Translesion Synthesis Past Acrolein-derived DNA Adducts by Human Mitochondrial DNA Polymerase γ

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Background: Mitochondria lack specialized DNA polymerases that in the nucleus can bypass acrolein-induced DNA adducts.

Results: Human mitochondrial DNA polymerase γ replicated past acrolein-induced deoxyguanosine adducts inefficiently and in an extremely error-prone manner.

Conclusion: Acrolein-induced deoxyguanosine adducts may inhibit replication and cause mutations in mitochondria.

Significance: The data suggest a role for pol γ in acrolein-induced mitochondrial DNA damage and mutagenesis.

Acrolein, a mutagenic aldehyde, is produced endogenously by lipid peroxidation and exogenously by combustion of organic materials, including tobacco products. Acrolein reacts with DNA bases forming exocyclic DNA adducts, such as γ-hydroxy-1,2-propano-2′-deoxyguanosine (γ-HOPdG) and γ-hydroxy-1,2-propano-2′-deoxyadenosine (γ-HOPdA). The bulky γ-HOPdG adduct blocks DNA synthesis by replicative polymerases but can be bypassed by translesion synthesis polymerases in the nucleus. Although acrolein-induced adducts are likely to be formed and persist in mitochondrial DNA, animal cell mitochondria lack specialized translesion DNA synthesis polymerases to tolerate these lesions. Thus, it is important to understand how pol γ, the sole mitochondrial DNA polymerase in human cells, acts on acrolein-adducted DNA. To address this question, we investigated the ability of pol γ to bypass the minor groove γ-HOPdG and major groove γ-HOPdA adducts using single nucleotide incorporation and primer extension analyses. The efficiency of pol γ-catalyzed bypass of γ-HOPdG was low, and surprisingly, pol γ preferred to incorporate purine nucleotides opposite the adduct. Pol γ also exhibited ~2-fold lower rates of excision of the misincorporated purine nucleotides opposite γ-HOPdG compared with the corresponding nucleotides opposite dG. Extension of primers from the terminus opposite γ-HOPdG was accomplished only following error-prone purine nucleotide incorporation. However, pol γ preferentially incorporated dT opposite the γ-HOPdA adduct and efficiently extended primers from the correctly paired terminus, indicating that γ-HOPdA is probably non-mutagenic. In summary, our data suggest that acrolein-induced exocyclic DNA lesions can be bypassed by mitochondrial DNA polymerase but, in the case of the minor groove γ-HOPdG adduct, at the cost of unprecedented high mutation rates.

Lipid peroxidation causes cell damage by oxidizing the lipid molecules in the cell membrane. One of the end products of lipid peroxidation is acrolein (CH₂ = CHCHO), the simplest α,β-unsaturated aldehyde (1–5). Acrolein is also a ubiquitous environmental contaminant formed via incomplete combustion of organic materials and is found in cigarette smoke, industrial emissions, and cooking oil fumes (5). Similar to other bifunctional aldehydes, acrolein reacts with DNA bases to form exocyclic DNA adducts, including γ-hydroxy-1,2-propano-2′-deoxyguanosine (γ-HOPdG)2 and γ-hydroxy-1,2-propano-2′-deoxyadenosine (γ-HOPdA), which are the products of Michael addition of the N²-amine of deoxyguanosine (dG) and the N⁶-amine of deoxyadenosine (dA), respectively (6–8). The mutagenic γ-HOPdG adduct has been found in cellular DNA after acrolein exposure and also as an endogenous lesion (2, 9–11). The γ-HOPdG adduct exists in equilibrium between ring-opened and ring-closed conformations (Fig. 1). The ring-closed form of γ-HOPdG exists mainly in the nucleoside and in single-stranded DNA, whereas the ring-opened form of γ-HOPdG, the N²-(3-oxopropyl)-2′-deoxyguanosine, predominates in duplex DNA (12–16). In contrast to the ring-closed form, the ring-opened form of the adduct retains the ability to interact with dC via Watson-Crick hydrogen bonds (14–16). The bulky γ-HOPdG adduct blocks the eukaryotic nuclear replicative DNA polymerases, pol δ and pol ε, during DNA replication in vitro (17). In contrast, the low fidelity Y-family translesion synthesis (TLS) DNA polymerases, Rev1 and pol ι, can accurately incorporate dC opposite γ-HOPdG, whereas pol κ can efficiently extend from dC opposite the adduct (18, 19). Pol ζ, a B-family DNA polymerase, is also proficient in the extension step (19). Another member of the Y-family, pol η, can

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2 The abbreviations used are: γ-HOPdG, γ-hydroxy-1,N²-propano-2′-deoxyguanosine; TLS, translesion DNA synthesis; dNTP, deoxyribonucleoside 5′-triphosphate; γ-HOPdA, γ-hydroxy-1,N⁶-propano-2′-deoxyadenosine; pol, polymerase.
Bypass of Acrolein-induced DNA Lesions by pol γ

![Diagram](image)

FIGURE 1. Structures of the acrolein-derived deoxyguanosine and deoxyadenosine adducts under investigation.

perform a relatively accurate bypass of γ-HOPdG, but only after the adduct is converted into the ring-opened form (20). Thus, several DNA polymerases can be involved in error-free replication past γ-HOPdG in the nucleus, thereby preventing this lesion from becoming highly blocking and mutagenic (21).

