Proliferating Cell Nuclear Antigen Promotes Misincorporation Catalyzed by Calf Thymus DNA Polymerase δ*

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A proliferating cell nuclear antigen (PCNA)-dependent complex, detectable after nondenaturing polyacrylamide gel electrophoresis, is formed between calf thymus DNA polymerase δ (pol δ) and synthetic oligonucleotide template-primers containing a mispaired nucleotide at the 3′-terminal position of the primer. This complex is indistinguishable in composition from that formed with a fully base paired template-primer. Extension of a mispaired primer terminus is a component of DNA polymerase fidelity. The fidelity of pol δ on synthetic oligonucleotide template-primers was compared with and without its specific processivity factor, PCNA. In the absence of PCNA, pol δ misincorporates less than one nucleotide for every 100,000 nucleotides incorporated correctly. Addition of PCNA to reactions reduces fidelity by at least 27-fold. PCNA also confers upon pol δ, the ability to incorporate (and/or not excise) the dTTP analog, 2′-deoxythymidine-5′-O-(α-phosphonomethyl)-β,γ-diphosphate. A model is proposed whereby the increased stability (decreased off-rate) of the pol δ-template-primer complex in the presence of PCNA facilitates unfavorable events catalyzed by pol δ. This model suggests an explicit mechanistic requirement for the intrinsic 3′-5′-exonuclease of pol δ.

DNA polymerase δ (pol δ)¹ is thought to be responsible for most DNA synthesis during mammalian replication (1). Together with its processivity factor, proliferating cell nuclear antigen (PCNA), pol δ is responsible for the bulk of leading strand synthesis as well as significant replication of the lagging strand. It is thought that PCNA enhances the processivity of pol δ by binding to both DNA and pol δ and by acting as a “sliding clamp” that stabilizes the interaction between pol δ and template-primer (2, 3). On model template-primers, PCNA stabilizes by nearly 2000-fold the pol δ-template-primer interaction (4, 5) and apparently increases the rate of single nucleotide incorporation by the polymerase (6).

The dramatically increased stability of the pol δ-template-primer interaction in the presence of PCNA has profound functional implications. For example, it was demonstrated by O’Day et al. (7) that PCNA could facilitate synthesis by yeast pol δ past a thymine dimer present in the template strand. Both cis-syn and trans-syn-1-thymine dimers could be bypassed. Similarly, the β-subunit of Escherichia coli pol III-holoenzyme, thought to be the functional prokaryotic homolog of eukaryotic PCNA (see Refs. 2, 8), also affects replicative bypass of lesions, although in a complex way (see e.g. 9, 10). Finally, thioredoxin, the processivity subunit of T7 DNA polymerase and thus analogous to PCNA, also has effects on polymerase fidelity (11). In light of these many observations, we reasoned that addition to pol δ incubations of homologous PCNA might lead indirectly to an increased incidence of any relatively unlikely event (e.g. incorporation versus misincorporation).

In the present study we analyzed the impact of PCNA on two different sorts of unlikely events catalyzed by pol δ, 1) misincorporation, i.e. incorporation of a normal dNMP opposite a noncomplementary template nucleotide, and extension without excision of a mismatched primer nucleotide; and 2) incorporation of an abnormal nucleotide, i.e. a nucleotide analog. For both, PCNA enhanced the activity of pol δ, essentially as was expected based on enhanced stability of the pol δ-template-primer complex. These observations suggest compelling evolutionary selection pressure to account for the active 3′-5′-exonuclease associated with pol δ.

