Fidelity of Eucaryotic DNA Polymerase δ Holoenzyme from Schizosaccharomyces pombe*

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The fidelity of Schizosaccharomyces pombe DNA polymerase δ was measured in the presence or absence of its processivity subunits, proliferating cell nuclear antigen (PCNA) sliding clamp and replication factor C (RFC) clamp-loading complex, using a synthetic 30-mer primer/100-mer template. Synthesis by pol δ alone was distributive. Processive synthesis occurred in the presence of PCNA, RFC, and Escherichia coli single strand DNA-binding protein (SSB) and required the presence of ATP. “Passive” self-loading of PCNA onto DNA takes place in the absence of RFC, in an ATP-independent reaction, which was strongly inhibited by SSB. The nucleotide substitution error rate for pol δ holoenzyme (HE) (pol δ + PCNA + RFC) was 4.6 × 10⁻⁴ for T-G mispairs, 5.3 × 10⁻⁵ for G-G mispairs, and 4.5 × 10⁻⁶ for A-G mispairs. The T-G misincorporation frequency for pol δ without the accessory proteins was unchanged. The fidelity of pol δ HE was between 1 and 2 orders of magnitude lower than that measured for the E. coli pol III HE at the same template position. This relatively low fidelity was caused by inefficient proofreading by the S. pombe polymerase-associated proofreading exonuclease. The S. pombe 3'-exonuclease activity was also extremely inefficient in excising primer-3'-terminal mismatches in the absence of dNTP substrates and in hydrolyzing single-stranded DNA. A comparison of pol δ HE with E. coli pol II HE (lacking the proofreading exonuclease subunit) showed that both holoenzymes exhibit similar error rates for each mispair.

The enzymes principally responsible for catalyzing prokaryotic and eucaryotic DNA replication share many common elements. Eucaryotic DNA polymerases δ and ε have the ability to proofread replication errors using polδ-associated 3'→5' exo-

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*This work was supported by National Institutes of Health Grants GM21422 (to M. F. G.), GM35559 (to J. H.), and GM38393 (to M. O'D.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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‡The abbreviations used are: pol, DNA polymerase; HE, holoenzyme comprised of DNA polymerase + processivity subunits, proliferating cell nuclear antigen (PCNA) sliding clamp, and replication factor C (RFC) clamp-loading complex for S. pombe, and β sliding clamp and γ clamp-loading complex for E. coli; SSB, E. coli single strand DNA-binding protein; RPA, replication protein A, eucaryotic single strand nuclease activity (1, 2), a property shared with Escherichia coli pols I–III (3). Each of these enzymes copies DNA with extremely low processivity, typically adding less than 30 nt before dissociating. There are closely analogous groups of eucaryotic and prokaryotic polymerase accessory proteins that interact with the non-processive core pols, forming highly processive polymerase holoenzymes. E. coli pol III HE and pol II HE bind to the β dimeric sliding clamp (4–6), whereas eucaryotic pol δ HE and pol ε HE bind to the PCNA trimeric sliding clamp (7). The processivity clamps are loaded on and off the DNA by clamp loading complexes, γ complex in E. coli (8–11) and RFC in eucaryotic cells (7, 12–14).

Extensive studies on the fidelity properties of core DNA polymerases have been reported over the past 3 decades focusing on biochemical and kinetic analysis of deoxynucleotide insertion specificity and the reduction in pol-generated errors by proofreading exonucleases (15–18), whereas there are but a paucity of experiments reporting on the fidelity properties of the more biologically relevant pol HE systems. Previous experiments employing pol HE systems have attempted to probe the fidelity of leading versus lagging strand synthesis using mutational reporter sequences (e.g. lacZ) (19, 20) and to visualize synthesis past DNA damage sites using two-dimensional gel electrophoresis (21, 22). We have recently generalized a gel kinetic assay originally designed to measure polymerase fidelity in the absence of proofreading (23, 24), enabling fidelity measurements to be made at arbitrary p/t DNA sites in the presence of proofreading and pol accessory proteins (25–27).

