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# 8-Hydroxyguanine, an Abundant Form of Oxidative DNA Damage, Causes G → T and A → C Substitutions*





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Mulations caused by oxidative DNA damage may contribute to human disease. A major product of that damage is 8-hydroxyguanine (oh$^8$Gua). Because of differences in experimental design, the base pairing specificity of oh$^8$G *in vivo* is not completely resolved. Here, oh$^8$dGTP and DNA polymerase were used in two complementary bacteriophage plaque color assays to examine the mutagenic specificity of oh$^8$Gua *in vivo*. The first is n reversion assay that detects all three single-base substitutions caused by misreading of guanine analogues inserted at a specific site. oh$^8$Gua at that site gave a mutation frequency of **0.7%**. Twenty-two of the **23** mutations were G → T substitutions.

The second assay, a forward mutation assay, tests the mispairing potential of any altered nucleotide 1) during incorporation as substrate nucleotide, and **2)** after multiple incorporations into a single-stranded DNA gap region of M13mp2. Substituting oh$^8$dGTP for dGTP during polymerization produced 16% mutants; two classes of mutations were observed, both caused by pairing of oh$^8$Gua with A. Seventy-six of **78** mutations were A → C substitutions, and two were G → T substitutions. These assays thus illustrate mutagenic replication of oh$^8$Gua as template causing G → T substitutions and misincorporation of oh$^8$Gua as substrate causing A → C substitutions, both caused by oh$^8$Gua·A mispairs.

Cellular DNA is damaged by oxygen free radicals generated during cellular respiration, cell injury, phagocytosis, and exposure to environmental oxidants (Kasai *et al.*, 1986; Fridovich, 1986; Floyd *et* al., 1986; Klebanoff, 1988; Breimer, 1990). The resultant damage to DNA bases may be a significant source of mutations that lead to cancer and other human pathology (Harman, 1981; Ames, 1983; Pryor, 1986). Because of the multiplicity of DNA modifications produced by oxygen lree radicals (Hutchison, 1981), it has been difficult to establish the frequency and specificity of mutations engendered by individual oxygen-induced DNA lesions.

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The guanine analogue 8-hydroxyguanine (oh$^8$Gua)[1] is an abundant base modification in mammalian DNA whose levels increase with oxidative stress. The amount of oh$^8$Gua in cellular DNA is higher in animals with a higher basal metabolic rate (Shigenaga *et al.*, 1989) and is higher in mitochondrial DNA than in nuclear DNA (Richter *et al.*, 1988). Increased levels of oh$^8$Gua are also observed in DNA from γ-irradiated mouse liver, X-irradiated HeLa cells, and $H_2O_2$-treated *Salmonella typhimurium* (Kasai *et al.*, 1986). It has been estimated that oh$^8$Gua is formed in human cellular DNA as a by-product of normal metabolic processes at the rate of 178 residues/cell/day (Shigenaga *et al.*, 1989). Of **13** DNA base adducts formed alter exposing purified mammalian chromatin to ionizing radiation-generated free radicals, oh$^8$Gua was the most abundant (Gajewski *et al.*, 1990). The accumulation of oh$^8$Gua is also observed in naked DNA exposed to mutagenic and carcinogenic agents that generate oxygen free radicals (Kasai and Nishimura, 1984; Dizdaroglu, 1985; Floyd *et al.*, 1986). The importance of oh$^8$Gua is underscored by the existence of cellular repair of this lesion, as suggested by the decrease in oh$^8$Gua over time in livers of y-irradiated mice (Kasai *et al.*, 1986), by the urinary excretion of oh$^8$Gua and oh$^8$dG in rodents and man (Shigenaga *et al.*, 1989), and by the finding of a glycosylase/endonuclease activity in *Escherichia coli* which removes oh$^8$Gua from DNA (Chung et *al.*, 1991). Based upon these findings and the existence of highly sensitive assays for its detection (Floyd *et al.*, 1986), oh$^8$Gua has been the most widely used marker for oxygen free radical damage to DNA (Shigenaga *et al.*, 1989).

Studies of the mutagenic potential of oh$^8$Gua in a variety of experimental systems have yielded different results. Kuchino *et al.* (1987) found that oh$^8$Gua not only has the potential to pair with any nucleotide *in vitro* but may also cause misinsertions at adjacent pyrimidines. Structural studies by Culp *et al.* (1989) showed that the C6,C8-diketo form of oh$^8$Gua predominates at physiological pH; in *syn* conformation, this structure is capable of pairing with cytosine, adenine, or thymine. Furthermore, the minor, 6-enolate-8-keto form, has the additional potential to pair with guanine (Culp et *al.*, 1989). In contrast, Shibutani et *al.* (1991) found that oh$^8$Gua pairs with either C or A during *in vitro* DNA synthesis and does not cause misinsertion at adjacent bases. Wood et *al.* (1990) demonstrated the ability of oh$^8$Gua to generate G → T transversions in E. coli. However, the phenotypic screen used, based upon the insertion of the oh$^8$dG in place of the G

[1] The abbreviations used are: oh$^8$Gua, 8-hydroxyguanine (also known as 8-oxo-7-hydroxyguanine, referring to the favored 6,8-diketo tautomer at physiological pH); oh$^8$dG, 8-hydroxydeoxyguanosine; oh$^8$dGTP, 8-hydroxydeoxyguanosine triphosphate; oh$^8$dGMP, 8-hydroxydeoxyguaosine monophosphate; TEAB, triethylammonium bicarbonate: HPLC, high pressure liquid chromatography; Pol, polymerase: HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

residue in a 5′-TAG-3′ amber codon, was not designed to detect targeted G → A transitions, which would yield a different stop codon, 5′-TAA-3′. More recently, Moriya et al. (1991) used phosphoramidite synthesis to generate a plasmid construct with a single-stranded gap containing oh⁸Gua at a specific site and, using an oligonucleotide hybridization screen, also detected G → T transversions in E. coli. However, the number of targeted mutations observed was insufficient to assess the possibility that oh⁸Gua also causes other mutations.

