exhibit higher fidelity than the WT enzyme (*37, 39*).

In our efforts to understand the contribution of Y222 of MuLV RT (equivalent to Y183 of HIV-1 RT) in the catalytic mechanism of the enzyme and its implication, if any, on the fidelity of DNA synthesis, we performed site-directed mutagenesis of Y222 and investigated the properties of the resulting mutants. Homologous, Y to F; neutral, Y to A; and polar, Y to S, substitutions were generated in order to understand the contribution of the aromatic ring and the phenolic hydroxyl group in the catalytic process. Preliminary results of the polymerase activity of MuLV RT mutants have indicated an absolute requirement of the phenyl ring at this position for the catalytic activity. This is in contrast to HIV-1 RT, where significant loss of activity is reported with Y→F substitution (*26, 39*). Most interestingly, replacement of Y222 with F shows a significant increase in the fidelity of DNA synthesis on both RNA and DNA templates. The possible molecular basis for the improved fidelity of Y222F of MuLV RT has also been inferred from the 3-D prepolymerase complex model of MuLV RT. This paper describes the results of this investigation.

MATERIALS AND METHODS

Materials

Mutagen-M13 in vitro mutagenesis kit was purchased from Bio-Rad Laboratories. Sequenase and DNA sequencing reagents were from Amersham Life Sciences. Expression vector pET28a and *Escherichia coli* BL21(DE3) were from Novagen. Restriction endonucleases, DNA-modifying enzymes, and HPLC-purified dNTPs were obtained from Promega or Boehringer Mannheim. ^{32}P- and ^{3}H-labeled dNTPs were purchased from Dupont/New England Nuclear Corp. and ICN, respectively. Fast-flow chelating Sepharose (iminodiacetic acid−Sepharose) for immobilized metal affinity chromatography (IMAC) was obtained from Pharmacia. Mutagenic oligonucleotides, sequencing primers, and synthetic template-primers were synthesized at the Molecular Biology Resource Facility at this school. Plasmid pHIV-PBS was a generous gift from Dr. M. A. Wainberg (*40*). All other reagents were of the highest purity grade and purchased from Fisher, Boehringer Mannheim, and Bio-Rad.

Methods

Site-Directed Mutagenesis and Construction of the Expression Plasmid. Site-directed mutagenesis was performed according to the procedure described by Kunkel et al. (*41*). The Y222F, Y222A, and Y222S mutations were introduced using a dU-containing bacteriophage M13mp19 template carrying the *Kpn*I and *Sal*I fragment (847 bp) of the MuLV RT polymerase coding sequence. Phosphorylation of the mutagenic primers, annealing, extension, and ligation were carried out in accordance with the manufacturer's protocol supplied with the in vitro mutagenesis kit. After ascertaining the mutation in M13 by DNA sequencing, the *Kpn*I and *Sal*I fragment was subcloned into the pET-28a-MRT expression cassette (*42, 43*). The recombinant plasmid was then introduced into *E. coli* BL-21(DE3) for induction and isolation of the enzyme (*42, 44*).

Expression and Purification of the Recombinant MuLV RT and Its Mutant Derivatives. The *E. coli* BL-21(DE3) strains carrying the recombinant plasmids were grown at 37 °C in Luria broth containing kanamycin (30 μg/mL). Induction was carried out at $OD_{595} = 0.3$ by the addition of 100 μM IPTG. Cells were further grown at 30 °C for 5 h and harvested by centrifugation at 12000*g* for 20 min at 4 °C. The cell pellet was washed with a buffer containing 50 mM Tris-HCl, pH 8.0, 200 mM NaCl, and 20 mM EDTA. Lysis of the cells and isolation of the enzyme were carried out by metal affinity chromatography as described by Chowdhury et al. (*42*). The purified enzyme preparations were found to be homogeneous as judged by SDS−PAGE analysis (*45*) and were stable at −20 °C for several months. Protein concentrations were determined by the Bradford colorimetric assay (*46*) as well as by densitometric scanning of the Coomassie blue stained protein band on the gel and comparing the intensity with known amounts of BSA.

Polymerase Assays. RT activity of the WT and mutant enzymes was determined on three different template-primers, poly(rA)·$(dT)_{18}$, poly(rC)·$(dG)_{18}$, and poly(dC)·$(dG)_{18}$, by monitoring the formation of radioactively labeled nucleic acid product using the TCA precipitation assay as described below. The reactions were carried out in a final volume of 100 μl containing 50 mM Tris-HCl, pH 8.0, 1 mM dithiothreitol, 100 μg/mL bovine serum albumin, 0.1% NP40, 250 nM template-primer, 60 mM KCl, 5 mM $MgCl_2$, 20 μM [^{3}H]-dNTP (1 μCi/assay) complementary to the homopolymeric template, and 6.6 nM enzyme. The reactions were initiated by the addition of $MgCl_2$, incubated for 7 min at 37 °C, and terminated by the addition of ice-cold 5% trichloroacetic acid containing 10 mM inorganic pyrophosphate. The acid-precipitated materials were then collected on Whatman GF/B filters and counted for radioactivity in a liquid scintillation counter, as described previously (*47*).

Pyrophosphorolysis Activity Assay. The template-primers used for this analysis consisted of poly(rA) ^{32}P-labeled $(dT)_{18}$, U5-PBS HIV-1 RNA primed with 5′-^{32}P 17-mer PBS primer

and 47-mer/5′-^{32}P-labeled 18-mer. The reaction mixture contained 50 mM Tris-HCl, pH 7.8, 1 mM DTT, 60 mM KCl, 0.1 mg of BSA/mL, 5.0 mM $MgCl_2$, 2 nM TP (10^6 Cerenkov cpm/pmol), 1.0 mM pyrophosphate, and 50 ng of enzyme in a total volume of 6 μL. Control reactions were carried out in the absence of pyrophosphate. The reactions were carried out at 25 °C for 30 min and quenched with an equal volume of Sanger's gel loading solution (*48*). The reaction products were analyzed on a 12% denaturing polyacrylamide−urea gel and visualized by autoradiography.

Assays To Determine rNTP Incorporation and ddNTP Sensitivity. To determine if substitution at tyrosine 222 may have altered the recognition of the sugar moiety of the nucleotide substrates, the ability of the mutant enzymes to utilize rNTPs and ddNTPs was assessed. In these experiments, U5-PBS HIV-1 RNA and U5-PBS HIV-1 DNA primed with 5′-^{32}P-labeled 19-mer and 21-mer PBS primers, respectively, were used. Reactions in a final volume of 5 μl contained 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 60 mM KCl, 1 mM DTT, 2 nM labeled primer termini, 0.1% BSA, and 50 ng of enzyme. For the rNTP incorporation experiments, a concentration of either 500 μM each of the four dNTPs, 500 μM each of the four rNTPs, or 500 μM each of 3dNTPs+1rNTP mix was used. For the dideoxynucleotide sensitivity experiments, the assay conditions were identical to those used for rNTP incorporation except that in addition to four dNTPs (500 μM each) the reaction mixture also contained four ddNTPs (250 μM each). After 30 min incubation at 25 °C, the reactions were terminated by the addition of an equal volume of Sanger's gel loading dye (*48*).The reaction products were resolved by denaturing 12% polyacrylamide gel electrophoresis and scanned on a phosphorImager (Molecular Dynamics Inc.).

