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Trace amounts of 8-oxo-dGTP in mitochondrial dNTP pools reduce DNA polymerase γ replication fidelity

Zachary F. Pursell[1], J. Tyson McDonald[1], Christopher K. Mathews[2] and Thomas A. Kunkel[1,*]

[1]Laboratory of Molecular Genetics and Laboratory of Structural Biology, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC 27709 and [2]Department of Biochemistry and Biophysics, Oregon State University, Corvallis, OR 97331-7305, USA

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ABSTRACT

Replication of the mitochondrial genome by DNA polymerase γ requires dNTP precursors that are subject to oxidation by reactive oxygen species generated by the mitochondrial respiratory chain. One such oxidation product is 8-oxo-dGTP, which can compete with dTTP for incorporation opposite template adenine to yield A-T to C-G transversions. Recent reports indicate that the ratio of undamaged dGTP to dTTP in mitochondrial dNTP pools from rodent tissues varies from ~1:1 to >100:1. Within this wide range, we report here the proportion of 8-oxo-dGTP in the dNTP pool that would be needed to reduce the replication fidelity of human DNA polymerase γ. When various *in vivo* mitochondrial dNTP pools reported previously were used here in reactions performed *in vitro*, 8-oxo-dGTP was readily incorporated opposite template A and the resulting 8-oxo-G-A mismatch was not proofread efficiently by the intrinsic 3′ exonuclease activity of pol γ. At the dNTP ratios reported in rodent tissues, whether highly imbalanced or relatively balanced, the amount of 8-oxo-dGTP needed to reduce fidelity was <1% of dGTP. Moreover, direct measurements reveal that 8-oxo-dGTP is present at such concentrations in the mitochondrial dNTP pools of several rat tissues. The results suggest that oxidized dNTP precursors may contribute to mitochondrial mutagenesis *in vivo*, which could contribute to mitochondrial dysfunction and disease.

INTRODUCTION

Mutations in mitochondrial DNA are associated with several diseases (1,2) and they accumulate with age (3). Mitochondrial DNA mutations can arise from different sources, including errors made by DNA polymerase γ (pol γ) (4), the enzyme that replicates the mitochondrial genome (5). Replication errors are normally rare when wild-type pol γ synthesizes DNA using undamaged substrates (6,7), partly because an intrinsic 3′ exonuclease can proofread mismatches made by pol γ (6–9). The biological importance of the 3′ exonuclease of pol γ to mitochondrial DNA integrity is illustrated by the fact that mice encoding an exonuclease-deficient form of pol γ have strongly elevated rates of base substitutions in mitochondrial DNA (10,11).

A potentially important source of replication infidelity is damage due to reactive oxygen species (12). The electron transport chain on the inner mitochondrial membrane is a rich source of reactive oxygen species capable of damaging macromolecules. The inner mitochondrial membrane surrounds the inner matrix that contains both the mitochondrial DNA and the dNTP pools needed for mitochondrial DNA synthesis. Thus, in addition to bases in the DNA, the mitochondrial dNTP pool is also a target of oxidation. Among several known oxidized dNTPs, one that is particularly common and potentially highly mutagenic is 8-oxo-dGTP (13). 8-oxo-dGTP can base pair correctly with a template C or incorrectly with template A (14), the latter via Hoogsteen base pairing with 8-oxo-G in the *syn* conformation (15). Incorrect 8-oxo-dGTP-A base pairing can lead to A-T to C-G transversions if the incorporated 8-oxo-dGMP escapes proofreading and any

*To whom correspondence should be addressed. Tel: +1 919 541 2644; Fax: +1 919 541 7613; Email: kunkel@niehs.nih.gov
Present address:
J. Tyson McDonald, Roy E. Coats Laboratories, Radiation Biology Experimental Division, Department of Radiation Oncology, David Geffen School of Medicine, University of California at Los Angeles, Los Angeles, CA 90095, USA