Methods used for the insertion of base analogues into DNA test constructs often necessitate conditions potentially degradative to unstable analogues. In both in vitro studies cited above (Kuchino et al., 1987; Shibutani et al., 1991) as well as the *in vivo* studies of Moriya *et al.* (1991), oh⁸Gua was introduced into DNA by phosphoramidite oligonucleotide synthesis, which exposes the DNA to low pH and high temperature during deprotection (Basu and Essigmann, 1988). Wood et al. (1990) achieved site-specific analogue insertion using enzymatic ligation rather than phosphoramidite synthesis, thereby avoiding deprotection steps. However, a 148-h RNA ligase incubation was required to achieve efficient ligation. Such manipulations may have contributed to mutations unrelated to the analogue.

We have synthesized oh⁸dGTP to allow DNA polymerase to insert the analogue into DNA; two independent and complementary bacteriophage assays were used to determine the mutagenic potential of oh⁸Gua in *E.* coli. In the first assay, called the M13G*1 assay[1] (Cheng et al., 1991), oh⁸Gua is site specifically incorporated opposite a template C; all three noncomplementary single-base substitutions can be detected after transfecting and replicating the DNA in E. coli. In the second, a forward mutation assay, oh⁸Gua is incorporated into a single-stranded DNA gap; this allowed us to detect multiple misinsertions of oh⁸dGTP which occur during an *in* vitro incorporation step, as well as to detect miscoding during DNA replication of analogue-containing template in *vivo*. Results from both assays indicate that during DNA replication in E. coli, oh⁸Gua most frequently codes correctly for C, but also has the monospecific mutagenic ability to pair with A to generate transversions at a frequency of about 1%.

## EXPERIMENTAL PROCEDURES

*Materials* and Enzymes—The four normal deoxynucleotide triphosphates and dGMP were obtained from Pharmacia LKB Biotechnology Inc. [γ-$^{32}$P]ATP was obtained from Amersham Corp. Oligonucleotides were synthesized and HPLC-purified by Operon Technologies. The DNA oligomers were 5′-end labeled using T4 polynucleotide kinase (U. S. Biochemical Corp.) and [γ-$^{32}$P]ATP (Sambrook *et al.*, 1989), with an unlabeled ATP chase, resolved by electrophoresis through a 15% denaturing polyacrylamide sequencing gel (Sambrook *et al.*, 1989), and purified by elution from Nensorb 20 columns (Du Pont) with 50% methanol. Uracil-containing single-stranded DNA was prepared by growing the desired phage strains in *E. coli* strain CJ236 (dut *ung*), as described (Sambrook *et al.*, 1989). The uracil-containing DNA was treated with bovine pancreatic RNase A (type IIA, Sigma) at 20 μg/ml for 30 min at 45 °C to hydrolyze contaminating RNA that otherwise hybridizes to the M13 template and serves as primers for DNA synthesis (data not shown). DNA grade Sephadex G-100 was obtained from Pharmacia and Bacto-Agar from Difco. Polyacrylamide was prepared from 40% 19:1 acrylamide:bisacrylamide stock from Fisher. Exonuclease-deficient large fragment mutant enzyme D424A of E. coli DNA Pol I (exo⁻ DNA Pol I, Derbyshire et al., 1988) was kindly sent by C. Joyce (Yale). Native bacteriophage T7 DNA polymerase was obtained from U. S. Biochemicol Corp.

[1] The name refers to the vector's M13mp2 background, and to this being the first assay we have developed specifically for guanine analogues ("G*").

E. *coli* Strains and Media—An F′ derivative of strain CSH50 (Δ(*pro-lac*) *thi ara strA*/F′ (*proAB lacIq-ZΔM15*)), used as plating bacteria, was received from T. Kunkel (NIEHS). Strain NR9161 (*mutL210*::Tn*10 hsdR*⁻ *hsdM*⁺ *araD* Δ(*ara,leu*) Δ*lacIPOZY lacU galK strA*; equivalent to MC1061 *mutL*), used as the competent calcium cells for infectious center plating, was kindly provided by R. Schaaper (NIEHS). Strain CJ236 (*ung-1 dut-1 relA1 spoT1 thi-1*, pCJ105), used to produce uracil-containing single-stranded M13mp2 and M13G*1 DNA, was obtained from Barbara Bachman (E. coli Genetic Stock Center, Yale University). M13G*1 was derived from M13mp2 as described (Cheng *et al.*, 1991). 2XYT medium contains, per liter, 16 g of Bacto-Tryptone, 10 g of Racto yeast extract, and 5 g of NaCl.