Mitochondria are highly susceptible to oxidative stress, and acrolein has been shown to directly stimulate the production of reactive oxygen species by compromising the function of the electron transport system in isolated brain mitochondrial tissues from guinea pig (22). Since acrolein readily reacts with DNA nucleobases, it is very plausible that acrolein-modified nucleotides would be formed in the mitochondrial genome both under normal conditions and as result of oxidative stress or following acrolein exposure. Acrolein adducts are not repaired by base excision repair, but normally are removed by nucleotide excision repair in both bacteria and the eukaryotic nucleus (21). However, because of the lack of nucleotide excision repair in mitochondria, these bulky adducts may persist in mtDNA and become substrates for the mtDNA polymerase. Although yeast Rev 1 and pol ζ have been shown to perform efficient and error-free replication past acrolein-derived adducts (19) and have been identified in yeast mitochondria (23, 24), no evidence exists to suggest that these DNA polymerases are also found in animal cell mitochondria. Thus, in contrast to the nucleus, which contains several DNA polymerases with specialized functions to efficiently bypass many different DNA lesions, the mammalian mitochondria appears to contain a single DNA polymerase, pol γ, which bears the burden to both replicate the 16.5-kb circular mitochondrial DNA and participate in DNA repair (17–20, 25–30).

The human DNA pol γ is a heterotrimeric holoenzyme complex composed of a catalytic subunit, encoded by POLG at chromosome locus 15q25, and a dimeric accessory subunit, encoded by POLG2 at chromosome locus 17q24.1. The catalytic subunit, a member of the A-family of DNA polymerases is a 140-kDa enzyme (p140) that possesses DNA polymerase, 3’ → 5’-exonuclease, and 5’-dRP lyase activities (30). The homodimeric accessory subunit, a 55-kDa protein (two p55 monomers), binds asymmetrically to the catalytic subunit, where the proximal p55 enables tight DNA binding and the distal subunit confers processive DNA synthesis to the catalytic subunit (31, 32).

In this study, we examined the ability of human mitochondrial DNA pol γ holoenzyme to incorporate nucleotides opposite the minor groove γ-HOPdG and major groove γ-HOPdA adducts and to extend primers post-incorporation. The ability of pol γ to bypass the reduced derivatives of the adducts (Fig. 1) was also investigated. The latter adducts cannot undergo ring closure and therefore represent reasonable models for the ring-opened forms of γ-HOPdG and γ-HOPdA.

**EXPERIMENTAL PROCEDURES**

*Enzymes-* Recombinant catalytic subunit of human DNA pol γ (exonuclease-proficient and exonuclease-deficient forms) containing a His6 affinity tag at its N terminus was overproduced in baculovirus-infected Sf9 cells, and the protein was purified to homogeneity as described previously (33–35). Two catalytic residues for exonuclease activity, the Asp-198 and Glu-200, were substituted by alanines to construct the exonuclease-deficient pol γ, which abolished all the 3’ → 5’-exonuclease activity (35). The p55 accessory subunit containing a His6 affinity tag at its C terminus was expressed in *Escherichia coli* and purified to homogeneity as described previously (33–36). The eluted protein samples were visualized with SDS-PAGE for purity, and the proteins were frozen in small aliquots in liquid nitrogen and stored at −80 °C.

*Substrates-* The 23-mer oligodeoxynucleotide containing γ-HOPdG, 5’-TCA C(γ-HOPdG)GAT CCT TAC GAG CCC CC-3’, was synthesized and purified as described elsewhere (13) and was the kind gift of Dr. Carmelo J. Rizzo (Dept. of Chemistry, Vanderbilt University). The oligodeoxynucleotide containing reduced γ-HOPdG was obtained by treatment of the γ-HOPdG-containing 23-mer with sodium borohydride according to the published procedure (37). The corresponding oligodeoxynucleotide with undamaged dG was purchased from Integrated DNA Technologies. These oligodeoxynucleotides served as templates. An18-mer oligodeoxynucleotide, 5’-GGG GGC TCG TAA GGA TTC-3’, served as primer for the single nucleotide incorporation and primer extension assays. For extensions from the primer termini opposite the lesion site and for exonuclease reactions, 19-mer oligodeoxynucleotides were used that contained an additional dA, dG, dC, or dT at the 3’-end.

The 30-mer oligodeoxynucleotide containing γ-HOPdA, 5’-GCT AGT ACT CGT CG(γ-HOPdA) CAA TTC CGT ATC CAT-3’, was synthesized as described elsewhere (13) and was the kind gift of Dr. Carmelo J. Rizzo (Dept. of Chemistry, Vanderbilt University). The preparation of the corresponding oligodeoxynucleotide that contained reduced γ-HOPdA was done as described previously (37). The oligodeoxynucleotide with undamaged dA with the same sequence was purchased from Integrated DNA Technologies. These oligodeoxynucleotides served as templates. A 19-mer oligodeoxynucleotide, 5’-AAA ATG CAT ACG GAA TTG T-3’, served as primer for the single nucleotide incorporation and primer extension assays. For extensions from the primer termini opposite the lesion site, 20-mer oligodeoxynucleotides were used that contained an additional dA, dG, dC, or dT at the 3’-end. All primer
oligodeoxynucleotides were purchased from Integrated DNA Technologies.