There are a variety of questions regarding the fidelity properties of holoenzymes that can be investigated systematically using gel kinetic methodology. Recently, measurements on the fidelity of calf thymus pol δ were made in the presence and absence of PCNA at normal (28) and abasic (29) template sites. In this paper, we report on the base substitution error rate of the Schizosaccharomyces pombe pol δ HE and core for comparison with data with the E. coli pol III HE-catalyzed error rates (26) determined in the same sequence context.

EXPERIMENTAL PROCEDURES

Materials

Proteins cloned S. pombe pol δ, PCNA, and RFC were purified as described (30). The enzyme reaction buffer contained 40 mM Tris-HCl, pH 7.8, 170 μg/ml bovine serum albumin, 0.5 mM dithiothreitol, and 7 mM MgCl₂. Bacteriophage T4 polynucleotide kinase was purchased from United States Biochemical Corp. or Amersham Pharmacia Biotech. T4 DNA ligase was purchased from Promega. E. coli single strand DNA-binding protein; p/t DNA, primer-template DNA; nt, nucleotide; ssDNA, single-stranded DNA.
DNA-binding protein and bovine serum albumin were purchased from Amersham Pharmacia Biotech.

**DNA Substrates**—The p/t DNA was made up of a synthetic 100-mer template annealed to complementary 30- or 35-mer primers or to a 35-mer primer containing a single noncomplementary base at its 3'-end. The 30-mer primer was annealed at the middle of the template leaving equal length (35 nt) ssDNA overhangs on each side. The matched 35-mer primer was annealed to the template leaving 35 nt of ssDNA at the 3'-end of the template and 30 nt of ssDNA at 5'-end. The mismatched 35-mer primer was identical to the matched 35-mer except that the nucleotide at the 3'-end contained an A in place of C. All oligomers were synthesized on an Applied Biosystems 392 DNA/DNA synthesizer (Perkin-Elmer) and gel-purified. The 100-mer was synthesized as two half-length oligomers and then ligated together.

The sequences for the 30-mer primer/100-mer template were as follows:

3′GCC CTATCCACATATAATGCGATAGCATGTCCTCAATCTATGCGC 3′

**ICN Radiochemicals**.

**Methods**

The primer was 5'-end-labeled with 32P using T4 polynucleotide kinase in enzyme reaction buffer at 37 °C for 60 min. p/t DNA was annealed in enzyme reaction buffer using a ratio of 1 primer to 1.2 templates by heating to 90 °C and gradually cooling to room temperature. The concentration of p/t DNA after annealing was 100 nM (primer termini).

**Assay for 3′-Exonuclease Activity of S. pombe pol δ**—10 nM either matched or mismatched 35/100-mer DNA were incubated at 37 °C with 10 µg/ml (0.2 unit/µl) S. pombe pol δ in reaction buffer in the presence and absence of all 4 dNTPs (0.5 mM each) if present in separate reactions containing 20 µl. One unit of pol δ supports the incorporation of 1 nmol of dTMP under the conditions specified above. The 35-mer primers were 5′-end-32P-labeled. The mismatched 35-mer primer (10 µM) was used as single-stranded DNA substrate and incubated at 37 °C with 10 µg/ml (0.2 unit/µl) S. pombe pol δ in reaction buffer (20 µl). Aliquots (4 µl) were removed from each reaction and quenched by mixing with 10 µl of 20 mM EDTA, 95% formamide at different time points. Reaction products were separated on a 12% denaturing polyacrylamide gel run for 2 h at 2000 V. The amount of primer extension catalyzed by pol δ (gel bands above the primer band) or degradation catalyzed by pol 3′-exonuclease activity (gel bands below the primer band) was measured as percentage of total gel band intensity in each lane using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

**Processive Synthesis on 30/100-Mer p/t DNA—p/t DNA was preincubated at 37 °C with different combinations of ATP and accessory proteins PCNA, RFC, and SSB in enzyme reaction buffer (16 µl) containing glycerol (4%) for 1 min. Following preincubation of enzyme reaction buffer (4 µl) containing glycerol (4%), the four dNTPs and pol δ were added to initiate the reaction. Aliquots (4 µl) were removed at different times and mixed with formamide/EDTA (10 µl) to quench the reaction. Final substrate concentrations in the reactions were p/t DNA (10 nM), pol δ (0.5 µg/ml), dATP (0.5 mM), dCTP (0.5 mM), dGTP (0.5 mM), and the accessory proteins, if present as SSB (20 µg/ml), PCNA, (90 µM), S. pombe pol δ, and dNTP concentrations as indicated in the figures. Control reactions were run for 5 min using just the running-start dATP to verify that misincorporation of the running-start nt opposite the target G site did not occur.

