Fidelity of DNA Polymerase δ Holoenzyme from *Saccharomyces cerevisiae*: The Sliding Clamp Proliferating Cell Nuclear Antigen Decreases Its Fidelity†

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ABSTRACT: DNA polymerases δ and ε (pol δ and ε) are the two major replicative polymerases in the budding yeast *Saccharomyces cerevisiae.* The fidelity of pol δ is influenced by its 3′−5′ proofreading exonuclease activity, which corrects misinsertion errors, and by enzyme cofactors. PCNA is a pol δ cofactor, called the sliding clamp, which increases the processivity of pol δ holoenzyme. This study measures the fidelity of 3′−5′ exonuclease-proficient and -deficient pol δ holoenzyme using a synthetic 30mer primer/100mer template in the presence and absence of PCNA. Although PCNA increases pol δ processivity, the presence of PCNA decreased pol δ fidelity 2−7-fold. In particular, wild-type pol δ demonstrated the following nucleotide substitution efficiencies for mismatches in the absence of PCNA: G·G, 0.728×10^{-4}; T·G, 1.82×10^{-4}; A·G, $<0.01 \times 10^{-4}$. In the presence of PCNA these values increased as follows: G·G, 1.30×10^{-4}; T·G, 2.62×10^{-4}; A·G, 0.074×10^{-4}. A similar but smaller effect was observed for exonuclease-deficient pol δ (i.e., 2−4-fold increase in nucleotide substitution efficiencies in the presence of PCNA). Thus, the fidelity of wild-type pol δ in the presence of PCNA is more than 2 orders of magnitude lower than the fidelity of wild-type pol ε holoenzyme and is comparable to the fidelity of exonuclease-deficient pol ε holoenzyme.

Genomic DNA is replicated with high fidelity by DNA replication enzymes and DNA mismatch repair enzymes (*1*). In eukaryotes, replicative DNA polymerases δ and ε (pol δ and ε)[1] insert correct deoxyribonucleoside triphosphates (dNTP) with relatively high accuracy, and misincorporated nucleotides are excised by associated 3′−5′ exonucleases. In the yeast *Saccharomyces cerevisiae*, three DNA polymerases (pol α, δ, and ε) are required for cell growth, chromosomal DNA replication (see ref *2* for review), and double-strand break repair (*3*). Pol α has four subunits [Pol1 (Cdc17), Pol12, Pri1, and Pri2] and functions as a DNA primase for the initiation of Okazaki fragments (see ref *2* for review). Pol δ and pol ε are required for the leading- and lagging-strand DNA synthesis. They bind at or near replication origins and move with the replication fork (*4*, *5*). The precise roles of these two polymerases during DNA replication have not yet been elucidated. However, several lines of evidence suggest that lagging-strand DNA synthesis is carried out by pol α and pol δ (*6*).

S. cerevisiae pol ε has four subunits (Pol2, Dpb2, Dpb3, and Dpb4). Both DNA polymerase and proofreading exonuclease reside in the largest subunit (Pol2) (*7*). The *POL2* gene encoding the catalytic subunit is essential for cell growth and is required for chromosomal DNA replication (*8*, *9*). Surprisingly, a deletion of the catalytic polymerase domain of the *POL2* gene (*pol2-16*) is viable (*10*, *11*). However, point mutations in the polymerase active site are lethal (*11*), the *pol2-16* allele is temperature-sensitive for growth and causes severe growth defects even at permissive temperatures, and the elongation step of DNA replication is significantly retarded, indicating that the DNA polymerase activity of pol ε is essential for normal cell growth and efficient and rapid chromosomal DNA replication (*12*). Thus, it has been suggested that the remaining DNA polymerase-(s) may substitute for the function of pol ε if the polymerase active domain was deleted. On the other hand, an inactivated polymerase polypeptide may prevent substitution of remaining DNA polymerase(s) at the restrictive temperature (*12*). The *pol2-4* allele has a mutation in the 3′−5′ exonuclease active domain, reducing exonuclease activity approximately 100-fold; however, *pol2-−4* retains wild-type polymerase activity (*13*). Yeast *pol2-4* cells have an ≈22-fold increase in spontaneous mutation rate (*14*). Another pol ε mutant (*pol2-C1089Y*) was isolated as a +1 frame-shift mutator within homonucleotide runs. In vitro studies show that this mutant has an ≈1000-fold increase in mutation frequency and the mutant enzyme pol ε-C1089Y has a lower intrinsic fidelity, independent of its 3′−5′ exonuclease activity (*15*, *16*). These results are consistent with the notion that pol ε plays a direct role in chromosomal DNA replication in vivo.

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[1] Abbreviations: dNTP, deoxyribonucleoside 5′-triphosphate; HE, holoenzyme; PCNA, proliferating cell nuclear antigen; pol, DNA polymerase; RFC, replication factor C; RPA, replication protein A; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; SSB, single-stranded DNA binding protein.

