Genetic effects of oxidative DNA damage: comparative mutagenesis of 7,8-dihydro-8-oxoguanine and 7,8-dihydro-8-oxoadenine in *Escherichia coli*

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Received July 17, 1992; Revised and Accepted October 26, 1992

ABSTRACT

A single 7,8-dihydro-8-oxoguanine (G^{8-oxo} ; 8-hydroxy-guanine) adduct in the *lacZ α* gene of bacteriophage M13 DNA induces a targeted G→T transversion after replication in *Escherichia coli* (Biochemistry, 29, 7024–7031 (1990)). This mutation is thought to be due to the facile formation during DNA synthesis of a G^{8-oxo} -base pair, where G^{8-oxo} is in the syn conformation about the deoxyglycosyl bond. A related modified purine, 7,8-dihydro-8-oxoadenine (A^{8-oxo} ; 8-hydroxy-adenine), is an abundant product found in irradiated and oxidized DNAs. Similar to G^{8-oxo} , as a mononucleoside A^{8-oxo} assumes the syn conformation. This work has assessed the relative mutagenicities of A^{8-oxo} and G^{8-oxo} in the same experimental system. A deoxypentanucleotide containing A^{8-oxo} [d(GCT- A^{8-oxo} G)] was synthesized. After 5'-phosphorylation with [γ - ^{32}P] ATP, the oligomer was ligated into a duplex M13mp19-derived genome at a unique *Nhe*I restriction site. Genomes containing either A^{8-oxo} (at position 6275, [+] strand) or G^{8-oxo} (position 6276) were denatured with heat and introduced into *E.coli* DL7 cells. Analysis of phage DNA from mutant plaques obtained by plating immediately after transformation (infective centers assay) revealed that G^{8-oxo} induced G→T transversions at an apparent frequency of ~0.3%. The frequency and spectrum of mutations observed in DNA sequences derived from 172 mutant plaques arising from the A^{8-oxo} -modified DNA were almost indistinguishable from those generated from transfection of an adenine-containing control genome. We conclude that A^{8-oxo} is at least an order of magnitude less mutagenic than G^{8-oxo} in *E.coli* cells with normal DNA repair capabilities.

INTRODUCTION

It is widely held that endogenously-mediated oxidative damage to cellular DNA may play a central role in both the aging process

and various age-related degenerative diseases, including cancer (1–4). Reactive oxidizing species such as superoxide, the hydroxyl radical, and hydrogen peroxide are formed *in vivo* as byproducts of normal aerobic metabolism (1–4). Although cells possess many defenses against oxidative damage, including chemical anti-oxidants, DNA repair enzymes, catalase, and superoxide dismutase (5–7), it has been estimated that, on average, the genome of a human cell receives *ca.* 10^4 oxidative hits/day (8). In addition to the effects of endogenous reactive oxygen species, DNA damage can also occur if cells are exposed to carcinogens that generate oxygen radicals, including ionizing radiation and some chemical oxidants (4,9). Consequently, these agents have been used extensively to study the spectrum of DNA lesions that are produced following oxidative damage to DNA (examples include strand breaks, DNA-protein cross-links, and base adducts). To date, however, only a few of these lesions have been rigorously characterized (9–11).

In general, little is known regarding the genetic effects of oxidative DNA damage. Recently, however, one adduct, 7,8-dihydro-8-oxoguanine (G^{8-oxo}), has received considerable attention (for a review, see Floyd [3]). This adduct, originally detected in γ - and x-irradiated DNA (12,13), has been shown to be mutagenic both *in vivo* (14–16) and *in vitro* (17,18). The predominant and possibly only mutation induced by G^{8-oxo} is a targeted G→T transversion (14–16,18). *Escherichia coli* cells possess at least one repair enzyme, formamidopyrimidine DNA glycosylase, that excises G^{8-oxo} from DNA (19,20). Recently, an *E.coli* mutator phenotype, *mutM*, has been associated with a defect in the gene encoding this enzyme (21). A possible association between oxidative DNA damage and neoplastic transformation has been suggested from studies that monitored G^{8-oxo} levels in the DNA of rodents being treated with organ specific carcinogens that mediate the formation of oxygen radicals (3). It was found that increases in genomic G^{8-oxo} levels were specific to the target tissue of the carcinogen used.