The primers for incorporation and extension assays were labeled at their 5'-ends with [γ-32P]ATP and T4 polynucleotide kinase and annealed to a 1.2-fold molar excess of various complementary templates by heating the mixture to 95 °C for 5 min followed by slow cooling to room temperature.

Single Nucleotide Incorporation/Extension Reactions—Steady-state kinetic parameters were determined using polynucleotide gel-based single nucleotide incorporation/extension assays. Reactions mixtures (10 μl) contained 25 mM HEPES-KOH (pH 7.5), 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 5 mM MgCl2, 50 nM radiolabeled DNA substrate, 10 nM exonuclease-deficient pol γ, and 20 nM p55. The reactions were started by the addition of one of the four dNTPs (at different concentrations depending on the substrate and analysis). After incubation at 37 °C for 10 min, reactions were terminated by the addition (10 μl) of 95% deionized formamide and 10 mM EDTA. Samples (3 μl) were boiled for 5 min at 95 °C and resolved by electrophoresis on 16% polyacrylamide gels containing 7 M urea. Gels were exposed to a phosphor screen; radioactive bands were detected with a Typhoon 9400 Phosphor-Imager (GE Healthcare), and band intensities were quantified with NIH Image software. Steady-state kinetic parameters, K\textsubscript{m} and k\textsubscript{cat}, were determined by fitting the data to the Michaelis-Menten model using KaleidaGraph (Version 4.1, Synergy).

Primer Extension Assays—Primer extension reactions were performed similar to the single nucleotide incorporation/extension assays but in the presence of all four dNTPs in the reaction mixture. The concentrations of dNTPs used for extension analyses are summarized in Fig. 2.

Exonuclease Activity Assay—Reactions mixtures (200 μl) contained 25 mM HEPES-KOH (pH 7.5), 2 mM 2-mercaptoethanol, 0.1 mM EDTA, 50 nM radiolabeled substrate, 10 nM exonuclease-proficient pol γ, and 20 nM p55. Reactions were started by the addition of 5 mM MgCl2 at 37 °C and terminated by removing 10 μl of the mixture at different time points (0–300 s) and adding it to tubes containing an equal volume of 95% deionized formamide and 10 mM EDTA. Gel electrophoresis and quantitation of intensities of radioactive bands were performed as described above. The rate of excision (k\textsubscript{ex}) of the 3'-primer terminus from each substrate was determined by plotting the loss of substrate against time (seconds) and fitting the data to a single-exponential equation using KaleidaGraph (Version 4.1, Synergy).

RESULTS

Error-prone Incorporation by Human DNA pol γ Opposite the γ-HOPdG and Reduced γ-HOPdG Adducts—The γ-HOPdG adduct is a minor groove DNA lesion that is formed by Michael addition of the N\textsuperscript{2}-amine of dG to acrolein, yielding the ring-opened aldehydic adduct, which further cyclizes with N\textsuperscript{1} (6, 7). To test the ability of human DNA pol γ to incorporate nucleotides opposite the γ-HOPdG adduct in DNA, single nucleotide incorporation reactions were performed. In parallel reactions, a DNA substrate was used that contained the ring-opened reduced γ-HOPdG adduct at the same position on the 23-mer template sequence. The control substrates contained template with an undamaged deoxyguanosine in an otherwise identical sequence. Single nucleotide incorporation reactions were performed as described under “Experimental Procedures” using exonuclease-deficient pol γ holoenzyme in the presence of dCTP (Fig. 2). The results demonstrated that even at the highest dCTP concentration used (20 μM), pol γ was unable to incorporate dC opposite γ-HOPdG (Fig. 2, lanes 7–9) and only weakly incorporated dC opposite reduced γ-HOPdG (Fig. 2, lanes 12–14). When primer extension reactions were performed in the presence of all four dNTPs, efficient incorporation was observed opposite both γ-HOPdG and reduced γ-HOPdG (Fig. 2, lanes 10 and 15). This suggested that pol γ performed the first incorporation step of TLS reaction in an error-prone fashion by inserting incorrect nucleotides instead of the cognate dC opposite these adducts. However, extension beyond the lesion sites was minimal at the dNTP concentrations used in the reactions (Fig. 2, lanes 10 and 15).

Preferential Incorporation of Purine Nucleotides Opposite the γ-HOPdG and Reduced γ-HOPdG Adducts—To determine the identity of the nucleotides incorporated opposite the γ-HOPdG and reduced γ-HOPdG adducts, steady-state kinetic analyses were performed using the same DNA substrates as before, but with each of the four dNTPs separately. The results from the kinetic analyses are summarized in Table 1. The data revealed that relative to the undamaged dG, pol γ incorporated dC opposite γ-HOPdG and its reduced derivative with 3100- and 120-fold lower efficiency, respectively (Table 1, compare k\textsubscript{cat}/K\textsubscript{m} values for dC incorporation opposite ND, γ-HOPdG, and reduced γ-HOPdG). Because previous studies have indicated that for many DNA polymerases the rapid binding equilibrium mechanism is valid and is followed by a single rate-limiting step, the K\textsubscript{m} and K\textsubscript{cat} values measured using pre-steady-state kinetics define the K\textsubscript{m} and k\textsubscript{cat} values, respectively.