**Control reactions**—for pol δ in the presence of processivity accessory proteins were performed as follows. Solution A contained 33 mM p/t DNA, 150 µg/ml RFCl, 270 nM (PCNA), 1 µM SSB, 2 mM ATP, and 4% glycerol in enzyme reaction buffer. Solution B consisted of the enzyme reaction buffer containing various concentrations of the dNTP to be incorporated opposite target site. Solution C contained 0.5 µg/ml pol δ, running start dATP (188 µM), and 4% glycerol in the enzyme reaction buffer. The reaction was performed as follows: solution A (3 µl) was mixed with solution B (3 µl) and incubated at 37 °C for 1 min to allow RFCl to load PCNA onto the DNA; then solution C (4 µl) was added to the mixture of A + B to initiate the primer extension reaction. The final concentrations in the 10-µl reaction mixture were 10 nM p/t DNA, 0.2 µg/ml pol δ, 75 µM dATP, 45 µg/ml RFCl, 80 nM (PCNA), 300 nM SSB, 0.6 mM ATP, and various concentrations of dNTP for incorporation opposite G. Control reactions were run for 20 min to ensure that the running-start dATP only to ensure that it did not misincorporate opposite G. The reactions, run at 37 °C for 2 min for both correct incorporation and misincorporations opposite G, approximately satisfied single-completed hit conditions, in which about 20% of the primers were extended, so that no further corrections were required in the kinetic analysis. Reactions were quenched by addition of formamide/EDTA (20 µl) to the reaction mixture. The samples were heated to 100 °C for 6 min, placed on ice for 3 min, and then loaded on a 16% polyacrylamide denaturing gel. The gel was run at 2000 V for 4 h to separate reaction products.

Integrated polyacrylamide gel band intensities were measured on a PhosphorImager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The nucleotide incorporation efficiency opposite the target site was obtained by measuring \( I_{2}^{z} / I_{1}^{z} \), where \( I_{2}^{z} \) is the integrated gel band intensities of primers extended to the target site and beyond, and \( I_{1}^{z} \) is the integrated gel band intensity of primers extended to the site just prior to the target site (26, 31).

A plot of the relative incorporation rate, \( I_{2}^{z} / I_{1}^{z} \), as a function of the dNTP substrate concentration, results in a rectangular hyperbola whose slope in the initial linear region is the apparent \( V_{max}/K_{m} \) value. Apparent \( K_{m} \) and relative \( V_{max} \) values were obtained using a least squares fit to a rectangular hyperbola. The relative \( V_{max} \) value is equal to the maximum value of \( I_{2}^{z} / I_{1}^{z} \) in reactions in which misincorporation opposite the target site was relatively inefficient, a plot \( I_{2}^{z} / I_{1}^{z} \) versus dNTP concentration showed little or no curvature, and apparent \( V_{max}/K_{m} \) values were obtained by a least squares fit of the data to a straight line. Apparent \( V_{max}/K_{m} \) values that were obtained under multiple-completed hit conditions were corrected to single-completed hit conditions as described in Ref. 27, but these corrections were essentially negligible. The misincorporation efficiency, \( f_{inc} \), which is the inverse of the fidelity, is given by the ratio shown in Equation 1,
Supporting Processive Synthesis by *Saccharomyces cerevisiae* PCNA (34). We observed that SSB strongly inhibited synthesis by pol δ in the presence of PCNA (Fig. 1, group 6), but processive synthesis was restored by the addition of RFC (Fig. 1, group 5), clearly demonstrating that RFC was active in the assay.

