Fidelity of Nucleotide Insertion at 8-Oxo-7,8-dihydroguanine by Mammalian DNA Polymerase δ

STEADY-STATE AND PRE-STEADY-STATE KINETIC ANALYSIS*

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Nucleotide insertion opposite 8-oxo-7,8-dihydroguanine (8-oxoG) by fetal calf thymus DNA polymerase δ (pol δ) was examined by steady-state and pre-steady-state rapid quench kinetic analyses. In steady-state reactions with the accessory protein proliferating cell nuclear antigen (PCNA), pol δ preferred to incorporate dCTP opposite 8-oxoG with an efficiency of incorporation an order of magnitude lower than incorporation into unmodified DNA (mainly due to an increased $K_m$). Pre-steady-state kinetic analysis of incorporation opposite 8-oxoG showed biphasic kinetics for incorporation of either dCTP or dATP, with rates similar to dCTP incorporation opposite G, large phosphorothioate effects (>100), and oligonucleotide dissociation apparently rate-limiting in the steady-state. Although pol δ preferred to incorporate dCTP (14% misincorporation of dATP) the extension past the A:8-oxoG mispair predominated. The presence of PCNA was found to be a more essential factor for nucleotide incorporation opposite 8-oxoG adducts than unmodified DNA, increased pre-steady-state rates of nucleotide incorporation by 2 orders of magnitude, and was essential for nucleotide extension beyond 8-oxoG. Pol δ replication fidelity at 8-oxoG depends upon contributions from $K_m$, $K_{dNTP}$, and rates of phosphodiester bond formation, and PCNA is an important accessory protein for incorporation and extension at 8-oxoG adducts.

High fidelity DNA replication is critical to the preservation of genomic stability and the avoidance of mutations that can disrupt the regulation of complex biological systems. Cells contain several DNA polymerases and complex DNA repair systems to preserve genomic integrity (1, 2). Accurate replication is disrupted by the presence of covalent DNA-chemical adducts, which can be misread and lead to mutations and cancer (3). Understanding the misreading events induced by modified DNA is important in understanding risks of environmental chemicals, as well as aspects of chemotherapeutic treatment. Misincorporation is primarily a kinetic phenomenon and not simply thermodynamic. Work with several DNA adducts and artificial DNA bases clearly indicates that both the identity of incorporated bases and their frequency of substitution are functions of which polymerase is used as a catalyst (4–10). Our own work on how polymerases influence misincorporation has been focused on 8-oxoG (9, 11–14). 8-OxoG is a relatively simple adduct in that the only chemical attached to the DNA is one atom of oxygen, and it was selected as a model because of its relatively high mutagenicity and lack of polymerase blockage. This lesion is generally regarded as being the most abundant of those induced by oxidative damage (15–17).

Polymerases derived from prokaryotic systems have been used extensively as models for mechanistic studies because of their availability, the general lack of need for complex accessory proteins, and the availability of structural and mechanistic information (18). The question arises as to how relevant findings made with these enzymes are to mammalian and other eukaryotic polymerases, particularly those polymerases that will copy past sites of DNA damage. Recent studies indicate the presence of low fidelity, distributive polymerases in both prokaryotes and eukaryotes that are involved in translesion DNA synthesis (6, 19, 20). However, the extent to which the mammalian forms are able to copy past chemical lesions other than photodamaged pyrimidines is largely unexplored, and the question of how lesions interact with replicative DNA polymerases is important. Recently we used purified calf thymus DNA pol δ, considered to be the main leading strand DNA polymerase (1, 2), in a series of steady-state and pre-steady-state kinetic experiments and concluded that the major features of the catalytic mechanism were very similar to those established in the prokaryotic models (21). However, questions about the processing of chemical-DNA adducts remain.

Some work has been done on the replication of mammalian pol δ past DNA adducts and misincorporation. O’Day et al. (22) reported that the presence of the accessory protein PCNA enabled pol δ bypass of cyclobutane thymine dimers, as judged by qualitative polyacrylamide gel electrophoresis experiments. Using similar approaches, Mozherin et al. (23) demonstrated that PCNA stimulated pol δ incorporation and bypass at abasic sites and 8-oxoG and C8(2-aminofluorenyl)guanine modifications (but not C8(2-acetamidofluorenyl)guanine). The increase in the amount of extended products was 2.5-fold for 8-oxoG and
C8(2-aminofluorenyl)guanine but more for abasic sites. The mechanism was concluded to involve extension of the new primer terminus (resulting from incorporation), facilitated by a decreased pol δ-DNA off-rate (koff) by PCNA. However, other mechanisms could not be excluded. Other work by Mozherin et al. (24) indicated that the presence of PCNA led to more errors in the incorporation of nucleotides in unmodified oligonucleotides, a result also rationalized in terms of a decreased DNA kcat for pol δ due to PCNA (24).