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Pol δ from *S. cerevisiae* has three subunits, Pol3 (Cdc2), Hys2 (Pol31), and Pol32, which are homologues of fission yeast *Schizosaccharomyces pombe* Pol3, Cdc1, and Cdc27, respectively (*17*−*19*). Pol δ from *S. pombe* has one additional subunit, Cmt1 (*19*). Pol δ only carries out processive DNA synthesis in the presence of PCNA (sliding clamp) and replication factor C (RFC; clamp loader) (*20*). The DNA polymerase and proofreading exonuclease activities of pol δ reside in the largest subunit, Pol3 (*13*, *21*). The *POL3* gene encoding the catalytic subunit is essential for cell growth and is required for chromosomal DNA replication (*22*, *23*). The mutant *pol3-01* has a mutation in the 3′−5′ exonuclease active site which elevates the spontaneous mutation rate 100-fold (*14*). This is a larger increase than in the case of a pol ϵ mutant *pol2-4* (22-fold). However, *pol3-01* may completely lack 3′−5′ exonuclease activity (*24*). Furthermore, *pol3-01* is a synthetic lethal with *rad27* (*fen1*), suggesting that the 3′−5′ exonuclease activity of pol δ plays a role in the maturation of Okazaki fragments (*24*, *25*).

To date, extensive biochemical and genetic studies have been carried out on pol δ and pol ϵ. However, their fidelity has not been studied comprehensively. Goodman and colleagues developed a gel-based kinetic assay that is very useful for evaluating polymerase kinetics and fidelity (*26*). In particular, kinetic parameters can be measured at a specific site on the primer/template DNA with a limited pool of dNTPs, so that rates of misinsertion can be determined for specific DNA mispairs. Goodman and collaborators used this assay to show that *S. pombe* pol δ, which had a weak editing 3′−5′ exonuclease activity, has an unexpectedly low fidelity (*27*) unlike a prokaryotic DNA replicase, *Escherichia coli* DNA polymerase III holoenzyme. In a previous study (*16*), we determined the nucleotide substitution efficiency of wild-type and mutant *S. cerevisiae* pol ϵ using the same kinetic assay and established that pol ϵ has higher fidelity than *S. pombe* pol δ. To complete our comprehensive studies of eukaryotic DNA replicases from the same organism, this study examines the fidelity of 3′−5′ exonuclease-proficient or -deficient pol δ holoenzyme purified from *S. cerevisiae* in the presence and absence of PCNA using the same kinetic assay.

EXPERIMENTAL PROCEDURES

Materials. Ultrapure deoxyribonucleoside triphosphates were purchased from Amersham Biosciences. T4 polynucleotide kinase was purchased from Takara Bio Inc. [γ-^{32}P]-ATP (111 TBq/mmol) was purchased from Perkin-Elmer Life Sciences. Polyacryamide gel electrophoresis-purified 30mer and 100mer DNAs were purchased from Sigma-Genosys Japan.

Template/Primer DNA. The template/primer DNA was a synthetic 100mer template annealed to a complementary 30mer primer. The primer is complementary to the DNA sequence in the middle of the template, forming a template/primer with a 35-nucleotide ssDNA overhang on both the 5′- and 3′-sides (*16*, *26*, *27*). The 5′-end of the primer was labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase at 30 °C for 30 min. DNA strands were annealed at a molar ratio of 1 primer to 2.4 templates by heating to 96 °C and gradually cooling to room temperature. The concentration of annealed DNA was 190 nM (primer termini). The DNA sequences were as follows: 5′-AGTCATTATCGGACTT-GCTCAGTGTCTGTCGTTTTACGAAACACCGGTTCTA-AGGATGGGTATACTAAACCTCCTGAGTACGGTGAT-ACACCTATTCCGG-3′ and 3′-TGCTTTGTGGCCAAG-ATTCCTACCCATATG-5′.

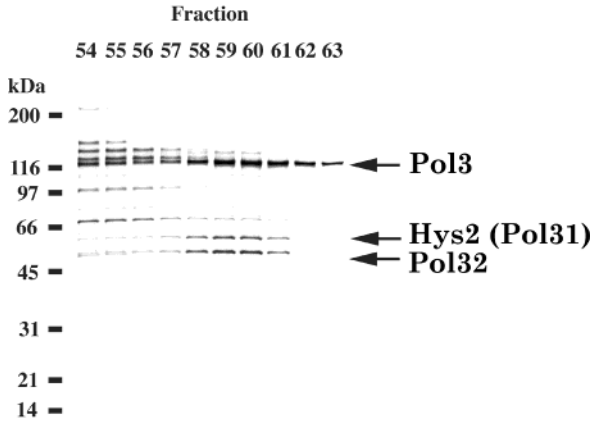

FIGURE 1: Purification of pol δ HE from *S. cerevisiae* CB001 cells. Fractions eluted from a Mono Q (HR5/5) column were analyzed by 4−20% SDS−polyacrylamide gel electrophoresis, and proteins were visualized by silver staining. Arrows indicate the Pol3, Hys2, and Pol32 subunits. Duplicate fractions were analyzed by Western blot, and the indicated species were reacted with the appropriate rabbit antibodies (ref *18* and our unpublished results). Fractions 60−62 were pooled and used for this study.