The apparent stimulation of the RFC-dependent reaction by SSB (compare Fig. 1, groups 4 and 5) was caused most likely by the inhibition of a 3'-exonuclease contaminant present in our purified preparation of RFC. This adventitious 3'-exonuclease appeared to digest the primer extension products causing a uniform reduction in the gel band intensities in group 4 bands relative to either groups 5 or 2, while maintaining similar processivity patterns for these three groups.

The assays shown in Fig. 1 were performed in the presence of ATP (1 mM) required for RFC-mediated loading of PCNA onto p/t DNA (12, 35–37). Further characterization of the effects of PCNA and RFC on pol δ synthesis was carried out by performing similar primer elongation experiments in the absence of ATP. We found that processive synthesis observed in the presence of PCNA (Fig. 1, group 2) was retained in the absence of ATP (data not shown), whereas ATP must be present to observe processive synthesis in presence of PCNA, RFC, and SSB (Fig. 1, groups 5 and 7). We conclude the following: (i) ATP is required for loading of PCNA onto DNA by RFC but that PCNA can also “thread” itself onto short linear p/t DNA in the absence of RFC, in a “passive” reaction not requiring ATP; (ii) SSB significantly inhibits the ATP-independent passive loading reaction but does not affect the loading of PCNA by RFC.

**RESULTS**

pol δ, thought to be one of the principal eucaryotic replication polymerases (32), forms a processive holoenzyme complex in the presence of the trimeric PCNA sliding clamp and RFC, which is responsible for loading PCNA onto DNA (7). Eucaryotic pol δ is analogous to *E. coli* pol III core which uses both the μ sliding clamp and the clamp loading γ complex to form the highly processive pol III HE. In this study, we have measured the fidelity of *S. pombe* pol δ HE in the same sequence context that was used previously to study the fidelity of *E. coli* pol III HE (26). Although similar in some respects, the biochemical properties of the *E. coli* and *S. pombe* pol holoenzymes revealed several unanticipated differences, particularly with respect to the contribution of proofreading to fidelity.

A 30/100-mer p/t DNA Serves as a “Minimal” Substrate Supporting Processive Synthesis by *S. pombe* pol δ HE—We chose to measure the fidelity of *S. pombe* pol δ HE when copying a synthetic 100-mer DNA template. The advantage of using relatively short synthetic oligonucleotide minimal p/t DNA is that pol fidelity can be measured at arbitrary template target sites in defined sequence contexts that can be easily varied. However, before such a system can be used, it is necessary to show that it recapitulates the properties observed with much longer biological substrates, e.g. SV40 DNA (33).

A time course showing extension of a 32P-labeled primer is arranged in seven groups of lanes to test the effects of PCNA, RFC, and SSB on pol δ processivity (Fig. 1). pol δ copied the synthetic 100-mer DNA template in a completely distributive manner in the absence of PCNA (Fig. 1, groups 1 and 3). A marked stimulation in pol δ processivity occurred in the presence of PCNA, PCNA + RFC, or PCNA + RFC + SSB (Fig. 1, group 2, group 4, and group 5, respectively). The observation that PCNA stimulated pol δ synthesis in the absence of RFC suggests that the processivity clamp can load onto the short DNA by itself and stabilize the pol δ/p/t DNA complex. Thread-
SSB does not imply that the 4-subunit pol δ core has higher fidelity than pol δ HE. Rather, the absence of target site misincorporation bands for the case of pol δ alone was caused by the distributive nature of the enzyme. The residence time on the primer-template DNA was simply too short to allow pol δ to catalyze the proofreading exonuclease activity for the 4-subunit pol δ core under synthesizing and non-synthesizing conditions on p/t DNA, using either matched or mismatched primer-3'-ends (Fig. 4A). Excision of the primer-3'-end containing an A-G mismatched base pair occurred more rapidly than removal of a C-G correctly matched pair both in the presence and absence of dNTP substrates. However, the pol δ exonuclease activity appeared extremely weak. Removal of a terminal A-G mismatch was detectable in a 3-min incubation in either the presence or absence of dNTP substrates (Fig. 4A), whereas in the presence of dNTPs, a low level of incorporation of a next correct dGMP occurred more rapidly than removal of a C-G mismatched base pair that was observed in a 7-min incubation.

