# Involvement of Phenylalanine 272 of DNA Polymerase Beta in Discriminating between Correct and Incorrect Deoxynucleoside Triphosphates†

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ABSTRACT: DNA polymerase $\beta$ is a small monomeric polymerase that participates in base excision repair and meiosis [Sobol, R., et al. (1996) *Nature 379*, 183−186; Plug, A., et al. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1327−1331]. A DNA polymerase $\beta$ mutator mutant, F272L, was identified by an in vivo genetic screen [Washington, S., et al. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 1321−1326]. Residue 272 is located within the deoxynucleoside triphosphate (dNTP) binding pocket of DNA polymerase $\beta$ according to the known DNA polymerase $\beta$ crystal structures [Pelletier, H., et al. (1994) *Science 264*, 1891−1893; Sawaya, M., et al. (1997*) Biochemistry 36*, 11205−11215]. The F272L mutant produces errors at a frequency 10-fold higher than that of wild type in vivo and in the in vitro HSV-*tk* gap-filling assay. F272L shows an increase in the frequency of both base substitution mutations and frameshift mutations. Single-enzyme turnover studies of misincorporation by wild type and F272L DNA polymerase $\beta$ demonstrate that there is a 4-fold decrease in fidelity of the mutant as compared to that of the wild type enzyme for a G:A mismatch. The decreased fidelity is due primarily to decreased discrimination between the correct and incorrect dNTP during ground-state binding. These results suggest that the phenylalanine 272 residue is critical for maintaining fidelity during the binding of the dNTP.

DNA polymerases play a central role in the replication, recombination, and repair of DNA (*1*). They catalyze the template-directed incorporation of a deoxyribonucleoside monophosphate (dNMP) substrate into a growing DNA polymer. DNA polymerases discriminate between the correct and incorrect dNTP mainly during binding of the substrate or by an induced-fit mechanism (*2*). Errors occur during DNA synthesis if a polymerase fails to discriminate between the correct and incorrect dNTP substrates. The incorporation of errors into the genome gives rise to mutations that may result in genetic disease or genomic instability, which contributes to neoplastic disease and aging (*3*, *4*). Therefore, it is important to understand the molecular mechanisms that DNA polymerases employ to synthesize DNA accurately.

DNA polymerase $\beta$ is a 39 kDa protein that catalyzes the synthesis of DNA and the removal of 5′-terminal deoxyribose phosphate (dRP) residues from incised apurinic and apyrimidinic sites (*5*). Pol $\beta$[1] functions in base excision repair (BER) by filling in gaps that result from excision of damaged bases (*6*). In fact, it has been shown that pol $\beta$ is most active and accurate when it utilizes a 1-base pair gapped DNA substrate containing a 5′-phosphate, suggesting that this is the physiological DNA substrate of pol $\beta$ (*7*, *8*). Pol $\beta$ also functions in meiosis (*9*). Although there is no evidence for a role for pol $\beta$ in the essential process of DNA replication, disruption of both copies of the pol $\beta$ gene in mice results in embryonic lethality, suggesting that pol $\beta$ is essential for embryonic viability or development (*10*).

The crystal structures of pol $\beta$ complexed with dideoxycytidine triphosphate (ddCTP) and 3′-recessed, 1-base pair gapped, and nicked DNA templates have been determined (*11*−*13*). These structures indicate that pol $\beta$ binds to DNA and to its dNTP substrate in an open conformation. Once the substrate is bound, the polymerase assumes a closed conformation. It has been postulated that this conformational change is one of the mechanisms by which pol $\beta$ discriminates between the correct and incorrect dNTP substrates (*13*). Other structural elements which may govern the fidelity of pol $\beta$ are (i) the interactions between key amino acid side chains and the minor groove of the DNA that function to position the DNA within the active site and (ii) a hydrophobic dNTP binding pocket that is proposed to participate in nucleotide selectivity of deoxyribose over ribose through steric exclusion by the protein backbone segment spanning Tyr271 to Gly274 (*14*, *15*). Pre-steady-state kinetic studies indicate that the rate-limiting step during dNTP incorporation is a conformational change preceding catalysis that most likely plays a major role in excluding the incorrect dNTP (*16*, *17*). It has also been suggested that ground-state binding of the substrate plays a role in discrimination between the correct and the incorrect dNTP (*17*−*19*).

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[1] Abbreviations: Pol, polymerase; $\beta$-wt, wild-type DNA polymerase $\beta$; F272L, mutant DNA polymerase $\beta$; Phe, phenylalanine; HSV-*tk*, Herpes simplex virus type 1 thymidine kinase; dNTP, deoxynucleoside triphosphate; dNMP, deoxynucleoside monophosphate; ddCTP, dideoxynucleoside triphosphate; ATP, adenosine 5′-triphosphate; dRP, deoxyribose phosphate; *E. coli*, *Escherichia coli*.

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To identify amino acid residues of pol $\beta$ that are critical for fidelity, we employed a genetic screen (*20*, *21*). This screen is based upon the discovery that rat pol $\beta$ substitutes for *Escherichia coli* (*E. coli*) DNA polymerase I (Pol I) in DNA replication (*22*). We have isolated several pol $\beta$ mutator mutants using this screen, including one that is altered from Tyr to Cys at position 265 (Y265C) and another that is changed from Phe to Leu at position 272 (F272L). The Y265C alteration appears to be in a hydrophobic hinge region that has the potential to participate in a conformational change of the enzyme that is important for fidelity (*13*, *23*). Phenylalanine 272 is located within the hydrophobic dNTP binding pocket of the enzyme. In this report, we characterize the F272L mutator mutant of pol $\beta$. To elucidate the role of the F272 residue in maintaining pol $\beta$ fidelity, we determined the in vivo and in vitro spontaneous mutation frequencies of the F272L variant and generated a mutation spectrum. Using transient-state kinetic methods and rapid chemical quench techniques, we determined directly the fidelity value for F272L and compared it to that of $\beta$-WT. Overall, we show that F272L has a spontaneous mutation frequency that is 10-fold greater than $\beta$-wt and that Phe 272 is critical for fidelity during ground-state binding.