Enzymes and Accessory Protein. Wild-type pol δ holoenzyme (HE), which consists of Pol3, Hys2 (Pol31), and Pol32, was purified from yeast strain *S. cerevisiae* CB001 (*MATa leu2 trp1 ura3 prb pep4::URA3*) as described previously (*18*), and its purity is shown in Figure 1. The 3′−5′ exonuclease-deficient pol δ holoenzyme, which also consists of three subunits, was purified from CG379 (*MAT*α *ade5-1 his7-2 leu2-3*, *-112 trp1-289 ura3-52*) carrying the *pol3-01* and Δ*pep4::URA3* mutations. The purification method was the same as for wild-type pol δ HE, except for the addition of single-stranded DNA−cellulose (Sigma) chromatography to remove contaminating 5′−3′ exonuclease activity. When analyzing *S. cerevisiae* pol δ-associated 3′−5′ exonuclease activity using 5′-end ^{32}P-labeled 30mer oligonucleotide, we noticed a contaminating phosphatase activity in both the wild-type and the 3′−5′ exonuclease-deficient mutant pol δ preparations (see Figure 2). However, this activity did not remove any phosphate residues from the 30mer primer annealed to the 100mer template polynucleotide used in this study and did not interfere our analyses of pol δ fidelity (data not shown). Yeast RFC and RPA were purified from *S. cerevisiae* CB001 as described previously (*18*, *28*), while yeast PCNA was purified from *E. coli* cells expressing yeast PCNA as described previously (*28*). The purity of these proteins was more than 90% as shown in Figure 3.

Assay for 3′−5′ Exonuclease Activity of Pol δ HE from S. cerevisiae. Thirty nanomolar 5′-end ^{32}P-labeled 30mer oligonucleotide was incubated at 30 °C with 0.2 unit of pol δ in a 20 μL reaction [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 25 μg/mL bovine serum albumin] in either the presence or absence of 200 ng of PCNA. One unit of pol δ HE supports the incorporation of 1 nmol of dTMP for 30 min under these conditions. The reaction was terminated by addition of 10 μL of formamide loading dye [80% (w/v) deionized formamide, 10 mM EDTA

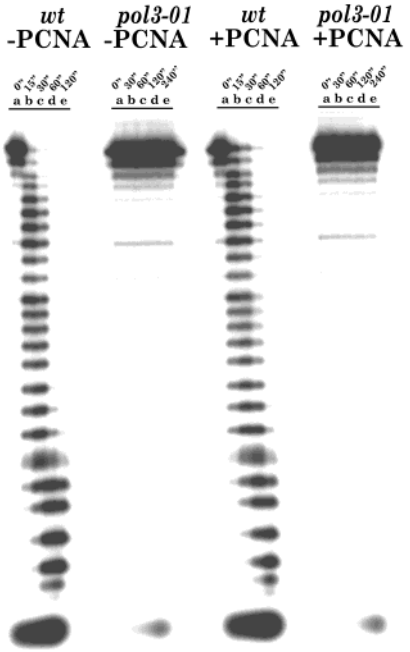

FIGURE 2: 3′−5′ exonuclease activity of wild-type and *pol3-01* pol δ HE. Thirty nanomolar 5′-end ^{32}P-labeled 30mer oligonucleotide substrate was incubated at 30 °C with 0.2 unit of wild-type pol δ HE for 0 (a), 15 (b), 30 (c), 60 (d), and 120 s (e) or with 0.2 unit of *pol3-01* pol δ HE for 0 (a), 30 (b), 60 (c), 120 (d), and 240 s (e). The samples were denatured and separated on a 12% denaturing polyacrylamide gel containing 8 M urea for 3 h at 1500 V. The gel was exposed to a BAS imaging plate (Fuji Film) as described in Experimental Procedures. The bottom band seen after incubation with enzyme could be the product liberated by contaminated 5′−3′ exonuclease activity, since it was seen from the beginning of the incubation.

(pH 8.0), 1 mg/mL xylene cyanol, and 1 mg/mL bromophenol blue], placed on ice, and heated to 96 °C. Two microliter aliquots were separated on a 12% denaturing polyacrylamide gel containing 8 M urea for 3 h at 1500 V. The gel was exposed to a BAS imaging plate (Fuji Film), and the data were analyzed using Image Reader software (Fuji Film).