EXPERIMENTAL PROCEDURES

Materials—A modified analog of dTTP, 2′-deoxythymidine-5′-O-(α-phosphonomethyl)-β,γ-diphosphate (dTCP-P₃, for structure, see Fig. 4A), was synthesized as described (12). Unlabeled deoxyribonucleoside triphosphates (dNTPs) were high performance liquid chromatography-purified and were from Pharmacia Biotech Inc. ³²P-Labeled dNTPs were from Amersham Corp. Synthetic oligonucleotides of defined sequence were prepared by conventional phosphoramidite chemistry and purified by PAGE in the presence of 7 M urea. (dT₄)₄ was also made synthetically by conventional phosphoramidite chemistry. PCNA and pol δ were both purified from calf thymus according to published protocols (6, 13, 14). Micrococcal nuclease was from Sigma. All other materials were obtained commercially; chemicals were of reagent grade and were used without further purification.

DNA Polymerase Assays—DNA polymerase assays were performed essentially as described previously (6, 15, 16). The standard reaction mixture for pol δ contained 40 mM bis-Tris, pH 6.7, 6 mM MgCl₂, 1 mM dithiothreitol, 10 mM glyceral, and 40 μg/ml bovine serum albumin. Additional details are provided in the figure legends. Fidelity assays were performed according to Creighton and Goodman (19). For kinetic measurements, 5% or less of the total primer present before incubation with enzyme was extended. Results of primer extension were quantified after PAGE of reaction products in the presence of 7 M urea using a Molecular Dynamics 445 SI PhosphorImager. Unless indicated other-

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wise, all kinetic constants were calculated as described previously (18). Inhibition reactions were performed within the linear region of product formation versus time. With poly(dA)-(dT)$_{16}$ as template-primer, inhibition was quantified by placing some of each reaction mixture on 1 × 1-cm squares of Whatman DE-81 filter paper and washing filter paper squares in a 5% solution of Na$_2$HPO$_4$·12H$_2$O to remove unincorporated dNTPs. Inhibition with other template-primers was quantified by phosphorimager analysis after denaturing PAGE. Kinetic constants were determined by least-squares nonlinear regression to a rectangular hyperbola (19).

**Nondenaturing PAGE Band Mobility Shift Assays**—Nondenaturing PAGE band mobility shift assays were performed essentially as described previously (4) but without MgCl$_2$ and otherwise as detailed in figure legends. EDTA was included in each incubation and in the gel electrophoresis buffer at 3 mM.

**RESULTS**

**Formation of a PCNA-dependent Complex Between Calf Thymus Pol δ and Mismatched Template-Primers**—We previously reported that PCNA stabilized the pol δ template-primer interaction nearly 2000-fold (5). To study the interaction of pol δ with mismatched template-primers, PAGE-band mobility shift assays were initially performed with a single 30-mer annealed to a mismatched template-primer (30-mer annealed to complementary 20-ddC-mer). The experiment shown in Fig. 1A was performed preparatively. Typically 65–100% of the 20-mer starting material was converted to ddNMP-terminated 21-mer during the incubation with terminal dideoxynucleotidyltransferase.

**In Situ Determination of DNA Polymerase Activity**—Determination of DNA polymerase activity in polyacrylamide gels in situ was performed as described previously (5) after standard nondenaturing PAGE-band mobility shift assays. Nucleic acid template-primers were unlabeled. After electrophoresis, entire gels were incubated for 30 min at 37°C in a standard pol δ-reaction mixture (4, 6) containing 6 mM MgCl$_2$, 40 μM dCTP, and 60 μCi of [α-32P]dTTP. After incubation, gels were washed extensively (24–48 h) in cold 5% trichloroacetic acid to remove unincorporated dTTP after which they were subjected to autoradiography.