## MATERIALS AND METHODS

*Bacterial Strains and Media. E. Coli* B/r SC18-12 has the genotype *recA718polA12uvrA155trpE65 lon-11 sulA1* and was used in the genetic screen for mutator mutants (*21*). DH5α with the genotype *mcrA* Δ*(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 (lacZYA-argF)U169 deoR recA1 endA1 phoA supE44thi-1gyrA96relA1* was used in cloning experiments. The FT334 strain with the genotype *recA13 upp tdk* was used to detect mutations in the herpes simplex virus type I thymidine kinase gene (HSV-*tk*) (*24*).

ET medium was E salts supplemented with 0.4% glucose and 20 $\mu$g/mL tryptophan. Eglu medium is ET without tryptophan (*25*). Transformants were selected on Luria-Bertani agar supplemented with 30 $\mu$g/mL of chloramphenicol (Cam) and 12 $\mu$g/mL tetracycline. HSV-*tk* mutant selection medium has been described by Eckert et al. (*24*).

*Chemicals and Reagents.* All of the deoxynucleoside triphosphates (dNTPs) and adenine triphosphate (ATP) were purchased from Sigma. The [$\gamma$-$^{32}$P]ATP (6000 $\mu$Ci/$\mu$mol) was purchased from Amersham. All of the oligonucleotides used in the study were synthesized by the Keck Molecular Biology Center at Yale University and purified using denaturing polyacrylamide gel electrophoresis (20% acrylamide, 8 M urea).

*Identification of Mutator Mutants using the Trp$^+$ Reversion Assay.* The F272L mutant was identified in a genetic screen developed in our laboratory to isolate pol $\beta$ mutator mutants (*21*). Briefly, we used the Trp$^+$ reversion assay to identify the mutator mutants from a library of random mutants constructed between nucleotides 763 and 855; this fragment encodes amino acids 255−285 of pol $\beta$ (*21*). DNA from the pol $\beta$ mutant library was used to transform the SC18-12 strain. Individual transformants were inoculated into 2 mL of LB broth containing 1 mM isopropyl $\beta$-D-thiogalactopyranoside (IPTG) and were incubated in 24-well microtiter plates (Falcon) with aeration for 16−24 h at 37 °C. Aliquots of these cultures were plated onto Eglu medium as described (*21*). In this screen, mutator mutants induce significantly more Trp$^+$ revertants than cultures containing the pol $\beta$ wild-type gene. The mutation of each pol $\beta$ mutant was identified by the dideoxy DNA sequencing method using Sequenase 2.0 (USB) according to the manufacturer's directions.

*Spontaneous Mutation Frequency of $\beta$-wt and F272L in the Trp$^+$ Reversion Assay.* To confirm that F272L was a mutator mutant, we compared the spontaneous mutation frequencies of $\beta$-wt and the F272L mutant in *E. coli*. Briefly, the SC18-12 bacteria containing the WT or mutant pol $\beta$ plasmid were cultured overnight in nutrient broth at 30 °C overnight. The overnight culture was diluted 1:10000 with LB broth containing 1 mM IPTG. The diluted culture was aliquoted into 24-well microtiter plates, and the bacteria were incubated at 37 °C overnight with aeration. After centrifugation and resuspension in 0.9% (w/v) NaCl (saline), 100 $\mu$L aliquots of the bacteria from each well were plated onto Eglu plates. Appropriate dilutions of the culture were plated onto ET to determine the total number of colonies assayed. The plates were incubated at 37 °C for 3 days and counted. The mutation frequency was calculated by dividing the number of Trp$^+$ colonies by the total number of colonies (*21*, *26*).

*Purification of $\beta$-wt and F272L Proteins.* To purify the F272L and $\beta$-wt proteins, we used $Ni^{2+}$ chelation chromatography. Briefly, the $\beta$-wt and F272L cDNAs were subcloned into the pET-28 vector (Invitrogen) to generate pHis$\beta$ and pHis$\beta$F272L. The resulting proteins carry 6 consecutive histidine residues fused to their N-termini. Protein expression was induced by the addition of 1mM IPTG and purified by using $Ni^{2+}$ chelation chromatography according to the manufacturer's instructions (Novagen). The protein was at least 90% homogeneous on the basis of a Commassie blue-stained SDS−PAGE gel. The yield was approximately (0.5 − 1 mg)/100 mL culture. A steady-state kinetic assay demonstrated that the $K_m$ values for dTTP and activated DNA and the $k_{cat}$ values for both native and His-tagged pol $\beta$ were virtually identical (data not shown). Therefore, the His-tagged proteins were used in the in vitro mutagenesis assays and kinetic studies reported here. We also measured the specific activities (pmol of dNTP incorporation $min^{-1}$ $ng^{-1}$) of $\beta$-wt and F272L proteins using a filter binding assay to detect the incorporation of a radiolabeled dNTP into activated DNA (*27*). By using this assay, we found that the specific activity of F272L is half that of $\beta$-wt.

*HSV-tk Forward Mutational Assay.* To determine if F272L has intrinsic mutator activity (*23*, *24*), we used the purified $\beta$-wt or F272L protein to fill a 203-nucleotide gap in a reaction that contained 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.2 mM DTT, 0.2 mg/mL BSA, 500 $\mu$M dNTPs, 10 pM gap DNA, and 100 pM WT or 200 pM F272L pol $\beta$. The reactions were incubated at 37 °C for 1 h before being quenched with 33 mM (final concentration) EDTA and electroporated into FT334 cells. The spontaneous mutation frequency was calculated as described earlier (*23*, *24*). To ensure the independence of selected mutant colonies for mutational spectra, we aliquoted FT334 cells into multiple tubes containing SOC broth immediately after the electroporation, incubated them at 37 °C for 2 h, and plated them separately on selection media. Mutation spectra were obtained by sequencing one or two mutants from each tube using the dideoxy sequencing method and Sequenase 2.0 according to the manufacturer's protocol (USB).