Although it has been the subject of less study, 7,8-dihydro-8-oxoadenine (A^{8-oxo}), has also been detected in DNA irradiated *in vitro* (22,23) as well as in urine and liver

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samples from *in vivo* sources (24,25). In irradiated DNA, this adduct is present at approximately one-third to one-half the level of G^{8-oxo} (22,23) although it is noteworthy that the relative amounts of the two lesions are dependent on the irradiation conditions used (22,23). Structural studies performed on 7,8-dihydro-8-oxoadenosine (26) have shown that A^{8-oxo}, like G^{8-oxo}, exists predominantly as the 6,8-diketo tautomer at physiological pH. The modified base is also in the syn conformation in this nucleoside. Since these two structural features appear central to the known miscoding properties of G^{8-oxo} (27–30), we speculated that A^{8-oxo} might also be a premutagenic lesion, and that its presence in DNA may contribute to the population of mutations that occur at A·T base pairs in the genomes of cells or viruses that have been exposed to ionizing radiation or chemical oxidation (31–34). Our aim in the work described below was to assess this possibility and also to compare the mutagenicity of A^{8-oxo} with G^{8-oxo}. To achieve this goal, oligonucleotides containing either a single A^{8-oxo} or G^{8-oxo} adduct were synthesized and inserted into the genome of an M13 bacteriophage. Following transfection and replication of these vectors in *E. coli*, mutant progeny were analyzed to determine the relative mutation frequencies and mutational specificities of the two DNA adducts. A^{8-oxo} was found to be less than 10% as mutagenic as its structurally related oxidized purine, G^{8-oxo}.

MATERIALS AND METHODS

Reagents

Bromine, 4-dimethylaminopyridine (DMAP), 4,4'-dimethoxytrityl chloride (DMTrCl), 2-cyanoethyl N,N,N',N'-tetraisopropyl phosphorodiamidite were obtained from Aldrich. Acetic anhydride (Ac₂O), sodium acetate, and pyridine were obtained from Malinkrodt. Pyridine was distilled before use from sodium pellets. Acetic anhydride was distilled neat and stored under an argon atmosphere. The dry acetonitrile used for phosphoramidite synthesis, along with the other chemicals and supports for oligonucleotide synthesis, were obtained from Applied Biosystems. Sephadex G10, T4 polynucleotide kinase, DNA sequencing reagents and modified T7 DNA polymerase were purchased from Pharmacia. Bacterial alkaline phosphatase was from BRL. 2'-Deoxyadenosine, Snake venom phosphodiesterase type II and Sepharose CL-4B were from Sigma. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), isopropyl β-D-thiogalactopyranoside (IPTG), calf intestinal phosphatase and the restriction endonucleases *Stu*I, *Nhe*I and *Bgl*II were obtained from Boehringer Mannheim. The restriction enzymes *Cla*I, *Eco*RI, *Pvu*II, and *Xba*I and T4 DNA ligase were supplied by New England Biolabs. [γ -³²P] ATP (6000 Ci/mmol) was bought from New England Nuclear and [α -³⁵S] dATP was obtained from Amersham. *E. coli* strain DL7 (*lac*⁻) was constructed in this laboratory (35) and *E. coli* GW5100 (*lacZ*, *supE*) was a gift from G. Walker (Department of Biology, MIT, Cambridge, MA).

General methods

Oligonucleotide synthesis by the phosphoramidite procedure was performed with an Applied Biosystems 381A automated DNA synthesizer. Reversed-phase high pressure liquid chromatography (HPLC) was conducted with a Phenomenex Ultremex 5-μm column (25×0.46 cm) that was connected to a Beckman system consisting of twin 114M pumps and a 421A gradient controller. The column eluant was monitored at 260 nm by using a Hewlett Packard 1040A diode-array detector. Strong anion-exchange

HPLC was performed with the same system but used a Spherisorb SAX 5-μm column (25×0.46 cm). Quantitative phosphorimaging analyses were performed with a Molecular Dynamics 400S PhosphorImager. Enzymatic digestion of oligonucleotides (~0.3 OD₂₆₀) with bacterial alkaline phosphatase and snake venom phosphodiesterase was carried out by the method of Fowler *et al.* (36). Restriction enzyme digestions and agarose gel electrophoresis were performed as described in Lasko *et al.* (37). DNA sequencing of single-stranded M13 DNA was carried out by using Pharmacia ³²P-Deaza Sequencing Mixes, modified T7 DNA polymerase and the M13 –40 sequencing primer by the method described by the manufacturer. Sequences were analyzed on denaturing 8% polyacrylamide/bisacrylamide (19:1) gels.