Fidelity of DNA Synthesis. Fidelity parameters were determined on U5-PBS HIV-1 RNA, U5-PBS 49-mer DNA, and 47-mer DNA templates (Chart 1) primed with the respective ^{32}P-labeled primers in the presence of only three dNTPs as described before (*15*, *49*). The reaction mixture in a final volume of 5 μL contained 50 mM Tris HCl, pH 7.8, 1 mM DTT, 0.1 mg/mL BSA, 60 mM KCl, 2.5 nM labeled TP, 5 mM $MgCl_2$ and only three dNTPs. The pattern of misincorporation and mispair extension was determined at 1, 50, and 500 μM substrate dNTP concentrations. Equivalent amounts of enzyme activity of the wild-type and mutant enzymes were used. The reaction mixture was incubated at 25 °C for 30 min and stopped by the addition of an equal volume of Sanger's gel loading solution (*48*). The products of misincorporation with the individual primers and their mispair extensions were analyzed by denaturing 12% polyacrylamide−urea gel electrophoresis followed by scanning and quantitation on the phosphorImager.

Single-Nucleotide Misincorporation Assays. Misinsertion and mispair extension with all four template bases were measured using the U5-PBS HIV-1 RNA and 49-mer U5-PBS DNA templates annealed with primers of variable lengths so as to provide four different template contexts. The templates were annealed with one of the four 5′-^{32}P-labeled PBS DNA primers of length 17, 19, 20, and 21 nucleotides (see Chart 1). Reaction conditions used to determine the 12 possible mismatches and their mispair extensions with the wild-type MuLV RT and the Y222F mutant were identical to those used in the presence of three dNTPs except that only a single nucleotide was used. The concentration of the individual dNTP substrate was kept constant at 500 μM for both RNA- and DNA-directed reactions. The reaction products were analyzed by 12% polyacrylamide−urea gel electrophoresis and quantitated on a phosphorImager. Percent misincorporation was determined as the ratio of the wrong nucleotide misincorporated and extended versus the total input labeled primer.

Chart 1: Sequence of Template-Primers Used for Activity Assays and Determining the Extent of Misincorporation and Extension with Three dNTP Substrates and a Single dNTP Substrate

47 / 18-mer DNA-DNA template-primer:

5'-CTT CCA TTC ACA CAC TGC-3'
3'-GAA GGT AAG TGT GTG ACG ATG TCT GAC CTT GTT TTT GTG ACA TTG AG-5'

Sequence of theU5-PBS RNA template:

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 UGU UGU CUG CCC GUG UGU GAU GAC UUC UGA GUU CCG UUC GAA AUA AUU CGU CAC CCA AGG GAU CAU CGG UUC UCG AUG GUC CGA GUC UAG A ----------5'RNA sequence of HIV-1

Sequence of the 49 mer DNA template:

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

17mer PBS –primer:

5'-GTC CCT GTT CGG GCG CC-3'

19 mer PBS -primer:

5'-GTC CCT GTT CGG GCG CCA C-3'

20 mer PBS –primer:

5'-GTC CCT GTT CGG GCG CCA CT-3'

21 mer PBS –primer:

5'-GTC CCT GTT CGG GCG CCA CTG-3'

22 mer PBS -primer:

5'-GTC CCT GTT CGG GCG CCA CTG C-3'

Molecular Modeling. The modeling of a prepolymerization ternary complex of MuLV RT was based on the crystal structures of DNA-bound HIV-1 RT (*50*) and the ternary complex of T7 DNA polymerase (*51*). The ternary complex was assembled using the catalytic core fragment of MuLV RT (*52*), 4 base pair A-type DNA, ddCTP, and two metal ions, available from the crystal structures of various DNA polymerases. The details of the modeling protocol will be presented elsewhere (Singh et al., in preparation). Briefly, a 4 base pair A-type DNA was constructed using SYBYL molecular modeling software (Tripos Associates Inc., St. Louis, MO). The A-type DNA and ddCTP from polymerase β structure were first used to model the ternary complex of HIV-1 RT (*53*). The Cα atoms of the palm domain of HIV-1 RT composed of β-strands β6, β9, and β10 and α-helices αE, and αF, were superimposed on the Cα atoms of the palm domain of MuLV RT (PDB file 1mml) which contains β7, β10, β11 and α-helices αH and αI. The side-chain conformation of the catalytic aspartates and the position of the metal ions in the active site were modeled such that the metals exhibited an octahedral coordination sphere. Modeling of the mutant enzyme (Y222F) of MuLV RT was performed by molecular modeling software LOOK 2.1 (Molecular Application, Palo Alto, CA). To obtain specific interactions between the desired side chains and TP and/or dNTP, a conformational search was performed using Kollman United charges on the protein and TP (*54*). Net atomic charges

Table 1: Specific DNA Polymerase Activity of the Wild-Type MuLV RT and Its Mutant Derivatives on Different Template-Primers

	% of WT polymerase activity		
enzyme	poly(rA)·$(dT)_{18}$	poly(rC)·$(dG)_{18}$	poly(dC)·$(dG)_{18}$
WT	100	100	100
Y222F	116	35	107
Y222A	4	1	13
Y222S	4	1	7

[a] The specific DNA polymerase activity of the wild-type MuLV RT and its mutant derivatives was examined on the indicated template-primers in the presence of Mg^{2+} as the divalent cation ion as described under Materials and Methods. The values shown above are the percent of the polymerase activity with respect to the WT enzyme. The 100% WT activities on poly(rA)·$(dT)_{18}$, poly(rC)·$(dG)_{18}$, and poly(dC)·$(dG)_{18}$ are 3.1×10^3, 5.69×10^3, and 2.73×10^3 units/mg, respectively. One unit is defined as the amount of enzyme activity necessary to incorporate 1 nmol of dNMP into acid-insoluble form in 10 min at 37 °C.

obtained from MOPAC calculations (QCPE, Indiana University, Bloomington, IN) were used on ddCTP with +2e charge on each of the metal ions.