*Primer-Template Preparation for Kinetic Study.* A one-nucleotide gapped DNA template−primer with a 5′-phosphate was used in all of the kinetic studies. The template−primer substrate shown below was used to study T:A incorporation and T:G misincorporation:

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5′GCCTCGCAGCCGTCCAACCAACTCA CCTCGATCCAATGCCGTCC
3′CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG
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The template−primer shown below was used to study A:T incorporation and A:G misincorporation:

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5′GCCTCGCAGCCGTCCAACCAAC CAACCTCGATCCAATGCCGTCC
3′CGGAGCGTCGGCAGGTTGGTTGAGTTGGAGCTAGGTTACGGCAGG
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The template−primers were prepared as described (*8*). Briefly, the primer oligonucleotides were end-labeled using T4 polynucleotide kinase (New England BioLabs) and [$\gamma$-$^{32}$P]ATP. The template, primer, and downstream DNA were annealed at an equimolar ratio. The annealing conditions used were 80 °C for 4 min followed by 50 °C for 30 min and immediate transfer to ice. The products of the annealing reaction were analyzed on a 20% native polyacrylamide gel followed by autoradiography (*8*).

*Single-Turnover Experiments.* To elucidate the mechanism by which F272L influences the fidelity of dNTP incorporation, we determined the equilibrium dissociation constant for dNTP binding, $K_d$, and the maximum rate constant for dNTP incorporation, $k_{pol}$, for correct and incorrect dNTPs using both $\beta$-wt and F272L. The kinetics of *correct* dNTP incorporation were determined under single-turnover conditions using rapid chemical quench experiments performed on a KinTek Instruments Model RQF-3 rapid-quench-flow apparatus thermostated at 37 °C (*28*). In this case, single-turnover conditions were determined empirically to be a ratio of enzyme to primer-template of 5:1 (data not shown). All single-turnover experiments were performed in buffer [50 nM Tris-Cl (pH 8.0), 10 mM $MgCl_2$, 2 mM DTT, 20 mM NaCl, 0.2 mg mL$^{-1}$ BSA, 2.5% glycerol] containing 250 nM pol $\beta$ with 50 nM primer-template substrate. Typically, experiments were carried out by loading 15 $\mu$L of the pol $\beta$−primer-template complex in buffer in one sample loop and 15 $\mu$L of a single dNTP (2.5−400 $\mu$M) in buffer in the second sample loop. Reactions were initiated by rapid mixing of the two reactant solutions, and reactions were quenched at various reaction times (0.005−15 s) with 0.3 M EDTA (final concentration).

The kinetics of *incorrect* dNTP incorporation were determined manually under the above single-turnover conditions. All manual single-turnover experiments were performed by preincubating 250 nM pol $\beta$ with 50 nM primer-template substrate in buffer for 3 min at 37 °C. Reactions were initiated by the addition of a single dNTP (50−4000 $\mu$M), and reactions were quenched at various reaction times (20 s−60 min) with 0.3 mM EDTA (final concentration). The reactions resulted in the addition of one dNTP onto the primer. The N (unextended) and N + 1 (extended by one nucleotide) DNA products were resolved on a 20% polyacrylamide gel. The N and N + 1 products were quantified to obtain the percentage of product formed at each reaction time.

*Data Analysis.* The data were fit by nonlinear regression using the program SigmaPlot version 4.14 (Jandel Scientific). The data from the single-turnover experiments were fit to a single-exponential equation: [product] = $A(1 - \exp(-k_{obsd}t))$, where $A$ is the amplitude and $k_{obsd}$ is the observed first-order rate constant for dNTP incorporation. The binding curve shown in Figure 2 was fit to the following hyperbolic equation: $k_{obsd} = k_{pol}[\text{dNTP}]/(K_d + [\text{dNTP}])$, where the $k_{pol}$ is the maximum first-order rate constant for dNTP incorporation and $K_d$ is the equilibrium dissociation constant. Fidelity values were calculated using the following equation: Fidelity = $[(k_{pol}/K_d)_{correct} + (k_{pol}/K_d)_{incorrect}]/(k_{pol}/K_d)_{incorrect}$.

## RESULTS

*DNA Polymerase $\beta$ Mutant F272L Shows an Increased Spontaneous Mutation Frequency in vivo.* To further our understanding of the molecular mechanism of fidelity of DNA polymerase $\beta$, we first identified amino acid residues that are critical for DNA polymerization fidelity. In this study, we used a genetic screen to identify pol $\beta$ mutator mutants from a library of pol $\beta$ mutants (*21*). Our initial screen detected several pol $\beta$ mutator mutants. One of the mutants carried a T to C change at nucleotide 814, resulting in phenylalanine 272 being altered to leucine (F272L). The F272 residue is located in the dNTP binding pocket of pol $\beta$ (*11*, *13*). To confirm that F272L was a mutator mutant, we determined the spontaneous mutation frequency of F272L in *E. coli* and compared it to $\beta$-wt using the Trp$^+$ reversion assay. The spontaneous mutation frequency of F272L is 3.9 $\times$ 10$^{-8}$ while that of WT is 3.9 $\times$ 10$^{-9}$. Thus, F272L shows a 10-fold increased spontaneous mutation frequency as compared to $\beta$-wt. Therefore, we have used our genetic screen to identify a mutator mutant that is located within the dNTP binding site, which we postulate may influence fidelity through the binding of the dNTP substrate.

*F272L Is a Mutator Mutant in vitro.* To confirm that F272L has an intrinsic mutator activity, we determined the in vitro spontaneous mutation frequencies of $\beta$-wt and F272L using the HSV-*tk* gap-filling assay. The HSV-*tk* gap-filling assay is a forward mutation assay. In this assay, either $\beta$-wt or F272L was used to fill a 203-nucleotide gap of the HSV-*tk* gene at the ATP binding site. The errors committed by the polymerase during gap filling can result in mutations that inactivate the HSV-*tk* gene. The inactive HSV-*tk* mutants confer resistance to the drug 5′-fluoro-2′deoxyuridine (FdUR) on the FT334 host bacterial cells. Therefore, this assay permits us to obtain the spontaneous mutation frequency for $\beta$-wt and F272L resulting from errors committed during gap-filling reactions (*24*). As shown in Table 1, the spontaneous mutation frequencies for $\beta$-wt and F272L are 9.6 $\times$ 10$^{-4}$ and 91 $\times$ 10$^{-4}$, respectively. Therefore, the F272L mutant has a spontaneous mutation frequency around 10 times higher than that of $\beta$-wt, which suggests that F272L has an intrinsic mutator activity.