subsequent repair. A variety of DNA polymerases can incorporate 8-oxo-dGTP into DNA (16–18), including pol γ, which was demonstrated to stably misincorporate 8-oxo-dGTP opposite template A in a complete DNA synthesis reaction *in vitro* (16). In that study, low-fidelity DNA synthesis was observed despite the presence of the intact proofreading exonuclease that strongly proofreads undamaged mismatches. This indicated that pol γ can indeed insert 8-oxo-dGTP opposite template A, and further suggested that the 8-oxo-GMP-A mismatch was not efficiently proofread. Recent kinetic analyses (19) have measured the rates at which pol γ inserts and excises 8-oxo-dGMP opposite both template C and template A and also clearly demonstrate inefficient proofreading, further supporting the idea that 8-oxo-dGTP is potentially a potent mitochondrial mutagen. Enzymes exist to minimize the mutagenic potential of 8-oxo-dGTP, such as bacterial MutT or mammalian MTH1, which hydrolyze 8-oxo-dGTP to prevent its incorporation into DNA (14,20). In addition to its role in the nucleus, mammalian MTH1 also localizes to the mitochondrial matrix, where it sanitizes the mitochondrial dNTP pool (21). Moreover, in mouse embryonic fibroblasts that are defective in MTH1, mitochondrial cristae degenerate in response to H_2O_2 treatment, and expression of MTH1 prevents this degeneration (22), thus revealing a direct link between 8-oxo-dGTP and mitochondrial dysfunction.

The possibility that 8-oxo-dGTP contributes to mitochondrial dysfunction by competing with dTTP for mutagenic incorporation opposite template adenine is particularly interesting in light of recent reports on mitochondrial dNTP pool sizes. In one study of mitochondrial dNTP pools from rat tissues (23), normal dGTP was found in excess over dTTP by factors of from 10-fold to >100-fold, depending on the tissue. A more recent study in mice (24) reported that dNTP pools of liver mitochondria are only slightly unbalanced, similar to mitochondrial dNTP pools isolated from cultured cells (25,26). Collectively, these studies (27) provide a valuable range of *in vivo* dGTP to dTTP ratios that can be used to examine how much of the dGTP pool would need to be oxidized to 8-oxo-dGTP in order to detect an effect on the error rate for A-T to C-G transversions generated during DNA synthesis by pol γ. To answer this question, we first confirm that, as observed earlier with exonuclease-proficient pol γ isolated from chicken embryos (16), human pol γ in the presence of all four correct dNTPs does indeed misinsert 8-oxo-dGTP opposite template A, and then fully extends the resulting mismatch, thereby generating A-T to C-G transversions. We then compare stable misincorporation of 8-oxo-dGTP by wild-type and exonuclease-deficient pol γ, thereby confirming in a complete DNA synthesis reaction. The conclusion is derived from kinetic analysis using single nucleotides (19), i.e. that the template A–8-oxo-dGMP mismatch is not efficiently proofread by the intrinsic 3′ exonuclease of pol γ. Most importantly, we then demonstrate the DNA synthesis fidelity is reduced when the amount of 8-oxo-dGTP is as little as 0.06% (imbalanced pools) to 0.6% (balanced pools) of the total dGTP available to pol γ. Finally, we confirm by direct nucleotide pool analysis that 8-oxo-dGTP is present in some rat tissues at levels shown by our *in vitro* analysis to be strongly mutagenic.

MATERIALS AND METHODS

DNA polymerase γ

His_6 affinity-tagged recombinant human DNA polymerase γ catalytic (p140) subunit (exonuclease-proficient and exonuclease-deficient forms) and the p55 accessory subunit were kindly supplied by W. Copeland (NIEHS). These proteins were purified separately to homogeneity and then used as described previously (28,29).

Fidelity assays

DNA polymerase γ fidelity was measured as described (7). Briefly, pol γ was used to copy a single-stranded region of the M13 *lacZ* α-complementation gene. Gap-filling reaction mixtures (25 µl) contained 25 mM HEPES•KOH (pH 7.6), 2 mM dithiothreitol, 2 mM $MgCl_2$, 50 µg/ml BSA, 0.1 M NaCl, ~150 ng gapped M13mp2 DNA, 40 ng of Exo^+ or Exo^- p140 pol γ and 1.3-fold molar excess of the p55 accessory subunit, and with dNTPs and 8-oxo-dGTP at the indicated concentrations. 8-oxo-2′-deoxyguanosine-5′-triphosphate was purchased from TriLink Bio Technologies Inc. (San Diego, CA, USA). Gap-filling was complete as monitored by agarose gel electrophoresis (30). M13mp2 DNA products were introduced by electroporation into the *Escherichia coli* host strain and plated and replication errors were scored as described (30). M13mp2 DNA samples from independent *lacZ* mutant plaques were sequenced to determine the types of polymerization errors, and A to C error rates were calculated as described (30). The statistical significance of differences was calculated using Fisher's Exact Test as described (31).