Analysis of Enzyme Kinetics and Fidelity. A gel-based activity assay was used to determine the kinetics of incorporation of each of the four dNTPs opposite a target template base (*27*). The template/primer DNA (5 nM) and a running-start dATP mix (50 μM) were mixed together in 20 μL of reaction buffer [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM dithiothreitol, 1 mM ATP, and 25 μg/mL bovine serum albumin]. PCNA (400 ng) was added to the reaction with the template/primer DNA and the running start dATP as indicated. Pol δ HE (0.2 unit) was incubated in the reaction mix for 1 min followed by addition of dNTP and incubation of 2.5 min. Control reactions used the running-start dATP mix but no dNTP; in this case, misincorporation of dA opposite the target G should not occur. (Misincorporation was detected in reactions with 3′−5′ exonuclease-deficient pol δ HE, with or without PCNA.) Reactions were terminated with 10 μL of formamide loading dye, placed on ice, and heated to 96 °C. Aliquots (1.5 μL) were separated on a 12% denaturing polyacrylamide gel containing 8 M urea for 3.5 h at 1500 V. The gel was exposed to a BAS imaging plate (Fuji Film) for several different times (between 5 and 20 h), and the data were analyzed using Image Reader software (Fuji Film). Integrated polyacrylamide gel band intensities were measured using Image Gauge software (Fuji Film). The nucleotide incorporation efficiency opposite the target site was calculated from I_T^{Σ}/I_{T-1}, whereby I_T^{Σ} is the integrated band intensity of primers extended to the target site and beyond (positions T, T + 1, T + 2, etc.) and I_{T-1} is the integrated band intensity of primers extended up to the target site (position T − 1) (*27*). The relative incorporation rate, I_T^{Σ}/I_{T-1}, was plotted vs the dNTP substrate concentration. This graph is a rectangular hyperbola whose slope in the initial linear region is the apparent V_{max}/K_m. Apparent K_m and relative V_{max} were computed using a least-squares fit to a rectangular hyperbola. The relative V_{max} is equal to the maximum value of I_T^{Σ}/I_{T-1} (*27*). When misincorporation opposite the target site is relatively inefficient, a plot of I_T^{Σ}/I_{T-1} vs dNTP concentration has little or no curvature, and the apparent V_{max}/K_m values are computed using a least-squares fit to a straight line. The misincorporation efficiency, f_{inc}, which is the inverse of the fidelity, is given by the ratio shown in eq 1, where the subscripts W and R refer to incorporation of incorrect or correct nucleotide, respectively.

$$f_{inc} = \text{fidelity}^{-1} = (V_{max}/K_m)_W/(V_{max}/K_m)_R \qquad (1)$$

Other Materials and Methods. Other materials and methods were described previously (*7*, *16*, *18*).

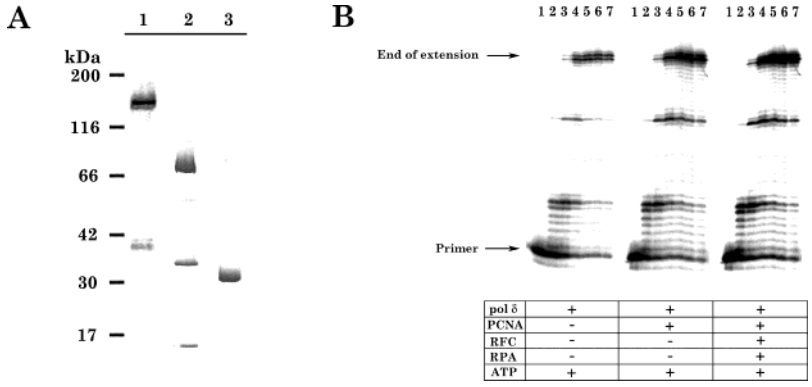

FIGURE 3: Effects of PCNA, RFC, and RPA on DNA synthesis by *S. cerevisiae* pol δ HE on a 30mer primer/100mer template. (A) RFC (0.5 μg), RPA (1 μg), and PCNA (1 μg) were analyzed by SDS−PAGE, and the gel was stained with Coomassie brilliant blue as described before (*7*). (B) Primer extension reactions were run with pol δ HE, four dNTPs, and the indicated proteins and ATP on a 30/100mer DNA. Six lanes in each group represent reaction products of seven time points of 0 s (1), 15 s (2), 30 s (3), 1 min (4), 2 min (5), 3 min (6), and 4 min (7). Final concentrations in the reactions (20 μL) were template/primer DNA (10 nM), pol δ HE (0.2 unit), and dNTPs (each 0.5 mM) and the accessory proteins and ATP were as follows: ATP (1.0 mM), RFC (54 μg/mL), PCNA (90 nM), and RPA (320 nM).

RESULTS

S. cerevisiae pol δ is one of the major replicative DNA polymerases in budding yeast (see ref *2* for review). The purified enzyme (holoenzyme, HE) has three subunits, Pol3, Pol31 (Hys2), and Pol32, and catalyzes highly processive DNA synthesis on φX174 single-stranded DNA primed with a short oligonucleotide in the presence of PCNA, RFC, and replication protein A (RPA) (ref *20* and our unpublished results).

Pol δ HE was purified to near homogeneity from wild-type (Figure 1) and *pol3-01* yeast cells (data not shown), and 3′−5′ exonuclease was measured using 5′-end ^{32}P-labeled 30mer primer in the presence and absence of PCNA (Figure 2). PCNA is a sliding clamp, which tethers pol δ HE to the end of DNA primer during polymerization and editing. Wild-type pol δ HE degraded the 30mer oligonucleotide from the 3′-end efficiently in the presence and absence of PCNA. However, the DNA substrate was degraded slightly less efficiently in the presence of PCNA, suggesting that PCNA may stabilize the enzyme in polymerization mode and slightly inhibit the exonuclease activity. On the other hand, 3′−5′ exonuclease activity was not detected in *pol3-01* exonuclease-deficient pol δ HE after incubation with the DNA substrate for 4 min (Figure 2) or 10 min (data not shown), although a contaminated phosphatase activity could be detected in both the wild-type and the mutant pol δ HE preparations (see the bottom bands in the figure). However, this activity was not detected when 5′-end ^{32}P-labeled 30mer primer annealed to the 100mer template polynucleotide was used (data not shown). Thus, we concluded that it would be a single-stranded oligonucleotide-specific phosphatase and it would not disturb our analyses of pol δ HE fidelity.