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PCNA Reduces the Fidelity of Pol δ

MgCl₂ and radiolabeled dNTP (5). Accordingly, complex was formed with unlabeled matched or mismatched template-primer; to monitor formation and mobility, complex was also formed with [32P]-labeled template-primer. Both labeled and unlabeled complexes were subjected to nondenaturing PAGE, and the portion of the gel containing complex formed with unlabeled template-primer was incubated in \([\alpha-32P]dTTTP, dCTP, and 6 \text{ mM MgCl}_2\). Nucleotide incorporation was readily detected coincident in PAGE mobility with the complex formed among pol δ, PCNA, and labeled template-primer (Fig. 2, B and C, compare lanes a; see also Fig. 2C, lane M). In contrast, mismatched template-primers, although supporting substantial complex formation when pol δ and PCNA were added (Fig. 2B, lanes b–d), supported much less nucleotide incorporation in the in situ DNA polymerase assay (Fig. 2C, lanes b–d). Curiously, primers with A-C and A-G mismatches at the 3’-position apparently supported more incorporation (mismatch extension) than a primer with an A-A mismatch at the 3’-position (Fig. 2C, lanes b–d). This observation is of uncertain significance.

PCNA Reduces the Fidelity with Which Calf Thymus Pol δ Replicates a Synthetic Oligonucleotide Template—It was established that extension of mismatched primer termini contributes significantly to errors made during DNA replication (for a review, see Ref. 17). Formation of a PCNA-dependent complex between pol δ and template-primer containing 3’-terminally mismatched nucleotides led us to determine, directly, the effect of PCNA on the fidelity of pol δ. The strategy of Creighton and Goodman (19) was employed. For this, we used the template-primers shown in Fig. 3A. Initially, this consisted of a 5’-32P-labeled 17-nucleotide primer (Fig. 3A, underlined) which was annealed to an unlabeled 64-mer template such that the next nucleotides to be incorporated were (in order) dGMP, dCMP, dTMP, and dAMP. All incubations contained 5 ng of pol δ and dGTP such that the first nucleotide specified could be incorporated. In addition, incubations contained either no PCNA (see Fig. 3B) or 70 ng of PCNA (Fig. 3C), dTTP at 10 μM (Fig. 3, B and C, lanes 2–6), or as indicated, and dCTP as indicated. Data from these experiments were quantified by phosphorimager.

To determine relative velocities for the correct nucleotide, results shown in Fig. 3, B and C, lanes 1–6, were quantified; radioactivity in the band resulting from dTMP incorporation plus radioactivity in the band resulting from dCMP incorporation was divided by radioactivity in the band resulting from dGMP incorporation. The results from Fig. 3C are plotted versus dCTP concentration (Fig. 3D). To determine relative velocity for the incorrect nucleotide, results shown in Fig. 3, C, lanes 1 and 7–9, were quantified; radioactivity in the band resulting from dTMP incorporation was divided by radioactivity in the band resulting from dGMP incorporation. The results are plotted versus dTTP concentration (Fig. 3E). There was no misincorporation of dTMP detected for pol δ alone (Fig. 3B, lanes 7–9) or in the presence of dGTP only (Fig. 3, B and C, lanes 1).

To determine fidelity (efficiency of incorporation (misincorporation), and extension (26)), \(V_{\text{max}}/K_m\) values were calculated as described previously (18) from the data shown (Fig. 3). As noted, with pol δ alone, there was no detectable misincorporation. We estimate that pol δ misincorporates less than once for every 10⁵ nucleotides incorporated correctly. In the presence of PCNA, pol δ catalyzed one misincorporation event (dTMP instead of dCMP) for every 3700 nucleotides incorporated correctly. In the absence of PCNA, pol δ recognized any primer with an A-A mismatch at the 3’-position (Fig. 2, B and C, lanes 1).

To investigate the effect of primer length on PCNA-induced misincorporation catalyzed by pol δ, each of the primers shown in Fig. 3A was 5’-32P-labeled and annealed to the template shown in Fig. 3A. Incubations were formulated as indicated; only results with 21-mer are shown (Fig. 3B). Identical results were obtained with 19-, 17-, 25-, 29-, 36-, 41-, and 46-mer (not shown). Only in the presence of both dTTP and PCNA was a second dTMP residue incorporated (Fig. 5, downpointing arrows), indicative of both misincorporation of dTMP in place of dCMP and extension without excision of the mismatched primer terminus.