*Preparation of oh⁸dGTP*—The general method of conversion of monodeoxyribonucleotide to 5′-triphosphate (Hoard and Ott, 1965) was used to prepare oh⁸dGTP. The rnonodeoxyribonucleotide oh⁸-5′-dGMP was prepared by oxidation of 5′-dGMP (800 mg) in 500 ml of an aqueous solution of ascorbic acid (20 mM), EDTA (20 mM), and $FeSO_4$ (2 mM) at 37 °C for 3 h with gentle shaking under air. The pH of the solution was adjusted to 3.0 and mixed with activated charcoal (5.7 g). The activated charcoal was then packed in a short column (2.5 × 5 cm), washed with 5 column volumes of water, and eluted with 1:1 acetone:water (v/v) in 400 ml. The eluate wns evaporated to dryness, the residue dissolved in 2 ml of water, and the resolubilized nucleotides loaded on a DEAE-Sephadex A-25 column (2.5 × 35 cm). Nucleotides were eluted by a 3-liter linear gradient of 0–0.3 M triethylammonium hicarhonate (TEAR). Elution of oh⁸dGMP, monitored by absorbance at 254 nm, was detected after a large 5′-dGMP peak. The 150-ml oh⁸dGMP fraction was evapoarted to dryness. Addition of water and evaporation was repeated to remove TEAB (yield, 5.8% from starting material).

The phosphorimidazolidate formed from oh⁸dGMP and 1,1′-carbonyldiimidazole was reacted with pyrophosphate to yield oh⁸dGTP (Hoard and Ott. 1965). The products were dissolved in 2 ml of water and loaded on a 2 × 20-cm column of DEAE-Sephadex A-25 pre-equilibrated with 0.05 M TEAB and eluted with a 500-ml linear gradient of 0.05–0.1 M TEAB, followed by another 200 ml of 0.1 M TEAB (Fig. 1*A*). The pooled oh⁸dGTP (57 ml), followed by absorbance at 254 nm, was evaporated to dryness. Addition of water and evaporation were repeated to remove TEAB. An aliquot representing 1/20 of this material was injected into an HPLC column (Beckman Ultrasphere ODS, 5 μm, 4.6 × 250 mm) and eluted with a solution containing 12.5 mM citric acid, 25 mM sodium acetate, 30 mM NaOH, and 10 mM acetic acid (flow rate 1 ml/min; Fig. 1*B*). The oh⁸dGTP fraction, collected in 1 ml, was evaporated to dryness, the residue dissolved in 0.1 ml of water, and the sample desalted by loading on the same HPLC column prewashed with 30 ml of water, and eluted with water. HPLC analysis of the purified product showed that contaminating dGTP (Fig. 1B; retention time 5 min) was completely separated from oh⁸dGTP (retention time 8 min). This HPLC purification was repeated (yield 40% from oh⁸dGMP). The maximal con-

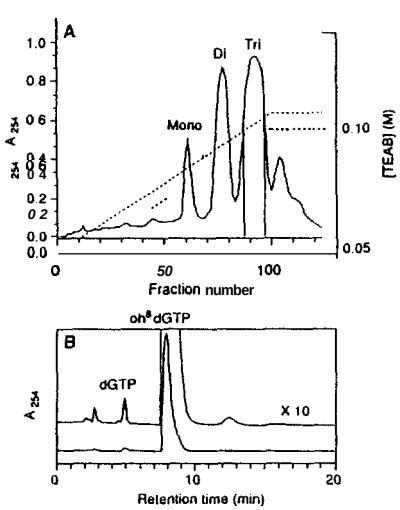


FIG. 1. Synthesis and purification of oh⁸dGTP. oh⁸dGTP was synthesized and purified as described under "Experimental Procedures." A, separation of nucleotide triphosphates by DEAE-Sephadex A-25 column chromatography. *B*, purification of oh⁸dGTP by HPLC.

tamination of final product with dGTP was less than 1 part in 100,000. UV spectra of the mono- and triphosphates matched those of other syntheses that were characterized further by NMR and direct mass spectral analysis by fast atom bombardment (data not shown; Ikehara et al., 1965; Kasai and Nishimura, 1984).

Site-specific Extension of Oligonucleotides—HPLC and gel-purified synthetic 17-mer 5′-d(GCTATTACGCCAGCTGG)-3′ (complementary to nucleotide positions 6321–6337 of the M13G*1 genome; see Cheng et al., 1991, for derivation from M13mp2) was 5′ end labeled with [$\gamma$-$^{32}$P]ATP (Sambrook et al., 1989), with a subsequent addition of unlabeled 5 $\mu$M ATP and incubation for 15 min at 37 °C followed by 10 min at 65 °C. Labeled 17-mer was hybridized to uracil-containing M13G*1 at a 2.5:1 primer:template ratio in 300 mM KCl, 100 mM HEPES, pH 7.3. One $\mu$g of primed template was incubated for 1 h at 30 °C in a 100-pl reaction mixture containing 20 mM Tris-HCl (pH 7.5). 10 mM $MgCl_2$, 23 mM KCl, 2 mM dithiothreitol, 1 unit of exo⁻ DNA Pol I, and 100 $\mu$M oh$^8$dGTP. Thereafter, each of the four normal deoxynucleoside triphosphates was added to the reaction mixture to a final concentration of 250 $\mu$M followed by incubation for 30 min at 37 °C. Four units of T7 native DNA polymerase were then added to the reaction mixtures followed by incubation for 20 min at 37 °C. The reaction was stopped by the addition of EDTA to 15 mM and stored at −20 °C until used for transformation of competent NR9161 cells. An aliquot of 5–20 $\mu$l of the reaction was directly added to 200 $\mu$l of competent *mutL* E. coli strain NR9161 (see below) without further processing.

