DNA Replication Fidelity with 8-Oxodeoxyguanosine Triphosphate†

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ABSTRACT: Oxidative metabolism is known to generate mutagenic compounds within cells, among which is 8-oxodeoxyguanosine. Here the mutagenic potential of the triphosphate form of this base analog (8-O-dGTP) is investigated during replication *in vitro* of the *lacZ* α-complementation sequence in M13mp2 DNA. Adding 8-O-dGTP at equimolar concentration with the normal dNTPs to polymerization reactions decreases the fidelity of DNA synthesis by exonuclease-deficient Klenow, T4, and *Thermus thermophilus* DNA polymerases. Sequence analysis of mutants suggests that 8-O-dGMP is misincorporated opposite template adenines, yielding A → C transversions. The degree of polymerase selectivity against this error is enzyme-dependent, with rates varying by >25-fold. To determine if the A·8-O-dGMP mispair is proofread, a direct comparison of the fidelity of proofreading-proficient and proofreading-deficient Klenow and T4 DNA polymerases was made. Although the exonuclease activity of Klenow polymerase did not substantially reduce overall misincorporation of 8-O-dGMP, misincorporation was lower for the proofreading-proficient T4 enzyme as compared to its proofreading-deficient derivative. These data suggest that the A·8-O-dGMP mispair can be proofread. The mutagenic potential of 8-O-dGTP with eukaryotic systems was also examined. Misincorporation of 8-O-dGTP opposite adenine was observed during SV40 origin-dependent replication of double-stranded DNA in HeLa cell extracts. When present during replication at a concentration equal to the four normal dNTPs, 8-O-dGTP was at least 13-fold more mutagenic for A·T → C·G transversions than was a 100-fold excess of normal dGTP. These data suggest that 8-O-dGTP could be highly mutagenic during nuclear genomic replication in eukaryotes, with a specificity similar to that in *Escherichia coli*. DNA polymerase γ, the replicative polymerase for mitochondrial DNA, also readily misincorporated 8-O-dGMP opposite adenine despite the presence of a highly active proofreading exonuclease activity. Given the amount of oxidative metabolism occurring in mitochondria, this result has implications for the stability of the mitochondrial genome and for the origin of degenerative diseases resulting from mitochondrial mutations.

Certain analogs of the four bases normally found in DNA are capable of provoking mutations during DNA synthesis due to their ambivalent pairing capacity (Freese, 1959). For many years they have been used as tools to examine DNA replication fidelity and the genetic consequences of inaccurate replication (Drake & Greening, 1970; Bessman *et al.*, 1974; Pavlov *et al.*, 1991). Interest in base analogs also stems from recent evidence that they are formed *in vivo* under both normal and abnormal physiological conditions and, thus, are a potential source of spontaneous mutations.

One process known to produce a base analog *in vivo* is oxidative metabolism, which generates reactive oxygen species that damage a variety of macromolecules. Among the many byproducts of oxidative metabolism, one that has received considerable recent attention is 8-oxodeoxyguanine, the so-called "GO"[1] lesion [Kasai & Nishimura, 1984; for reviews, see Kasai and Nishimura (1991) and Michaels and Miller (1992)]. A series of studies in *Escherichia coli* have demonstrated that this modified guanine nucleotide can be mutagenic by base pairing with either cytosine or adenine, the latter possibly occurring when GO is in the *syn* conformation (Kouchhakdjian *et al.*, 1991). Thus, *E. coli* strains defective in repair of the GO lesion are among the strongest mutators known. For example, the products of the *mutM* and *mutY* genes are involved in repair of base pairs containing the GO lesion, and the double mutant is a >10000-fold mutator for G → T transversions (Michaels *et al.*, 1992a). These result when the modified guanine is present as a template base and mispairs with incoming dATP (Wood *et al.*, 1990; Shibutani *et al.*, 1991: Moriya *et al.*, 1991; Moriya, 1993).

Alternatively, as a triphosphate precursor for synthesis by *E. coli* DNA polymerases (Maki & Sekiguchi, 1992; Cheng *et al.*, 1992), 8-O-dGTP is incorporated opposite template adenine, leading to A·T → C·G transversions. The product of the *mutT* gene has nucleoside triphosphatase activity (Bhatnagar & Bessman, 1988; Akiyama *et al.*, 1989) that hydrolyzes 8-O-dGTP to 8-O-dGMP (Maki & Sekiguchi, 1992). Because a *mutT*⁻ strain is a 100–10000-fold mutator for A·T → C·G transversions (Yanofsky *et al.*, 1966) that result from misincorporation of some form of dGTP opposite template A (Schaaper & Dunn, 1987; Akiyama *et al.*, 1989), Maki and Sekiguchi (1992) suggested that the 8-O-dGTPase activity eliminates this potentially mutagenic base analog from the dNTP precursor pool used for DNA replication.