Extraction and analysis of mitochondrial dNTP pools

Methods for isolation and extraction of mitochondria from rat tissues were similar to those described previously (23). Briefly, adult male Wistar rats were anesthetized with isofluorane and killed by decapitation. Organs were rapidly removed and chilled in 0.9% sodium chloride on ice. Organs were weighed, minced, homogenized and subjected to differential centrifugation as described previously (23). Each mitochondrial pellet was then washed by re-suspension in mitochondrial isolation buffer. Mitochondria were aliquoted and stored at –20°C as centrifugal pellets, with each aliquot representing about one gram of the original tissue.

For nucleotide analysis, one pellet from each mitochondrial preparation was suspended in cold 60% aqueous methanol, with each suspension having a volume slightly >2.0 ml. Each suspension was subdivided into two 1.0 ml portions, with the remainder saved for assay of total protein. To one portion was added 500 pmol of authentic 8-oxo-dGTP, for subsequent correction for incomplete nucleotide recovery during extraction. Both portions were held at –20°C for 2 h, with occasional shaking. Next, both suspensions were placed in a boiling water bath for 3 min,

then chilled and centrifuged. The supernatants were taken to dryness in a Speed-Vac centrifugal concentrator. Each residue was dissolved in 200 µl of MilliQ water and any remaining insoluble material was removed by centrifugation.

Nucleotides were resolved by reversed-phase HPLC with ion pairing, as described previously (32). The HPLC system used was a Hitachi model D-7000, with dual-channel detection. One channel monitored UV absorbance, while the other monitored the output from an ESA Coulochem II electrochemical detector set at +425 mV. Measurement of the canonical dNTP pools (dATP, dTTP, dCTP and dGTP) was carried out by the DNA polymerase-based enzymatic assay, as described previously (23). We thank Linda J. Wheeler of the Mathews laboratory for carrying out these analyses.

RESULTS

Measuring the rate of stable misincorporation of 8-oxo-dGTP into DNA

In this study, the error rate for misincorporation of 8-oxo-dGTP opposite template adenine was determined using the M13mp2 forward mutation assay (30). The human pol γ holoenzyme (p140 catalytic subunit plus p55 accessory subunit) was used to fill a 407-nt single-stranded DNA gap in M13mp2 DNA, and DNA synthesis errors were scored as light blue or colorless plaques (see Materials and methods section). The number of A-T to C-G transversions among total sequence changes was then determined by sequencing DNA samples prepared from independent *lacZ* mutants. This proportion and the *lacZ* mutant frequencies were then used to calculate the average rate for A-T to C-G changes, expressed as errors per detectable adenine copied (see Materials and methods section). Scoring an error in this way requires both misinsertion of 8-oxo-dGTP (or dGTP) opposite any of 19 different template adenines in the *lacZ* template where this error leads to a change in plaque color, and then multiple additional correct incorporation events to embed the 8-oxo-G-A mismatch into duplex DNA. The A to C error rates described here are for complete synthesis reactions in the presence of all four normal dNTPs plus 8-oxo-dGTP, and therefore differ from kinetically determined rates of misinsertion and mismatch extension, which are typically performed using a single correct or incorrect dNTP.

Effect of 8-oxo-dGTP equimolar with dGTP on the fidelity of wild-type pol γ

In the absence of 8-oxo-dGTP, DNA synthesis by wild-type (i.e. exonuclease proficient) pol γ is highly accurate, as evidenced by a *lacZ* mutant frequency (11×10^{-4}, Table 1, Experiment 1, line 1) that is close to the background mutant frequency of the assay (5 to 7×10^{-4}). In this reaction containing only the four undamaged dNTPs at equimolar concentration (1 mM each dNTP), sequence analysis of 127 *lacZ* mutants revealed no A to C substitutions. From this, we calculate that the average error rate for A to C substitutions that would result from undamaged dGMP pairing with template adenine is $\leq 0.1 \times 10^{-5}$ (Table 1, Experiment 1). Inclusion of an equal amount of 8-oxo-dGTP in the DNA synthesis reaction (Table 1, Experiment 2) increased the overall mutant frequency by more than 45-fold (to 500×10^{-4}) and increased the average error rate for A to C substitutions to 400×10^{-5}. This 4000-fold increase ($P \leq 0.001$) clearly demonstrates that human pol γ can indeed stably incorporate 8-oxo-dGTP into DNA opposite template adenine. This conclusion with the human enzyme is consistent with our initial study of avian pol γ (16), and with more recent kinetic studies of 8-oxo-dGTP misinsertion and mismatch extension by human pol γ (19).