Preparation of Capped *M13mp2* DNA—The gapped M13mp2 DNA used was composed of an intact single-stranded circular uracil-containing plus (+) strand DNA and a linear minus (−) strand with a 631-base deletion opposite positions 5962–6323 of the Vecbase sequence of M13mp2. This deletion extends from outside the promoter into the amino-terminal portion of the *lacZ*$\alpha$ gene of M13mp2 (to position +145 relative to the start site of transcription). Since this assay scores for decreases or loss of a nonessential gene function ($\beta$-galactosidase a-complementation), a wide variety of mutations, including single-base substitutions, insertions, and deletions, are detected (Kunkel, 1986). In the gapped DNA assay used here, mutations would be detectable from bases −68 to +145 relative to the start site of transcription of the *lacZ*$\alpha$ gene. The incomplete strand was derived from M13mp18, which is essentially the same as M13mp2 except for a restriction enzyme polylinker at the *Eco*RI site in the deleted region. Since the deleted region is very small compared with full-length DNA, we used an M13mp18 construct containing a 1.7-kilobase insert in the deleted region to increase agarose gel resolution between single cut and fully cut plasmid DNA. The desired double-stranded 6835-base pair *Pvu*II restriction endonuclease fragment was combined with uracil-containing single-stranded M13mp2 DNA to produce gapped DNA. Annealing was accomplished by dialyzing the combined DNAs in progressive dilutions of formamide (Lundquist and Olivera, 1982). The desired *Pvu*II fragment and gapped DNAs were purified from 0.8% preparative agarose gels by electroelution (Sambrook et al., 1989) without exposure to potentially mutagenic UV light or ethidium bromide. To do so, 0.8-cm strips were cut from each side of the preparative gels and stained in 0.5 $\mu$g/ml ethidium bromide. The desired bands were visualized in the stained strips and marked by cuts above and below the bands; the corresponding bands were then excised from the unstained preparative gels using the cuts for alignment.

Gap Filling in the *lacZ*$\alpha$ Region of *M13mp2*—The reaction mixtures in the gap-filling assay contained 400 ng of gapped DNA (above), 20 mM Tris-HCl (pH 7.5). 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 units of exo⁻ DNA Pol I, and the indicated concentrations of dNTPs (see Table II). The reactions were incubated for 8 min at 37°C and stopped by adding EDTA to 13 mM. One-$\mu$l aliquots or 1 $\mu$l of 1:10 dilutions were used to transform 200 $\mu$l of competent E. coli (below).

Competent Cell Preparation, Plating, and *Plaque* Purification—Competent NR9161 cells were prepared using $CaCl_2$, and transfection and plating were performed as described previously (McBride et al., 1991). Multiple transformations and platings yielded similar mutation frequencies. In the M13G*1 assay, dark blue plaques representing revertants were scored, purified, and sequenced. In the gap filling assay, light blue and white mutant plaques were restreaked on bacterial lawns for purification but otherwise processed identically to the M13G*1 assay.

## RESULTS

DNA polymerase-mediated analogue insertion was used in two different bacteriophage assays to determine the mutagenic potential of oh$^8$Gua. Our assays differ from the earlier ones in requiring neither oligonucleotide synthesis nor lengthy ligase incubations. The synthesis of oh$^8$dGTP (see "Experimental Procedures") allowed the rapid DNA polymerase-mediated introduction of oh$^8$Gua into bacteriophage constructs. In addition, both assays utilize a uracil-containing template strand to bias for replication of the analogue-containing strand lacking uracil (Kunkel, 1985; Reid *et al.*, 1990). The first assay, the M13G*1 assay, detects all three single base substitutions opposite a single site-specifically incorporated guanine analogue (Figs. 2 and 3*A*). The second scores for forward mutations after the insertion of modified bases at multiple positions within a defined region of a DNA template (Fig. 3*B*).

***Site-specific Mutagenesis*** by *oh$^8$Gua*—To determine the coding specificity of oh$^8$Gua residues in DNA during replication in E. coli we first used a reversion assay, the M13G*1 assay (Figs. 2 and 3*A*). The assay is based upon restoration of @-galactosidase activity by any single-base substitution at the target site, resulting in a change in plaque color from white to blue (Fig. 2). Site-specific incorporation of oh$^8$Gua in M13G*1 was accomplished in a series of reactions (Fig. 3*A*). The uracil-containing single-stranded circular M13G*1 template DNA was first hybridized to an oligonucleotide such that the next base to be added onto the 3′ primer terminus was opposite the target cytosine at position 141. Site-specific insertion of oh$^8$Gua opposite C141 was accomplished using oh$^8$dGTP as the sole nucleotide precursor for exo⁻ DNA Pol I. During this first step, more than 90% of the 17-mer primer was elongated to an 18-mer, with no further extension observed; 10% of the primer remained unextended (data not shown). The nucleotide analogue was then sealed into the nascent strand by the subsequent addition of all four normal dNTPs at high concentration followed by T7 DNA polymerase to generate fully double-stranded product. Native T7 DNA polymerase was chosen because it is highly processive and exhibits little strand displacement during DNA synthesis (Lechner and Richardson, 1983), in contrast to the modified versions of T7 polymerase (Tabor and Richardson, 1989 "Sequenase"). The DNA product was directly transformed into competent E. *coli* to minimize delay and manipulations between analogue insertion and transformation. In control experiments (not shown), we determined that further extension by T7 DNA polymerase caused an increase in transformation efficiency by at least 2 orders of magnitude. Revertant blue plaques were scored, purified, and sequenced (Fig. 3*A*).