RESULTS

Construction and Purification of the WT MuLV RT and Its Mutants. The gene construct pET-28a-MRT encoding the wild-type MuLV RT containing hexahistidine at the N-terminus region was used for site-directed mutagenesis as described by Chowdhury et al. (*42*). Three single mutants of the Y222 residue were constructed. A homologous phenylanine, neutral alanine, and polar serine, lacking the aromatic ring structure but keeping the hydroxyl moiety of tyrosine, replaced the tyrosine side chain. Induction and purification of the enzymes using a metal affinity IMAC column were carried out as described under Materials and Methods. The enzyme protein was dialyzed against storage buffer containing 50 mM Tris-HCl, pH 7.0, 1 mM DTT, 100 mM NaCl, and 50% glycerol. The pH of the storage buffer (pH 7.0) was found to be a critical factor for the stability of the His-tag containing recombinant MuLV RT. The enzyme preparations were homogeneous as judged by SDS−polyacrylamide gel analysis. The levels of protein expression, yield, and solubility and chromatographic characteristics of the mutant proteins were identical to the wild-type enzyme, suggesting no significant change in the overall structure of the mutant enzymes.

Differential Polymerase Activity of the Mutant Enzymes on RNA and DNA Templates. The polymerase activity of the wild-type MuLV RT and its mutant derivatives was assessed on homopolymeric poly(rA)·$(dT)_{18}$, poly(rC)·$(dG)_{18}$, and poly(dC)·$(dG)_{18}$ as described under Materials and Methods, in the presence of Mg^{2+} as the effective divalent cation. The results of these assays are summarized in Table 1. The polymerase activity of the Y222F mutant was increased by 5−20% over the WT enzyme activity with poly(rA) and poly(dC) templates. In contrast, the activity with poly(rC)·$(dG)_{18}$ was only about 35% of the WT. Substitution of Tyr with a nonconservative residue such as Ala or Ser resulted in drastic reduction in the polymerase activity ranging between 2 and 7% of the WT. These results suggest that an aromatic ring at position 222 is essential for efficient polymerase function of the enzyme since its substitution by homologous Phe but not by Ala or Ser is well tolerated.

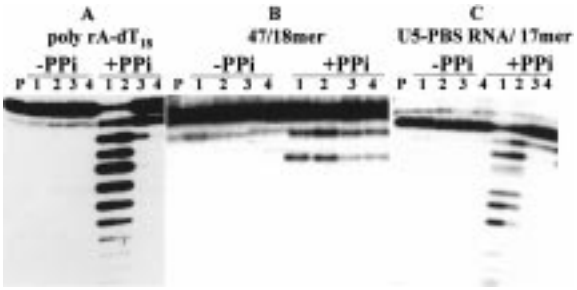

FIGURE 1: Pyrophosphorolysis activity of the wild-type MuLV RT and its Y222 mutant derivatives. Three template primers [(A) poly(rA), 5′-^{32}P-labeled dT_{18}; (B) 47-mer 5′-^{32}P-labeled 18-mer primer; and (C) 5′-^{32}P-labeled 17-mer PBS annealed to the 496 base U5-PBS HIV-1 RNA template] were used to determine the pyrophosphorolysis activity of the wild-type MuLV RT and its mutant derivatives. Fifty nanograms of the individual enzyme protein was incubated with the desired template primer in the presence or absence of 1 mM PP_i at 25 °C for 30 min in a standard reaction mixture as described under Materials and Methods. Lanes 1−4 represent reactions carried out in the presence of the wild-type MuLV RT, Y222F, Y222A, and Y222S mutant enzymes, respectively.

Pyrophosphorolysis Activity of Y222 Mutants. Pyrophosphorolysis may be considered as the reversal of the polymerase reaction, and, therefore, residues essential in the forward reaction may be expected to participate in the reverse reaction. Since nonconservative substitutions at Y222 result in severely impaired polymerase function of the enzyme, the effect of these mutations on the reverse reaction, namely, pyrophosphorolysis was also examined. The results are shown in Figure 1. As seen in the figure, a significant reduction in the pyrophosphorolysis activity on RNA templates by both Y222A and Y222S mutant enzymes was observed. In contrast, the conserved Y222F mutant enzyme displayed a wild-type pyrophosphorolysis activity. We have consistently observed that the WT enzyme utilizes the DNA template rather poorly compared to an RNA template in the reverse reaction. Consequently, no significant change in the pyrophosphorylysis activity of the mutants on this template-primer was observed. These results suggest that tyrosine or a phenyl ring is required in both the forward (polymerase) and the reverse (pyrophosphorolysis) polymerase function of the enzyme on an RNA template.

RNA Replicase Activity Exhibited by the Mutants of Y222. Minor structural changes in the Y/F side chains at specific locations in both DNA and RNA polymerases have been implicated in alteration of the specificity of the individual enzyme for the nucleotide substrates (*55*−*63*). Earlier, Georgiadis et al. noted the presence of Phe 155 near the 2′-deoxyribose moiety of dNTP substrate in their ternary complex model (*52*). Subsequently, Goff and colleagues demonstrated that mutation of F155 to V155 expanded the recognition of MuLV RT to rNTPs, thus confering RNA polymerase activity to MuLV RT (*57*). Tyrosine in the YXDD motif is highly conserved among RNA-dependent RNA polymerases as well as reverse transcriptases (*30, 31*). We, therefore, examined the ability of WT and Y222 mutant derivatives to utilize rNTPs as substrates. In this analysis, the ability of the wild-type enzyme and its mutant derivatives to catalyze the incorporation of rNTPs was tested using both heteropolymeric DNA and RNA templates (Figure 2). A rather interesting pattern of rNTP incorporation by the various substituents at tyrosine 222 emerged. First of all, to