**Weak Proofreader**—We measured 3'→5' proofreading exonuclease activity for the 4-subunit pol δ core under synthesizing and non-synthesizing conditions on p/t DNA, using either matched or mismatched primer-3'-ends (Fig. 4A). Excision of the primer-3'-end containing an A-G mismatched base pair occurred more rapidly than removal of a C-G correctly matched pair both in the presence and absence of dNTP substrates. However, the pol δ exonuclease activity appeared extremely weak. Removal of a terminal A-G mismatch was detectable in a 3-min incubation in either the presence or absence of dNTP substrates (Fig. 4A), whereas in the presence of dNTPs, a low level of incorporation of a next correct dGMP onto an A-G mismatched base pair was observed in a 7-min incubation.

The S. pombe pol δ exonuclease-to-polymerase ratio is about 1 to 30. That is, the rate of extending a correct dCMPG terminus is roughly 30 times greater than the rate of removal of a dAMP-G mismatched terminus in the absence of dNTP substrates. Indeed, the excision of dAMP from a terminal A-G mispair was remarkably inefficient with greater than 90% of the input primer/template DNA remaining following a 40-min reaction. In contrast, degradation of ssDNA occurred more rapidly than p/t DNA (Fig. 4B). The degradation reaction appeared to be dis-
tributive, showing removal of about 6 nt during a 6-min reaction.

The extremely low nuclease/polymerase ratio suggests that 3′-exonuclease of the pol δ may not be effective in eliminating nucleotide substitution errors. We tested this supposition by measuring $f_{inc}$ (dTMP-G) for pol δ at different concentrations of the next correct dGTP substrate. We found no measurable change in the T-G misincorporation ratios ($f_{inc} = 4.6 \times 10^{-4}$) for pol δ, when varying dGTP concentrations between 0 and 160 μM (data not shown). Since a decrease in fidelity with increasing next correct dNTP concentration is a well established hallmark of proofreading (38, 39), the absence of a dependence of fidelity on dNTP concentration implies that the 3′-exonuclease of pol δ may not be effective in eliminating polymerase-catalyzed base substitution errors.

**DISCUSSION**

pol δ is believed to be the primary replicative enzyme in eucaryotic cells responsible for carrying out processive DNA synthesis in the presence of PCNA, RFC, and RPA (32). Despite the importance of this enzyme, little is known regarding its fidelity properties in vitro and in vivo. In this paper, we have used a gel kinetic assay (24, 26, 31) to measure fidelity at an arbitrary template G site using the pol δ HE purified from S. pombe (30).

**Processive Synthesis by pol δ Using a Synthetic p/t DNA Oligomer**—It is convenient to synthesize relatively short DNA templates to investigate DNA polymerase fidelity using defined sequence contexts. However, prior to performing a fidelity analysis using S. pombe pol δ HE on a 30/100-mer p/t DNA, it was necessary to demonstrate that the PCNA sliding clamp stimulated pol δ processivity, dependent on the presence of RFC and ATP, since the presence of ATP is required for loading of PCNA onto DNA by the RFC clamp loading complex (12, 35–37). This requirement is potentially important because PCNA can also diffuse onto linear but not circular DNA in the absence of RFC and ATP (34).

Synthesis by pol δ alone was distributive on the 30/100-mer p/t DNA with the addition of about 6 nt following a 3-min reaction and increasing to just 7 nt at 8, 18, and 40 min (Fig. 1, group 1). The enzyme remained active during the 40-min time course as shown by the increased primer extension band intensities at the later time points. In contrast, synthesis by the pol δ HE was much more processive, with the addition of 35 nt to reach the end of the template strand well within the first time point taken at 3 min (Fig. 1, group 5). Processive synthesis does not occur in the absence of either PCNA or ATP (Fig. 1, groups 3 and 7, respectively). One can also clearly observe the PCNA-independent passive clamp loading reaction, with full-length synthesis also occurring in less than 3 min (Fig. 1, group 2).