We studied the incorporation of dCTP and dATP opposite 8-oxoG in an oligonucleotide primer-template complex with purified calf thymus pol δ and recombinant human PCNA, using steady-state and pre-steady-state kinetic approaches. The results show a strong dependence on the presence of PCNA for the polymerization rate constant (kpol) and extension beyond the lesion, and the effects of phosphorothioate substitution of dNTPs suggest that the rate-limiting step may be phosphodiester bond formation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Primer and template oligonucleotides were purchased from Midland Certified Reagent Co. (Midland, TX) and purified as described previously (9, 21) or were purchased (gel-purified) from Operon Technologies (Alameda, CA). UltraPure Grade dNTPs were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). The thio-substituted dNTPs, (S)-3′-dNTPs, were purchased from U. S. Biochemical Corp. (Cleveland, OH), and [32P]dATP and [3H]dGTP were obtained from PerkinElmer Life Sciences (Boston, MA). 8-Oxo-dGTP was prepared and rigorously characterized as described elsewhere (9). First trimester fetal calf thymus was purchased from Pel-freez (Rogers, AR) and the sources of reagents used for the purification of pol δ were described elsewhere (21). The bacterial expression vector pET/PCNA (expressing human PCNA) was a generous gift of Dr. B. Stillman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY) obtained from Prof. E. Fanning (Vanderbilt University). Enzymes—Human PCNA was expressed in E. coli and purified to electrophoretic homogeneity as described (25) with modifications (21). The concentration of PCNA was estimated by the calculated ε280 value of 17.7 mm M⁻¹cm⁻¹.

pol δ was purified from first trimester fetal calf thymus as described by Podust et al. (28) with modifications (21). The amount of pol δ was determined by quantitative amino acid analysis of the individual polypeptides of pol δ in the Vanderbilt facility following separation by SDS-polyacrylamide gel electrophoresis and transfer to a polyvinylidene fluoride membrane (21). The preparations obtained using the five-step procedure contained three polypeptides, molecular mass 50, 116, and 125 kDa (21). The 125-kDa protein is the catalytic subunit of pol δ (27), the 50-kDa protein is an accessory protein reported to be required for PCNA binding (28), and the 116-kDa protein is a 125-kDa proteolytic product of the p125 subunit. p116 lacks ~80 amino acids of the N terminus of p125 and has been found in many thymus pol δ preparations (21, 27, 29). In a recent report, Schumacher et al. (29) found that the 116-kDa proteolytic protein retained polymerase activity to the same extent as the full-length p125 for replication factor C-independent incorporation of nucleotides into primer/template DNA, in support of the work of Wu et al. (29). The concentrations of pol δ in the reactions in this report were determined by the limiting amount of subunit (p50 or p116 + p125).

**End Labeling of Primer and Primer/Template Annealing**—Oligonucleotide primers (Table I) were 5′-end-labeled with [γ-32P]dATP (3000 Ci mmol⁻¹) and T4 polynucleotide kinase and annealed to the template in a ratio of 1.15 (primer-template) (9).

**Steady-state Reactions**—pol δ (containing 0.031 pmol of the limiting subunit of pol δ) was added to a mixture (5 μl volume) containing annealed 5′-32P-labeled 24/36-mer or 24/36 8-oxoG-mer primer-template (200 nm), 72 nm PCNA, 0.4 mg of bovine serum albumin ml⁻¹, 50 mM bis-Tris-HCl (pH 6.5), and 2 mM dithiothreitol. The reactions were initiated with the addition of a mixture of BuflF4A (50 mM bis-Tris-HCl (pH 6.5) and 12.5 mM MgCl2) and dNTP (2 mM final concentration) at varying concentrations. Final concentrations were 50 mM bis-Tris-HCl (pH 6.5), 0.2 mg of bovine serum albumin ml⁻¹, 100 nm 24/36-mer or 24/36 8-oxoG-mer, 3.1 nmol pol δ, 36 nm PCNA, 6 mM MgCl2, and 1 mM dithiothreitol in 10 μl. The reactions were run at 37 °C in triplicate and quenched with 20 μl of 20 mM EDTA (pH 7.4) after 5 min. The products were separated by denaturing polyacrylamide gel electrophoresis (16% acrylic acid (w/v), 1.5% biacrylamide (w/v), 8.0 M urea) and the amount of primer extended was quantitated utilizing a Molecular Dynamics Model 4000 PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and Image Software version 3.3. kpol, koff, and Kd values were determined by nonlinear regression using a lcgat computer program (Bioinformatics, Princeton, NJ). In steady-state reactions performed in the absence of PCNA, the pol δ concentration was increased to 27 nm and incubation time to 10 min.

A similar procedure was used for incorporation of 8-oxo-dGTP opposite A and C. A complementary oligonucleotide to the 24/36-mer was used with a C or A in the position of the incoming dNTP (9). The concentrations of pol δ and PCNA were 3.1 and 72 nm, respectively, in the buffer described above. Reactions were done in the presence of varying concentrations of 8-oxo-dGTP for 5 min (opposite C) or 10 min (opposite A), and the results were analyzed as for other steady-state experiments.