*F272L Is a General Mutator Mutant.* To determine if F272L is a general type of mutator mutant or if it commits predominantly specific types of frameshift or base substitution mutations, we characterized the mutational specificity of F272L and compared it to $\beta$-wt. We sequenced *68 and 72* HSV-*tk* mutants generated from the gap-filling assay for

Table 1: Error Specificity by Class of F272L and β-WT in the HSV-*tk* Forward Assay

| | frequency × $10^{-4}$ (# observed) | | |
|---|---|---|---|
| | β-wt | F272L | F272L/WT |
| $MF_{obs}$[a] | 9.6 (68) | 91.6 (73) | 9.5 |
| single | 9.0 (64) | 91.6 (73) | 10.2 |
| multiple | 0.7 (4) | 0 | |
| base substitution | 3.2 | 21.3 | 6.7 |
| frameshift[b] | 4.8 | 60.0 | 12.5 |

[a] $MF_{obs}$ is the average observed mutation frequency from at least three independent determinations. [b] Frameshifts include 1-base deletions or insertions.

Table 2: Frameshifts Generated by DNA Pol β WT and F272L at Different Lengths of Homonucleotide Runs

| | error rate[a] ($10^{-5}$) (no. of mutants) | | |
|---|---|---|---|
| length of runs (bp) | β-wt | F272L | F272L/WT |
| 1 | 0.048 (4) | 0.32 (3) | 7 |
| 2 | 0.52 (10) | 3.7 (8) | 7 |
| 3 | <0.18 (0) | 4.6 (3) | >25 |
| 4 | 7.1 (20) | 106 (34) | 15 |
| error frequency | 48 (34) | 700 (48) | 14.5 |

[a] Error rates were calculated by multiplying the total frameshift error frequency by the proportion of 1-base frameshifts at each repeat length per total frameshift and then dividing by the number of occurrences of each repeat length in the target sequence.

β-wt and F272L, respectively. The mutational spectra for β-wt and F272L are shown in Figure 1. A summary of the spectra is shown in Table 1. We used the Adams and Skopek algorithm (*29*), available on the Worldwide Web (*30*), to compare our β-wt spectrum with that of Opresko et al. (*23*). We found that $p = 0$ when the base substitutions of the two spectra are compared; because a $p$ value of 0.05 indicates that we can reject the hypothesis that the two spectra are the same, our $p$ value of 0 indicates that the spectra of base substitutions produced by β-wt in the two different studies are different. When we employed the algorithm to compare our β-wt spectrum of 1-base frameshifts to that of Opresko et al., we found $p = 0.06$, suggesting that the two spectra are similar. At least two possibilities exist to account for this discrepancy. First, we employed a 203-bp gapped DNA molecule as the substrate in our reactions, whereas Opresko utilized a primed single-stranded circular molecule. Pol β is known to have different catalytic efficiencies and fidelities on different types of DNA substrates (*8*); thus, the spectrum obtained in this study may not be directly comparable to the spectrum obtained in the Opresko study. Second, the characterization of limited numbers of HSV-tk mutants generated by β-WT could contribute to the differences observed in the spectrum generated by Opresko and the β-WT spectrum generated in this study.

*F272L commits base substitution errors at higher frequencies than β-wt.* The majority of the errors committed by F272L and β-wt are single errors; very few multiple mutations were detected. For β-wt, 34% (23/68) of the total mutations are base substitution mutations, and for F272L, 23% (17/73) of total mutations are base substitutions. When we compared the spectra of base substitutions produced by β-wt and F272L, using the Adams and Skopek algorithm, we obtained a $p$ value of 0.18, indicating that, overall, both β-wt and F272L produce similar types of base substution mutations. However, the base substitution frequency for F272L is elevated 6.7-fold over that of β-wt. On the basis of our data set, these results suggest that F272L is a general mutator with regard to base substitutions.

F272L also commits frameshift errors at a higher frequency than that of β-wt. In fact, the majority of mutations we detected are single-base insertions or deletions. In general, the frameshift error rate increased as a function of the length of the homonucleotide run, as shown in Table 2, which is consistent with previous observations for β-wt (*23*, *24*, *31*). However, we did not identify any insertions or deletions within runs of three homonucleotides for β-wt, whereas we did detect 3/48 single-base deletions within two different runs of three homonucleotides for F272L. There are a total of 8 runs of three homonucleotides throughout the target. All of the frameshift mutations detected at runs of three homonucleotides for F272L are single-base deletions. Four of the runs of three homonucleotides are CCC, three are GGG, and one is TTT. Deletion at TTT (212−214) and CCC (273−275) has been observed for F272L. This suggests that there is no sequence specificity for deletion at runs of three homonucleotides for F272L. We employed the Adams and Skopek algorithm to compare the frameshift spectra of β-wt and F272L. We obtained a $p$ value of 0.004, indicating that they are different. When we eliminated, one by one, frameshift positions from the spectra and reanalyzed the data, we found that elimination of β-wt frameshifts at postion 248 had the most significant impact on the $p$ value, increasing it to 0.15. This analysis indicates that the frameshift spectra for β-wt and F272L differ mainly at position 248. At this position, β-wt produced seven 1-bp deletions, whereas no deletions were produced by F272L.

The 2-base deletion mutation frequency for F272L pol β is $9 \times 10^{-4}$, which is 20 times higher than that of β-wt, $0.44 \times 10^{-4}$. There are a total of 28 two-nucleotide repeats in the targets. All of the 2-base deletions committed by β-wt and F272L pol β occur at the $(TA)_3$ dinucleotide repeat at position 214−219, a hotspot for 2-base deletions observed by Eckert et al. (*24*). However, using the algorithm to analyze the 2-bp deletions produced by β-wt and F272L yielded a $p$ value of 1, strongly suggesting that β-wt and F272L are similar, with regard to producing 2-bp deletions. Our data indicate that F272L is a general mutator for misalignment-initiated frameshifts. Because the F272L produces a variety of base substitution and frameshift mutations, our data set suggests that F272L is a general mutator.