![Bypass of Acrolein-induced DNA Lesions by pol γ](attachment:image.png)
TABLE 1
Steady-state kinetic parameters for nucleotide incorporation by exonuclease-deficient form of human pol γ opposite the γ-HOPdG and reduced γ-HOPdG adducts

| Adduct in template | Incoming nucleotide | $K_{\text{cat}}$ | $k_{\text{cat}}$ | $K_{\text{m}}$ | $f_{\text{rel}}$ |
|--------------------|---------------------|-----------------|-----------------|----------------|-----------------|
| ND                 | dATP                | 15 ± 1 μM       | 0.67 ± 0.01     | 0.04           | 0.014           |
|                    | dGTP                | 33 ± 2 μM       | 0.48 ± 0.02     | 0.01           | 0.005           |
|                    | dCTP                | 0.20 ± 0.03 μM  | 0.62 ± 0.07     | 3.1            | 1               |
|                    | dTTP                | 20 ± 2 μM       | 0.43 ± 0.03     | 0.02           | 0.007           |
|                    | dATP                | 8 ± 1 μM        | 0.40 ± 0.01     | 0.05           | 48              |
| γ-HOPdG            | dGTP                | 29 ± 9 μM       | 0.36 ± 0.04     | 0.01           | 13              |
|                    | dCTP                | 182 ± 39 μM     | 0.18 ± 0.02     | 0.001          | 1               |
| Reduced γ-HOPdG    | dTTP                | 62 ± 14 μM      | 0.08 ± 0.01     | 1.2            |                 |
|                    | dATP                | 3 ± 2 μM        | 0.22 ± 0.04     | 0.07           | 3               |
|                    | dGTP                | 20 ± 8 μM       | 0.23 ± 0.01     | 0.01           | 0.5             |
|                    | dCTP                | 8 ± 1 μM        | 0.22 ± 0.01     | 0.03           | 1               |
|                    | dTTP                | 28 ± 3 μM       | 0.08 ± 0.00     | 0.003          | 0.1             |

Although we cannot equate $K_{\text{m}}$ to binding affinity in the steady-state experiment, it is fair to assume that $K_{\text{m}}$ values obtained in our study are equivalent to the $K_d$ values observed using pre-steady-state conditions (34, 38). Hence, we can define the catalytic efficiency of the reaction as $k_{\text{cat}}/K_{\text{m}}$, a value equivalent to the $K_{\text{cat}}/K_d$ ratio.

DNA pol γ preferred to incorporate the purine nucleotides opposite γ-HOPdG; relative to the cognate dC, the efficiency of dA and dG incorporation was ∼48- and 13-fold higher, respectively. When reduced γ-HOPdG was present in the template, pol γ did not show a strong preference for either dA or dG incorporation compared with dC incorporation. Surprisingly, pol γ displayed a lower $K_m$ for dA and preferred to misincorporate dA opposite the adducts compared with the control dG (Table 1, compare $K_m$ and $k_{\text{cat}}$ values for dA incorporation opposite the adducts or undamaged dG). To illustrate the major effect of $K_m$, the pol γ-catalyzed nucleotide incorporations were conducted at the incoming dNTP concentrations near the $K_m$ values (Fig. 3).

Thus, pol γ incorporated nucleotides opposite the γ-HOPdG and reduced γ-HOPdG adducts ineffectively and in an extremely error-prone manner, and the ring-opening did not significantly improve the efficiency and fidelity of the reaction.

**Reduced Efficiency of Excision of Mismatched Nucleotides Opposite γ-HOPdG by DNA pol γ—**The nucleotides misincorporated opposite the γ-HOPdG, and reduced γ-HOPdG adducts could be substrates for the exonuclease activity of pol γ prior to further primer extension. Therefore, to investigate the fate of the (mis)incorporated nucleotides opposite these lesions, exonuclease assays were performed with exonuclease-proficient human DNA pol γ holoenzyme. The substrates were prepared by annealing primers containing different 3’-ends (either dA, dG, dC, or dT) opposite the adducts or undamaged dG in template. The rate of excision ($k_{\text{exc}}$) of the 3’-primer terminus nucleotide in each substrate was monitored over time as described under “Experimental Procedures” and tabulated in Table 2. Relative to the control value, the excision efficiencies of dA opposite γ-HOPdG and reduced γ-HOPdG were decreased 0.4- and 0.8-fold, respectively. Thus, the enzyme preferred to cleave the dA:dG mismatch better than the dA:γ-HOPdG mismatch. Similarly, the dG:dG and dT:dG mismatches were the preferred substrates relative to the corresponding dG:γ-HOPdG and dT:γ-HOPdG mismatches. In contrast, the rates of exonuclease activity were very similar for the dC:dG in comparison with the dC:γ-HOPdG pair. Thus, the presence of the dG opposite the template inhibited pol γ-catalyzed incision of the mismatched nucleotides but not the correctly paired dC.