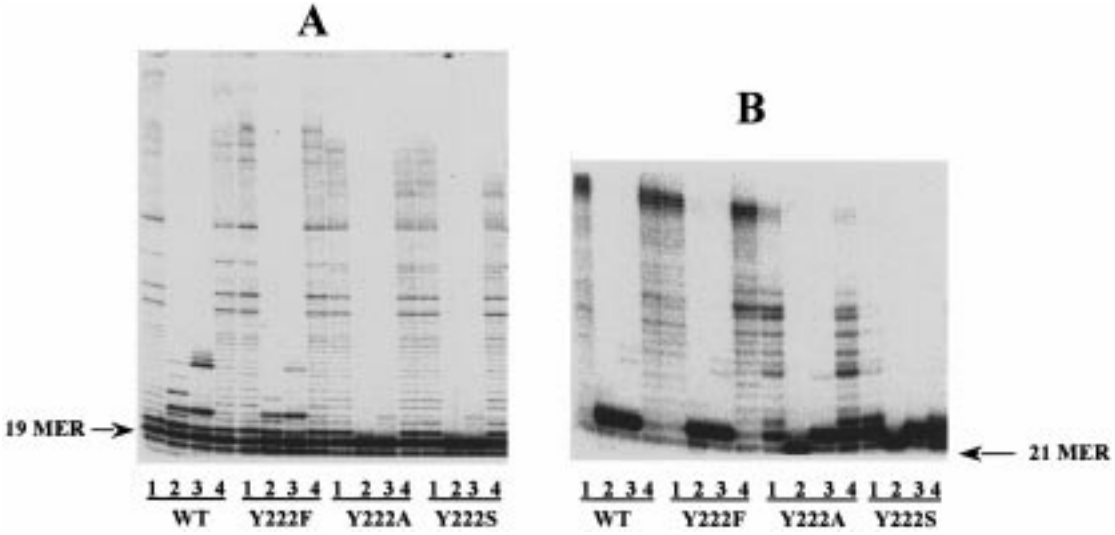

FIGURE 2: Utilization of rNTPs and sensitivity to ddNTPs in RNA- and DNA-directed reactions by the wild-type MuLV RT and Y222 mutant derivatives. The ability of the wild-type MuLV RT and its indicated Y222 mutant derivatives to catalyze incorporation of rNTP as substrates as well as their sensitivity toward dideoxynucleotides was examined on U5-PBS RNA template annealed with 5′-^{32}P-labeled 19-mer (A) or 49-mer U5-PBS DNA template annealed with 5′-^{32}P-labeled 21-mer (B). Reactions were carried using 2 nM labeled primer termini and 50 ng of enzyme as described under Materials and Methods. The products were analyzed on a denaturing 12% polyacrylamide gel and scanned on a phosphorImager. Lane 1, four dNTPs (500 μM each); lane 2, four rNTPs (500 μM each); lane 3, three dNTPs + rUTP (500 μM each); and lane 4, four dNTPs (500 μM each) + four ddNTPs (250 μM each).

our surprise, the wild-type enzyme appeared to carry out limited synthesis with rNTPs as sole substrate on RNA-PBS template. This is the first demonstration of RNA-dependent RNA polymerase (RNA replicase) activity of a reverse transcriptase. The Y222F mutant displayed a significantly reduced extent of rNTP incorporation compared to the WT enzyme. The Y222F mutant enzyme incorporated only about 5 ribonucleotides compared to about 10 ribonucleotides incorporated by the WT MuLV RT (Figure 2A, lanes 2 of Y222F and WT enzyme). However, in the presence of a mixture 3dNTP+1rNTP substrates, both the WT and the Y222F mutant displayed near similar ability to catalyze extended synthesis (Figure 2A, lanes 3 of Y222F and WT enzyme). It is probable that this extended synthesis may be due to the incorporation of one of three dNTPs (−A) and not due to the rNTP (UTP) against the template nucleotide dA. However, comparison of lane 3 of Figure 2A with lane 1 of Figure 3A suggests that the prominent pauses seen in Figure 2A (lane 3) at positions 20, 23, 29, and 30 correspond to the template nucleotide A against which the rUTP is incorporated. Although both Y222A and Y222S mutant enzymes could incorporate only a single ribonucleotide on U5-PBS RNA template, they were unable to extend it further (Figure 2A, lanes 2 of Y222A and Y222S mutants). In the presence of a mixture of 3dNTP+1rNTP substrates, these mutants displayed drastically reduced ability to extend the 3′-ribonucleotide primer terminus (Figure 2A, lanes 3 of Y222A and Y222S mutants). In contrast to an RNA template, a different pattern of ribonucleotide incorporation by the WT and the Y222 mutants was observed on DNA template. Unlike the pattern of ribonucleotide incorporation seen on an RNA template by the WT and Y222F mutant, both these enzymes could incorporate only a single ribonucleotide on a DNA template (Figure 2B, lane 2). Moreover, extension of ribonucleotide-incorporated primer terminus was catalyzed to a negligible extent by both these enzymes when supplemented with dNTPs (Figure 2B, lane 3). The Y222A and Y222S mutants displayed a qualitatively similar pattern, although the extent of incorporation of a single ribonucleotide on a DNA template primer was extremely small. These results suggest that the functional side chain of Y222 may permit limited recognition of the ribonucleotides as substrates for polymerization on an RNA template. Interestingly, the removal of the OH group at this position (substitution of Y to F) significantly reduces the ability of the enzyme to recognize ribonucleotides. The observation that the WT enzyme as well as mutants derivatives of Y222 exhibits a rather limited extent of ribonucleotide incorporation on a DNA template suggests that Y222 may strongly influence the selection/discrimination of ribonucleotides versus deoxyribonucleotides on a DNA template.

Sensitivity to ddNTPs. Among the various RNA and DNA polymerases studied to date, the F and the Y residues near the active site have been implicated in discrimination against deoxy- and dideoxy-containing nucleotides (*59, 61*−*63*). In the case of HIV-1 RT, dideoxynucleotides have been shown to be competitive inhibitors of the enzyme (*64, 65*). However, MuLV RT is insensitive to ddNTP inhibitors. The residue responsible for this discrimination in MuLV RT is not yet known. Our observation that the wild-type MuLV RT could use rNTP substrates with RNA template and that the Y222F mutant has reduced ability to utilize rNTPs suggested that it may play a role in the recognition of the 3′-OH moiety of the sugar. We therefore tested the sensitivity of the various mutants of Y222 to ddNTP inhibitors. The analysis was carried out on RNA-PBS template annealed to a labeled 19-mer primer and 47-mer DNA with labeled 18-mer primer. The ratio of ddNTP to dNTP was maintained at 1:2. The desired enzymes were incubated with the template-primer, and the reactions were initiated by the simultaneous addition of dNTPs and ddNTPs. A control lane for each enzyme consisted of polymerization reaction carried out in the presence of dNTP substrates only. The results of this analysis are presented in Figure 2A,B). A comparison of the pattern of synthesis by the wild type and mutant enzymes, in the presence or absence of ddNTP (lanes 1 and 4, respectively),