Table 1. Effect of equimolar 8-oxo-dGTP on the fidelity of wild type and exonuclease-deficient pol γ

	Exo-deficient pol γ	Wild-type pol γ
Experiment 1: normal dNTPs only[a]		
Mut. Freq. ($\times 10^{-4}$)	62	11
Total sequenced mutants	140	127
Mutants with A to C	0	0
A to C rate ($\times 10^{-5}$)	≤0.53	≤0.1
Experiment 2: normal dNTPs + 8-oxo-dGTP		
Mut. Freq. ($\times 10^{-4}$)	720	500
Total sequenced mutants	ND	20
Mutants with A to C	ND	18
A to C rate ($\times 10^{-5}$)	ND	400

[a]Taken from (23).

8-oxo-dGTP-dependent errors using highly imbalanced dNTP pools as found in the rat heart mitochondria

The above polymerization reactions can be viewed as 'proof-of-principle' experiments, as they contained equimolar concentrations of 8-oxo-dGTP and the four undamaged dNTPs, a situation that is unlikely to be physiologically relevant for at least two reasons. First, the ratio of 8-oxo-dGTP to the undamaged dNTPs is likely to be low *in vivo*, at least partly due to hydrolysis of 8-oxo-dGTP by MTH1 (20,33). Secondly, the concentrations of the four undamaged dNTPs in mitochondria are reported to differ from one another (23,24,27). The most extreme case is for mitochondrial dNTP pools from subsarcolemmal rat heart tissue (23), where the dGTP concentration was estimated at 110 µM. This high dGTP concentration provides a large target for potential oxidation to 8-oxo-dGTP. In contrast, the concentration of dTTP, the nucleotide that competes with 8-oxo-dGTP for incorporation opposite template adenosine, was estimated to be only 0.7 µM. Thus, oxidation of a relatively small proportion of the dGTP pool could yield sufficient 8-oxo-dGTP to effectively compete with dTTP for incorporation opposite template adenine.

To test how little 8-oxo-dGTP is needed to reduce pol γ fidelity under such a highly imbalanced dNTP conditions, we performed reactions that contained the biased dNTP pools observed in subsarcolemmal rat heart mitochondria [110 µM dGTP, 0.7 µM dTTP, 13 µM dCTP and 3.6 µM dATP, from (23)], either without 8-oxo-dGTP (Table 2, Experiment 1) or with 8-oxo-dGTP at 110 µM (equimolar to dGTP, Experiment 2), 0.7 µM (0.6% of dGTP,

Table 2. 8-oxo-dGTP effects with the highly imbalanced rat heart mitochondrial dNTP pools

	Exo-deficient pol γ	Wild-type pol γ
Experiment 1: normal dNTPs only[a]		
Mut. Freq. ($\times 10^{-4}$)	160	23
Total sequenced mutants	38	23
Mutants with A to C	1	1
A to C rate ($\times 10^{-5}$)	4.4	0.98
Experiment 2: dNTPs + equimolar 8-oxo-dGTP (110 μM)		
Mut. Freq. ($\times 10^{-4}$)	5200	7700
Total sequenced mutants	24	69
Mutants with A to C	23	68
A to C rate ($\times 10^{-5}$)	4900[b]	7400[b]
Experiment 3: dNTPs + 0.6% 8-oxo-dGTP (0.7 μM)		
Mut. Freq. ($\times 10^{-4}$)	520	390
Total sequenced mutants	ND	23
Mutants with A to C	ND	23
A to C rate ($\times 10^{-5}$)	ND	380
Experiment 4: normal dNTPs + 0.06% 8-oxo-dGTP (0.07 μM)		
Mut. Freq. ($\times 10^{-4}$)	330	97
Total sequenced mutants	37	14
Mutants with A to C	17	11
A to C rate ($\times 10^{-5}$)	160	62

[a]$A = 3.6\,\mu M$, $T = 0.7\,\mu M$, $C = 13\,\mu M$, $G = 110\,\mu M$ [from (23)].
[b]These two values are not statistically different by Fisher's Exact Test.