PCNA Promotes the Stable Incorporation of Nucleotide Analog dTCH₂PP₄ by Calf Thymus Pol δ—The structure of dTCH₂PP₄ is shown in Fig. 4A. dTCH₂PP₄ has been used diagnostically to distinguish different DNA polymerases (16). Incorporation of this analog in place of dTMP was evaluated using the synthetic template-primer shown (Fig. 4B). Extension of this primer with either pol δ alone (Fig. 5, lanes 2–6) or pol δ plus PCNA (Fig. 5, lanes 7–11) revealed that PCNA
Relative velocities were calculated from the ratio, \((19-\text{mer})/(20-\text{mer})\). The efficiency of dCTP incorporation was determined from experiments in which PCNA was not added. When PCNA was present, 70 ng was added. Incubations were for 10 min at 37 °C after which products were analyzed by PAGE performed in the presence of 7 M urea. Results demonstrated that after digestion of the dTMP-containing 24-mer (Fig. 6, lane 1), only \(^{32}\text{P}\)-labeled dTMP was recovered (Fig. 6, lane 7). This species was presumably 5'-dAMP-dTCH2P-terminated primer generated after incorporation from a dTCH2P2 precursor (not shown).

Identical conclusions were reached after considering results from gel thin layer chromatography (not shown).

Quantitative comparison of dTMP and dTCH2P incorporation (Fig. 7) revealed that dTTP was much more efficiently utilized than dTCH2P2. Efficiency is defined as V_{max}/K_m, see Ref. 26.) V_{max} and K_m were calculated as described previously (18). The V_{max} and K_m for dTTP were 4.6 and 0.6 μM, respectively. The V_{max} and K_m for dTCH2P2 were 1.6 and 18.2 μM, respectively. Hence, in the presence of PCNA, dTTP is utilized by pol δ 87-fold more efficiently than dTCH2P2. In the absence of PCNA, dTTP is utilized by pol δ 17-fold more efficiently than dTCH2P2. In the absence of PCNA, dTTP is utilized by pol δ 17-fold more efficiently than dTCH2P2. In the absence of PCNA, dTTP is utilized by pol δ 17-fold more efficiently than dTCH2P2. In the absence of PCNA, dTTP is utilized by pol δ 17-fold more efficiently than dTCH2P2.
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misincorporation of dTMP opposite template dGMP (see Fig. 3). dTCH2P incorporation, or indirect, through increased stability by pol γ. Incubations were for 20 min at 37°C after which products were analyzed by PAGE performed in the presence of 7 M urea and a portion of each 24-mer digested to completion with micrococcal nuclease. Subsequent PAGE was performed in the presence of 7 M urea followed by autoradiography. Migration positions of the unmodified 21-mer primer of the autoradiogram. Horizontal arrow between lanes 7 and 8 designates the species in lane 8 resulting from PCNA-promoted misincorporation of dTMP opposite template dGMP (see Fig. 3).

FIG. 6. Micrococcal nuclease treatment of reaction products formed by pol δ and PCNA in the presence of dTTP versus dTCH2PP2. Unlabeled 21-mer primer was annealed to complementary template (see Fig. 4B for structure and sequence) and incubated for 30 min at 37°C with pol δ (20 ng), PCNA (140 ng), [γ-32P]dCTP (1.4 μM; 3000 Ci/mmol), and either dTTP (10 μM) or dTCH2PP2 (100 μM). 23P-Labeled 24-mer reaction product was PAGE-purified in the presence of 7 M urea and a portion of each 24-mer digested to completion with micrococcal nuclease. Subsequent PAGE was performed in the presence of 7 M urea; lane 1, product of incubation with dTTP present; lane 2, product of incubation with dTCH2PP2 present; lane 3, product of incubation with dTTP present after micrococcal nuclease treatment; and lane 4, product of incubation with dTCH2PP2 present after micrococcal nuclease treatment. The species in lane 4 was presumably 5'-dA-HO3PCH2O-32P[dT]-OH-3'. Marker positions are indicated to the left of the autoradiogram.