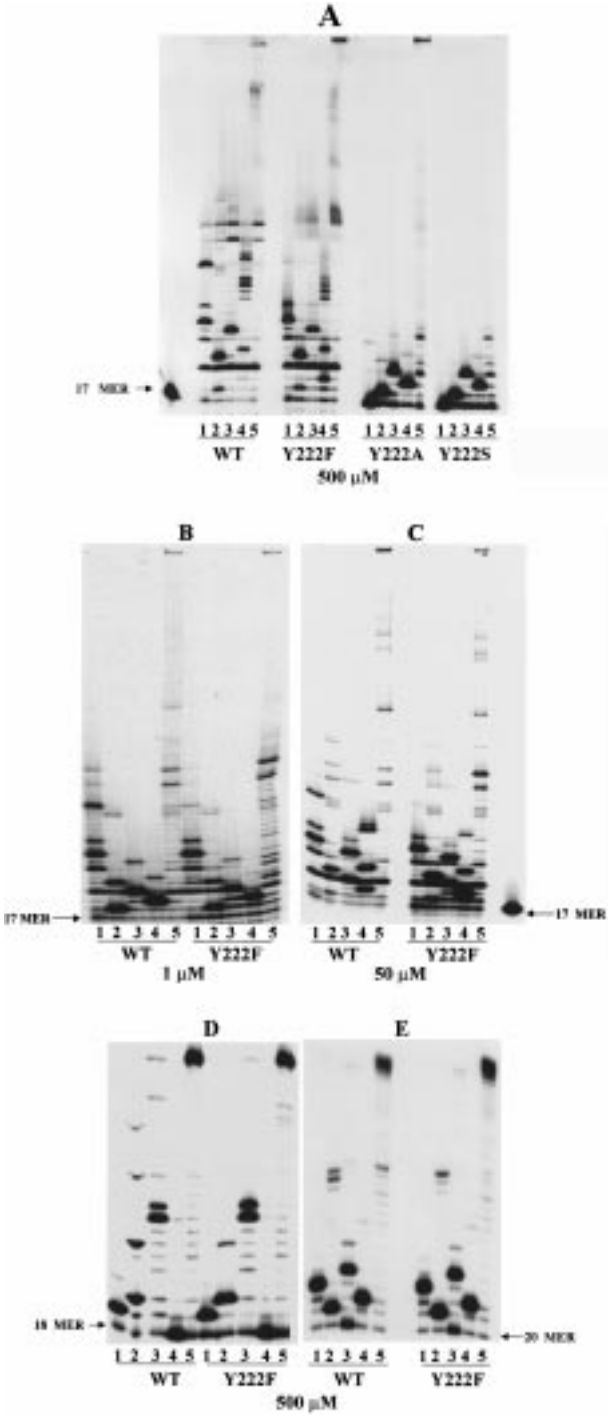

FIGURE 3: Assessment of fidelity of the wild type and Y222 mutant derivatives of MuLV RT with RNA and DNA templates in the presence of three dNTPs. Fidelity of the WT and mutant RTs was determined using the U5-PBS HIV-1 RNA template primed with ^{32}P-labeled 17-mer PBS primer (frames A, B, and C), a 47-mer ^{32}P-labeled 18-mer TP (D), and a 49-mer U5-PBS DNA annealed with a ^{32}P-labeled 20-mer PBS TP (E). The desired enzymes were incubated at 25 °C for 30 min with 2 nM labeled template-primer and the indicated dNTP substrates as described under Materials and Methods. The products were resolved on a 12% polyacrylamide−urea gel and scanned. In frames A, B, and C, three dNTPs were present at concentration levels of 500, 1, and 50 μM, respectively, while in frames D and E only 500 μM concentration of the three dNTPs was used. In all sets, lanes 1 through 4 represent reactions carried out in the absence of dATP, dCTP, dGTP, and dTTP, respectively. Lane 5 represents DNA synthesis in the presence of all 4 dNTPs.

with RNA template-primer shows no change. An insignificant amount of discrimination against ddNTPs on the DNA template-primer was observed. This suggests that Y222 of MuLV RT is not involved in the discrimination against ddNTPs on an RNA template. This is in contrast to analogous mutants of HIV-1 RT where both Y183F and Y183A mutants have been shown to exhibit a high degree of resistance to ddNTPs (*39*).

Fidelity of DNA Synthesis. Recent reports on HIV-1 RT mutants of Y183 have indicated improved fidelity characteristics in spite of significant reduction in the catalytic activity (*23, 26, 37, 39*). In MuLV RT, the status of Y222 and its mutant derivatives needed thorough investigation of this aspect. For our studies, we selected a modification of the gel-based assay, also described as the minus sequencing assay (*15, 49*). The advantage of this assay is that it allows the quantitative determination of the net extent of misincorporation and mispair extension. In addition, the gel-based assay permits qualitative determination of the nature of the fidelity pattern of the individual enzyme. Template-primers used in this study comprised the U5-PBS RNA and 49-mer U5-PBS DNA annealed with the 5′-^{32}P-labeled PBS primers and the 47-mer (5′-^{32}P)-18-mer DNA−DNA TP. Fidelity of DNA synthesis on RNA template was evaluated in the presence of three dNTPs at concentrations of 1, 50, and 500 μM with the WT and Y222F mutant enzymes. Due to very low activity of Y222A and Y222S mutants, we used only 500 μM dNTP concentration to determine the fidelity parameters of these mutants. The results of this analysis are presented in Figure 3. Our results indicate that all the mutants of Y222 have higher fidelity on U5-PBS RNA template than the wild-type enzyme (Figure 3A−C). Lanes 1 through 4 of Figure 3 represent the reaction products obtained in the absence of a single nucleotide, namely, dATP, dCTP, dGTP, and dTTP, respectively, from the reaction mixtures. Lane 5 represents synthesis in the presence of all four dNTPs. Generally, the absence of one of the four dNTPs is expected to result in the accumulation of the product preceding the site of the missing nucleotide. The appearance of products at and following the site of the missing nucleotide indicates infidelity representing misincorporation and mispair extension. Our data indicate that Y222F is the best of the three mutants in terms of fidelity parameters for its catalytic activity is similar to the wild type (Figure 3A−C, lane 5) and yet it is significantly less error-prone, as seen by the reduced misincorporation and mispair extension at all substrate concentrations tested. We would like to point out that the extent of misincorporation and mismatch extension for the individual missing nucleotides is different. For instance, both −A and −T lanes (Figure 3A−C) show significantly higher fidelity of the Y222F mutant compared to the WT enzyme. In the case of the −C and −G lanes, although the Y222F mutant is capable of extending the misincorporated primer to the same length as the WT enzyme, the total amount of misincorporation and mismatch synthesis is less. Y222S and Y222A are poor catalysts (Figure 3A, lane 5); nonetheless, these also show extremely low levels of misincorporation and mispair extension (Figure 3A, lanes 1−4). With regard to the fidelity pattern on DNA template, the Y222F mutant exhibited contrasting results on two different DNA templates. When 47−18-mer DNA was used, Y222F showed significantly reduced misincorporation and mismatch DNA synthesis than the WT enzyme (Figure

3D). This was evident specifically in the case of the −C and −G missing nucleotides. In contrast to these results, when 49−20-mer DNA having the same sequence as the U5-PBS HIV-1 RNA was used, both the Y222F mutant and the WT enzyme exhibited near identical extents of misincorporation and extension, thereby implying that the fidelity of this mutant enzyme may, to some extent, be governed by the sequence and nature of the TP (Figure 3E). In the case of the Y222S and Y222A mutants, their low catalytic activity with both DNA template-primers did not permit a reliable estimate of their fidelity characteristics on these templates.