Table 3. 8-oxo-dGTP effects on the fidelity of wild-type and exonuclease-proficient pol γ using slightly imbalanced dNTP pools reported in rodent liver

Condition	Wild-type pol γ
Experiment 1: normal dNTPs only[a]	
Mut. Freq. ($\times 10^{-4}$)	12
Total sequenced mutants	19
Mutants with A to C	0
A to C rate ($\times 10^{-5}$)	≤ 0.5
Experiment 2: dNTPs + 60% 8-oxo-dGTP (0.24 μM)	
Mut. Freq. ($\times 10^{-4}$)	160
Total sequenced mutants	33
Mutants with A to C	29
A to C rate ($\times 10^{-5}$)	120
Experiment 3: dNTPs + 6% 8-oxo-dGTP (0.024 μM)	
Mut. Freq. ($\times 10^{-4}$)	27
Total sequenced mutants	29
Mutants with A to C	16
A to C rate ($\times 10^{-5}$)	13
Experiment 4: dNTPs + 0.6% 8-oxo-dGTP (0.0024 μM)	
Mut. Freq. ($\times 10^{-4}$)	22
Total sequenced mutants	23
Mutants with A to C	4
A to C rate ($\times 10^{-5}$)	3.3

[a]$A = 0.87\,\mu M$, $T = 0.53\,\mu M$, $C = 1.1\,\mu M$, $G = 0.4\,\mu M$ [adapted from (24)].

Experiment 3) or 0.07 μM (0.06% of dGTP, Experiment 4). When 8-oxo-dGTP was equimolar to dGTP, the rate of A to C substitution increased by >1000-fold ($P \leq 0.001$) (Experiment 2) compared to the control reaction lacking 8-oxo-dGTP (Experiment 1). Similar rates were observed for wild-type and exonuclease-deficient pol γ, again indicating inefficient proofreading of 8-oxo-dGMP-A mismatches. When 8-oxo-dGTP was present at 0.7 μM (Experiment 3), which is only 0.6% of dGTP but equimolar to dTTP, wild-type pol γ generated A to C substitutions at a rate of 380×10^{-5}, about 400-fold higher ($P \leq 0.001$) than in the control reaction lacking 8-oxo-dGTP (0.98×10^{-5}, Experiment 1). Finally, when 8-oxo-dGTP was present at an even 10-fold lower concentration (0.07 μM, Experiment 4), exonuclease-deficient and wild-type pol γ generated A to C substitutions at rates of 160×10^{-5} and 62×10^{-5}, respectively. Thus, as little as 70 nM 8-oxo-dGTP promotes A to C transversions at rates that are much higher than when 8-oxo-dGTP is absent.

8-oxo-dGTP-dependent errors using slightly imbalanced dNTP pools as found in mouse liver mitochondria

The degree to which mitochondrial dNTP pools are imbalanced varies depending on the rodent tissue examined. For example, while the pools in rat heart and skeletal muscle are highly imbalanced, those in rat liver are less imbalanced (23). Moreover, a recent study of mitochondria isolated from mouse liver (24) reported that no dNTP was in excess over any other by >2.8-fold. To determine how little 8-oxo-dGTP is needed to reduce pol γ fidelity under the latter, more balanced conditions, we performed reactions that contained the dNTP pool observed in mouse liver mitochondria (0.4 μM dGTP, 0.53 μM dTTP, 1.1 μM dCTP and 0.87 μM dATP), either without 8-oxo-dGTP (Table 3, Experiment 1) or with 8-oxo-dGTP at 0.24 μM (60% of the dGTP concentration and 45% of the dTTP concentration, Experiment 2), or a 10-fold (Experiment 3) or a 100-fold (Experiment 4) lower concentration of 8-oxo-dGTP. Compared to the rates of A to C substitutions seen in the absence of 8-oxo-dGTP ($\leq 0.5 \times 10^{-5}$), 8-oxo-dGTP reduced fidelity at all concentrations tested, including by 6.6-fold ($P \leq 0.05$) when as little as 2.4 nM 8-oxo-dGTP was present.