We initiated the present study of the fidelity of DNA synthesis *in vitro* in the presence of 8-O-dGTP to address

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[1] Abbreviations: GO, 8-oxodeoxyguanine; 8-O-dGTP, 8-oxodeoxyguanosine triphosphate; 8-O-dGMP, 8-oxodeoxyguanosine monophosphate; dNTPs, deoxyribonuclotide triphosphates; *Tth*, *Thermus thermophilus*.

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three unresolved issues. First, we wanted to quantitatively establish the mutagenic potential of 8-O-dGTP in model polymerization reactions. In a previous study (Cheng *et al.*, 1992), 8-O-dGTP was shown to be incorporated opposite template adenine by the exonuclease deficient large fragment of *E. coli* DNA polymerase I. However, this was detected only when the analog was substituted completely for dGTP and in 10–100-fold excess over the other three normal dNTPs. Likewise, the catalytic subunit of *E. coli* DNA polymerase III also incorporated 8-O-dGTP opposite template adenine (Maki & Sekiguchi, 1992), but that study examined insertion (but not mispair extension) at a single template site in a reaction containing only dATP and 8-O-dGTP. Given the potential importance of this analog *in vivo* and the lack of information on its possible concentration relative to the four normal dNTPs, we wanted to extend the earlier studies to establish mutagenic potential in quantitative terms in reactions in which the analog was present in equimolar concentration with all four normal dNTPs. The fidelity assay used here also permits an examination of the effects of local sequence context on misincorporation.

Second, we wanted to determine if, once formed by a DNA polymerase, the A·8-O-dGMP mispair was subject to exonucleolytic proofreading. In a study of the miscoding potential of 8-O-dG as a template nucleotide during DNA synthesis *in vitro*, Shibutani *et al.* (1991) concluded that misinsertions of dAMP opposite template 8-O-G were not proofread by the 3′ → 5′ exonuclease of *E. coli* DNA polymerase I. They suggested that this is because the exonuclease does not recognize the 8-O-dG·dAMP mispair as a substrate. However, the situation in the present study is different in that the modified nucleotide is the incoming dNTP. It is well-known that DNA polymerases discriminate to different extents against substitutions involving the same two nucleotides depending on which is the template and which is the incoming triphosphate [for a review, see Kunkel and Bebenek (1988)]. This could result in differential proofreading. In fact, from a more recent study of revertants at *trpA* sites using mutator strains of *E. coli*, Fowler *et al.* (1992) suggested that proofreading by the *dnaQ* gene product (the 3′ → 5′ exonuclease subunit of DNA polymerase III holoenzyme) does reduce the frequency of A·T → C·G transversions in *mutT* strains *in vivo*. Because these transversions have been suggested to result from misincorporation of 8-O-dGTP opposite template adenine (Maki & Sekiguchi, 1992), we wanted to determine whether 8-O-dGMP, once inserted opposite adenine, would be removed by the exonucleolytic proofreading activity associated with DNA polymerases. This determination is made possible by the availability of mutant DNA polymerases that have normal DNA polymerase activity but lack proofreading activity due to amino acid substitutions within the exonuclease active sites [*e.g.*, Derbyshire *et al.* (1988) and West Frey *et al.* (1993)].

The identification of a human enzyme functionally equivalent to the MutT protein (Mo *et al.*, 1992) and the recent cloning and expression of its cDNA (Sakumi *et al.*, 1993) suggest that eukaryotes may need protection against mutagenic forms of dGTP generated by oxidative stress. Since the mutagenic potential of 8-O-dGTP has not been examined beyond the studies in the powerful *E. coli* genetic system, we wanted to determine whether 8-O-dGTP is mutagenic during eukaryotic DNA replication. To do so, we performed reactions with proteins involved in replication of either of two genomes. The first uses the well-characterized *in vitro* model for duplication of nuclear DNA, the SV40 origin-dependent replication system (Kelly, 1988); Stillman, 1989; Hurwitz *et al.*, 1990). Here circular, double-stranded DNA can be completely replicated in a series of complex reactions catalyzed by a number of host cell proteins. We have previously found that such replication in HeLa cell extracts is accurate for base substitution errors (Roberts & Kunkel, 1988; Thomas *et al.*, 1991), including the A·T → C·G substitutions characteristic of 8-O-dGTP misincorporation. This provides a low background, such that analog-induced errors can be readily detected. The second is gap-filling, single-stranded DNA synthesis catalyzed by DNA polymerase γ, the central enzyme for replication of mitochondrial DNA. DNA polymerase γ is normally very accurate for substitution errors due to the presence of a highly active proofreading exonuclease (Kunkel & Soni, 1988). This determination is particularly intriguing because oxygen radicals are a natural byproduct of the extensive oxidative phosphorylation that occurs in mitochondria and hence might generate mutagenic nucleotide precursors in this organelle. Mutagenesis induced by 8-O-dGTP during mitochrondrial DNA replication could contribute to the degenerative diseases associated with mitochondrial DNA mutations [for a review, see Wallace (1992)].