Mispair Extension Fidelity in the Presence of a Single dNTP Substrate. Nucleotide misinsertions contribute to the overall polymerase fidelity especially in the case of the RTs, which lack proofreading ability. The fidelity parameters studied in the presence of three nucleotides represent a qualitative assessment of fidelity. To investigate quantitatively the extent and nature of misincorporation and mispair extension with 12 possible nucleotide mismatches, we used 4 different primers to provide 4 different template base contexts. Since the nonconservative mutants Y222A and Y222S exhibited extremely low catalytic activity, our analysis was restricted to the Y222F mutant and the WT enzyme with both RNA and DNA templates at saturating (500 μM) single substrate concentration. The formation of 12 possible misinsertions by the mutant and WT enzymes was assessed under identical reaction conditions. One representative analysis using U5-PBS RNA 19-mer TP is shown in Figure 4A. Lanes 1 through 4 represent the extent of misincorporation and its mispair extension in the presence of dATP, dCTP, dGTP, and dTTP, respectively. Lane 5 represents the total synthesis in the presence of all four dNTPs. The results obtained with various mismatches and mispair extension are plotted in Figure 4B, which shows a comparative picture of the percent misincorporation and mispair extension of the individual mismatch for the WT and the Y222F mutant on RNA template. As shown in the figure, the percent misinsertion and mispair extension was lowest with the Y222F mutant for each of the 12 possible individual mismatches. Both the WT and Y222F enzymes exhibited the maximum misinsertion and mispair extension for the mispairs U:C, U:G, and U:T, respectively, whereas the least was seen for the C:C mispairs. Interestingly, with U5-PBS DNA template, both the WT and Y222F enzymes exhibited a similar pattern with a negligible extent of misincorporation and mispair extension. These results suggest that Tyr 222 of MuLV RT may selectively influence the substrate binding pocket with RNA templates but not with DNA templates.

DISCUSSION

Tyr 222 of MuLV RT is a component of the YXDD motif present among all retroviral RTs. The importance of this motif comes from the fact that the two aspartates constitute the catalytically essential divalent cation coordinating centers that are conserved in all polymerases (*20*−*26*). The role of the first two members, Y and X, is somewhat unclear, although indications from HIV-1 RT studies have implicated Y in the catalytic process (*23*, *25*, *26*, *39*). Our preliminary studies indicate an important catalytic role for Y222 as judged by the fact that substitution of Tyr to Ser or Ala results in severely impaired catalytic activity of the enzyme while Tyr to Phe substitution retained the WT activity (Table 1). This significant decrease in the enzyme activity in the case of the nonconservative substitutions was found to be due to changes in both K_m and k_{cat} (unpublished results). In contrast, Tyr to Phe substitution at the corresponding 183 position of HIV-1 RT resulted in significant reduction of the polymerase activity (*26*, *39*). Thus, in MuLV RT, a phenyl ring seems to be sufficient for efficient catalysis whereas in HIV-1 RT the ring as well as the phenolic-OH group appears to be necessary for complete catalytic activity. A similar pattern was observed in the reverse polymerase reaction (pyrophosphorolysis reaction) with both wild-type MuLV RT and its Y222 mutant derivatives (Figure 1), suggesting an identical role for this residue in both the forward and backward reactions.

Curiously, tyrosine in the YXDD motif not only is conserved in retroviral RTs but also is invariant in RNA-dependent RNA polymerases (*30*, *31*). Therefore, the existence of a common mechanism for substrate nucleotide selection in all polymerases may be predicted/expected. The basic difference in the substrates for an RNA polymerase and a reverse transcriptase lies at the 2′-OH position of the sugar moiety. Recent studies for several nucleic acid polymerases have indicated that the presence of phenylalanine and/or tyrosine residues at the dNTP binding site plays an important role in discriminating between ribo- and deoxyribonucleotides (*55*−*58*, *60*). F155 in MuLV RT and F644 and F667 in T7 RNA polymerase have been shown to be partly responsible for the discrimination between ribo- and deoxyribonucleotides (*57*, *66*). In the present investigation, we have shown that a Tyr or Phe residue at position 222 of MuLV RT permits limited incorporation of rNTPs selectively with RNA templates. We propose that this activity represents a cryptic RNA replicase activity. Furthermore, the observation that in the presence of a mixture of 3dNTPs+1 rNTP rNTP can partially substitute for the missing dNTP in the polymerase reaction suggests the possible utility of such an activity. Most interestingly, even a conservative substitution of Tyr→Phe results in a significant reduction in the incorporation of rNTPs with RNA template, whereas Tyr→Ser and Tyr→Ala substitutions caused near complete abolishment of ribonucleotide utilization. These observations suggest that both the phenyl ring and the OH group of Tyr may be required in the recognition of rNTP. This is in contrast to the requirement of only the aromatic ring for the recognition of dNTPs.

It may be recalled here that the F155V mutant of MuLV RT has been shown to incorporate rNTPs on both RNA and DNA templates (*57*). The position of F155 in modeled MuLV RT ternary complex is clearly in close vicinity of the sugar moiety (*52*), and therefore its mutation can justify the effect on the sugar recognition. For Y222, this does not appear to be the case. The molecular model of the polymerase ternary complex of MuLV RT, DNA, and dNTP (Figure 5) shows Y222 within interacting distance from the two bases of the primer nucleotides with its OH group pointing toward the base moiety of the template. It appears that this interacting environment indirectly allows for the binding of both rNTP and dNTP with RNA template. Furthermore, Phe substitution at position 222 selectively reduces the incorporation of rNTPs but not that of dNTPs, suggesting that the phenolic OH interaction with the template base is somehow required for