Fidelity of Wild-Type Pol δ HE with and without PCNA. In earlier work, the fidelity of *S. cerevisiae* pol ϵ HE (*16*), *S. pombe* pol δ HE in the presence of RFC, PCNA, and *Escherichia coli* SSB (*27*), and *E. coli* pol III and the effects of β, γ processivity and ϵ proofreading subunits (*26*) were characterized using a 30mer primer/100mer template. It was shown that *S. pombe* pol δ HE copies the synthetic 100mer DNA template in a completely distributive manner in the absence of PCNA (*27*). However, *S. cerevisiae* pol δ HE does not require PCNA, RFC, and RPA for DNA synthesis on a short DNA substrate, such as a 30mer primer/100mer template used in the previous studies (*16*, *26*, *27*). Therefore, it is possible to examine the fidelity of wild-type and exonuclease-deficient pol δ HE using the same DNA substrate without RFC and RPA. A time course showing extension of a ^{32}P-labeled primer is arranged in three groups of lanes to test the effects of PCNA on pol δ HE processivity (Figure 3). Pol δ HE copied the synthetic 100mer DNA template in a processive manner in the absence of PCNA (Figure 3). A slight stimulation in pol δ HE processivity occurred in the presence of PCNA (Figure 3, +PCNA). A similar stimulation of pol δ HE processivity could be observed in the presence of yeast PCNA, RFC, RPA, and ATP (Figure 3, +PCNA, RFC, RPA, and ATP). However, if we used oligo(dT)$_{10}$/poly(dA)$_{500}$ (1:20) primer/template as a primer/template, pol δ HE DNA synthesis activity was stimulated 5−7-fold by addition of PCNA (data not shown). These observations that PCNA stimulated pol δ HE synthesis in the absence of RFC suggest that the processivity clamp

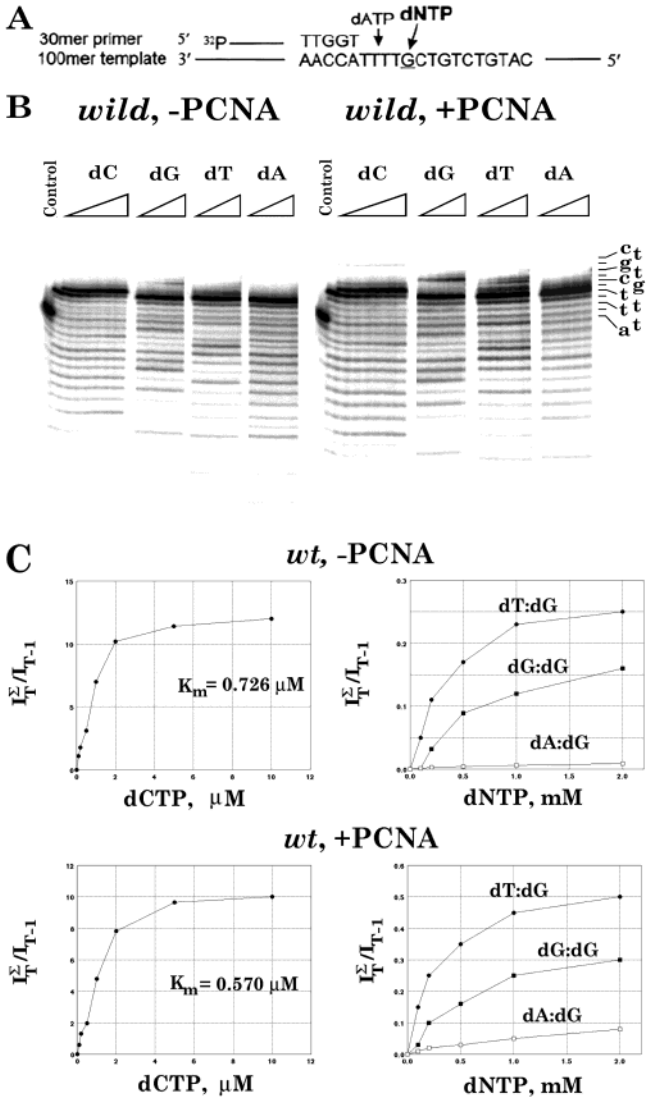

FIGURE 4: Fidelity of wild-type pol δ HE with and without PCNA. (A) Structure of the 30mer primer/100mer template DNA used in this study. (B) Primer extension reactions with wild-type pol δ HE were carried out in the absence or presence of PCNA and analyzed as described in Experimental Procedures. The incorporation of dCMP opposite G and the three misincorporation reactions were run under multiple hit conditions [90% primers used (correction factor for exonuclease-proficient enzyme, 1.70); see Appendix in ref *27*] to detect a significant misincorporation of dNTPs. The dNTP concentrations were 0.1, 0.2, 0.5, 1, 2, 5, and 10 μM for dCTP and 0.1, 0.2, 0.5, 1, and 2 mM for dTTP, dGTP, and dATP. (C) Gel band intensities in (B) were measured, and their ratio I_T^{Σ}/I_{T-1} was plotted versus dNTP concentration. The misincorporation efficiency f_{inc} was computed using eq 1 (see Experimental Procedures).