EXPERIMENTAL PROCEDURES

Bacterial Strains and Reagents. *E. coli* strains CSH50, NR9099, NR9162, and MC1061 were as described (Kunkel & Soni, 1988). *E. coli* strain CC104 *mutM*⁻ (*ara* Δ(*gpt-lac*)$_5$ *rpsL* [*F′lacI378 lacZ461 proA*⁺*B*⁺] *mutM*) was from Jeffrey H. Miller (UCLA). NR9373 (*ara thi* Δ(*pro-lac*) *mutY*::*Tn5*) was provided by Roel M. Schaaper (NIEHS). A mutant strain deficient in both *mutM* and *mutY* was made by P1 transduction of *mutM* with a P1 lysate grown on NR9373, selecting for tetracycline and kanamycin resistance. Resulting transductants were colony-purified and tested for spontaneous mutation rate, which was found to be 1000-fold higher than in the parent strain, in agreement with a previous study (Michaels *et al.*, 1992a). The 8-O-dGTP was prepared as described (Mo *et al.*, 1992) using a 4-μm Waters Radial NovaPack column instead of a Spherisorb SAX column. The sources of all other reagents and the preparation of DNA substrates were as described (Kunkel & Soni, 1988; Roberts & Kunkel, 1993).

DNA Polymerases. The wild-type large (Klenow) fragment of *E. coli* DNA polymerase I and its exonuclease-deficient variant (D355A, E357A changes; Derbyshire *et al.*, 1988) were from U.S. Biochemical Corp. (Cleveland, OH). The thermostable DNA polymerase from *Thermus thermophilus* (Bechtereva *et al.*, 1989) was from Biomaster (Moscow, Russia) or Amersham. Wild-type T4 DNA polymerase and its exonuclease-deficient derivative (containing a D219A change; West Frey *et al.*, 1993) were gifts from Michelle West Frey and Stephen J. Benkovic (Pennsylvania State University). Chick embryo DNA polymerase γ was described previously (Kunkel & Soni, 1988). For replication studies, HeLa cell cytoplasmic extract was prepared according to the method of Li and Kelly (1985). SV40 T antigen was purchased from Molecular Biology Resources.

DNA Synthesis Reactions. Reactions (25 μL) contained equimolar concentrations of dATP, dGTP, dCTP, TTP, and 8-O-dGTP, either 50 μM (for Klenow, T4, and *Tth* polymerases) or 100 μM (for pol γ and SV40 replication reactions). DNA polymerase reactions contained 150 ng (~67 fmol) of gapped M13mp2 DNA; the replication reaction contained 40 ng of double-stranded M13mp2 SV DNA. Other reaction components, incubation temperatures, and time were as follows. Klenow polymerase: 20 mM Hepes (pH 7.8), 2 mM

Table 1: Fidelity of Exonuclease-Deficient Klenow Polymerase in the Presence of 8-O-dGTP[a]

strain	8-O-dGTP	plaques total	plaques mutant	mutant freq ($\times 10^{-4}$)
wild-type	–	12570	116	92
	+	2152	107	500
mutM$^-$	–	7250	53	73
	+	2387	96	400
mutM$^-$*Y*$^-$	–	4215	39	93
	+	2923	47	160

[a] Reactions, electroporations, and platings were performed as described under Experimental Procedures.

dithiothreitol, 10 mM $MgCl_2$, and 0.7 pmol of polymerase; 37 °C; 10 min. T4 DNA polymerase: 67 mM Tris-HCl (pH 8.8), 10 mM β-mercaptoethanol, 5 mM $MgCl_2$, 60 mM NaCl, and 0.9 pmol of polymerase; 37 °C; 30 min. *Tth* polymerase: 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 10 mM $MgCl_2$, and 0.2 pmol of polymerase; 70 °C; 10 min. DNA polymerase γ: 20 mM Tris-HCl (pH 8.0), 2 mM dithiothreitol, 10 mM $MgCl_2$, and 5 units of polymerase; 37 °C; 30 min. Replication reaction: 30 mM Hepes (pH 7.8), 7 mM $MgCl_2$, 4 mM ATP, 200 μM CTP, GTP, and UTP, [α-^{32}P]dCTP (4000 cpm/pmol), 40 mM creatine phosphate, 100 μg of creatine phosphokinase/mL, 15 mM sodium phosphate (pH 7.5), 1 μg of SV40 T antigen, and 10 μL of extract; 37 °C; 6 h.