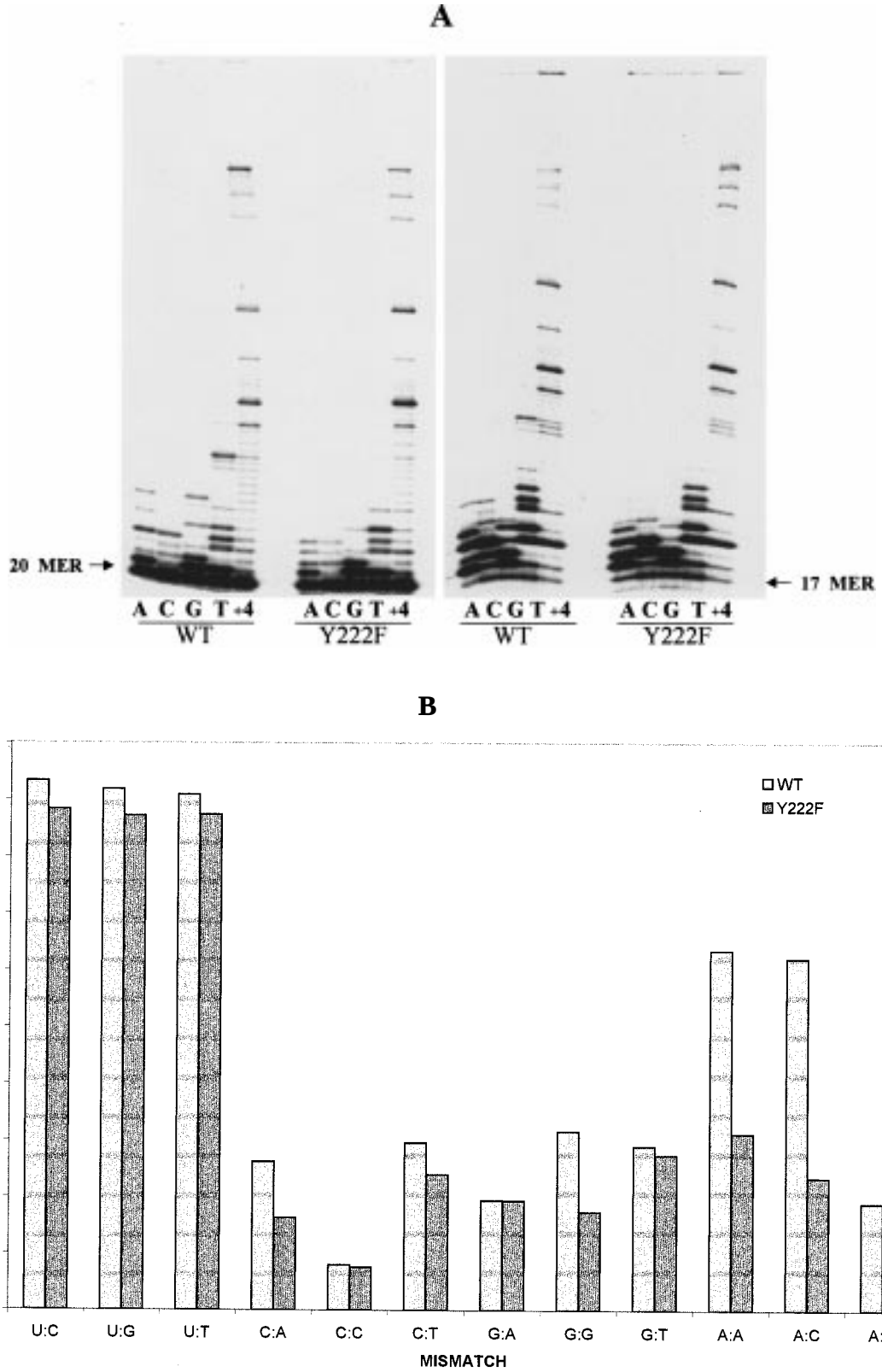

FIGURE 4: Misinsertion and mispair extension catalyzed by the wild-type and Y222F MuLV RT in the presence of a single dNTP with different template context (A) and its graphic representation (B). The ability of the enzymes to generate and extend mispairs in the presence of only a single dNTP was assessed on U5-PBS RNA primed with one of the four 5′-^{32}P-labeled PBS DNA primers of lengths 17, 19, 20 and 21 nucleotides (Chart 1). Each set of experiments was carried out in the presence of all four dNTPs as well as in the presence of a single indicated dNTP at a final concentration of 500 μM each for 30 min at 25 °C as described under Materials and Methods. The products synthesized under these conditions were resolved by 12% polyacrylamide−urea gel electrophoresis, scanned, and quantitated using phosphorImager analysis. Panel A represents the pattern of misinsertion and mispair extension on RNA-PBS template primed with PBS primers 17-mer and 20-mer. Lanes marked A, C, G, and T represent reactions carried out in the presence of dATP, dCTP, dGTP, or dTTP, respectively. Lanes marked +4 represent the products synthesized in the presence of all four dNTPs. (B) Fidelity assays were carried out on a U5-PBS RNA primed with one of the four different primers having A, C, G, or T as the first template nucleotide (Chart 1) in the presence of a single dNTP substrate as described in frame A. Band intensities were scanned on a phosphorImager and quantitated. The percentage of both misincorporation and mispair extension with each enzyme was calculated and plotted for each mismatch as described under Materials and Methods.

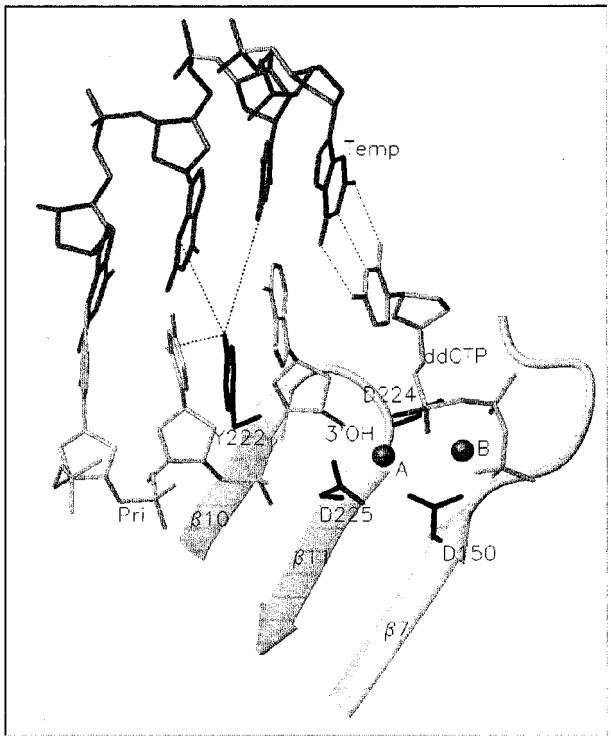

FIGURE 5: A prepolymerization ternary complex model of MuLV RT showing the interactions of Y222 with the bases of template-primer. The template nucleotides are in dark gray and marked 'Temp'. The primer nucleotides are in light gray and labeled 'Pri'. The dNTP is represented by ddCTP. The dotted lines between base moieties of ddCTP and template represent a complementary Watson−Crick hydrogen bonding pattern. β7, β10, and β11 (gray arrows) are components of the palm subdomain of MuLV RT and are taken from the crystal structure of Georgiadis et al. (1995). The protein side chains of Y222, D150, D224, and D225 together with two metal ions (A and B) are shown in dark shade. Note that the side chain of Y222 makes two types of interactions with the template-primer: (i) the hydrophobic base stacking interaction due to its intercalated position between two primer bases; and (ii) three possible hydrogen bond interactions with the base moieties of two template and penultimate primer nucleotides. Interaction of Y222 with DNA template base shown here is expected to be valid with RNA template base as well, since both templates assume 'A' DNA like geometry at the active center. Due to the near-equidistant location of the OH among three potential hydrogen acceptor atoms, the hydrogen bonding of phenolic Y222 is proposed to have a resonating character; i.e., the hydrogen bond formation capability of Y222 will continually shift among the three acceptors. This will result in occasional change in the positions of the template or primer nucleotide which in turn can favor accommodation of noncomplementary dNTP giving rise to error-proneness of the enzyme.

the incorporation of rNTPs. This property also seems to be related to the alteration in the fidelity characteristics of Y222F (see below).