**Pre-steady-state Kinetics**—Pre-steady-state rapid-quench experiments were performed utilizing a KinTek Quench Flow Apparatus (Model RQF-3, KinTek, Austin, TX). Reactions were initiated by rapid mixing of dNTP in Buffer A with a primer/template/poly δ solution with or without PCNA at 37 °C. The final concentrations of the reactants were 10–95 nm pol δ (based on the limiting subunit of pol δ), 50 mM bis-Tris-HCl (pH 6.5), 6 mM MgCl2, 1 mM dithiothreitol, 180 or 400 nM PCNA, 0.2 mg bovine serum albumin ml⁻¹, and 100 nm primer/template. The reactions were quenched with equal volumes of 0.6 mM EDTA at times varying from 5 ms to 10 s. The products were analyzed as described for steady-state assays. kpol (maximum rate of nucleotide incorporation) was determined by a fit of the data to the burst equation:

\[ y = A(1 - e^{-kt}) + k_{off}t, \]

where \( A \) = burst amplitude, \( k_{off} \) = first-order rate constant, \( t \) = time, and \( k_{pol} \) = steady-state rate of nucleotide incorporation.

Kd Determination of dCTP or dATP Binding to pol δ-PCNA-DNA—The kinetic Kd value for dCTP or dATP binding to pol δ-PCNA was estimated by pre-steady-state rapid-quench analysis. The dNTP concentration dependence of the pre-steady-state burst rates was examined by varying the concentration of dNTP and measuring the pre-steady-state burst rates of dNTP incorporation into the 24/36 8-oxoG-mer duplex DNA. The values of kpol were determined as described above. The pre-steady-state rates were plotted against the dNTP concentration, and the data was fit to the hyperbola \( k_{pol} = k_{pol}(dNTP) \cdot k_{d} + k_{d} \) to determine Kd (30).

**Determination of KdNA for pol δ-24/36 8-Oxo-G Mer Complexes**—The Kd values for productive pol δ binding to 24/36 8-oxoG-mer or 24/36 8-oxoG mer complexes in the presence of PCNA were determined by pre-steady-state rapid quench analysis. The DNA concentration was varied and the changes in the burst amplitude were measured to determine enzyme active site concentration and the Kd for binding DNA. The Kd was determined by plotting the burst amplitudes against the concentration of DNA and then fitting the data to the quadratic equation:

\[ [ED] = (0.5K_{d} + E_{A} + D) + 0.25(K_{d} + E_{A} + D)^{2} - E_{A} - D_{A}^{2}, \]

where \( E_{A} = [\text{total enzyme}], D_{A} = [\text{total DNA}], \) and Kd = dissociation constant for the reaction E + D ↔ E-D.

**RESULTS**

**Steady-state Kinetics of dCTP or dATP Incorporation Opposite 8-Oxoguanine**—The steady-state kinetics of elongation of the 5′-32P-labeled 24/36 8-oxoG-mer duplex DNA (Table I) to 25/36 8-oxoG-mer product were measured as a function of dCTP or dATP.
TABLE II
Steady-state kinetic parameters for pol δ

|        | $k_{cat}$ (s$^{-1}$) | $K_m$ (mM) | $k_{cat}/K_m$ (mM$^{-1}$s$^{-1}$) |
|--------|---------------------|-----------|---------------------------------|
| C:G    | dCTP +PCNA          | 0.0035 ± 0.0001 | 0.067 ± 0.011 | 52 ± 9 |
|        | −                   | 0.0020 ± 0.0001 | 1.2 ± 0.2 | 1.7 ± 0.5 |
| C:8-oxoG | dCTP +PCNA    | 0.0055 ± 0.0002 | 1.3 ± 0.2 | 4.2 ± 1.0 |
|        | −                   | 0.0040 ± 0.00008 | 510 ± 37 | 0.008 ± 0.002 |
| A:8-oxoG | dATP +PCNA     | 0.0055 ± 0.0003 | 4.1 ± 0.9 | 1.3 ± 0.33 |
|        | −                   | 0.00035 ± 0.00005 | 19 ± 0 | 0.018 ± 0.0006 |

*a Lines represent nucleotide sequences; see Table I.
  b G*, 8-oxoG.

$\text{dATP concentration dependence using a large excess of } 24/36 \text{ 8-oxoG-mer (100 nM) relative to pol } \delta (3.1 \text{ nM}).$ The steady-state $k_{cat}$ values for dCTP or dATP incorporation opposite 8-oxoG in the presence of PCNA were 0.0055 s$^{-1}$, similar to the $k_{cat}$ for dCTP incorporation into unmodified DNA (0.0035 s$^{-1}$) (21). $K_m$ values for dCTP and dATP incorporation opposite 8-oxoG were determined to be 1.3 and 4.1 μM, respectively, an order of magnitude higher than the $K_m$ for correct nucleotide incorporation into unmodified DNA, 0.067 μM (Table II).2

PCNA has been reported to increase the processivity of pol δ replication and the bypass of abasic sites and DNA adducts, such as 8-oxoG, C$^6$(2-aminofluorenyl)guanine, and cyclobutane thymine dimers (22, 23). When PCNA was omitted, the $k_{cat}$ for dCTP and dATP incorporation opposite 8-oxoG was either similar or 16-fold lower (0.0040 and 0.00035 s$^{-1}$, respectively) than in reactions containing PCNA (Table II). The $K_m$ values were 400 and 5-fold higher (510 and 19 μM) for dCTP and dATP incorporation, respectively.