**Extension from the Site Opposite γ-HOPdG Is Achieved Only Following Purine Nucleotide Incorporation Opposite the Adduct—**Because the efficiency of exonucleolytic activity of pol γ was typically low for incorrect nucleotides opposite the adducts, these mismatched 3’ termini could serve as substrates for further primer extension. To evaluate whether these mismatched primer termini could be extended by pol γ, extension assays were performed in the presence of dGTP, which is complementary to the next downstream template nucleotide. Incorporations of dG (Fig. 4 and Table 3) from the mismatched and the correctly paired primer termini were characterized by steady-state kinetic experiments. The analyses showed that relative to
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TABLE 2

| DNA substrate | Adduct in template | $k_{cat}$ | $f_{rel}$ |
|---------------|--------------------|-----------|-----------|
| 5′-A 3′-GCACT- | ND                 | 0.048 ± 0.033 | 1.0       |
|                | γ-HOPdG            | 0.017 ± 0.001 | 0.4       |
|                | Reduced γ-HOPdG    | 0.036 ± 0.011 | 0.8       |
| 5′-G 3′-GCACT- | ND                 | 0.012 ± 0.005 | 1.0       |
|                | γ-HOPdG            | 0.006 ± 0.001 | 0.5       |
|                | Reduced γ-HOPdG    | 0.020 ± 0.009 | 1.7       |
| 5′-C 3′-GCACT- | ND                 | 0.004 ± 0.001 | 1.0       |
|                | γ-HOPdG            | 0.003 ± 0.001 | 0.9       |
|                | Reduced γ-HOPdG    | 0.006 ± 0.001 | 1.6       |
| 5′-T 3′-GCACT- | ND                 | 0.029 ± 0.015 | 1.0       |
|                | γ-HOPdG            | 0.012 ± 0.002 | 0.4       |
|                | Reduced γ-HOPdG    | 0.020 ± 0.004 | 0.7       |

FIGURE 4. Single nucleotide extensions catalyzed by the exonuclease-deficient form of DNA pol γ from various 3′-end terminated primers annealed to templates containing the γ-HOPdG and reduced γ-HOPdG adducts. A schematic representation of the substrate used, with the location of adduct (X) in DNA template, is shown on the left side of each autoradiogram, and the asterisk denotes the radiolabeled strand. Panels A–D show reactions that contain primers terminating with dA, dG, dC, or dT, respectively. Extension reactions past γ-HOPdG were carried out as described under “Experimental Procedures.” In all panels, lanes 1–3, 4–6, and 7–9 have substrates with templates containing no damage (ND), or γ-HOPdG, or reduced form of γ-HOPdG, respectively. In all panels, lanes 1, 4, and 7 contain no dNTP. Lanes 2 and 3 contain 10 and 80 μM dGTP, respectively (panels A and B), 10 and 80 μM dGTP, respectively (panel C), and 1 and 8 μM dGTP, respectively (panel D). In all panels, lanes 5 and 6 contain 100 and 800 μM dGTP, respectively. The concentrations of dGTP in lanes 8 and 9 are the same as in lanes 5 and 6, respectively, in all panels. The concentrations of dGTP used in this representative gel were determined based on the $K_m$ values from Table 3.

the control mismatched substrates, pol γ was able to extend from the dA:γ-HOPdG and dG:γ-HOPdG mismatches with only 2.5- and 1.7-fold lower catalytic efficiencies, respectively (Table 3, compare $k_{cat}/K_m$ values of dG incorporation past the dA:γ-HOPdG and the dG:γ-HOPdG mismatches with the dA:dG and the dG:dG mismatches, respectively). However, the extension efficiencies measured for the mismatched primers were at least an order of magnitude lower for substrates containing the reduced γ-HOPdG adduct relative to the control and γ-HOPdG-containing templates. Surprisingly, no extension could be detected from a cognate dC:γ-HOPdG pair, even at very high concentrations of the incoming dGTP (Table 3; Fig. 4, panel C, lanes 5 and 6). These data provide further evidence for a possible role of pol γ in the mutagenic bypass of the γ-HOPdG adduct in the mtDNA.

High Fidelity Incorporation by DNA pol γ Opposite the γ-HOPdA and Reduced γ-HOPdA Adducts—The γ-HOPdA adduct is a major groove DNA lesion that is formed by Michael addition of the N6-amine of dA to acrolein, followed by cyclization at N7 (8). To test whether these adducts can be bypassed by DNA pol γ, single nucleotide incorporation assays were performed as described above to determine the specificity of nucleotides that could be incorporated opposite the γ-HOPdA and reduced γ-HOPdA adducts. Steady-state kinetic analyses revealed that the catalytic efficiency of pol γ-catalyzed incorporation of the cognate dT opposite either the γ-HOPdA or reduced γ-HOPdA adduct was ~20-fold lower relative to control unadducted substrates (Table 4, compare $k_{cat}/K_m$ of dT incorporation opposite dA, γ-HOPdA, and reduced γ-HOPdA). Pol γ preferred to incorporate the correct dT over any of the other nucleotides (dA, dG, and dC) on all three substrates implying a high fidelity mechanism of insertion opposite the γ-HOPdA and reduced γ-HOPdA adducts (Table 4). Representative results of the various nucleotide incorporations opposite dA adducts are shown in Fig. 5, with the incoming nucleotide concentrations approaching the $K_m$ values.