The Effect of PCNA on Utilization of dTCH2PP2 by Pol δ Occurs through an Indirect Mechanism—The ability of PCNA to alter significantly inhibition of incorporation by pol δ of dTCH2PP2 was a potent inhibitor of pol δ activity; inhibitory potency was not affected by PCNA (Fig. 8B). On a different template-primer that directed dTMP incorporation, poly(dA)-(dT)16. dTCH2PP2 was also a potent inhibitor of dTMP incorporation, and inhibition was not affected by PCNA (Fig. 8C). PCNA is without significant effect on the affinity of pol δ for dTTP (not shown).

Discussion

Under static conditions, PCNA decreases dissociation of pol δ from the pol δ-template-primer complex nearly 2000-fold (4, 5). This is reflected under dynamic conditions, i.e., during template-directed dNMP incorporation, in decreased Km for appropriate template-primers (6), and dramatically increased processivity of DNA synthesis (see e.g. Ref. 14). Although the PCNA-induced increase in the stability of the pol δ-template-primer complex is necessary for efficient DNA replication (see e.g. Ref. 27), our results demonstrate that it also causes substantial decreases in the fidelity with which pol δ catalyzes replication as well as increases incorporation (and/or inhibits excision) of a nucleotide analog, dTCH2P2, relative to unmodified dTMP.

We propose that increased efficiency of both unlikely events results from prolonged residence of pol δ at the template-primer junction induced specifically by PCNA and that this is intrinsic to the mechanism of "clamp-type" processivity factors. Similar proposals have previously been advanced for prokaryotes (see e.g. Refs. 9–11). We demonstrated directly that PCNA stabilizes the interaction of pol δ with a template-primer containing a 3' terminally mismatched primer (Fig. 1) in a complex that is indistinguishable from that formed with a fully base-paired template-primer (Fig. 2). Moreover, the terminally mispaired primer can apparently be extended, at least under some circumstances (Fig. 2). These observations correlate with at least a 27-fold decrease in the fidelity of pol δ brought about by the addition of PCNA (Fig. 3).

Formally, the apparent increase in misincorporation of dTMP as measured in our assay could result if the dTTP used was contaminated with dCTP and PCNA acted to reduce the Km of pol δ for dCTP. Given the vendor-stated purity of the dTTP used, we considered this highly unlikely. Nevertheless, to evaluate this possibility directly, we determined the Km of pol δ for dCTP both with and without PCNA. Km values of 7.5 and 12 μM, respectively, were determined, thus ruling out Km effects as a mechanism to account for the apparent misincorporation of dTMP. We also considered the possibility of template contamination to account for dTMP misincorporation. However, if this was the case, the ratio of dTMP incorporation versus dCMP incorporation would not be expected to change dependent on the presence or absence of PCNA.

We think it noteworthy that in the assay used to evaluate pol δ fidelity, we did not distinguish among nucleotide misincorporation, mismatch excision, and extension of a 3'-terminally mispaired primer. It is clear from the data shown that PCNA promotes complex formation with a mispaired primer terminus (Fig. 1) and, at least under some conditions, can allow such a primer to be extended (Fig. 2). In several contexts, we were unable to demonstrate any effect of PCNA on mismatch excision.3 There is currently no information pertinent to effects of PCNA on misincorporation of normal nucleotides (as distinct from mispaired primer extension) by pol δ. However, PCNA clearly promotes the misincorporation (and/or inhibits excision) of a nucleotide analog by pol δ (Figs. 4–8; see below). This is

3 D. Ju. Mozherin and P. A. Fisher, unpublished data.

4 M. McConnell and P. A. Fisher, unpublished data.
certainly an area for future research that will have considerable bearing on understanding the effects of PCNA on replication by pol δ. We would also like to note that the conditions used to delineate PCNA-induced infidelity by pol δ differ significantly from those found in vivo. In our assays, only a single dNTP, dTTP, is provided, potentially biasing our system toward misincorporation.