MuLV RT, unlike HIV-1 RT, but similar to prokaryotic polymerases, has been shown to exhibit strong resistance to ddNTPs. In prokaryotes, a specific Phe, e.g., Phe 762 in *E. coli* DNA polymerase I, is responsible for discrimination against ddNTPs (*59, 63*). In HIV-1 RT, resistance to ddNTPs is acquired by mutation of several residues, some of which are far away from the dNTP binding pocket (*12*−*19, 32*−*35, 64, 65*). A decrease in the binding affinity and turnover, due to mutation, has been postulated as the mechanism of resistance development (*17, 18, 67*). The results obtained with some of the naturally occurring drug-resistant variants of HIV-1 RT (e.g., M184V and Q151M) provide support for this conclusion. Since no ddNTP-sensitive mutant phenotype of MuLV RT has been reported, we examined if the Y222 mutant derivatives of MuLV RT exhibited any altered sensitivity toward ddNTPs. We found that both the WT and the Y222 mutants exhibit a similar pattern of resistance to ddNTPs on both RNA and DNA templates (Figure 2). These observations suggest that Y222 of MuLV RT is not involved in conferring sensitivity or resistance to ddNTPs, although its counterpart in HIV-1 RT exhibits some change from sensitive to resistance phenotype upon mutation at the equivalent site (*39*).

Several studies have suggested that both HIV-1 RT and HIV-2 RT are more error-prone than MuLV RT and AMV RT (*49, 68*−*72*). Generally, two types of errors may be encountered during the replication of a template strand; one relates to insertion fidelity and the other to mispair extension. Both of these properties have been implicated in the low fidelity observed with HIV-1 and HIV-2 RTs (*70, 73*). With MuLV RT and its Y222F mutant, we had noted a decrease in the ability of the latter to incorporate rNTPs, which may also be argued as an indication of alteration in fidelity. An examination of the error-prone DNA synthesis in the presence of three dNTPs by the wild-type MuLV RT and its Y222 mutant derivatives clearly shows a significant decrease in the error-prone DNA synthesis by the mutant enzymes. When synthesis was monitored in the presence of three dNTPs (Figure 3), Y222F, which is catalytically as active as the wild-type enzyme, extended the primer to a much smaller extent than the WT enzyme. This observation suggests that Tyr to Phe substitution results in significant reduction of both misinsertion and mispair extension as compared to the wild-type enzyme. Further, fidelity examination using both RNA and DNA templates in the presence of a single dNTP substrate, with all four template nucleotide contexts, confirmed the overall superiority of Y222F as judged by the significantly lower extent of misinsertion and extension. Among the various combinations, U template appeared to provide the maximum misincorporation environment whereas C templates appeared to be copied with little or no errors (Figure 4B). Qualitatively similar results were also reported for HIV-1 RT's Y183F mutants, albeit the catalytic activity of Y183F is significantly less than that of the WT HIV-1 RT (*39*).

To obtain some physicochemical justification for the observed changes in the fidelity characteristics of Y222F compared to wild-type enzyme, we compared two ternary complex models of MuLV RT containing Y222 and F222. A prepolymerization ternary complex model of MuLV RT containing DNA and ddNTP shows that the aromatic ring of Tyr 222 stacks between the bases of the last two primer nucleotides, and the phenolic-OH is well within hydrogen bonding distance from bases of two successive template nucleotides as well as the base of the penultimate primer nucleotide (Figure 5). The stacking of the aromatic ring appears to stabilize the primer terminus at the appropriate position required in the nucleotidyl transfer reaction. However, when the OH of the tyrosine is removed by substitution with Phe, an interaction with template bases is lost. There are two plausible mechanisms by which the loss of OH (from tyrosine) would influence the position of the template or that of the primer nucleotide. The net atomic and π-charges on the aromatic ring atoms of both tyrosine and phenylalanine

Table 2: Net Atomic and π-Charges on the Ring Atoms[a] of Tyrosine and Phenylalanine in the Presence and Absence of DNA[b]

	Phe				Tyr			
	with DNA		without DNA		with DNA		without DNA	
atom	net	π	net	π	net	π	net	π
CG	−0.08	0.95	−1.66	1.03	−0.05	1.03	0.12	1.06
CE1	−0.09	0.89	−0.82	1.03	−0.11	0.92	−0.13	1.03
CD1	−0.13	0.86	−0.12	1.03	−0.07	0.92	−0.05	1.03
CG	−0.97	0.88	−0.24	1.05	−0.05	1.08	−0.15	1.04
CD2	−0.19	0.88	−1.07	1.03	−0.10	0.84	−0.05	1.03
CE2	−0.04	0.87	−1.77	1.03	−0.18	0.95	−0.19	1.03

[a] The ring atoms of Phe/Tyr are represented in standard PDB nomenclature. [b] The PM3 semiempirical quantum chemical method (*74*) was employed for the calculation of the net atomic and π-charges. These charges were calculated for an extracted fragment containing only Y/*F*222 and two terminal base pairs from a ternary complex of MuLV RT, template-primer, and ddNTP (Figure 5).

in the presence of template-primer (see model in Figure 5) as calculated by the PM3 method (*74*) have shown that the charges on the ring atoms of Phe are greater than for the corresponding atoms of Tyr (Table 2). This may result in stronger base-stacking interaction between the bases of the primer nucleotides and the ring of F222 compared to that with Y222. This may imply that Y222 may provide relatively higher flexibility in positioning the primer terminus, resulting in an occasional compromise in the Watson−Crick base pairing based DNA synthesis. In the second scenario, we envisage a stable primer terminus due to the base stacking of either the Tyr or the Phe, but the OH group of Tyr may slightly destabilize two template bases and penultimate primer base by transiently bonding with O2 and N3 of the template base moieties and O2 of the primer base. Because of three possible hydrogen bond centers, the aromatic OH may be expected to resonate among the three bases, causing a slight local destabilization near the active site. This in turn may allow an occasional mismatch or error-prone synthesis. In conclusion, we have reported here the catalytic importance of the aromatic ring of Tyr of the YXDD in MuLV RT and have shown it to be one of the important players in the RNA replicase activity as well as error-prone synthesis of DNA. A possible mechanism based on the molecular model of the ternary complex suggests a higher destabilization of the template-primer base as the probable cause for the error-prone synthesis.

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