can load onto the short DNA by itself and stabilize the pol δ HE primer/template DNA complex. Threading of PCNA onto linear DNA in the absence of RFC has been reported previously for *S. cerevisiae* PCNA (*29*). Thus, base substitution fidelity of wild-type pol δ HE was measured in the absence or in the presence of PCNA in this study (Figure 4). When the reaction was initiated in the presence of dATP, the enzyme incorporated four residues of A opposite four template T residues up to the template target G (template DNA sequence is shown in Figure 4A). When dCTP was added to a reaction lacking PCNA, pol δ HE incorporated a C opposite the target G. When dCTP was added to a reaction with PCNA, pol δ HE also extended the primer beyond the

Table 1: Fidelity of Pol δ HE from *S. cerevisiae*

base pair	V_{max}	K_m	V_{max}/K_m	f_{inc}
		Wild-Type Pol δ, −PCNA		
dC·dG	4.21	0.726	5.80	
dG·dG	0.089	212	4.22×10^{-4}	0.728×10^{-4} (1)
dT·dG	0.234	163	14.4×10^{-4}	1.82×10^{-4} (1)
dA·dG	<0.01	>2000	$<0.05 \times 10^{-4}$	$<0.01 \times 10^{-4}$ (1)
		Wild-Type Pol δ, +PCNA		
dC·dG	3.84	0.570	6.74	
dG·dG	0.320	365	8.75×10^{-4}	1.30×10^{-4} (1.78)
dT·dG	0.536	246	21.8×10^{-4}	2.62×10^{-4} (1.44)
dA·dG	0.072	2000	0.36×10^{-4}	0.074×10^{-4} (>7.4)
		Pol δ from *pol3-01*, −PCNA		
dC·dG	5.81	0.210	27.6	
dG·dG	1.74	368	47.2×10^{-4}	1.68×10^{-4} (1)
dT·dG	3.24	243	133×10^{-4}	4.83×10^{-4} (1)
dA·dG	1.45	904	16.0×10^{-4}	0.572×10^{-4} (1)
		Pol δ from *pol3-01*, +PCNA		
dC·dG	4.94	0.327	15.1	
dG·dG	1.16	127	92.7×10^{-4}	6.13×10^{-4} (3.65)
dT·dG	1.15	127	90.6×10^{-4}	9.83×10^{-4} (2.03)
dA·dG	0.332	561	5.92×10^{-4}	1.07×10^{-4} (1.87)

target G; faint bands were detected at the next template C and at the T five nucleotides from the target G. Primer was also degraded to the first upstream template G with increasing frequency at higher concentrations of dCTP. When dGTP was added to a reaction lacking PCNA, a faint band was detected at the template T three nucleotides from the target G at higher concentrations of dGTP. This reaction product was more abundant in reactions with PCNA and also increased at higher concentrations of dGTP. When dTTP was added to reactions lacking PCNA, a faint band was detected at the target G. In the presence of PCNA, faint bands were also detected beyond the target G at higher concentrations of dTTP. dTTP was also incorporated opposite downstream template nucleotides C and T. In reactions with dGTP, pol δ HE shortened a fraction of the primer by one nucleotide. In all reactions with each dNTP, pol δ HE generated DNA fragments shorter than the primer DNA (compare a control lane). These results indicate that 3′−5′ exonuclease activity of pol δ HE acts as a proofreading exonuclease during DNA synthesis. Previous studies showed that pol ϵ HE possesses an efficient 3′−5′ exonuclease that removes misincorporated nucleotides during in vitro DNA synthesis (*16*). Thus, the above results suggest that the pol δ 3′−5′ exonuclease is less efficient than pol ϵ 3′−5′ exonuclease during in vitro DNA synthesis; however, the pol δ and pol ϵ 3′−5′ exonucleases degrade a single-stranded oligonucleotide with similar efficiency (Figure 2 and ref *16*).

The data shown in Figure 4B was used to estimate the nucleotide misincorporation efficiency (f_{inc}) of pol δ HE in the absence of PCNA as follows: G·G, 0.728×10^{-4}; T·G, 1.82×10^{-4}; A·G, $<0.01 \times 10^{-4}$. f_{inc} of pol δ HE in the presence of PCNA was estimated as follows: G·G, 1.30×10^{-4}; T·G, 2.62×10^{-4}; A·G, 0.074×10^{-4} (Figure 4C, and Table 1). Thus, nucleotide misincorporation increases severalfold in the presence of PCNA. In addition, nucleotide misincorporation in the presence and absence of PCNA is almost 2 orders of magnitude higher for pol δ HE than for pol ϵ HE [i.e., pol ϵ HE f_{inc} is 0.047×10^{-4} for G·G, 0.015×10^{-4} for T·G, and 0.0015×10^{-4} for A·G mismatches (*16*)]. Furthermore, pol ϵ HE also efficiently degrades DNA synthesis products containing misincorporated dGTP or dTTP