Product Analysis. Polymerase reactions were terminated with EDTA, and a portion of each reaction was analyzed by agarose gel electrophoresis (Kunkel, 1985a) to monitor the extent of synthesis. In all cases, the products migrated coincident with the full-length circular DNA standard, indicating that the 390-nucleotide gap molecules had been filled. Replication reaction products were analyzed by diagnostic restriction endonuclease digestion and agarose gel electrophoresis as described (Roberts & Kunkel, 1993). Aliquots of each reaction were used for electroporation of competent cells as described (Roberts & Kunkel, 1993).

Fidelity Assay. The assay scores errors during synthesis of the minus-strand of the wild-type *lacZ*α gene sequence in M13mp2. Correct polymerization during gap-filling synthesis by purified polymerases or during SV40 origin-dependent replication of double-stranded DNA produces DNA that yields dark blue M13 plaques upon transfection of an appropriate *E. coli* host strain followed by plating on indicator plates. Errors are scored as lighter blue or colorless plaques. All possible types of substitutions as well as frameshifts at many different sites can be scored (Roberts & Kunkel, 1993).

RESULTS

Misincorporation of 8-O-dGTP during Synthesis by Exonuclease-Deficient Klenow Polymerase. To examine the mutagenic potential of 8-O-dGTP during DNA synthesis *in vitro*, we began with the well-defined enzyme studies by Cheng *et al.* (1992) of the Klenow fragment of *E. coli* DNA polymerase I. This polymerase (Table 1) is devoid of exonucleolytic proofreading activity by virtue of the double amino acid change D355A,E357A within the exonuclease active site. The enzyme is otherwise normal both structurally and kinetically (Derbyshire *et al.*, 1988), and its fidelity with normal dNTP substrates has been extensively characterized (Bebenek *et al.*, 1990; Joyce *et al.*, 1992; Eger & Benkovic, 1992).

In reactions using M13mp2 DNA with a 390-nucleotide single-stranded gap containing the *lacZ* α-complementation sequence, gap-filling DNA synthesis with the D355A,E357A Klenow polymerase and normal dNTPs generates reaction products that, upon electroporation of a wild-type *E. coli* α-complementation strain, yield a mutant frequency of 92×10^{-4} (Table 1). Sequence analysis of over 100 independent mutants demonstrated that none contained an A → C transversion. Thus, this polymerase rarely misincorporates dGTP opposite template A.

In a parallel reaction, inclusion of 8-O-dGTP at a concentration equal to that of the four normal dNTPs yielded a 5-fold increase in mutant frequency (Table 1). Sequence analysis of 19 mutants demonstrated that 17 contained one or more A → C substitutions. These data are consistent with formation of A·8-O-dGMP mispairs during synthesis *in vitro*. They also demonstrate that the mispair survived transfection and was expressed, *i.e.*, that the newly incorporated 8-O-dGMP templated the incorporation of dCTP during subsequent replication *in vivo*. These data are completely consistent with observations reported by Cheng *et al.* (1988).

At least two repair activities in *E. coli* could influence recovery of mutants. The *MutM* gene encodes a glycosylase that removes 8-O-dGMP from a C·8-O-dGMP pair (Tchou *et al.*, 1991). The *MutY* gene encodes an adenine glycosylase (Au *et al.*, 1988) that removes the adenine from a A·8-O-dGMP mispair (Michaels *et al.*, 1992a,b; Moriya & Grollman, 1993). Introduction of aliquots of the polymerase reaction products into a *mutM*$^-$ strain yielded mutant frequencies that were similar to those obtained with the wild-type strain (Table 1). Thus, the MutM gene product does not substantially affect the ability to score mutants originating from misincorporation of 8-O-dGTP during synthesis *in vitro*. Introduction of an aliquot of the 8-O-dGTP-containing reaction products into a *mutM*$^-$*mutY*$^-$ double-mutant strain yielded mutant frequencies lower than those obtained with the wild-type strain (Table 1, compare 160×10^{-4} to 500×10^{-4}). This antimutator effect is consistent with the possibility that, upon transfection of DNA into a wild-type strain, the glycosylase removes the "correct" (*i.e.*, adenine) nucleotide from the A·8-O-dGMP mispair made *in vitro*, which is then replaced by dCMP during repair synthesis *in vivo*. Thus, *MutY* action in the wild-type strain actually enhances the ability to score misincorporation of 8-O-dGTP *in vitro*.