*Decreased Fidelity of F272L pol β Is Due to Decreased Discrimination between Correct and Incorrect dNTPs.* To understand the role of the Phe 272 residue in DNA synthesis fidelity, we studied both correct and incorrect dNTP incorporation by β-wt and F272L under single-turnover reaction conditions. This methodology permits us to observe directly the events occurring at the active site of pol β, including ground-state binding of the dNTP, conformational changes, and the chemical step of the nucleotidyl transfer reaction.

We observed in our mutational spectra studies that the frequency of misincorporation of G opposite A for F272L ($50 \times 10^{-5}$) is increased over that of β-wt ($2.8 \times 10^{-5}$). We only observed misinsertion of G opposite T for F272L and β-wt, one and zero times, respectively, indicating that these enzymes rarely insert G opposite T. This also seemed to be the case in an earlier study of β-WT (*23*). Therefore, we

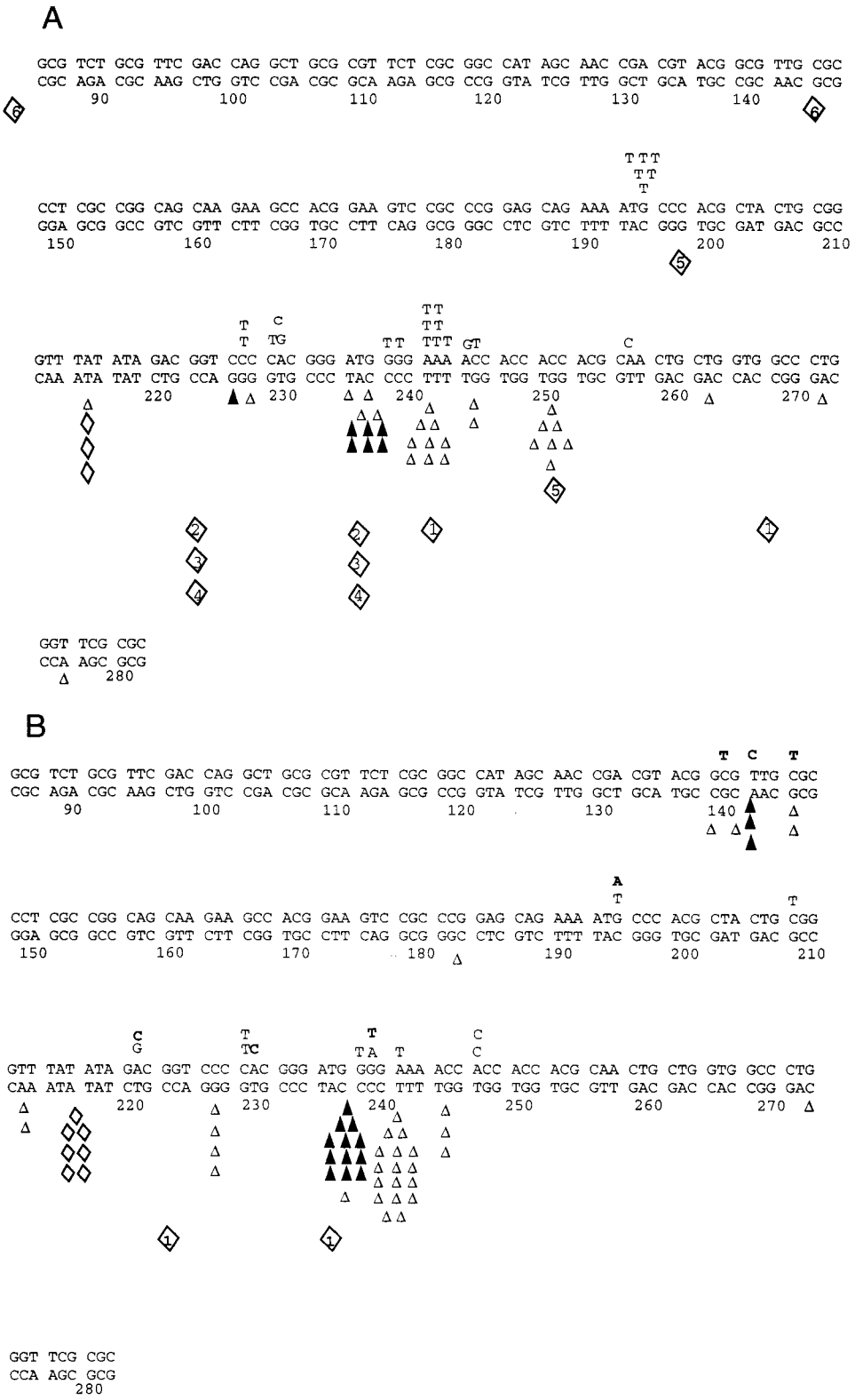


FIGURE 1: Mutational spectra of $\beta$-wt (A) and F272L (B) determined by using HSV-*tk* gap-filling assays. Frameshift mutations, including 1-base additions (▲), 1-base deletions (△), 2-base deletion (◇), and big deletions (◇ with numbers inside) are shown under the HSV-*tk* target sequence. Base substitution mutations are shown above the HSV-*tk* target sequence. Tandem multiple mutations, defined as being separated by 15 bases or less, are not shown (*22*). For $\beta$-wt, three mutants contained tandem multiple mutations; all three had a 1-base insertion at postion 237 and a 1-base deletion at position 241. One multiple mutation produced by $\beta$-wt carried an insertion at position 237 and a G to T mutation at position 195. Because these mutations are nontandem, they are shown in the spectrum. The F272L mutant did not produce any multiple mutations.

examined the fidelity of A:G and T:G misincorporation by F272L and compared it to that of $\beta$-wt.