Steady-state Kinetics of 8-Oxo-dGTP Incorporation Opposite Template C or A—Previous studies with other polymerases had shown asymmetry in the pairing of 8-oxoG with C and A, depending on whether the 8-oxoG was in the DNA template or the incoming nucleotide (9). These studies were repeated with pol δ, in the presence of PCNA. The steady-state $k_{cat}$ values estimated for incorporation of 8-oxo-dGTP opposite C and A were 0.0030 (± 0.0003) and 0.0019 (± 0.0004) s$^{-1}$, respectively. As in the case of the prokaryotic polymerases (9), the $K_m$ values for incorporation of 8-oxo-dGTP were much higher than for incorporation of dCTP or dATP opposite 8-oxoG, 0.65 (±0.16) mM for 8-oxo-dGTP opposite C and 13 (±5) mM opposite A. Thus, the efficiency ($k_{cat}/K_m$) of 8-oxo-dGTP incorporation is 3 orders of magnitude lower than for dCTP or dATP incorporation opposite 8-oxoG.3

Pre-steady-state Kinetics of Nucleotide Incorporation Oppo-

2 Using the $k_{cat}$ and $K_m$ values, a “misincorporation frequency” (f) of 0.33 can be estimated, with caveats of the limitations of these single nucleotide incorporation experiments, defined as $f = \left(1 - \frac{k_{incorporation}}{k_{correct}}\right) \times 100$. The preference for incorporation of dCTP opposite 8-oxoG is reflected in a misincorporation frequency (f$^\text{pol}$) of 0.33, which corresponds to 24% misincorporation for dATP.

3 A misincorporation frequency (f) of 0.03 can be calculated, again with the caveats of limitations of single nucleotide incorporation experiments (see footnote 2), corresponding to 3% misincorporation (of 8-oxo-

site 8-OxoG—Pre-steady-state kinetic analysis of dCTP or dATP incorporation opposite 8-oxoG by pol δ was performed to discern contributions of specific steps in the catalytic cycle important for fidelity of incorporation opposite 8-oxoG. The rate of polymerization ($k_{pol}$) can be measured by rapid quench kinetics and includes rates of dCTP binding, any associated conformational changes, and phosphodiester bond formation. The incorporation of dCTP or dATP into the 24/36 8-oxoG-mer by pol δ in the presence of PCNA showed biphasic kinetics, fitted to an initial burst followed by a linear phase (Fig. 1). The burst phase represents the rate of single nucleotide incorporation in the first turnover of the enzyme. $k_{pol}$ was determined to be 22 s$^{-1}$ for dCTP incorporation and 21 s$^{-1}$ for dATP incorporation. These values were similar to the rate of dCTP incorporation opposite unmodified G (28 s$^{-1}$). The amplitude of the burst corresponds to the concentration of “active” enzyme present in the reactions. The amplitude was ~4 nM for dCTP incorporation and ~2 nM for dATP incorporation, indicating that 8% and 4% of the pol δ is in an active conformation in these reactions, respectively. These values were calculated based upon the 11 nM burst product formed in reactions containing 51 nM pol δ for dCTP incorporation opposite unmodified G, or 36%, from this preparation of pol δ (results not shown).

When PCNA was excluded from the pre-steady-state reactions, no apparent burst of dCTP or dATP incorporation opposite 8-oxoG was observed, even at pol δ concentrations of 95 nM (Fig. 2). The apparent rates of polymerization in the absence of
PCNA were 0.032 and 0.018 s\(^{-1}\) for dCTP and dATP incorporation, respectively, 3 orders of magnitude less than with PCNA and indicating that PCNA is essential for formation of an active pol \(\delta\) complex capable of nucleotide incorporation opposite 8-oxoG.

**Determination of Phosphorothioate Elemental Effect**—The rate of polymerization was examined for a phosphorothioate elemental effect to determine if the rate of a single nucleotide incorporation event is influenced by phosphodiester bond formation (chemistry step). Replacement of the \(\alpha\)-phosphate group of a dNTP with a phosphorothioate can reduce the rate of \(k_{\text{pol}}\) by \(10^2\) fold if the rate of polymerization is a direct function of the chemistry step. The rates of dCTP and dATP incorporation by pol \(\delta\) in the presence of PCNA were reduced by a factor of 900–1000 (Fig. 1). The results are interpreted to mean that phosphodiester bond formation may be the rate-limiting step for both dCTP and dATP incorporation opposite 8-oxoG and that the rate of polymerization (\(k_{\text{pol}}\)) is a direct reflection of this chemical step.