Estimation of intramitochondrial 8-oxo-dGTP concentrations

The data above demonstrate that even low concentrations of 8-oxo-dGTP can significantly affect replication error rates when present with the canonical dNTPs at their approximate intramitochondrial concentrations. Is 8-oxo-dGTP present within mitochondria at concentrations comparable to those shown here to be mutagenic? Previous attempts to detect and quantitate 8-oxo-dGTP in extracts of *E. coli* used HPLC with electrochemical detection. The instrument used contained an amperometric detector, with a lower detection limit for 8-oxo-dGTP of about 6 pmol. With that instrument Tassotto and Mathews (32) were unable to detect 8-oxo-dGTP. To improve sensitivity, here we used a coulometric detector that can detect 0.5 pmol or less of 8-oxo-dGTP and which gave a linear response over a several 100-fold concentration range (data not shown). With this instrument it was possible to detect in extracts of rat tissue mitochondria as little as 0.3 pmol of 8-oxo-dGTP. Panels A and B in Figure 1 depict analysis of rat liver and heart mitochondrial extracts, respectively. A peak appearing at about

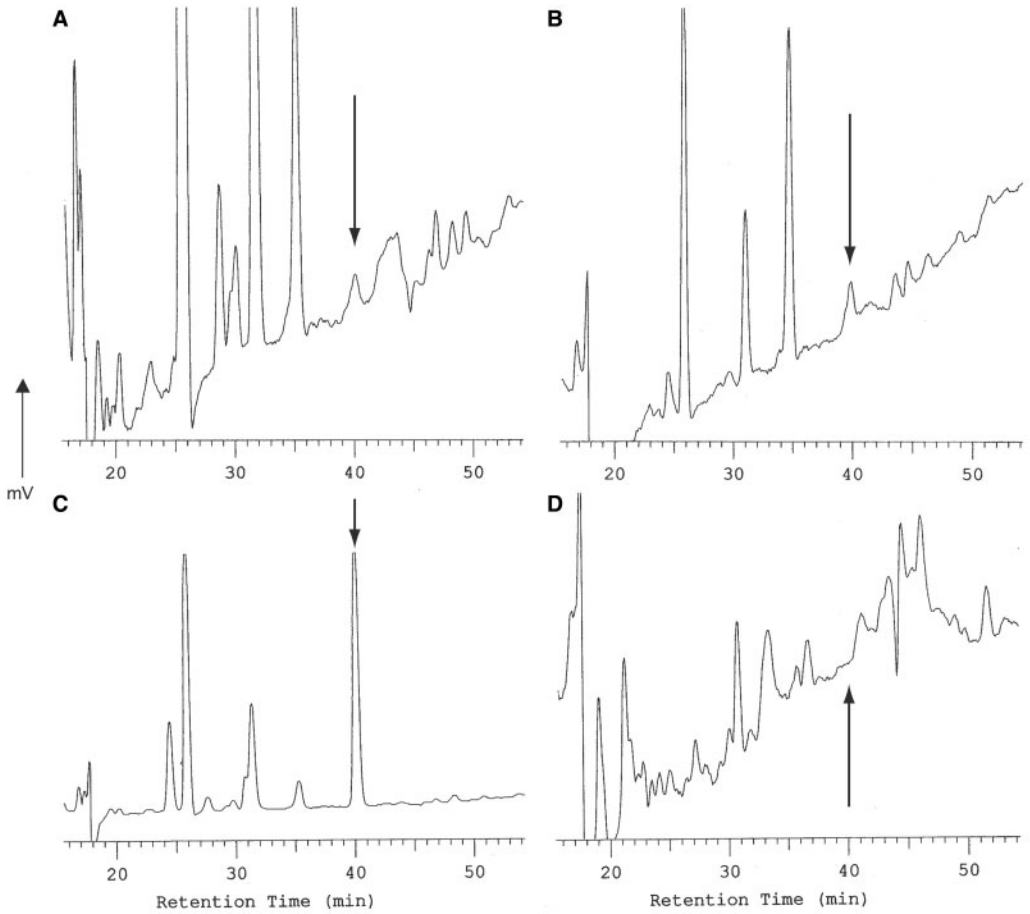

Figure 1. Resolution and detection of 8-oxo-dGTP by HPLC. The four panels depict HPLC elution profiles monitored by electrochemical detection. (**A**) Analysis of a rat liver mitochondrial extract. (**B**) Analysis of rat heart mitochondria. (**C**) Analysis of a rat liver mitochondrial extract; identical to panel A, except that 100 pmol of authentic 8-oxo-dGTP was present, after addition of standard to the mitochondria prior to extraction and analysis. (**D**) Analysis of rat skeletal muscle mitochondria. Each arrow points to a species eluted at about 40.2 min, identified as 8-oxo-dGTP by virtue of its coelution with the standard nucleotide.