To determine the fidelity of $\beta$-wt and F272L for misincorporation of G opposite template A, the $K_d$ and $k_{pol}$ values were measured for both correct and incorrect dNTP incorporation. These values were determined by measuring the observed rate constant, $k_{obsd}$, at various dNTP concentrations. Figure 2A shows timecourses for correct incorporation of

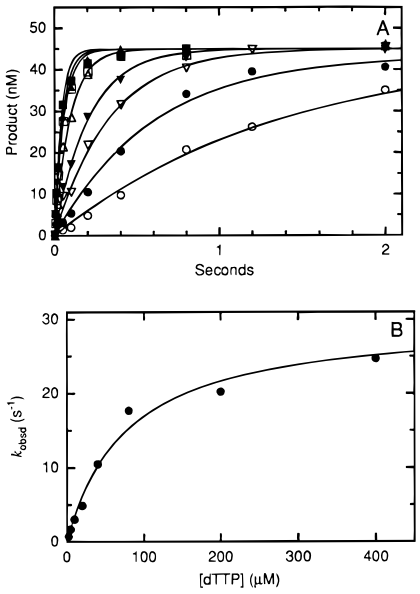


FIGURE 2: Kinetics of a single-turnover experiment to monitor the incorporation of dTMP opposite A by F272L. (A) Time courses using 2.5 $\mu$M (○), 5 $\mu$M (●), 10 $\mu$M (▽), 20 $\mu$M (▼), 40 $\mu$M (□), 80 $\mu$M (■), 200 $\mu$M (△), and 400 $\mu$M (▲) dTTP. A solution containing enzyme (250 nM) preincubated with 50 nM DNA was mixed with various concentrations of dTTP. The reactions were terminated by quenching with 0.3 mM EDTA, and formation of the product was monitored on a 20% denaturing polyacrylamide gel. Time courses were fit to a single-exponential equation to obtain the observed rate of product formation, $k_{obsd}$, at each dTTP concentration, and the *solid lines* were generated using these $k_{obsd}$ values. (B) The observed rate constants for product formation, $k_{obsd}$, were plotted against the corresponding dTTP concentrations and fit to a hyperbolic equation as described to determine the maximum rate constant for polymerization, $k_{pol}$, and the equilibrium dissociation constant for dTTP, $K_d$.

dTMP opposite A for F272L at various concentrations of dTTP. By fitting the data from each timecourse to a single-exponential equation, we obtained a set of $k_{obsd}$, values at various substrate concentrations. The $K_d$ and $k_{pol}$ values were determined by plotting the $k_{obsd}$ values against concentrations of dTTP and fitting the plot to a hyperbolic curve, as shown in Figure 2B.

In a similar manner, the $K_d$ and $k_{pol}$ values were determined for G opposite A, A opposite T, and G opposite T. These values were used to calculate the fidelities of $\beta$-wt and F272L. Table 3 shows the summary of A:G and T:G misincorporation of F272L compared to $\beta$-wt. The F272L variant shows an overall 4-fold decrease in fidelity (26 000) for A:G misincorporation compared to that of $\beta$-wt (104 000). This decrease in fidelity for F272L is mainly due to a decreased discrimination between the correct and incorrect dNTP during binding of the dNTP; $\beta$-wt is 15 times less likely to bind to the incorrect versus the correct dNTP whereas F272L is only five times less likely to do so. In contrast to the decreased fidelity of A:G misincorporation, within error, F272L exhibits fidelity similar to $\beta$-wt for T:G misincorporation. The $k_{pol}$ and $K_d$ values for misincorporation of dGMP opposite T by F272L are not changed significantly compared to the $\beta$-wt enzyme.

## DISCUSSION

In this study we employed a genetic screen to identify a mutator mutant of pol $\beta$ in which the phenylalanine residue at position 272 is mutated to leucine. We used three different assays to demonstrate that F272L is a mutator protein. First, the F272L mutant confers a mutator phenotype on the SC18-12 *E. coli* strain. Second, the F272L protein has a spontaneous mutation frequency that is 10-fold higher than that of the $\beta$-wt protein in the in vitro HSV-*tk* gap-filling assay. Third, F272L has a 4-fold lower fidelity than that of $\beta$-wt for misincorporation of G opposite A in a kinetic assay. Therefore, we conclude that our genetic screen is able to identify pol $\beta$ variants that synthesize DNA inaccurately. We also conclude that the phenylalanine residue at position 272 is critical for the fidelity of DNA synthesis by pol $\beta$.

The analysis of the in vitro mutation spectra comparing $\beta$-wt and F272L revealed that the F272L enzyme is a general mutator because it commits all types of base substitutions and frameshift mutations at frequencies above those of the $\beta$-wt protein. Kinetic analysis of the mutant enzyme demonstrates that F272L has decreased fidelity for misincorporating G opposite template A as compared to $\beta$-wt. The major determinant of the decreased fidelity of misincorporation of G opposite A of F272L is decreased discrimination between correct and incorrect dNTP at ground-state binding. The mutation spectra and kinetic studies also showed that there is no significant difference between F272L and $\beta$-wt in their fidelity of misincorporation of G opposite template T.

*The Aromatic Ring of F272 Serves as a Geometric Sensor for Ground-State Binding Discrimination.* F272L is located in the dNTP binding motif within the active site of pol $\beta$ as shown in Figure 3. Before binding to the dNTP, pol $\beta$ assumes an open conformation. In this conformation, Y271 and F272 face each other and form a deep and narrow pocket along with amino acid residues 273−276 and the template base. The binding of a dNTP and the binding of the second $Mg^{2+}$ ion triggers two conformational changes (*18*, *19*), the second of which is rate-limiting. After binding to dNTP pol $\beta$ assumes a closed conformation. It is evident that in the closed conformation the dNTP binding motif rotates so that the aromatic rings of F272 and Y271 move more than 5 Å, the hydroxyl group of Y271 forms a hydrogen bond with the O2 atom of the primer residue, the backbone atoms of Y271, F272, and G274 form close van der Waals contacts with the ribose ring carbon of the ddCTP substrate, and the side chain carbon of Asp276 forms a van der Waals contact with the base ring carbon atom C4 (*13*, *14*). It is postulated that this rotation of the dNTP binding pocket helps to align the incoming dNTP to form hydrogen bonds with the templating base and to position the α-phosphate for attack by the primer 3′-OH group (*13*). It has been proposed that the protein backbone segment of Tyr271 to Gly274 may participate in nucleotide selectivity of deoxyribose over ribose through steric exclusion (*11*, *15*). However, no structural information is available for mispair formation within the active site of pol $\beta$. Our data suggest that the alteration of phenylalanine 272 to leucine in this binding pocket results in decreased discrimination between the correct and the incorrect dNTP. This indicates that side chains within the pocket exclude the formation of mispairs. We postulate that the aromatic ring of F272 may obstruct a bulky mispair such as A:G by imposing a steric constraint on the sugar ring. Elimination of the aromatic ring of F272 may result in a less geometrically constrained dNTP binding pocket that is less exclusive of bulky mispairs. We postulate that, because