Incorporation of 8-O-dGTP opposite template C during DNA synthesis *in vitro*, followed by misincorporation of dAMP opposite template 8-O-dGMP during subsequent replication *in vivo*, would yield C·G → A·T transversions. When the products of the Klenow polymerase reactions were used to transfect the wild-type *E. coli* strain and DNAs from 40 independent mutants were sequenced, none contained C·G → A·T transversions. This does not exclude the possibility that 8-O-dGTP may be incorporated opposite template C, since this base pair could be converted to a normal C·G pair *in vivo* by MutM action or by replication. Alternative approaches will be required to study misincorporation of 8-O-dGTP opposite template C. Nonetheless, the data with the exonuclease-deficient Klenow polymerase validate the M13mp2 forward mutation assay for addressing several issues on misincorporation of 8-O-dGMP opposite adenine.

Contribution of Base Selectivity to 8-O-dGTP Misincorporation Error Rate. We first wanted to examine the role of DNA polymerase selectivity in determining the degree of 8-O-dGTP-induced infidelity, in the absence of proofreading activity. To do so, we performed reactions with and without 8-O-dGTP using three different proofreading-deficient DNA polymerases, the 3′ → 5′ exonuclease-deficient mutant forms

Table 2: Differential Mutagenic Potential of 8-O-dGTP with Prokaryotic DNA Polymerases[a]

DNA polymerase	mutant frequency ($\times 10^{-4}$)			
	exonuclease-deficient		exonuclease-proficient	
	− 8-O-dGTP	+ 8-O-dGTP	− 8-O-dGTP	+ 8-O-dGTP
Klenow	92	500	27	340
T4 (expt 1)	70	120	7	22
(expt 2)	46	120	8	19
Tth	73	1100		

[a] Reactions were performed as described under Experimental Procedures. All frequencies are derived from scoring several thousand plaques per variable. The data in Table 1 represent typical results.

of Klenow polymerase and bacteriophage T4 DNA polymerase, and the normally exonuclease-deficient thermostable *Tth* DNA polymerase. All three enzymes generate higher mutant frequencies in reactions containing the base analog than in reactions that do not (compare the second and third columns in Table 2). The differences were 5.4- and 15-fold for Klenow and *Tth* polymerases, respectively. Since the difference was only 1.7-fold in the initial experiment with T4 DNA polymerase (120×10^{-4} vs 70×10^{-4}), a second experiment was performed. This experiment (T4 experiment 2 in Table 2) confirmed the small 8-O-dGTP-dependent effect. It also illustrates, as in a number of previous studies [see Thomas *et al.* (1991)], that M13-based transfection assays are reproducible, with repeat experiments seldom varying by more that 50% and multiple determinations yielding standard deviations less than 20% of mean values.

Previous studies have shown that DNA polymerases generate a variety of errors during synthesis with normal dNTPs. To quantitatively establish the full miscoding potential of 8-O-dGTP, sequence analysis of mutants obtained from reactions with and without the analog was performed. The results (Table 3) revealed that the increases resulted predominantly from A → C substitutions. The analog-dependent increases in error rate were ≥600-, ≥70-, and ≥730-fold for the exonuclease-deficient Klenow, T4, and *Tth* polymerase, respectively. Thus, these three DNA polymerases all misincorporate 8-O-dGTP opposite template adenine. However, they do so at rates that vary by more than 25-fold, ranging from 2.8×10^{-4} for exonuclease-deficient T4 DNA polymerase to 73×10^{-4} for *Tth* DNA polymerase (Table 3). In this type of fidelity assay, these rates reflect the probability of misinsertion followed by successful extension to fix the mispair. Thus, the observed enzyme-dependent differences could result from rate differences at one or both steps in the reaction.

As of this writing, there are 25 template adenines in the *lacZ* α-complementation reporter gene used in this study at which A → C substitutions can be detected as a mutant plaque phenotype. The sequence analysis of mutants from reactions containing 8-O-dGTP revealed a distinctly nonrandom distribution of misincorporation of the base analog. For example, 4 of the 17 A → C substitutions (24%) recovered from reactions with the exonuclease-deficient Klenow polymerase were at one position, a template adenine at position 109 (where +1 is the first transcribed nucleotide of the gene). Similarly, 4 of the 16 A → C substitutions (25%) recovered from reactions with the exonuclease-deficient T4 DNA polymerase were at a single site. However, this was a different nucleotide, a template adenine at position 130.