**Next Correct Nucleotide Insertion Beyond C:8-OxoG or A:8-OxoG Base Pairs in the Presence or Absence of PCNA**—Pre-steady-state kinetic analysis of the next correct base (dGTP) incorporation beyond C:8-oxoG or A:8-oxoG pairs was examined to determine whether pol \(\delta\) has a preference for extension of either base pair. In the presence of PCNA (Fig. 3A), pol \(\delta\) efficiently extended the A:8-oxoG base pair (\(k_{\text{pol}} = 27 \text{ s}^{-1}\)) an order of magnitude faster than C:8-oxoG base pairs (\(k_{\text{pol}} = 1 \text{ s}^{-1}\)). When PCNA was omitted (Fig. 3B), rates of next correct base pair insertion decreased 70-fold for A:8-oxoG base pairs (0.38 s\(^{-1}\)) and 4-fold for C:8-oxoG base pairs (0.24 s\(^{-1}\)) and no bursts of nucleotide incorporation occurred.

**Determination of \(K_d^{\text{NTP}}\) for Binding of Nucleotides to pol \(\delta\)-PCNA-24/36 8-OxoG-mer Complexes**—The pre-steady-state rate of nucleotide incorporation into the 24/36 8-oxoG-mer primer/template was determined as a function of the concentration of dCTP (results not shown) or dATP (Fig. 4). The \(K_d^{\text{NTP}}\) for DNA modified with 8-oxoG was 8 \(\mu\text{M}\) and the \(K_d^{\text{ATP}}\) was 42 \(\mu\text{M}\). These values are considerably higher than the \(K_d^{\text{NTP}}\) for dCTP binding to unmodified DNA (0.93 \(\mu\text{M}\)) (21).

**Determination of \(K_d^{\text{DNA}}\) for pol \(\delta\)-24/36 8-OxoG-mer**—\(K_d^{\text{DNA}}\), which describes the reaction \(E\)-PCNA + DNA \(\rightarrow\) \(E\)-DNA-PCNA, was determined by examining the DNA concentration dependence on the burst amplitude of dCTP incorporation by pol \(\delta\). A fixed amount of pol \(\delta\) was incubated with increasing concentrations of 24/36G-mer or 24/36 8-oxoG-mer and the reactions were initiated with the addition of saturating dCTP for 100 ms, at which maximal burst amplitudes were observed with negligible effect from multiple turnovers of the enzyme (Fig. 5). The \(K_d^{\text{DNA}}\) for the reaction pol \(\delta\)-PCNA + 24/36G-mer \(\rightarrow\) pol \(\delta\)-PCNA-24/36G-mer was determined to be \(64 \pm 10 \text{ nM}\) (21) and the \(K_d^{\text{DNA}}\) was 2-fold higher, \(124 \pm 24 \text{ nM}\), with the 8-oxoG-modified DNA. When PCNA was omitted, no appreciable burst of nucleotide incorporation occurred even with 1 \(\mu\text{M}\) DNA (results not shown).

**DISCUSSION**

The major features of the catalytic mechanism of correct nucleotide insertion into unmodified DNA by pol \(\delta\) are similar to those of the prokaryotic DNA polymerases (21). A single nucleotide incorporation reaction is defined by several consecutive steps involving DNA binding, nucleotide binding, conformational changes, and phosphodiester bond formation (Fig. 6). The kinetic parameters of normal nucleotide incorporation by pol \(\delta\) are similar in magnitude to prokaryotic polymerases and the rate-limiting step is most likely a conformational change preceding phosphodiester bond formation (21, 30–32). The goals of this study were to examine the kinetics of nucleotide incorporation opposite 8-oxoG by mammalian DNA pol \(\delta\), to establish the steps in the polymerase catalytic cycle important for fidelity, and to determine the roles of the pol \(\delta\) accessory protein PCNA.
Experimental Procedures.

mE; 25 d

tial component for efficient nucleotide incorporation opposite prokaryotic polymerases (11, 12). PCNA was also found to be an essential component for efficient nucleotide incorporation opposite 8-oxoG, as found previously for prokaryotic polymerases (11, 12). PCNA was also found to be an essential component for efficient nucleotide incorporation opposite prokaryotic polymerases (11, 12). PCNA was also found to be an essential component for efficient nucleotide incorporation opposite

deated G. This decrease in nucleotide insertion efficiency at 8-oxoG is within the 1–3 orders of magnitude range of the decrease seen with prokaryotic polymerases (11, 12). The decrease in insertion efficiency at 8-oxoG adducts is dominated by an increased $K_m$ value for dCTP or dATP insertion (Table II). A direct interpretation of this change is unknown because of the complexity of the catalytic mechanisms of polymerases (18).

In the absence of PCNA, the efficiency of dCTP incorporation opposite unmodified G or 8-oxoG was affected primarily at the level of $K_m$. The $K_m$ value of dCTP incorporation opposite unmodified G and 8-oxoG increased by a factor of 18 and 300, respectively, whereas the $k_{cat}$ value was not as affected. The absence of PCNA decreased the insertion efficiency of pol δ by 30- and 500-fold for dCTP incorporation opposite G and 8-oxoG, respectively. These results indicate that PCNA is an important factor for efficient incorporation of the correct nucleotide not only opposite unmodified bases but even more importantly for insertion opposite DNA adducts, such as 8-oxoG.