Table 3: Misincorporation of G Opposite A and G Opposite T by $\beta$-wt and the F272L Mutant

| | | $K_d$ ($\mu$M) | $k_{pol}$ ($s^{-1}$) | $(K_d)_{inc}/(K_d)_c$[a] | $(k_{pol})_{inc}/(k_{pol})_c$[b] | $k_{pol}/K_d$ | fidelity[c] |
|---|---|---|---|---|---|---|---|
| $\beta$-wt | A:T[d] | 41 ± 8 | 40 ± 2 | | 0.0001 | 0.97 | |
| | A:G[e] | 650 ± 150 | 0.0060 ± 0.0005 | 15 | | $9.2 \times 10^{-6}$ | 104000 |
| | T:A[f] | 27 ± 4 | 47 ± 2 | | 0.03 | 1.7 | |
| | T:G[g] | 670 ± 110 | 1.4 ± 0.1 | 24 | | $2.0 \times 10^{-3}$ | 874 |
| F272L | A:T | 77 ± 10 | 30 ± 1 | | 0.0001 | 0.42 | |
| | A:G | 360 ± 160 | 0.0050 ± 0.0006 | 5 | | $1.4 \times 10^{-5}$ | 26000 |
| | T:A | 18 ± 6 | 27 ± 2 | | 0.02 | 1.5 | |
| | T:G | 780 ± 350 | 0.5 ± 0.1 | 42 | | $6.0 \times 10^{-4}$ | 2000 |

[a] The $K_d$ for incorrect dNTP (inc) divided by correct (c). [b] The $k_{pol}$ for incorrect dNTP (inc) divided by correct (c). [c] The fidelity was calculated as described in experimental procedures. [d] Kinetic parameters for incorporation of T opposite A. [e] Kinetic parameters for misinsertion of G opposite A. [f] Kinetic parameters for incorporation of T opposite A. [g] Kinetic parameters for misinsertion of G opposite T.

the shape of a T:G mispair resembles correct Watson−Crick base pair geometry more than an A:G mispair does, formation of a T:G mispair is permitted by F272L to the same extent as it is by the wild-type enzyme. Tyr271 also forms part of the hydrophobic dNTP binding motif, and work from Tsai's group indicates that the ring structure at this position is important for discrimination during the binding of the dNTP (*16*). They show that, when Tyr is altered to Ser or Ala, the binding affinity for the incorrect dNTP is increased compared to the wild-type protein, whereas when Tyr 271 is changed to Phe, there is no difference in the binding affinity of the resulting enzyme compared to wild type. The altered binding affinities for dNTP observed for Y271S also result in decreased fidelity for A:G misincorporation. However, the Y271S enzyme has not been studied for its ability to commit errors when it is filling in a large or small gap opposite the HSV-*tk* or *lacZ*α target genes, so it is not known whether Y271S is in fact a mutator protein; the Y271A protein does not commit errors at a greater frequency than the wild-type protein in the *lacZ*α reversion assay. Nevertheless, our data combined with the results from Tsai's group suggest that the aromatic rings of both Y271 and F272 are important for the discrimination of correct from incorrect dNTP at ground-state binding and that steric exclusion by the *backbone* of the nucleotide binding motif is not the only mechanism pol $\beta$ employs for maintaining fidelity during ground-state binding fidelity.

Sawaya and colleagues proposed that, in the open conformation of the enzyme prior to dNTP binding, Arg258 and Asp192 form an ionic bond. Once the dNTP binds and induces a conformational change, the F272 side chain is translocated between Arg258 and Asp192 and is postulated to break the salt bridge between Asp192 and Arg258, allowing Asp192 to bind to the catalytic $Mg^{2+}$ ion. Asp192 must interact with the $Mg^{2+}$ ion for catalysis to occur (*13*). Since a phenylalanine and a leucine residue have a similar van der Waals volume, the alteration of F272 to L should not alter the rate of catalysis. This is consistent with our kinetic studies which demonstrate that the maximum rate of catalysis, $k_{pol}$, of F272L mutant is very similar to that of WT. We speculate that radical alteration of F272 to a residue with considerably less van der Waals volume may result in an inactive pol $\beta$ mutant, which will not be detected in our genetic screen.

*Pol β Fidelity and Structure of the Primer-Template.* We have employed two different in vitro assays to demonstrate that the F272L protein possesses intrinsic mutator activity. In the HSV-*tk* assay the spontaneous mutation frequency of F272L is increased 10-fold over that of the wild-type enzyme. However, our kinetic studies indicate that F272L catalyzes the synthesis of DNA with 4-fold less fidelity than the $\beta$-wt enzyme. The differences in the magnitudes of fidelity observed for F272L in these assays are most likely due to the primer-template employed in the study. Our group recently demonstrated that pol $\beta$ has the most activity and fidelity when it employs a 1-bp gapped DNA substrate with a 5′ phosphate compared to a 3′ recessed or 5-bp gapped substrate (*8*). The 1-bp gapped substrate with a 5′ phosphate is probably also a physiologically relevant DNA substrate for pol $\beta$. In the HSV-*tk* assay, the F272L enzyme must fill in a 203-nucleotide gap in order for us to detect a mutation. Therefore, the HSV-*tk* DNA substrate most likely resembles a 3′ recessed primer-template, which appears not to be the optimum DNA substrate for pol $\beta$. The DNA substrate employed in our kinetic studies was a 1-bp gap with a 5′ phosphate, which is the optimum DNA substrate for pol $\beta$ activity and fidelity. Therefore, we suggest that the different magnitudes of mutator activity for the F272L variant we observed in our in vitro assays resulted from the different DNA substrates we employed.