Contribution of Exonucleolytic Proofreading to 8-O-dGTP Misincorporation Error Rate. We next wanted to use this model system to determine whether 8-O-dGMP, once inserted opposite adenine, would be removed by the exonucleolytic proofreading activity associated with DNA polymerases. To examine this possibility, we made pairwise comparisons using two different wild-type DNA polymerases and their exonuclease-deficient counterparts. For the first comparison we used the Klenow polymerase, where the proofreading deficiency of the mutant enzyme results from two amino acid differences in the exonuclease active site. Since these changes affect neither the structure of the polymerase active site nor polymerization activity (Derbyshire *et al.*, 1988), any differences in fidelity are interpreted as reflecting the proofreading defect rather than altered base selectivity, although the latter cannot be completely excluded.

Using identical reaction conditions, both wild-type and exonuclease-deficient Klenow polymerases yield a high rate of A → C errors in reactions performed with 8-O-dGTP (Tables 2 and 3). However, the rate is only slightly higher for the exonuclease-deficient enzyme (compare 19×10^{-4} to 30×10^{-4} in Table 3), suggesting that proofreading of A·8-O-dGMP mispairs by the exonuclease activity of Klenow polymerase is inefficient.

The second comparison uses T4 DNA polymerase. The wild-type enzyme has a 3′ → 5′ exonuclease, while its mutant counterpart does not (West Frey *et al.*, 1993) as a result of a change of an amino acid (D219A) that is highly conserved among DNA polymerases containing associated 3′ → 5′ exonuclease activity. Under identical reaction conditions, both forms of the T4 polymerase yield A → C errors in reactions performed with 8-O-dGTP (Table 3). However, the rate with the mutant polymerase is 4.5-fold higher than for the wild-type enzyme (compare 0.6×10^{-4} to 2.7×10^{-4} in Table 3). This suggests that more than 80% of misinsertions of 8-O-dGMP opposite template adenine by the T4 DNA polymerase are proofread by its associated exonuclease activity. (Again, this interpretation assumes no difference in polymerase base selectivity between the wild-type and mutant enzyme.) However, even in a polymerization reaction where proofreading would contribute greatly to accuracy for mispairs involving normal dNTPs, 8-O-dGTP retains a high mutagenic potential.

8-O-dGTP Misincorporation Error Rates during Eukaryotic Replication. To examine whether 8-O-dGTP is mutagenic during eukaryotic DNA replication, we used the simian virus 40 (SV40) replication system. Inclusion of 8-O-dGTP in a replication reaction at a concentration equal to that of the other dNTPs reduces replication efficiency by less than 2-fold, as measured by incorporation of radiolabeled dCTP. When analyzed by agarose gel electrophoresis, the products of reactions containing 8-O-dGTP (not shown) are indistinguishable from those obtained with normal dNTPs [e.g., see Figure 1 in Roberts and Kunkel (1988)]. The monomer-length circular products are resistant to digestion by the restriction endonuclease *DpnI* and are thus inferred to be the hemimethylated products of semiconservative replication.

The results of transfection of these products are shown in Table 4. In the first experiment, the mutant frequency is 2.8-fold higher for products of reactions containing 8-O-dGTP than for replication with normal dNTPs (compare 23×10^{-4} to 8.2×10^{-4} in Table 4). This 8-O-dGTP-dependent increase is T antigen-dependent (not shown), demonstrating that it reflects replication errors. We had previously found that replication using normal dNTPs is accurate for base substitution errors (Roberts & Kunkel, 1988; Thomas *et al.*, 1991), including the A·T → C·G substitutions characteristic of misincorporated 8-O-dGTP. In contrast, sequence analysis of mutants from reactions containing 8-O-dGTP suggests that

Table 3: Error Rates for A→C Transversions with Several DNA Polymerases

	without 8-O-dGTP			with 8-O-dGTP		
	mutants sequenced			mutants sequenced		
DNA polymerase	total	A→C	error rate[a] for A→C (×10⁻⁴)	total	A→C	error rate[a] for A→C (×10⁻⁴)
wild-type Klenow	ND[b]	ND		20[c]	17	19
exo⁻ Klenow	118	0	≤0.05	19[c]	17	30
wild-type T4	13	0	≤0.04	32	15	0.6
exo⁻ T4	104	0	≤0.04	47	16	2.7
Tth	50	0	≤0.10	20[c]	20	73
poly γ	162[d]	1[d]	~0.01	20[c]	20	37

[a] The error rate was calculated by multiplying the mutant frequencies from Table 2 by the proportion of mutants containing A→C substitutions, dividing by 0.6 to correct for expression of errors in these *E. coli* cells (Kunkel & Soni, 1988), and dividing by 25, the number of sites at which A→C substitutions can be detected. [b] ND, not determined. [c] Because some mutants contained more than one A→C substitution, the error rate is a minimum estimate. [d] From Kunkel (1985b).