The steady-state $k_{cat}$ value is dominated by the rate of enzyme dissociation ($k_{off}$) from the DNA or some other step following product formation, as clearly demonstrated by the burst kinetics in the biphasic pre-steady-state plots of the reaction course (Figs. 1 and 3). It is interesting to note that in the absence or presence of PCNA, the $k_{cat}$ value is similar to that for correct nucleotide incorporation opposite G or 8-oxoG (Table II). This result suggests that the rate of dissociation ($k_{off}$), if described by $k_{cat}$, is probably not affected by PCNA in correct nucleotide incorporation reactions. However, in the case of the misincorporation of dATP opposite 8-oxoG, the $k_{cat}$ is decreased 16-fold when PCNA is omitted, indicating that the enzyme is either dissociating at a slower rate or that other steps in the cycle have been perturbed and become rate-limiting. These results suggest that PCNA may be more important for the activity of pol δ in misincorporation than normal incorporation reactions (23). However, the results do not support a view that PCNA acts primarily by lowering the $K_d$ for the complex between pol δ and DNA (33–35), where a lower $k_{cat}$ would be expected in the presence of PCNA. The lack of a burst of nucleotide incorporation without PCNA for both dCTP and dATP incorporation reactions opposite 8-oxoG (Fig. 2) indicates that the rate-limiting step is at or prior to phosphodiester bond formation. The lower $k_{cat}$ for dATP incorporation compared with dCTP suggests that there may be differences in $K_d^{DNA}$ (step 2, Fig. 6) and/or conformational changes (steps 3 or 8, Fig. 6) for the misincorporation reaction.

Pre-steady-state Reactions—A burst of nucleotide incorporation opposite 8-oxoG occurred for incorporation of either dCTP or dATP ($k_{cat} = 22$ and $21$ s$^{-1}$, respectively) with rates similar to dCTP incorporation opposite unmodified G (28 s$^{-1}$). The difference in the efficiency of nucleotide insertion opposite 8-oxoG compared with unmodified G is due to an increase in $K_d^{DNA}$ (reduction in dNTP binding affinity compared with dCTP insertion opposite G, 7- and 45-fold for dCTP and dATP incorporation opposite 8-oxoG, respectively) and less to changes in $K_d^{DNA}$ values (2-fold) (Table III). pol δ incorporated

protein PCNA on replication at DNA adducts. The results presented here indicate that replication by pol δ at 8-oxoG adducts was most notably affected at the levels of phosphodiester bond formation, nucleotide binding, and efficiency of base pair extension beyond 8-oxoG, as found previously for prokaryotic polymerases (11, 12). PCNA was also found to be an essential component for efficient nucleotide incorporation opposite 8-oxoG and base pair extension beyond the adduct.

Steady-state Reactions—In steady-state reactions with PCNA, the efficiency of nucleotide incorporation by pol δ was decreased by at least a factor of 12 for dATP and dCTP incorporation opposite 8-oxoG compared with reactions with unmodified G. This decrease in nucleotide insertion efficiency at 8-oxoG is within the 1–3 orders of magnitude range of the decrease seen with prokaryotic polymerases (11, 12). The decrease in insertion efficiency at 8-oxoG adducts is dominated by an increased $K_m$ value for dCTP or dATP insertion (Table II). A direct interpretation of this change is unknown because of the complexity of the catalytic mechanisms of polymerases (18).

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protein PCNA on replication at DNA adducts. The results presented here indicate that replication by pol δ at 8-oxoG adducts was most notably affected at the levels of phosphodiester bond formation, nucleotide binding, and efficiency of base pair extension beyond 8-oxoG, as found previously for prokaryotic polymerases (11, 12). PCNA was also found to be an essential component for efficient nucleotide incorporation opposite 8-oxoG and base pair extension beyond the adduct.

Steady-state Reactions—In steady-state reactions with PCNA, the efficiency of nucleotide incorporation by pol δ was decreased by at least a factor of 12 for dATP and dCTP incorporation opposite 8-oxoG compared with reactions with unmodified G. This decrease in nucleotide insertion efficiency at 8-oxoG is within the 1–3 orders of magnitude range of the decrease seen with prokaryotic polymerases (11, 12). The decrease in insertion efficiency at 8-oxoG adducts is dominated by
dCTP 6-fold more efficiently \((k_{\text{pol}}/K_{d}^{\text{NTP}})\) than dATP. The misinsertion frequency \(f = \{k_{\text{pol}}/K_{d}^{\text{ATP}}\} / \{k_{\text{pol}}/K_{d}^{\text{NTP}}\}\) was determined to be 0.17, which corresponds to 14% misincorporation of dATP opposite 8-oxoG (Table III).

PCNA increased the affinity of the pol δ for unmodified DNA interaction by only 5-fold in our earlier work (21). Due to the lack of a burst of nucleotide incorporation opposite 8-oxoG in the absence of PCNA (Fig. 2), the affinity of the pol δ interaction with DNA \(K_{d}^{\text{DNA}}\) could only be estimated to be >1000 nM (>3-fold greater than the \(K_{d}^{\text{DNA}}\) value for pol δ-unmodified DNA interaction). However, the presence of 8-oxoG in the template reduced the \(K_{d}^{\text{DNA}}\) only minimally (2-fold) in the presence of PCNA (Table III), as in the case of the prokaryotic polymerases (11, 12). The possibility does exist that PCNA may have a major role in the interaction of pol δ with modified DNA (see below), although further experiments are needed to discern this.