The mutation frequency obtained after insertion of oh$^8$Gua at C141 was 32-fold greater than that obtained in controls; 22 of the 23 mutations were G → T transversions (Table I). In contrast, with dG at position 141, eight of the nine mutations were G → A transitions, and none were G → T transversions

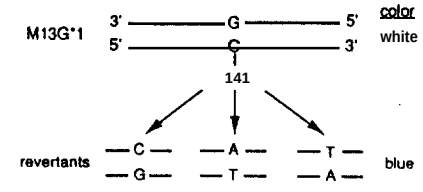


FIG. 2. The M13G*1 site-specific reversion assay for the determination of mutagenic spectrum of guanosine analogues. All three single-base substitutions at the site of analogue insertion at Cys$^{141}$ are detected by a change in plaque color.

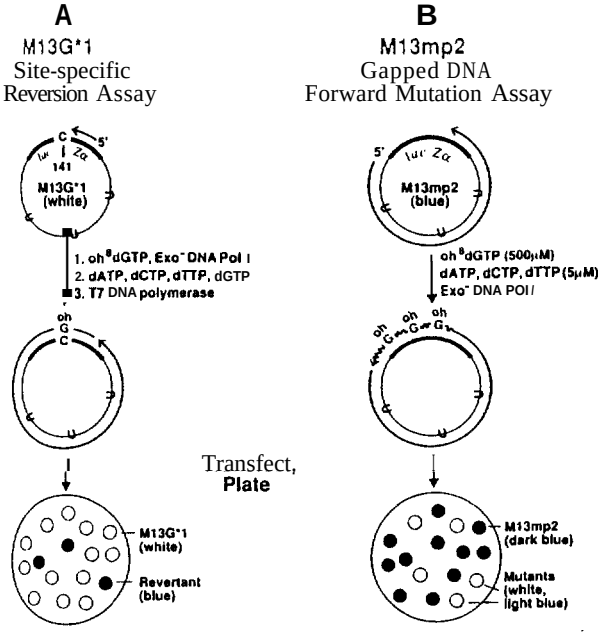


FIG. 3. **Two *in uiuo*** assays for oh⁸Gua mutagenesis. ***Panel A,*** the M13G*1 assay. Uracil-containing plus-strand **M13G*1** was primed with a 17-mer such that the next template nucleotide was $C^{141}$. oh⁸Gua was then inserted opposite $C^{141}$ using DNA polymerase **and** oh⁸dGTP as the only nucleotide substrate. After analogue insertion, all four normal dNTPs and finally native T7 DNA polymerase were added to complete extension. The copied circle was then transfected into competent E. ***coli*** and plated in the presence of isopropyl 1-thio-β-D-galactopyranoside and x-Gal for detection of blue revertant plaques. The revertants were sequenced to determine which of the three possible single-base substitutions gave restoration of β-galactosidase activity. ***Panel B,*** insertion of oh⁸Gua into gapped M13mp2 DNA. Gapped DNA containing uracil in the plus strand was prepared and reactions carried out as described under "Experimental Procedures." Incorporation of oh⁸Gua into the single-stranded gap was catalyzed by exo⁻ DNA Pol I in the presence of oh⁸dGTP, dATP, dCTP, and dTTP. After nucleotide incorporation, the DNA was transfected into competent E. ***coli,*** and mutant DNA from white and light blue plaques was sequenced to localize the inactivating mutations. In both ***A*** and ***B, arrowheads*** outside the ***circles*** represent 3′ DNA termini. In the template plus strand DNAs about **10%** of the thymines have been replaced **by** uracil (Sagher and Strauss, **1983).**

TABLE I

***Site-specific mutagenesis by oh⁸Gua***

In the normal G control, the guanine base was positioned at the target site by hybridization of an 18-mer that contained a 3′-terminal dG. Copying reactions, transfections, platings, mutant purification, and sequencing were performed as outlined in Fig. 2B and detailed under "Experimental Procedures."

| Base at position 141 | No. of plaques screened | % Mutants | Mutation | No. | $10^4$ × Mutation frequency |
|---|---|---|---|---|---|
| oh⁸Gua | 3,010 | 0.76 | G → T | 22 | 72 |
| | | | G → C | 0 | <3 |
| | | | G → A | 1 | ~3 |
| Gua | 37,000 | 0.024 | G → T | 0 | <0.3 |
| | | | G → C | 1 | ~0.3 |
| | | | G → A | 8 | 2 |

(Table I). A combination of experiments, yielding a total of 38,000 plaques, indicates that the background frequency of G → T substitutions at this position in the M13G*1 assay is about $0.13 \times 10^{-4}$, more than 500-fold less than the frequency of $72 \times 10^{-4}$ caused by oh⁸Gua. We sequenced about 175 nucleotides in each **of** the 23 oh⁸dGTP-induced mutations; no untargeted nucleotide sequence alterations were observed. In other experiments, SOS induction did not affect the frequency or spectrum of mutations (data not shown).