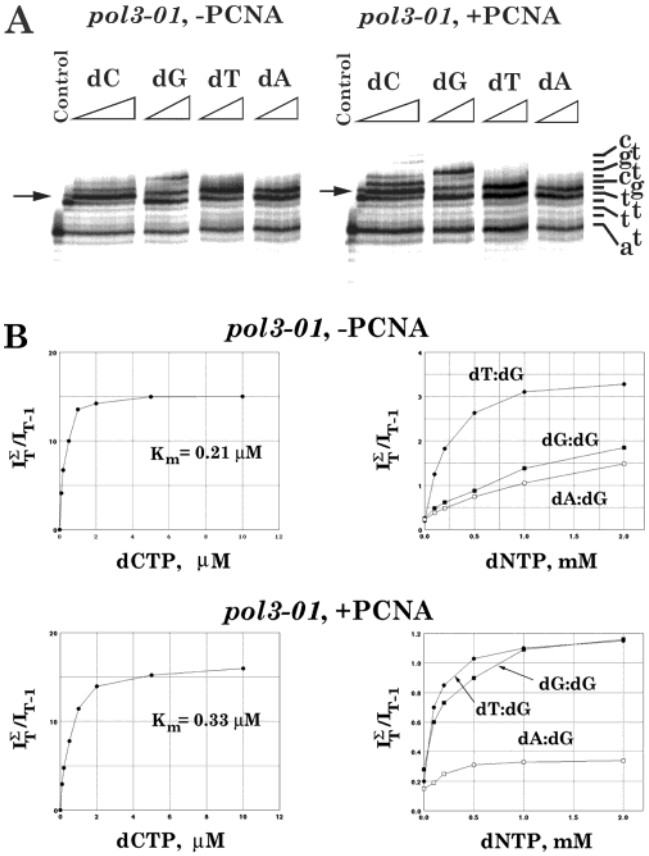

FIGURE 5: Fidelity of *pol3-01* pol δ HE with and without PCNA. (A) Primer extension reactions with 3′−5′ exonuclease-deficient *pol3-01* pol δ HE were carried out in the presence or absence of PCNA as described in Figure 4. The incorporation of dCMP opposite G and the three misincorporation reactions were run under multiple hit conditions [70% primers used (correction factor for exonuclease-deficient enzyme, 1.75); see Appendix in ref *27*]. The dNTP concentrations were 0.1, 0.2, 0.5, 1, 2, 5, and 10 μM for dCTP and 0.1, 0.2, 0.5, 1, and 2 mM for dTTP, dGTP, and dATP. An arrow indicates misincorporation in the presence of dATP. Such misincorporation was not detected in reactions with wild-type pol δ HE. (B) Gel band intensities in (A) were measured, and their ratio I_T^{Σ}/I_{T-1} was plotted versus dNTP concentration as in Figure 4C.

(*16*). Thus, these results are consistent with the observation that the fidelity of *S. cerevisiae* pol ϵ HE is considerably higher than the fidelity of *S. cerevisiae* or *S. pombe* pol δ HE (*27*).

Fidelity of Proofreading Exonuclease-Deficient Pol δ HE. Base substitution fidelity of exonuclease-deficient pol δ HE was also measured in the absence or presence of PCNA as described above for wild-type pol δ HE (Figure 5). During preincubation in the presence of dATP, the enzyme incorporated four A residues opposite four template T residues up to the template target G. At high concentrations of dATP, a band was detected at the target G in the absence and presence of PCNA. When dCTP was added to a reaction lacking PCNA, the mutant pol δ HE incorporated a C opposite the target G. In addition, faint bands were detected at the next template nucleotides C and T [these products were not produced by wild-type pol δ HE (Figure 4B)]. When a high concentration of dCTP was added to a reaction with PCNA, the enzyme incorporated dCTP or dATP to the C residue at least five nucleotides beyond the target G. Incorporation at the template nucleotide C next to the target

G could be nontemplate extension of the primer, because 3′−5′ exonuclease-deficient DNA polymerases tend to catalyze this reaction at the end of duplex DNA (*30*). However, this reaction is not observed for an exonuclease-proficient enzyme, because of its proofreading 3′−5′ exonuclease. When a high concentration of dGTP was added to a reaction lacking PCNA, faint bands were detected at the target G and at the template T three nucleotides from the target G, but the latter species migrated more slowly than the correct product of the same size (Figures 4B and 5A). This may be due to misincorporation of dGTP at the template T (mutant pol δ HE) instead of dATP (wild-type pol δ HE). Addition of PCNA enhanced synthesis of this product as well as a product terminating at the template T nine nucleotides from the target G. When dTTP was added to a reaction without PCNA, incorporation at the target G was much higher than in reactions only with dATP. Similar results were observed in the presence of PCNA (Figure 5A).

The nucleotide misincorporation efficiency of *pol3-01* pol δ HE in the absence of PCNA was estimated as follows: G•G, 1.68×10^{-4}; T•G, 4.83×10^{-4}; A•G, 0.572×10^{-4}. In the presence of PCNA, f_{inc} was estimated as follows: G•G, 6.13×10^{-4}; T•G, 9.83×10^{-4}; A•G, 1.07×10^{-4} (Figure 5B and Table 1) Thus, f_{inc} of *pol3-01* pol δ increased severalfold in the presence of PCNA for all mismatches examined (Table 1). These results suggest that nucleotide misincorporation efficiency increases with increasing processivity and shows that the nucleotide misincorporation efficiency is higher for *pol3-01* than for wild-type pol δ HE.