40 min coincides with authentic 8-oxo-dGTP, which was added to a liver mitochondrial extract and run under identical conditions, as shown in panel C. An extract of rat skeletal muscle revealed little or no such material (Panel D).

Using this procedure, we detected 8-oxo-dGTP in mitochondrial extracts from rat liver, heart, brain, skeletal muscle and kidney and compared these data with measurements of the four canonical dNTPs in the same extracts. The estimated concentrations of the oxidized nucleotide in liver, heart and kidney were in the 1–2 μM range (Table 4), while comparable measurements in brain and muscle mitochondria gave lower values, approaching our limits of detection. As reported previously (23), the pools for the four canonical dNTPs were highly asymmetric, with dGTP being the most abundant, followed by dCTP and dATP and then dTTP. Since publication of our previous report (23), Ferraro *et al.* (24) have questioned the validity of our measurements, based upon the possibility that cells and mitochondria were anaerobic during harvesting and extraction of the organs. Keys to evaluating this possibility are the levels of adenine nucleotides in the mitochondrial extracts. Because the HPLC instrument has dual-channel detection, it was possible to determine these levels from the UV absorbance trace that was generated simultaneously with the electrochemical signal used to quantify 8-oxo-dGTP. Figure 2 shows the UV trace for one of the liver extracts analyzed. Peaks corresponding to ATP, ADP and AMP were identified and quantified with respect to standards. In the experiment shown, ATP, ADP and AMP comprised 44, 35 and 21%, respectively, of the total adenine nucleotide pool, and the intramitochondrial ATP concentration was estimated to be 2.6 mM. Due to incomplete resolution of the ATP and ADP peaks, these values are only estimates. However, they are comparable to adenine nucleotide pool data reported by Ferraro *et al.* (24) for mouse liver mitochondria, and they suggest that the dNTP asymmetries that we reported (23) and confirm here are not an artifact of ATP depletion during isolation and extraction of mitochondria. Our data reveal that in most of the tissues analyzed the estimated intramitochondrial concentration of 8-oxo-dGTP is comparable to that of dTTP, such that it could compete effectively for incorporation opposite template A. On the other hand, competition with dGTP for incorporation opposite

Table 4. Estimated intramitochondrial concentrations of dNTPs

Tissue	Estimated intramitochondrial concentration, μM ± SD				
	dATP	dTTP	dCTP	dGTP	8-oxo-dGTP
Liver	1.7 ± 1.1	1.7 ± 1.5	3.9 ± 0.7	12.1 ± 5.9	1.2 ± 0.4
Heart	2.1 ± 1.5	3.2 ± 2.4	5.6 ± 2.8	69.3 ± 8.2	1.5 ± 1.2
Brain	3.5 ± 2.1	0.5 ± 0.2	2.8 ± 0.3	39.0 ± 0.2	0.4 ± 0.2
Skeletal muscle	1.6 ± 0.3	1.6 ± 2.5	4.5 ± 3.8	28.4 ± 5.8	0.2 ± 0.1
Kidney	2.4 ± 1.4	3.3 ± 3.8	5.7 ± 3.4	69.0 ± 63.8	1.7 ± 1.2

Data are averages of measurements with three adult male Wistar rats, with the exception of brain, which involved two measurements.