***Mutagenic Spectrum of oh⁸Gua in a Forward Mutation*** Assay—To test the mispairing potential of oh⁸Gua in multiple sequence contexts and to examine the potential ability of oh⁸Gua to cause insertions, deletions, or mutations of adjacent bases, we have also scored forward mutations after analogue insertion at multiple sites in a defined region of the *lacZα* gene of M13mp2. Gapped DNA was used to 1) incorporate analogue over a defined region of DNA; 2) increase transformation efficiency of product DNA without further polymerase incubations; and 3) protect phage genes essential for viral viability by making those regions double stranded; polymerization into the double-stranded DNA regions would then require strand displacement synthesis.

By using unbalanced concentrations of nucleotides one can favor the misincorporation of noncomplementary nucleotides (Zakour ***et al.***, 1984). Modified nucleoside triphosphates can be used for DNA polymerase-mediated incorporation into DNA (Eadie ***et al.***, 1984; Preston ***et al.***, 1986). These two principles, together with the use of a 3′ → 5′ exonuclease-deficient DNA polymerase, exo⁻ DNA Pol I (Derbyshire ***et al.***, 1988), and biasing for replication of analogue-containing strand (Kunkel, 1985; Reid ***et al.***, 1990), were combined here in a forward mutation bacteriophage assay that examines 1) base pairing potential of oh⁸dGTP as a substrate, and 2) miscoding potential of analogue as template at multiple sites during DNA replication in E. ***coli*** (Fig. 3*B*). After transfection into *E.* coli, ***lacZα*** mutants are identified by their white or light blue plaque color against a background of nonmutant dark blue plaques.

We first examined the dependence of transformation and mutation frequencies on the ratio of oh⁸dGTP to normal nucleotides (Table II). Substitution of oh⁸dGTP for dGTP in the extension reactions resulted in a decrease in relative transformation efficiency to that obtained for unextended DNA (Table II). This decrease is consistent with poor elongation. Increasing the concentration of oh⁸dGTP increased the frequency of mutations. This concentration dependence suggests that the mutations were caused by the presence of oh⁸dGTP rather than the absence of dGTP. When the concentration of oh⁸dGTP was 100-fold higher than dATP, dCTP, and dTTP, the mutation frequency was 16%. Of 61 mutants sequenced from this reaction, 47 contained single mutations, and 14 contained two to four mutations each. The mutations showed striking specificity and localization (Fig. 4).

Among the 78 base substitutions observed in the gapped DNA forward mutation assay, **76** were A·T → C·G transversions opposite plus (+) strand template adenines within the gap region. Two mutations represented G·C → T·A transversions at positions where the plus strand base was cytosine; these occurred in separate mutants, each containing multiple mutations. No mutations were observed in bases adjacent to putative oh⁸dGMP insertion sites, and no insertions or deletions were detected. The number of mutations was largest near the 3′ primer end of the gap (the ***right side*** of the gap in Fig. 3B) and decreased with distance; the farthest observed was 71 nucleotides from the 3′ end of the gap even though the gap continued for another 290 nucleotides (Fig. 4). This distribution of mutations is consistent with poor elongation from 3′-oh⁸dG termini suggested by the transformation data (see above).

TABLE II

*Transformation efficiencies and mutation frequencies in the M13mp2 gap assay*

**Reactions and transfections were done as detailed under "Experimental Procedures" and outlined in Fig. 2B. The percentages of each type of mutation were calculated on the basis of the total of 78 single-base substitutions among the 61 sequenced mutants from the reaction containing 500 μM oh⁸dGTP. The relative efficiency of transformation refers to the number of plaque-forming units/ng of DNA, divided by the value obtained for the gapped DNA reaction to which no nucleotides were added.**

| [Deoxynucleotide triphosphate] | Relative efficiency of transformation | Mutants | No. of plaques screened | % Mutants | Mutations |
|---|---|---|---|---|---|
| None | 1 | 0 | 319 | <0.3 | |
| 5 μM dATP, dCTP, dTTP, dGTP | 10 | 0 | 1,222 | <0.08 | |
| 5 μM dATP, dCTP, dTTP; 5 μM oh⁸dGTP | 1 | 0 | 417 | <0.2 | |
| 5 μM dATP, dCTP, dTTP; 50 μM oh⁸dGTP | 1 | 2 | 398 | 0.5 | |
| 5 μM dATP, dCTP, dTTP; 500 μM oh⁸dGTP | 1 | 61 | 385 | 16 | 97% A·T → C·G<br>3% C·G → A·T |

' Not sequenced.