To examine whether the chemistry step is rate-limiting in a single turnover reaction by pol δ (at an 8-oxoG site), the \(\alpha\)-phosphorus of dCTP or dATP was replaced with a sulfur group and the rate of nucleotide insertion was measured by pre-steady-state kinetics. The rate of incorporation of these \(S\)-dNTPs \((k_{\text{pol}})\) was 103-fold less than for dATP or dCTP incorporation, suggesting that phosphodiester bond formation becomes rate-limiting during replication reactions at 8-oxoG. Similar results have also been obtained with the prokaryotic pol T7 and E. coli pol I and II (11, 12). (Except in the case of E. coli pol II exonuclease, no thio effect is observed during normal incorporation.)

PCNA has been shown to be important for pol δ extension beyond DNA adducts, including thymine dimers, C\(^\ast\)\(^\ast\) (2-acetamido-2-(2-aminofluorenyl)guanine), and 8-oxoG (22, 23). The results presented here confirm that PCNA is critical for nucleotide insertion past both 8-oxoG:C and 8-oxoG:A base pairs (Fig. 3). There is a preference for extension of 8-oxoG:A mispairs (27-fold), as for pol T7 exonuclease, E. coli pol I exonuclease and II exonuclease, and HIV-1 RT (11, 12). The rate of the next correct base incorporation beyond 8-oxoG:A base pairs (27 s\(^{-1}\)) was similar to rates of normal base pair insertion (28 s\(^{-1}\)). Although C is the preferred base inserted opposite 8-oxoG, the ability of pol δ to extend the mispair can contribute to the mutagenicity of 8-oxoG.

**Effects of Accessory Proteins**—Some of the effects of PCNA on incorporation have already been described. A considerable body of literature has accrued to demonstrate physical interactions of PCNA with both the p50 (28) and p125 (36) subunits of pol δ. The PCNA trimer appears to form a circular “clamp” around DNA (37). The stimulatory effect of PCNA is seen with short primer-template complexes (Figs. 1–3) (21) as well as longer nucleic acids (for which “loading factors,” e.g. replication protein A, replication protein C (38), are required). Exactly how PCNA functions is still unresolved.

PCNA has been demonstrated to promote pol δ-catalyzed DNA replication past thymidine dimers (22) and, more recently, past abasic sites, C\(^\ast\)\(^\ast\) (2-acetamido-2-(2-aminofluorenyl)guanine, and 8-oxoG but not C\(^\ast\)\(^\ast\) (2-acetamido-2-(2-aminofluorenyl)guanine (23). Misincorporation in normal DNA (with the four normal dNTPs) is increased in *in vitro* pol δ experiments by the presence of PCNA (24). This effect could be due to promotion of extension beyond incorrect base pairs or to increased catalysis of misincorporation per se. This study clearly indicates that the extension beyond the A:8-oxoG base pair predominates (over C:8-oxoG pairs) and that PCNA stimulates this reaction 70-fold. Mozzherin *et al.* (24) and McConnell *et al.* (28) proposed that the principal effect of PCNA stimulation of pol δ is an increased affinity of pol δ for DNA (2000-fold). Electrophoretic gel shift mobility results were presented as evidence, but exactly how these assays relate to events in catalysis is yet unknown. Interestingly, in steady-state assays the \(K_{m}\) value was mainly affected in correct nucleotide incorporation (Table I) (21). Is there a direct effect of PCNA on processivity, as proposed by others (23, 24)? In assays in which single base incorporation is measured (e.g. this work), \(k_{\text{cat}}\) is probably approximated by \(k_{\text{cat}}\) (Fig. 6), the \(k_{\text{off}}\) rate (see below). However, in steady-state kinetic experiments in which a single dNTP is incorporated, there is little effect of PCNA on \(k_{\text{cat}}\) (Table II). McConnell *et al.* (28) estimated the \(t_{\text{1/2}}\) of the pol δ oligonucleotide complex to be 2.65 h, in the presence of PCNA, using a filter binding assay. In that work the authors did not express “\(V_{\text{max}}\)” in quantitative terms, so no direct interpretation can be made. However, this \(t_{\text{1/2}}\) corresponds to \(k_{\text{cat}} < 0.3 \text{ h}^{-1}\), which is 2 orders of magnitude slower than the steady-state \(k_{\text{cat}}\) in our own experiments (Table II). Qualitative evidence also indicates that the presence of dNTP significantly destabilizes the pol δPCNA-DNA complex (33).

We did, as in Ref. 23, find that PCNA stimulated polymerization beyond 8-oxoG lesions. The extent of stimulation by PCNA was estimated by Mozzherin *et al.* (23) to be 2.5-fold. In our pre-steady-state experiments, the stimulation of dNTP incorporation beyond either 8-oxoG:A or 8-oxoG:C pairs by PCNA was much greater (Fig. 3). The extent of stimulation by PCNA might have been underestimated in the previous steady-state
work (23) because of the contribution of the inherent exonuclease activity of pol δ (21), which can obscure steady-state experiments. Another possibility for the difference seen is that Mozherin et al. (23) used a mixture of dNTPs in their experiments, the effect of preferential extension beyond an 8-oxoG:A pair in the presence of PCNA (Fig. 3A) might have been diminished because of the lower efficiency of incorporation of dATP opposite 8-oxoG (Table II).