Table 4: Mutagenic Potential of 8-O-dGTP during Replication of Double-Stranded DNA

	plaques				
addition	total	mutant	mutant freq (×10⁻⁴)	sequencing (A·T→C·G per total sequenced)	error rate[a] (×10⁻⁶)
expt 1					
normal dNTPs[b]	47 635	39	8.2	2/78	≤0.9[c]
plus 8-O-dGTP	11 988	28	23	13/27	46
100 × dGTP	45 830	79	17	0/20	≤3.5
expt 2					
normal dNTPs	16 699	27	16[d]	ND[e]	
plus 8-O-dGTP	12 802	74	58	25/59[f]	100

[a] Calculated as described in the footnote to Table 3, but expression of errors upon transfection is 50% (Roberts & Kunkel, 1988) and there are 48 detectable sites for A→C errors, 25 on the plus strand and 23 on the minus strand (Roberts & Kunkel, 1993; and this study). [b] From Thomas *et al.* (1991). [c] This is a "≤" value because it differs by less than 2-fold from the background frequency obtained with unreplicated DNA [see Thomas *et al.* (1991)]. [d] This frequency is from an original plating of transfected cells. Typically when mutant candidates are replated, some are found to be plating artifacts. In addition, a few mutants are sequenced and found to have no change in the gene from position −84 to 170. Thus, this frequency may slightly overestimate the actual α-complementation mutant frequency. [e] ND, not determined. [f] Slightly fewer than half the mutants analyzed from 8-O-dGTP-containing replication reactions contained A·T→C·G substitutions. Of the remaining 48 mutants from experiments 1 and 2, 41 had no change within the sequenced analyzed. These could have had A→C substitutions or other changes downstream in the unsequenced region of the *lacZ* α-complementation gene in the vector.

the rate for A·T → C·G substitutions increases at least 50-fold when 8-O-dGTP is present during replication. When the experiment was performed a second time (Table 4, experiment 2), the estimated error rate with 8-O-dGTP was slightly higher. These A·T → C·G substitution rates with equimolar 8-O-dGTP are much higher than that obtained from a reaction containing no 8-O-dGTP but a 100-fold excess of normal dGTP (1000 μM) over the other three normal dNTPs (10 μM each). This illustrates the substantial mutagenic potential of 8-O-dGTP during replication of double-stranded DNA.

Because oxygen radicals are a natural byproduct of the extensive oxidative phosphorylation that occurs in mitochondria and hence might generate mutagenic nucleotide precursors in this organelle, we also inquired whether 8-O-dGTP was mutagenic with the replicative DNA polymerase for the mitochrondrial genome, DNA polymerase γ. Transfection of the DNA products of reactions performed with and without 8-O-dGTP yielded mutant frequencies of 25 × 10^{-4} and 560 × 10^{-4}, respectively. Sequence analysis of mutants (Table 3) indicates that the A → C substitution error rate for chick embryo pol γ is increased more than 1000-fold for synthesis in the presence of the analog at a concentration equal to that of the normal dNTP. This increase occurs despite the presence of an associated proofreading exonuclease (Kunkel & Soni, 1988) which renders the polymerase highly accurate during DNA synthesis with normal dNTPs.

DISCUSSION

The present study addresses several issues regarding the mutagenic incorporation of 8-O-dGTP. First, the data in Tables 2 and 3 extend earlier studies by Cheng *et al.* (1992) and Maki and Sekiguchi (1992) by demonstrating that this modified nucleotide is primarily misincorporated opposite template adenine by a variety of DNA polymerases. The data provide a quantitative estimate of the average rate of stable misincorporation at 25 detectable template adenines, when 8-O-dGTP is present at a concentration equimolar to the four normal dNTPs. This provides a basis for a future estimation of the mutagenic potential of 8-O-dGTP *in vivo* in relation to the concentration of the normal dNTPs, if indeed 8-O-dGTP is found to be stably present in the dNTP pool.