V_{max}/K_m was also compared for *pol3-01* and wild-type pol δ HE. For correct nucleotide incorporation (dCTP) in the absence of PCNA, V_{max}/K_m was 27.6 for *pol3-01* and 5.80 for the wild-type enzyme (Table 1). In the presence of PCNA, V_{max}/K_m for dCTP incorporation was 15.1 for *pol3-01* and 6.74 for the wild-type enzyme. As discussed above, nucleotide misincorporation efficiency is higher for A•G, G•G, and T•G for *pol3-01* than for wild-type pol δ HE (Table 1). However, because the V_{max}/K_m of exonuclease-deficient pol δ for C•G was 2.24-fold higher than for the wild-type enzyme, it was calculated that the G•G, T•G, and A•G misincorporation efficiencies of the *pol3-01* pol δ HE were approximately 10-, 8-, and 30-fold higher than for wild-type pol δ HE, respectively (Table 1).

DISCUSSION

Pol δ is one of the major eukaryotic replicative polymerases, and it was expected that its fidelity would be high (*1*). Goodman and collaborators (*27*) showed that the fidelity of *S. pombe* pol δ HE is 1−2 orders of magnitude lower than the fidelity of *E. coli* pol III (*26*). The fidelity of an another eukaryotic replicative DNA polymerase pol ϵ is comparable to that of *E. coli* pol III (*16*) and, therefore, higher than the fidelity of *S. pombe* pol δ HE. This study measures the fidelity of wild-type and exonuclease-deficient *S. cerevisiae* pol δ HE and enables us to directly compare its fidelity to that of the other major replicative polymerase in *S. cerevisiae*, pol ϵ HE. Wild-type *S. cerevisiae* pol δ HE has 3′−5′ exonuclease activity whereas the activity of *S. pombe* pol δ HE was inefficient, suggesting that *S. cerevisiae* pol δ HE might have a higher fidelity (*27*). However, this study shows that the fidelity of *S. cerevisiae* pol δ HE is significantly lower than the fidelity of pol ϵ HE (i.e., comparable to the fidelity of *S. pombe* pol δ HE) and that the pol δ 3′−5′ exonuclease removes errors inefficiently from a template/primer during in vitro DNA synthesis (Figure 4B). Therefore, this study supports the conclusion that the intrinsic fidelity of pol δ HE is low and that this is due primarily to the inherent fidelity of its DNA synthesis activity but only to a small extent on its 3′−5′ exonuclease activity.

Previous studies showed that the fidelity of *S. cerevisiae* pol ϵ HE was enhanced by its 3′−5′ exonuclease (*16*). Especially for G•G and T•G mismatches, the nucleotide misincorporation efficiency was approximately 100-fold higher for the 3′−5′ exonuclease-deficient enzyme than for the wild-type enzyme. Thus, nucleotide misincorporation efficiency was similar for exonuclease-deficient pol ϵ HE and wild-type pol δ HE (Table 1). The nucleotide misincorporation efficiency of exonuclease-deficient pol δ HE was approximately 10-fold higher than of wild-type pol δ HE in the presence of PCNA (Table 1). By comparison, pol ϵ HE is an extremely accurate DNA polymerase with an efficient 3′−5′ proofreading exonuclease. The fidelity of pol δ HE is low, and the contribution of the 3′−5′ exonuclease activity to its fidelity is relatively small. If pol δ synthesizes DNA with low fidelity in vivo in the presence of PCNA, it is possible that other enzymes might correct misincorporation errors introduced by pol δ HE.

Previous studies examined the spontaneous mutator phenotypes of exonuclease-deficient *pol3-01* pol δ and *pol2-4* pol ϵ (*14*). The *pol3-01* mutation elevated the spontaneous mutation rate by approximately 100-fold, and the *pol2-4* mutation elevated the spontaneous mutation rate by approximately 10-fold (assessed by *URA3* forward mutation and *his7-2* reversion assays). However, in vitro results demonstrate that the fidelity of exonuclease-deficient pol δ HE was reduced approximately 10-fold and the fidelity of exonuclease-deficient pol ϵ HE was reduced approximately 100-fold (this study and ref *16*). This discrepancy suggests that the in vivo spontaneous mutation rates of *pol3-01* and *pol2-4* strains are determined by another factor (or factors) in addition to the proofreading activity of the DNA polymerase. For example, misincorporation errors by 3′−5′ exonuclease-deficient pol ϵ HE might be corrected by mismatch repair, such that fewer misincorporation errors persist in vivo than in vitro. However, in *pol3-01* yeast strain, the situation might be more complicated. The low fidelity of wild-type pol δ HE might be rescued by mismatch repair activities, but the even lower fidelity of exonuclease-deficient pol δ may cause enough mutations to exceed the capacity of mismatch repair. Also, pol δ HE and pol α primase participate in lagging-strand synthesis (*6*) and pol α primase lacks intrinsic 3′−5′ exonuclease; thus, misincorporation errors introduced by pol α primase may be corrected in vivo by pol δ HE, exo1, and/or mismatch repair activities (*25*). Therefore, it is possible that inactivation of pol δ 3′−5 exonuclease may severely reduce the fidelity of lagging-strand DNA synthesis, and the spontaneous mutation rate in *pol3-01* might reflect the misincorporation rate of pol δ HE and pol α primase.

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