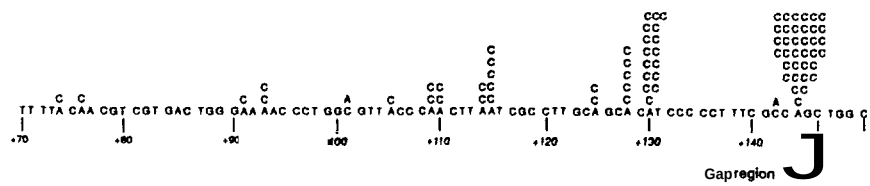


FIG. 4. **Spectrum of oh⁸Gua-induced mutations. A total of 61 mutants obtained from the gapped DNA assay (Fig. 2B) were sequenced. Some of the mutants contained more than one mutation, yielding a total of 78 mutations, all single-base substitutions. These mutations (plus strand changes shown) are indicated above the wild-type sequence shown here, which represents the template plus (+) strand in the *lacZα* gene; the *numbers* refer to position from the start of *lacZ* transcription. Although the gap is 361 nucleotides long (further than the sequence shown here), all of the mutations were present within the first 71 nucleotides of the start of the gap.**

## DISCUSSION

The nonspecific coding of oh⁸Gua and loss of coding specificity at adjacent pyrimidines, observed by Kuchino et al. (1987) in ***vitro,*** and the promiscuous base pairing potential of oh⁸Gua suggested by Culp et ***al.*** (1989) make it important to establish whether a similarly wide spectrum of mutations may also be induced ***in vivo.*** Studies in E. **coli** by Wood ***et al.*** (1990) and Moriya ***et*** al. (1991), which demonstrate targeted G → T transversions, partially answer this question. To ascertain the mutagenic potential of oh⁸Gua during replication of oh⁸Gua-containing DNA in ***uiuo*** we have used two genetic assays in which the placement of oh⁸Gua in double-stranded DNA was accomplished using oh⁸dGTP as substrate for DNA polymerase (Fig. 3). In a site-specific assay, the M13G*1 assay (Figs. 2 and ***3A)*** we observed that a single oh⁸Gua raised the mutation frequency more than ***30-fold*** above background to 0.7%; 22 of 23 mutations were G → T transversions (Table I). No G → T transversions appeared among nine control mutants.

in the second assay, utilizing a double-stranded DNA with a single-stranded gap (Fig. 3*B*), mutations were scored after polymerase-mediated incorporation of analogue into the minus strand of the promoter/amino-terminal region of the *lacZα* gene. To bias for replication of the analogue-containing strand during replication in E. ***coli,*** uracil is present in the template (+) strand but not in the gapped (–) strand (Kunkel, 1985; Reid et *al.*, 1990). In this forward mutation assay, insertions, deletions, and single base substitutions are detectable, as are multiple mutations in single mutants. We observed only two categories of single-base substitutions, however. In the first category, mutagenic pairing of the analogue with template adenine occurred ***in vitro*** during incorporation of analogue as incoming base. In the second category of mutants, mutagenic pairing of A with template oh⁸Gua occurred during ***in vivo*** DNA replication after correct insertion in multiple sequence contexts.

The first and most abundant category of oh⁸Gua mutants in the gapped DNA assay was at plus strand **A** positions. Here, oh⁸Gua as an incoming base paired with template adenine during the ***in vitro*** DNA replication step. The insertion of oh⁸Gua in the new minus strand was likely followed by pairing of the oh⁸Gua with C during replication in E. **coli** to generate A → C transversions (Table **II** and Fig. **4). Other** sources of these mutations are unlikely. Pairing between **the** new minus strand oh⁸Gua with dATP during E. ***coli*** replication would restore the original sequence and not register as **a** mutation. Repair **of oh⁸Gua to G appears unlikely because** of resistance of oh⁸Gua to excision by oh⁸Gua DNA glycosylase when paired with A (Tchou ***et al.,*** 1991), as well as to the resistance of 3′-terminal A to 3′ → 5′ exonucleolytic proofreading by DNA polymerase (Shibutani ***et al.***, 1991).

The second type of mutation in the gapped DNA assay is analogous to those occurring in the M13G*1 assay; these occurred at plus strand C positions. Here, oh⁸Gua was first inserted ***in vitro*** in the minus strand across from plus strand C positions. During DNA replication in E. **coli** the minus strand oh⁸Gua miscoded for A, producing G → T transversions.

From the numbers and types of mutations within the gap region, one may derive an ***in vivo*** estimate of the average frequency of mutations during replication of oh⁸Gua-containing DNA. Consider a hypothetical mutant (below) with a single mutation M opposite template A, in which 1) sequence between the mutation and the 3′ end of the original single stranded DNA gap is shown; 2) the lower strand is the plus strand; and 3) X in the plus strand represents any nucleotide except C.

3′. . .MXXXGXXXGXXXXXXXGXXX. . .5′

5′. . .AXXXCXXXCXXXXXXXCXXX. . .3′

During synthesis of the upper, minus strand, DNA polymerase

encountered three template Cs as it proceeded from the start of the gap on the right to the mutation site M on the left. Since normal dGTP was not present during DNA synthesis, oh⁸Gua was presumably inserted at each of the plus strand template C sites. In this example, oh⁸Gua coded for C during subsequent DNA replication or was repaired, since no mutations were observed at those template C sites. In our experiments we totaled the number of template Cs between the mutation and the start of the gap in each mutant; for mutants with more than one mutation, we counted from the mutation farthest to the left as drawn above. The sum of all putative oh⁸Gua incorporations opposite template Cs in the 61 mutants (Table II) was 338. Since two template C mutations were observed, the calculated mutation rate is 2/338, or about **0.6%.** This frequency is similar to the mutation frequency of 0.7% observed in the M13G*1 assay.