In vivo, all four dNTPs are present. It remains to be determined if PCNA would promote misincorporation by pol δ during in vivo replication.

The other unfavorable event catalyzed by pol δ that PCNA promotes is incorporation of the dTMP analog, dTCH2P (Figs. 4–8). This analog was used diagnostically to distinguish DNA polymerase α from DNA polymerase ε (16). In light of our current observations, such results must be interpreted with caution since, in vivo, it is possible that hypothetical and as yet undiscovered accessory factors could influence dramatically analog utilization by DNA polymerases. Inhibition data (Fig. 8) demonstrate that PCNA enhancement of dTCH2P incorporation by pol δ occurs through an indirect mechanism rather than a direct effect of PCNA on the interaction of pol δ with dTCH2PP2. The ability of dTCH2PP2 to compete with dTTP is unaffected by PCNA (Fig. 8). Accordingly, it is our conclusion that PCNA can promote stable nucleotide misincorporation (as opposed to extension of mismatched primer termini) indirectly through stabilization of interaction of pol δ with the primed template.

Ultimately, it is, in our estimation, the effect of PCNA on dissociation of pol δ from the template-primer that provides the evolutionary selection pressure necessary to maintain the 3’–5’-exonuclease intrinsic to pol δ. Any DNA polymerase that functions processively by virtue of decreased dissociation from the primed template (enhanced template-primer binding) is inherently more likely to misincorporate, immortalize a misincorporated nucleotide, or incorporate a nucleotide analog in place of a normal nucleotide. In all instances, the 3’–5’-exonuclease is ideally suited to deal efficiently with any errors committed by pol δ. In contrast, DNA polymerases α and β, which are not highly processive, lack intrinsic 3’–5’-exonuclease activities. However, we would also like to note that in our in vitro assays, the 3’–5’-exonuclease of pol δ is demonstrably active (see Ref. 6), yet both effects of PCNA, enhanced misincorporation/mismatched primer extension and greater incorporation of

Fig. 7. Use of dTCH2PP2 as a substrate by pol δ; quantitative analysis. A, standard reaction mixtures contained 5 ng of pol δ, 70 ng of PCNA, 0.4 μM 40-mer template annealed to an equal amount of 5’-32P-labeled 21-mer primer (see Fig. 4B for structure and sequence), and varying concentrations of either dTTP (○) or dTCH2PP2 (●) as indicated. Incubations were for 30 s at 37°C during which less than 5% of the primer was extended. After incubation, nucleic acids were subjected to PAGE in the presence of 7 M urea, and products were quantified. B, Lineweaver-Burk double-reciprocal plots of the data shown in A.

Fig. 8. Effect of PCNA on dTCH2PP2 inhibition of dTTP utilization by pol δ. Standard reaction mixtures contained 10 ng of pol δ, either with 70 ng of PCNA (●) or without PCNA (○) and dTCH2PP2, as indicated on the abscissae. A, 0.4 μM 64-mer template was annealed to an equal amount of 5’-32P-labeled 17-mer primer (see Fig. 3A for structure and sequence); this template-primer directed dGMP incorporation. 10 μM dGTP was included in all incubations. B, 0.4 μM 40-mer template was annealed to an equal amount of 5’-32P-labeled 21-mer primer (see Fig. 4B for structure and sequence); this template-primer directed dTMP incorporation. 10 μM dTTP was included in all incubations. C, 0.4 μM poly(dA) template was annealed to unlabeled (dT)16 primer (200:1 ratio of nucleotide:nucleotide); this template-primer directed dTMP incorporation. 10 μM [α-32P]dTTP was included in all incubations.
dTCH₂P, were easily observed. In cells, there are presumably other factors that contribute to the accuracy of pol δ. The assays described here may be useful to identify such factors in vitro.

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