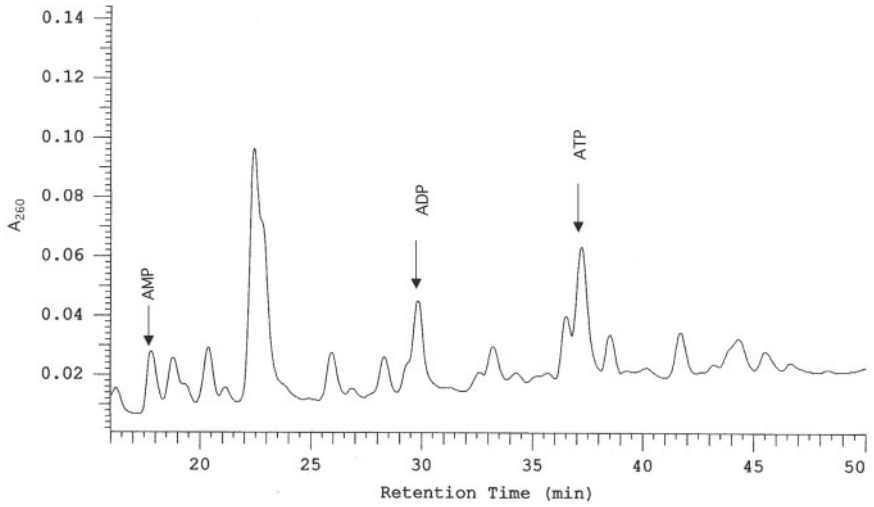

Figure 2. Estimation of adenine nucleotide levels in a rat liver mitochondrial extract. The figure shows an ultraviolet absorption profile (260 nm) obtained simultaneously with the electrochemical detection profile of a rat liver mitochondrial extract. The peaks corresponding to ATP, ADP and AMP were identified by analysis of standard nucleotide solutions.

template C would be expected to be ineffective because of the high concentration of dGTP.

DISCUSSION

The 8-oxo-dGTP-dependent A to C error rate reported here with human pol γ (Table 1) and earlier with avian pol γ (16) is observed despite the fact that wild-type pol γ has an intrinsic 3′ exonuclease activity that strongly proofreads natural base–base mismatches made by the polymerase (6,9), e.g. undamaged dGMP inserted opposite template thymine (7). This suggests that the exonuclease activity of human pol γ does not efficiently proofread 8-oxo-dGMP misinserted opposite adenine, a conclusion also reached from elegant kinetic analysis of insertion and mismatch extension by pol γ performed in the presence of individual dNTPs (19). Like Hanes *et al.* (19), we conclude that once 8-oxo-dGMP is incorporated opposite adenine by pol γ it is preferentially extended rather than excised, which increases its mutagenic potential. Inefficient proofreading of 8-oxo-dGMP opposite adenine by pol γ, an A family DNA polymerase, is reminiscent of the inefficient proofreading of the same mismatch in the opposite symmetry, i.e. dAMP inserted opposite template 8-oxo-guanine, by another A family enzyme, T7 DNA polymerase (34,35). In that case, structural studies indicate that the damaged mismatch, when present at the primer terminus, has geometry and minor groove interactions with the polymerase that are similar to those of normal Watson–Crick base pairs and therefore largely escape proofreading.

Our data indicate that at levels that are detected in mitochondrial dNTP pools, 8-oxo-dGTP promotes pol γ replication infidelity. This is readily explained by 8-oxo-G-A mismatch mimicry of a correct base pair, thereby degrading the two main mechanisms by which pol γ normally achieves high-fidelity, high-nucleotide selectivity and exonucleolytic proofreading. Thus, misincorporation of 8-oxo-dGTP, and by extrapolation, possibly other oxidized dNTPs, should contribute to mitochondrial genome instability *in vivo*, which may in turn contribute to aging and mitochondrial diseases. For example, two studies (10,11) have shown that mice with a homozygous defect [but not a heterozygous defect (36)] in the exonuclease activity of pol γ age prematurely, and mutations in the motifs encoding the exonuclease as well as the polymerase activities of human pol γ are both linked to mitochondrial diseases (5,37). In fact, several of these disease-associated mutant pol γ's have been demonstrated to have reduced nucleotide selectivity. Among these, the Y955C mutant pol γ is particularly interesting.

The Y955C substitution in pol γ is clearly linked to severe autosomal dominant progressive external opthalmoplegia, with significant cosegregation of Parkinsonism and in some cases, with symptoms of premature ovarian failure. The Y955C polymerase itself has strongly reduced nucleotide selectivity yet retains the ability to efficiently proofread natural base–base mismatches (37). However, we show here that the 8-oxo-dGMP-A mismatch is refractory to proofreading, while a recent study has shown that Y955C pol γ has 100-fold reduced discrimination against misinsertion of 8-oxo-dGTP opposite template adenine (38). This may explain why transgenic mice that specifically express Y955C cDNA in heart have increased levels of 8-oxoG in heart mitochondrial DNA (39). These transgenic mice have decreased mitochondrial DNA and aberrant mitochondria and they exhibit cardiomyopathy. The analogous mutation in the gene encoding yeast pol γ results in loss of mitochondrial DNA, a high frequency of petite mutants, and increased levels of lesions in mitochondrial DNA that are consistent with Y955C-associated oxidative stress (40,41).

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