Accurate and Proficient Primer Extension Following Nucleotide Insertion Opposite the γ-HOPdA and Reduced γ-HOPdA Adducts by DNA pol γ—Additional experiments were designed to test whether DNA pol γ could effectively perform primer extension from the site opposite the γ-HOPdA and reduced γ-HOPdA adducts after accurate incorporation of dT opposite the adduct. To determine the efficiency of extension from matched and mismatched primer termini opposite the adducted site, single nucleotide extension assays were performed with the exonuclease-deficient form of DNA pol γ (Fig. 6). Steady-state kinetic analyses revealed that in the presence of dT opposite γ-HOPdA and reduced γ-HOPdA, extensions with the incoming dCTP that were complementary to the downstream template dG, were only 1.7- and 2.2-fold less efficient compared with the control substrate (Table 5, compare $k_{cat}/K_m$ values for extension from dT opposite dA, γ-HOPdA, and reduced γ-HOPdA). The catalytic efficiencies of extension from any of the mismatched termini were significantly lower than from the corresponding correct dT, irrespective of the identity of the dA on template (Table 5, see relative efficiency, $f_{rel}$ values, less than 1% in all cases). Thus, the γ-HOPdA adduct could be bypassed by the human DNA pol γ with relatively high efficiency and fidelity.
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TABLE 3
Steady-state kinetic parameters for dGTP extension past the γ-HOPdG and reduced γ-HOPdG adducts by exonuclease-deficient form of human DNA pol γ

The $K_m$ and $k_{cat}$ values for incorporating dGTP from 3’-terminally matched (dC) or mismatched (dA, dG, and dT) primer opposite either dG (ND), or γ-HOPdG, or reduced γ-HOPdG on a 3’-terminally matched primer-template substrate (Fig. 5) were determined as described under “Experimental Procedures.” The average values of at least two independent experiments are shown with S.D. $f_{cat}$, relative efficiency ($f_{cat}/K_m$) of dGTP incorporation from 3’-terminally mismatched primer-template substrate ($K_m$, $k_{cat}$) of dGTP incorporation from 3’-terminally matched primer-template substrate, $f_{cat}$, is template-specific. ND, no damage in template; nd, none detected.

| Adduct in template | Nucleotide opposite adduct in primer | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ | $f_{cat}$ |
|-------------------|-------------------------------------|------------|------------------------|---------------|---------|
| ND                | dA                                  | 124 ± 23   | 0.37 ± 0.03            | 0.002         | 0.0001  |
|                   | dG                                  | 84 ± 3     | 0.27 ± 0.01            | 0.003         | 0.0001  |
|                   | dC                                  | 0.02 ± 0.00| 0.73 ± 0.02            | 0.001         | 0.0001  |
|                   | dT                                  | 4.4 ± 0.4  | 0.27 ± 0.04            | 0.006         | 0.0015  |
| γ-HOPdG           | dA                                  | 192 ± 3    | 0.19 ± 0.01            | 0.001         | 0.0001  |
|                   | dG                                  | 125 ± 6    | 0.24 ± 0.01            | 0.002         | 0.0002  |
|                   | dC                                  | nd         | nd                     | nd            | nd      |
|                   | dT                                  | 187 ± 4    | 0.19 ± 0.00            | 0.001         | 0.0001  |
| Reduced γ-HOPdG   | dA                                  | 320 ± 113  | 0.08 ± 0.00            | 0.0002        | 0.2     |
|                   | dG                                  | 145 ± 6    | 0.12 ± 0.01            | 0.0008        | 0.6     |
|                   | dC                                  | 111 ± 8    | 0.15 ± 0.01            | 0.0013        | 1       |
|                   | dT                                  | 138 ± 18   | 0.10 ± 0.02            | 0.0007        | 0.5     |

TABLE 4
Steady-state kinetic parameters for nucleotide incorporation by exonuclease-deficient human pol γ opposite the γ-HOPdA and reduced γ-HOPdA adducts

The $K_m$ and $k_{cat}$ values for incorporating the correct (dTTP) or incorrect (dATP, dGTP, and dCTP) nucleotide opposite either dA (ND), or γ-HOPdA, or reduced γ-HOPdA on a 3’-terminally matched primer-template substrates (Fig. 5) were determined as described under “Experimental Procedures.” The average values of at least two independent experiments are shown with S.D. $f_{cat}$, relative efficiency ($f_{cat}/K_m$) of incorrect nucleotide incorporation ($K_m$, $k_{cat}$) of correct nucleotide incorporation, $f_{cat}$ is template-specific. ND, no damage in template.

| Adduct in template | Incoming nucleotide | $K_m$ (μM) | $k_{cat}$ (min$^{-1}$) | $k_{cat}/K_m$ | $f_{cat}$ |
|-------------------|---------------------|------------|------------------------|---------------|---------|
| ND                | dATP                | 1.9 ± 0.2  | 0.03 ± 0.01            | 0.28          | 0.006   |
|                   | dGTP                | 106 ± 11   | 0.53 ± 0.02            | 0.005         | 0.0001  |
|                   | dCTP                | 53 ± 3     | 0.49 ± 0.00            | 0.009         | 0.0002  |
|                   | dTTP                | 0.01 ± 0.00| 0.54 ± 0.02            | 47.1          | 1.00    |
| γ-HOPdA           | dATP                | 5.2 ± 0.3  | 0.66 ± 0.03            | 0.13          | 0.055   |
|                   | dGTP                | 118 ± 11   | 0.24 ± 0.03            | 0.002         | 0.0009  |
|                   | dCTP                | 209 ± 65   | 0.18 ± 0.01            | 0.001         | 0.0004  |
|                   | dTTP                | 0.16 ± 0.01| 0.38 ± 0.00            | 2.3           | 1.00    |
| Reduced γ-HOPdA   | dATP                | 2.9 ± 0.1  | 0.65 ± 0.02            | 0.22          | 0.085   |
|                   | dGTP                | 107 ± 6    | 0.24 ± 0.00            | 0.002         | 0.0009  |
|                   | dCTP                | 147 ± 6    | 0.16 ± 0.01            | 0.001         | 0.0004  |
|                   | dTTP                | 0.15 ± 0.01| 0.37 ± 0.01            | 2.5           | 1.00    |