The different error rates obtained with exonuclease-deficient DNA polymerases (Table 3) illustrate that the analog's mutagenic potential in model polymerization reactions depends on the DNA polymerase catalyzing the reaction. The limited error specificity data described above, *i.e.*, the "hot spots" observed with two different DNA polymerases, also suggest that, as for errors with normal dNTPs [e.g., Joyce *et al.* (1992); for reviews, see Kunkel and Bebenek (1988) and Echols and Goodman (1991)], the rate of misincorporation of 8-O-dGTP depends on the surrounding sequence. Extrapolating to the *in vivo* situation, 8-O-dGTP-dependent mutagenesis may vary depending on where it is misincorporated and on which of the several replicative and repair DNA polymerases utilizes it. The unique signature of 8-O-dGTP-dependent mutagenesis, A·T → C·G transversions, makes this compound an ideal choice for model studies to try to understand how sequence context affects base selectivity and perhaps proofreading activity and to examine fidelity during different phases of replication (Kunkel, 1992). The high rate of A·T → C·G transversions generated in reactions catalyzed by the *Tth* DNA polymerase at 72 °C suggests that this base analog may also be useful for

in vitro mutagenesis of genes using the polymerase chain reaction.

The observation that the rate for A·8-O-dGMP errors is 4.5-fold higher for exonuclease-deficient T4 DNA polymerase than for its proofreading-proficient counterpart (Table 3) is consistent with the possibility that 8-O-dGMP misinserted opposite adenine can be excised by the proofreading exonuclease associated with this polymerase. This possibility is also supported by a recent study (Fowler *et al.*, 1992) suggesting that proofreading by the *E. coli dnaQ* gene product (the 3′ → 5′ exonuclease subunit of DNA polymerase III holoenzyme) reduces the frequency of A·T → C·G transversions in *mutT* strains *in vivo*. Comparison of mutant vs wild-type Klenow polymerase, which has a less active 3′ → 5′ exonuclease than does the T4 DNA polymerase, revealed little difference in error rates (Table 3). Interestingly, Shibutani *et al.* (1991) concluded that misinsertions of dAMP opposite template 8-O-G were not proofread by the 3′ → 5′ exonuclease of *E. coli* DNA polymerase I. Perhaps a difference might be observed if the T4 DNA polymerase were used in their approach. However, the situation in the present study is different from their study in that here the modified nucleotide is the incoming dNTP. It is well-known that DNA polymerases can discriminate to different extents against substitutions involving the same two nucleotides depending on which is the template and which is the incoming triphosphate [for a review, see Kunkel and Bebenek (1988)]. Differential polymerase discrimination at the extension step for the two mispairs or different rates of excision of dAMP vs 8-O-dGMP as terminal nucleotide could result in differential proofreading.

The A·T → C·G transversions generated during replication in HeLa cell extracts containing equimolar concentrations of 8-O-dGTP and the four normal dNTPs (Table 4) suggest that it will be mutagenic in human cells if it is indeed stably generated by oxidative stress *in vivo*. The magnitude of the increase shown in Table 4 should not be overinterpreted, because the extract may contain proteins that process the nucleotide analog before or after incorporation. For example, the human protein with 8-O-dGTPase activity (Mo *et al.*, 1992) is likely to be present in HeLa cell extracts and may degrade the analog. Nonetheless, degradation would lead to an underestimate of the mutagenic potential of 8-O-dGTP, and the analog is clearly highly mutagenic during SV40 origin-dependent replication. In fact, at equimolar concentration, it is much more mutagenic for A·T → C·G transversions than is a 100-fold excess of dGTP. This high degree of mutagenesis occurs despite observations suggesting that mispairs involving undamaged nucleotides are efficiently proofread during replication (Roberts & Kunkel, 1991). It remains to be determined whether A·8-O-dGMP mispairs are proofread during replication in human cells.

The 8-O-dGTP-dependent mutagenesis observed during DNA synthesis catalyzed by eukaryotic DNA polymerase γ is consistent with the possibility that 8-O-dGTP is mutagenic during mitochondrial DNA replication *in vivo*. Again, the high rate of A → C transversions is striking in light of the large contribution to fidelity with normal mispairs provided by the 3′ → 5′ exonuclease activity associated with DNA polymerase γ (Kunkel & Soni, 1988). It is also interesting in light of the substantial oxidative metabolism occurring in mitochondria. Mutagenesis resulting from such metabolism has been suggested to contribute to the degenerative diseases associated with mitochondrial DNA mutations [for a review, see Wallace (1992)]. The present study suggests that one route to mitochondrial mutagenesis may be misincorporation of modified dNTPs. This raises the question of whether enzymes exist to sanitize mitochondrial dNTP pools.

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