*DNA Polymerase β Fidelity May Be Maintained through More than One Pathway.* Several mechanisms have been proposed for pol $\beta$ fidelity maintenance. Correct positioning of template-primer DNA is crucial for fidelity and catalytic efficiency (*12*, *13*, *15*). It is clear that the protein interacts with the O2 of pyrimidines and N3 of purines in the minor groove, which is hypothesized to result in proper geometric positioning of the template-primer in the active site. There is also a 90° bend in the template DNA as it moves through the active site; this permits the interaction of the n + 1 template residue with the 8 kDa domain of pol $\beta$, resulting in displacement of the n + 1 residue out of the active site and away from catalysis. Residue R283 is proposed to stabilize the templating base and is critical for fidelity (*32*). The alteration of R283, especially to Ala, results in a significant decrease in both catalytic efficiency and fidelity (*17*, *32*).

Here we show that discrimination during ground-state binding of the dNTP also influences the fidelity. In this study, we identified a mutator mutant, F272L, that influences the fidelity by decreasing the discrimination between correct and incorrect dNTP during the ground-state binding. Multiple conformational changes are also critical for fidelity. The structural and biochemical information shows that pol $\beta$ undergoes a conformational change from an open complex before the dNTP substrate is bound to a closed complex once

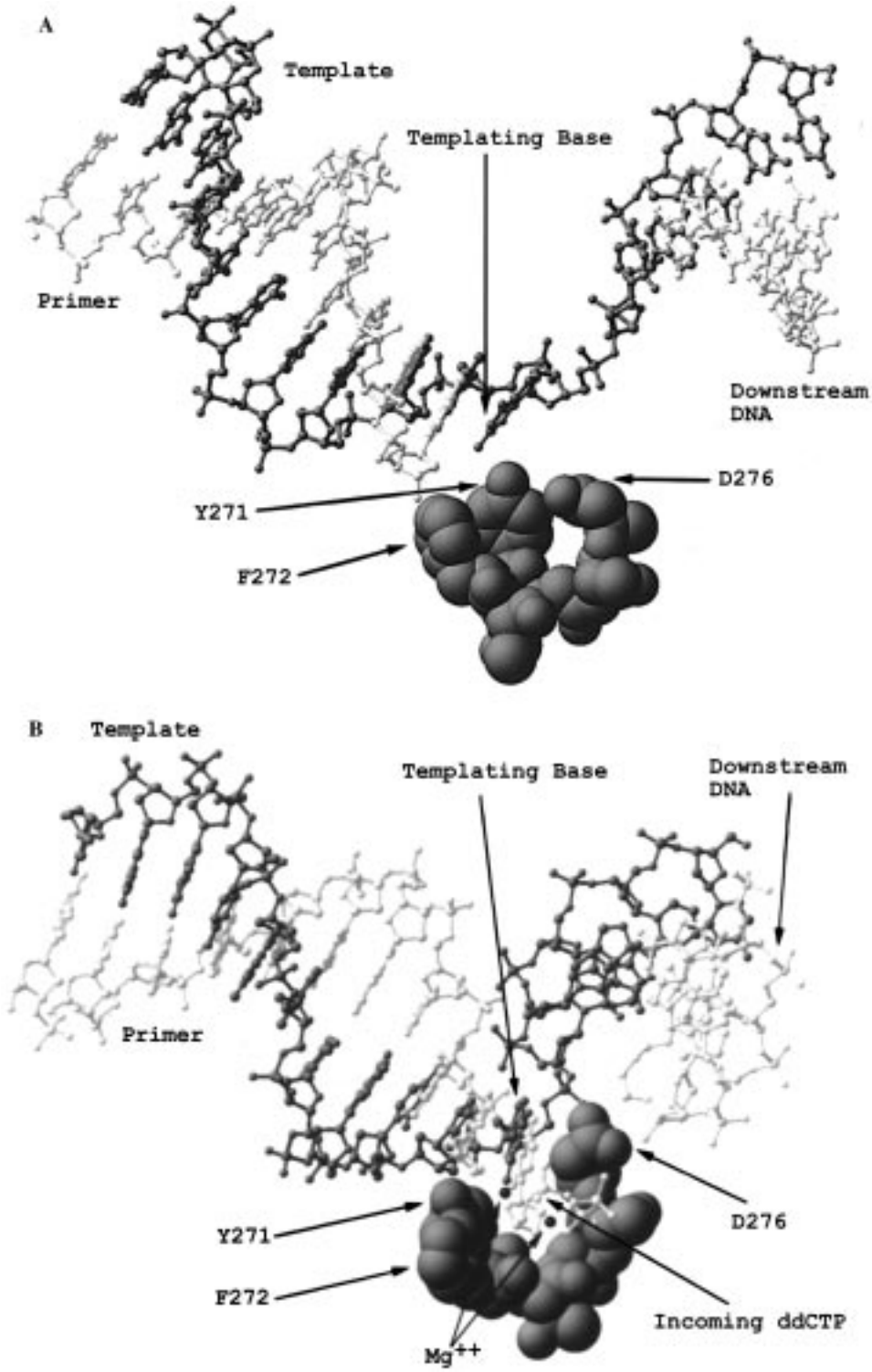


FIGURE 3: Deoxyribonucleotide triphosphate (dNTP) binding pocket in the open conformation (A) before dNTP binding and in the closed conformation after the ddCTP binding (B). Amino acid residues Y271, F272, D276, template, primer, downstream DNA, templating base, incoming dNTP, and $Mg^{2+}$ ion are indicated. The pictures shown here were generated using Ribbons software and the X-ray crystal structure coordinates published by Sawaya et al. (*13*).

the correct dNTP is in the active site (*11*, *13*, *15*, *18*, *19*). The conformational change is proposed to align the primer, template, dNTP, magnesium ions, and the catalytic residues of the protein in the optimum geometric configuration for the nucleotidyl transfer reaction to occur. It has been proposed that the Y265 residue, which is located at a hydrophobic hinge region in the finger domain, may influence fidelity by altering the rate or amount of the closing of the carboxyl-terminal domain of pol $\beta$ to form the closed active conformation. Indeed, the Y265C mutator mutant identified in our lab is defective in both catalytic efficiency and fidelity (*21*).

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