FIGURE 5. Single nucleotide incorporations catalyzed by the exonuclease-deficient form of DNA pol γ opposite the γ-HOPdA and reduced γ-HOPdA adducts. A schematic representation of the substrate used, with the location of the γ-HOPdA adduct (X), is shown at the top of the autoradiogram, and the asterisk denoting the radiolabeled strand. Incorporation opposite the adducts was performed as described under “Experimental Procedures.” Lanes 1–5, 6–10, and 11–15 have substrates with templates containing no damage (ND), or γ-HOPdA, or reduced form of γ-HOPdA, respectively. Lanes 1, 6, and 11 contain no dNTP; lanes 2, 7, and 12, contain 2, 5, and 3 μM dATP, respectively; lanes 3, 8, and 13 contain 100 μM dGTP; lanes 4, 9, and 14 contain 50, 200, and 160 μM dCTP, respectively; lanes 5, 10, and 15 contain 15, 150, and 150 μM dTTP, respectively. The concentrations of dNTP used in this representative gel were determined based on the $K_m$ values from Table 4.

DISCUSSION

Mitochondria are the cellular source of endogenous oxidative stress due to respiration and natural electron leakage from complex I and III of the electron transport chain. Steady-state levels of oxidative damage to mitochondrial DNA are high relative to that sustained by nuclear DNA (39). The peroxidation of lipids results in the formation of various aldehydic species, such as acrolein and malondialdehyde (1–5). Because mitochondria represent the site for the generation of endogenous reactive oxygen species, the mtDNA is likely to be exposed to acrolein and accumulate acrolein-induced lesions. The $N^2$-acrine modification of dG by acrolein results in formation of γ-HOPdG, the minor groove DNA adduct, whereas the $N^2$-acrine modification of dA produces γ-HOPdA, the major groove DNA adduct (6–8). Although acrolein-induced DNA lesions have yet to be measured in mtDNA, one of these adducts, γ-HOPdG, was found in cellular DNA samples isolated from human and rodent tissues (2, 9–11). Furthermore, acrolein damage to mitochondrial proteins has been documented, and mitochondrial function is impaired upon acrolein exposure suggesting the evidence of acrolein in mitochondria (22, 40–42).

In this study, the human mitochondrial DNA polymerase, pol β, was tested with respect to its ability to perform TLS past the γ-HOPdG and γ-HOPdA adducts. When pol γ encountered the γ-HOPdG adduct, it preferred to incorporate purine nucleotides (dA>dG) opposite the lesion and further primer
extension was appreciable only when the purine nucleotides were present at that position. The $K_m$ concentrations of dATP and dGTP required for incorporation opposite γ-HOPdG were within the levels of nucleotide concentrations reported in the mitochondria (43). In contrast, the nucleotide incorporation opposite the γ-HOPdA adduct was accurate and followed by efficient extension. Thus, pol γ appeared to possess the ability to perform mutagenic TLS past the minor groove adduct, while catalyzing generally nonmutagenic TLS past the major groove adduct.

The previously tested eukaryotic DNA polymerases were either almost completely blocked by γ-HOPdG or could insert the correct dC opposite the lesion with various degrees of fidelity (21). Although error-prone nucleotide incorporation opposite γ-HOPdG was observed in pol δ-catalyzed reactions, the products were formed very inefficiently and only in the presence of proliferating cell nuclear antigen and at high concentrations of dNTPs (17). Thus, relative to other eukaryotic DNA polymerases, pol γ manifested unprecedented high potential to cause mutations during replication past γ-HOPdG. Preferential incorporation of dA opposite γ-HOPdG has been shown for T7 DNA polymerase (44), but the relative efficiency of dA incorporation versus dC incorporation was significantly less than that measured for pol γ. The efficiency of extension from dA opposite the adduct was not addressed in the T7 polymerase study. Preferential incorporation of purine nucleotides opposite the γ-HOPdG adduct was also observed for the E. coli pol I Klenow fragment (45, 46). Thus, DNA polymerases of the A-family tend to insert purine nucleotides opposite this minor groove adduct. In contrast, pol γ incorporated dT opposite the major groove γ-HOPdA adduct more efficiently than any other nucleotide. Although replication bypass of this adduct by other members of the A-family of DNA polymerases has not been studied, human pol θ and Klenow fragments could perform high fidelity TLS past the major groove acrolein-derived DNA-peptide cross-links (47, 48). It remains to be determined whether another member of the A-family, human pol θ, could perform TLS past these acrolein-derived DNA adducts.