The role of PCNA is probably more complex than simply increasing the affinity of pol δ with oligonucleotides. A possible explanation (Fig. 6) is that PCNA alters the rates of conversion of the pol δ-DNA complex to and from an inactive complex. Further analysis will be required to address the hypothesis. Another issue is that of other accessory proteins. Additional components of yeast pol δ have been identified (39), and Lee and associates (35) have presented evidence that an additional 12- and two 25-kDa subunits may be associated with mammalian pol δ, as well as the 125- and 50-kDa proteins and PCNA (40, 41). The work to date has involved only association studies (40) and no function has been associated with these gene products yet. These additional subunits may have contributions to the activity and fidelity of pol δ, particularly when blocking lesions are a consideration. However, we do feel that the 3-protein pol δ system we are using here (125- and 50-kDa proteins and PCNA) is a useful one in that up to 90% burst amplitudes with some pol δ preparations are observed in the pre-steady-state experiments (Fig. 3A), and appreciable bursts are seen even at incorporation opposite 8-oxoG (Fig. 1).

Incorporation of 8-Oxo-dGTP by pol δ—There is potential for 8-oxo-dGTP to be incorporated by DNA polymerases (42, 43) and cause A → C transversions (43). Efficiencies of 8-oxo-dGTP incorporation opposite A or C by prokaryotic polymerases are 4 orders of magnitude lower than normal dGTP incorporation opposite C (9). The ability of pol δ to incorporate 8-oxo-dGTP opposite A or C was also examined using steady-state kinetics, and a similar low nucleotide insertion efficiency of 8-oxo-dGTP was found for pol δ. As in the case of the prokaryotic polymerases (9), the very poor kinetic efficiency for incorporation opposite C or A is the result of an unfavorable $K_n$. We would presume that the fraction of the dGTP pool accounted for by 8-oxo-dGTP is similar to the situation in DNA, suggesting that $\sim 1/100$ of the dNTP pool is 8-oxo-dGTP (15–17, 44). The actual 8-oxo-dGTP pool size has not been reported but preliminary estimates are very low. With a catalytic efficiency for 8-oxo-dGTP incorporation $\sim 10^{-3}$ that of dGTP by pol δ (this work), the probability of inserting 8-oxo-dGTP should be $\sim 10^{-9}$. The significance of the MutT “repair” enzyme in mammalian systems (45) is an issue in that the tendency to insert 8-oxo-dGTP appears to be low. We raise the possibility that the critical substrate for this enzyme may not be 8-oxo-dGTP but instead a yet unknown nucleotide. However, an alternative explanation we cannot rule out is that other mammalian polymerases insert 8-oxo-dGTP with high efficiency.

Conclusions—We have studied the replication events at 8-oxoG catalyzed by mammalian pol δ because there is rather general agreement that this is the main polymerase involved in leading strand DNA replication (1, 2). Also, conservation of sequence identity among all of the components of the system is extensive. However, the 3-protein system we used here is a simplification of the cellular situation, in which coupling with pol α and possibly other proteins exist. Nevertheless, the system we use does provide a means of studying the interactions of pol δ with DNA and carcinogen-modified DNA. Another point is that a number of relatively nonprocessive polymerases have been identified and probably make important contributions to the outcome of the processing of modified DNA (6, 46). At this time it is not clear exactly which of these become involved in replication past 8-oxoG lesions in mammalian cells.

In pre-steady-state experiments, pol δ incorporated both dCTP and dATP opposite 8-oxoG with similar rates as for correct nucleotide incorporation into unmodified DNA. The efficiency of incorporation opposite 8-oxoG was dominated by a reduced $K_{\text{dNTP}}$ and there was a preference for incorporation of dCTP opposite 8-oxoG. Although pol δ displayed a preference for correct incorporation, extension beyond A:8-oxoG pairs predominated. An issue central to many experiments presented in this paper is the role of PCNA. The presence of PCNA was more essential to the efficiency of nucleotide incorporation opposite 8-oxoG than reactions with unmodified DNA (21). PCNA was also found to be essential to the extension beyond A:8-oxoG or C:8-oxoG base pairs. The mechanistic role of PCNA in these reactions could involve alterations of $k_{\text{off}}$ ($k$ of Fig. 6), dNTP binding, or conformational effects of the enzyme. Our work can be interpreted in terms of a role for altering rates of conversion of pol δ-DNA complexes to unproductive forms and vice versa. Evidence for the existence and importance of such unproductive complexes with prokaryotic replicative polymerases has been presented from this (14) and other laboratories (47–50). We propose that such inactive complexes are also an issue with mammalian polymerases such as pol δ. With all of these postulated unproductive complexes, there is no structural information available. Another current deficit is the lack of ability to monitor changes in real time, although some possibilities exist (13, 32) and are the subject of future efforts.

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