However, it is important to note that the passive clamp loading reaction failed to occur in the presence of SSB (Fig. 1, group 7), ensuring that our fidelity measurements made with pol δ HE in the presence of SSB, required PCNA, RFC, and ATP to carry out processive primer elongation. Experiments in which RPA (human or S. pombe RPA) was substituted for E. coli SSB showed no significant differences in either the rates or fidelity of DNA synthesis (data not shown). Therefore, a specific requirement for eucaryotic SSB has not been demonstrated in our in vitro model system and remains an open question requiring further investigation.

**Fidelity of S. pombe pol δ HE**—Nucleotide misincorporation values for S. pombe pol δ were found to be $f_{inc} = 4.6 \times 10^{-4}$ (T-G), 5.3 $\times$ 10$^{-5}$ (G-G), and 4.5 $\times$ 10$^{-6}$ (A-G) (Fig. 3 and Table 1). The pol δ HE error rates can be compared with values obtained with E. coli pol III HE and proofreading-defective E. coli pol IIIα HE (26) containing the β sliding clamp (analogous to PCNA), γ clamp loading complex (analogous to RFC), and SSB in the same p/t DNA sequence context (Table 1). The fidelity of pol δ HE is considerably lower than pol III HE for each mispair. The reduction in fidelity compared with pol III HE is 82-fold (T-G), 76-fold (G-G), and 11-fold (A-G).

The higher nucleotide misincorporation rates for pol δ HE appear to be attributable almost entirely to a severely compromised ability to proofread insertion errors made by the polymerase catalytic subunit. Indeed, a comparison of $f_{inc}$ for pol IIIα $^{a}$

$^{a}$ In the fidelity comparison for G-G misincorporations, the template used for E. coli pol III HE contains the base A in place of C immediately downstream from the target G site because pol III HE can incorporate dGMP opposite the downstream C by a primer-template slippage mechanism called “dNTP-stabilized” misalignment (26). In contrast, S. pombe pol δ HE misincorporates dGMP directly opposite G when C is located at the 5′-side of the target.

**TABLE I**

Comparison of misincorporation efficiencies for DNA polymerase holoenzymes

| T-G          | G-G          | A-G          |
|--------------|--------------|--------------|
| pol δ HE     | 4.6 $\times$ 10$^{-4}$ | 5.3 $\times$ 10$^{-5}$ | 4.5 $\times$ 10$^{-6}$ |
| pol III HE$^{a}$ | 5.6 $\times$ 10$^{-4}$ | 7.0 $\times$ 10$^{-5}$ | 4.2 $\times$ 10$^{-7}$ |
| pol IIIα HE$^{a}$ | 1.8 $\times$ 10$^{-4}$ | 3.5 $\times$ 10$^{-5}$ | 5.6 $\times$ 10$^{-6}$ |

$^{a}$ $f_{inc}$ values for the pol III HE and α HE, measured in the same p/t DNA sequence context used for pol δ HE, are from Ref. 26.

$^{b}$ The base downstream of the target G site was changed from C to A. See text, Footnote 2.
HE (containing the 3′ polymerase subunit in the absence of the ɛ proofreading and δ subunits) shows that the nucleotide misinsertion rates for pol δ and pol III are essentially the same (Table I). The reduction in fidelity for pol δ HE compared with pol IIIa HE is only 2.6- and 1.5-fold for T-G and G-G mismpairs, respectively, whereas pol δ HE may be slightly (1.2-fold) more accurate in forming A-G mismpairs. These small differences are not statistically significant.

The apparent “absence” of effective proofreading for S. pombe pol δ in the in vitro experiments is quite puzzling. By using the same assay and p/t DNA sequence to measure E. coli proofreading of mispaired A-T, we observed an 8-fold reduction in fidelity as in vivo proofreading was readily apparent using the same gap filling assay. Although we are unaware of any in vivo repair pathway in S. pombe that has been shown to excise mispaired bases (48), in addition to damaged DNA bases, raises the possibility that this repair pathway might compensate for a lack of effective proofreading by pol δ.

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