The mechanism of replication bypass of the γ-HOPdG adduct has been studied for several Y-family DNA polymerases. When pol η, pol κ, or E. coli pol IV were tested, the efficiency and fidelity of nucleotide incorporation were greatly improved by conversion of the adduct into the ring-opened derivative (20, 25, 49). This was not the case for pol γ; the reduced γ-HOPdG

![FIGURE 6. Single nucleotide extensions catalyzed by the exonuclease-deficient form of DNA pol γ from various 3’-end terminated primers annealed to templates containing the γ-HOPdA and reduced γ-HOPdA adducts. A schematic representation of the substrate used, with the location of adduct (X) in DNA template, is shown on the left side of each autoradiogram and with the asterisk denoting the radiolabeled strand. Panels A–D show reactions that contain primers terminating with dA, dG, dC, or dT, respectively. Extension reactions past added site γ-HOPdA was carried out as described under “Experimental Procedures.” In all panels, lanes 1–3, 4–6, and 7–9 have substrates with templates containing no damage (ND), or γ-HOPdA, or reduced form of γ-HOPdA, respectively. In all panels, lanes 1, 4, and 7 contain no dNTP. Lanes 2 and 3 contain 5 and 40 μM dCTP, respectively (panels A and C), 50 and 400 μM dCTP, respectively (panel B), and 5 and 40 μM dCTP, respectively (panel D). In panels A–D, the concentrations of dCTP in lanes 5 and 6 and in lanes 8 and 9 are the same as in lanes 2 and 3, respectively. The concentrations of dCTP used in this representative gel were determined based on the $K_m$ values from Table 5.](image)

**TABLE 5**

| Adduct in template | Nucleotide opposite to adduct in primer | $K_m$ | $k_{cat}$ | $k_{cat}/K_m$ | $f_{rel}$ |
|-------------------|----------------------------------------|-------|----------|-------------|----------|
| ND                | dA                                    | 48 ± 3 | 0.26 ± 0.02 | 0.005 | <0.001 |
|                  | dG                                    | 246 ± 10 | 0.10 ± 0.01 | 0.0004 | <0.0001 |
|                  | dC                                    | 25 ± 0.1 | 0.29 ± 0.01 | 0.12 | 0.005 |
|                  | dT                                    | 0.01 ± 0.00 | 0.28 ± 0.01 | 24 | 1 |
| γ-HOPdA           | dA                                    | 86 ± 6 | 0.27 ± 0.02 | 0.003 | >0.001 |
|                  | dG                                    | 186 ± 7 | 0.05 ± 0.00 | 0.0003 | <0.0001 |
|                  | dC                                    | 11.1 ± 1.7 | 0.32 ± 0.01 | 0.03 | 0.002 |
|                  | dT                                    | 0.02 ± 0.01 | 0.30 ± 0.00 | 14 | 1 |
| Reduced γ-HOPdA  | dA                                    | 79 ± 20 | 0.26 ± 0.01 | 0.003 | <0.001 |
|                  | dG                                    | 134 ± 24 | 0.06 ± 0.01 | 0.0005 | <0.0001 |
|                  | dC                                    | 9.7 ± 0.4 | 0.27 ± 0.00 | 0.03 | 0.002 |
|                  | dT                                    | 0.02 ± 0.01 | 0.24 ± 0.03 | 11 | 1 |
adduct posed a significant block to pol γ-catalyzed DNA synthesis, and it was bypassed in an extremely error-prone manner. The biological implication of this observation is that γ-HOPdG would inhibit replication of mtDNA and potentially cause mutations regardless of its ring-opening status.

The mutagenic TLS past γ-HOPdG by pol γ is not typical of other DNA lesions in the mitochondria. Compared with other lesions in DNA that arise from either oxidative stress or environmental sources, the TLS synthesis past γ-HOPdG may represent one of the most mutagenic events documented (39). Of the many lesions studied, abasic sites are probably one of the most common and have been shown to inhibit more than 80% of DNA synthesis by the Xenopus laevis pol γ (50). The oxidative lesion, 8-oxo-dG, blocks more than 95% of synthesis by pol γ, and the amount of TLS that does occur appears to be accurate in ~90% of the bypass events (51, 52). Benzopyrene adducts and cisplatin intrastrand cross-links principally act as a block to pol γ (53, 54). When pol γ encounters cyclobutane pyrimidine dimers generated from ultraviolet radiation, the majority of DNA replication stalls (55). However, an insignificant amount of the correct dA incorporation and extension was observed across the cyclobutane pyrimidine dimer adduct (55). Unlike nuclear DNA, complete blockage of mtDNA synthesis is not necessarily lethal because of the high copy number of mtDNA genomes per cell. Blocking a small percentage of mtDNA replication may only transiently lower the mtDNA pool size in the cell. The multicopy nature of mtDNA gives the cell an advantage of dispensing with damaged genomes and dysfunctional mitochondria instead of attempting to repair them through actions of mitophagy (56, 57). However, the mutagenic TLS past the acrolein-derived dG adduct by pol γ observed in this study can represent a significant challenge to mtDNA genomic stability. Our data suggest that the γ-HOPdG adduct could be responsible for the G to T as well as the G to C transversions in mtDNA. Although the mismatch repair protein, Msh1, has been found in yeast mitochondria (58, 59), no corresponding proteins have been found in animal cell mitochondria, and no mismatch repair activity has been documented in the literature. Thus, the errors caused by mutagenic bypass of γ-HOPdG by pol γ would likely not be corrected and lead to the fixed transversion mutations. A similar mutagenic TLS may also be occurring past the malondialdehyde-induced M1dG adduct (39). Thus, γ-HOPdG and possibly M1dG may be responsible for the majority of transversions observed in the evolutionary drift of mtDNA in nature. The combined mutagenic effects of these adducts could also contribute to accumulation of somatic mtDNA mutations and age-dependent neurodegenerative disorders.

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