The gapped DNA assay (Fig. 3B) can be applied to adenine, cytosine, thymine, or guanine deoxynucleoside triphosphate analogues. This approach is dependent upon the ability of the polymerase both to utilize the modified deoxynucleoside triphosphate as a substrate and then to extend DNA from 3' termini containing the modified base. Since mutations in genes necessary for phage production can prevent the detection of concurrent mutations in the *lacZα* target gene, we limited incorporation to the region of the nonessential target gene by the use of gapped DNA, whose double-stranded region covers essential phage genes.

The data from the two assays used here suggest that in ***E. coli,*** oh⁸Gua is nonmutagenic about 99% of the time but at a <1% frequency, yields G·C → T·A transversion mutations by base pairing with A. The G·C → T·A transversions observed here were unlikely to be the result of depurination (Schaaper *et al.,* 1983) or of copying past bulky base lesions (Strauss *et al.,* 1982). Mutagenesis via depurination is SOS dependent; the mutations obtained here were SOS independent. This analogue does not appear to act as a bulky derivative since it can be readily inserted and copied by DNA polymerase. Moreover, the base pairing observed is also consistent with NMR studies showing that oh⁸dG *(anti)* stably pairs with either C *(anti)* (Oda *et al.,* 1991) or, with A *(anti)* in *syn* conformation (Kouchakdjian *et al.,* 1991). Our results are entirely consistent with the *in uitro* results of Shibutani *et* al. (1991), the *in uiuo* results of Wood *et* al. (1990), and the poor repair of oh⁸Gua·A pairs by E. *coli* oh⁸Gua-DNA glycosylase reported by Tchou *et al.* (1991). The relevance of this lesion and its associated mutagenesis *in vivo* is also supported by the G·C → T·A transversion mutator phenotype of a probable mutant in the E. *coli* oh⁸Gua repair enzyme, *mutM* (Cabrera *et al.,* 1988). The *mutM* gene turns out to be the same as *fpg-1,* which encodes the strand-nicking glycosylase for both oh⁸G and the ring-opened form of guanine, **2,6-diamino-4-hydroxy-5*N*-methyl-formamidopyrimidine** (Boiteux *et al.,* 1987; Boiteux and Huisman, 1988; Bailly *et al.,* 1989; Michaels *et al.,* 1991). The radiation-sensitive yeast ***Saccharomyces cereuisiae*** mutant *rad18,* which also exhibits a G·C → T·A transversion mutator phenotype (Kunz *et al.,* 1991), may also be deficient in this enzyme although other mutator mechanisms for G·C → T·A transversions exist (Nghiem *et al.,* 1988).

The A → C substitutions produced by oh⁸dGTP in the gapped DNA assay may have direct relevance *in uiuo.* The E. *coli mutT* mutator, first isolated by Treffers *et al.* (1954), specifically increases the rate of spontaneous A → C substitutions 2–3 orders of magnitude over that of wild type (Schaaper and Dunn, 1987). The 15-kDa protein encoded by *mutT* was initially found to catalyze the hydrolysis of some form of dGTP to dGMP and to suppress the misincorporation of dGTP on poly(dA) by E. *coli* DNA Pol III *in uitro* (Akiyama *et al.,* 1989). The model for A·G mispairing of Topol and Fresco (1976) and work by Bhatnagar *et al.* (1991) with nucleotide analogues suggested that the enzyme might prefer a *syn* tautomer of dGTP, which is the predominant form of oh⁸dG (Culp *et al.,* 1989). In fact, H. Maki and M. Sekiguchi[3] have recently found that the MutT protein hydrolyzes oh⁸dGTP to monophosphate much more efficiently than dGTP. Therefore, this specificity not only accounts for the mutagenic specificity of *mutT* mutators but also accounts for the appearance of oh⁸dG as nucleoside in urine (Shigenaga *et al.,* 1989); *mutM/fpg-1* glycosylase activity, in contrast, releases free base (Bailly *et al.,* 1989). The results from the gapped DNA assay thus implicate oh⁸dGTP as a possible source of oxygen-induced A·T → C·G transversions.

The low mutagenic potential of oh⁸Gua may be caused by repair of oh⁸Gua (Tchou *et* al., 1991) or to low miscoding potential, or both. This low mutagenic potential allows greater survival in the face of the putatively high steady-state number of oh⁸Gua residues in cellular DNA (about $10^5$/cell; Fraga *et al.,* 1990). The presence of this modified base in cellular DNA suggests that excisional repair of oh⁸G is not complete. Hence, oh⁸Gua may still play a major role in the generation of oxygen-induced G·C → T·A transversion mutations.

The mutagenic specificity of oh⁸Gua provides a potential tag for assessing the role of oxygen free radical mutagenesis in human cancer. It is of interest that the spectrum of p53 tumor suppressor gene mutations includes G·C → T·A transversions in about half of non-small cell carcinomas of the lung and nearly three-quarters of primary liver carcinomas (Hollstein et *al.,* 1991). In primary liver carcinoma, which is associated with hepatitis B virus infection, G·C → T·A transversions are the predominant p53 gene mutation. Oxygen free radicals, which are a side product of chronic inflammation (Cerruti and Trump, 1991), may be produced by the many inflammatory cells present in the liver during chronic hepatitis B virus infection (Cotran *et al.,* 1989). If these free radicals contribute to G·C → T·A transversions by the production of oh⁸Gua in liver DNA, therapies designed to reduce damage by oxygen free radicals (Ames, 1983; Imlay and Linn, 1988) during chronic hepatitis would be predicted to delay the onset of primary liver cancer.

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