Gas chromatography-mass spectrometry (GC-MS) was performed on a Model 5971A mass selective detector interfaced to a Model 5890 Series II gas chromatograph (both from Hewlett Packard). The injection port, ion source and interface were maintained at 240°C. Separations were carried out on fused silica column (12.5 m×0.20 mm) coated with cross-linked methyl silicone gum phase (film thickness 0.33 μm) (Hewlett Packard). Helium was the carrier gas at an inlet pressure of 37.5 psi. Scan mode of monitoring was performed at 70 eV using the electron-impact mode of ionization. The A^{8-oxo} base fragment was obtained by hydrolyzing the dA^{8-oxo} nucleoside with 0.5 ml of formic acid (98%) in an evacuated and sealed tube at 150°C for 30 min follow by lyophilization and subsequent conversion to the trimethylsilyl (TMS) derivative in a Teflon-capped vial with 40 μl of N,O-bis(trimethylsilyl)-trifluoroacetamide (BSTFA)/acetonitrile/pyridine (2:1:1 v/v) at 130°C for 20 min. The TMS derivatized nucleoside was formed by treated of dA^{8-oxo} with BSTFA/acetonitrile (2:1 v/v) at 85°C for 30 min. After cooling, the derivatized products were injected directly onto the GC without further treatment.

Proton, ¹³C and ³¹P NMR spectra were recorded on 300- and 301-MHz Varian instruments with chemical shifts reported relative to solvent unless otherwise noted. Melting points are uncorrected. Thin layer chromatography (TLC) were performed with Baker-flex silica gel IB-F plates, and all column chromatography was performed with Merck grade 60 (230–400 mesh), with elution under pressure.

8-Bromo-2'-deoxyadenosine. Bromination of 2'-deoxyadenosine was carried out by a modification of the method used by Ikehara and Kaneko (38). 2'-Deoxyadenosine (2.0 g) was suspended in 20 ml 1 M sodium acetate (pH 5.4) and stirred at room temperature. Bromine (2 ml) was dissolved in 40 ml sodium acetate buffer and added drop-wise to the suspension. The reaction was stirred for an additional 2 h at which time it was quenched by adding solid NaHSO₃ until the brown color of the reaction faded. The buffer was adjusted to pH 7.0 and chilled at –20°C for 20 min. The brown solid was collected by filtration, washed with water, and dried on a Kugelrohr apparatus (Aldrich) (60°C, 0.3 mm Hg) overnight: Yield 1.7 g (65%). TLC (CH₃OH/CH₂Cl₂ 10:90), R_f 0.42; UV (CH₃OH), λ_{max} 265 nm (ε 15,500) (lit. [39], 265 nm, 15,900).

2'-Deoxy-7,8-dihydro-N⁶-acetyl adenosin-8-one. 8-Bromo-2'-deoxyadenosine (0.8 g), DMAP (0.08 g), and sodium acetate (1.6 g) were suspended in 23 ml Ac₂O and maintained under an argon atmosphere. The reaction was refluxed for 2 h and cooled for 30 min. At this time the reaction was quenched with saturated Na₂CO₃ (50 ml) and the solution extracted with 2×50

ml ethyl acetate. The combined organic phase was extracted with 2×75 ml saturated Na₂CO₃. The aqueous washes were back extracted with 25 ml ethyl acetate and the combined organic solution was dried over Na₂SO₄, filtered, and concentrated in vacuo. The resulting solid was evaporated twice from toluene prior to being dissolved in 100 ml pyridine/CH₃OH/water (35:10:5). Upon addition of 2 M NaOH (5 ml) the brown solution darkened. After 15 min the reaction was quenched by adjusting the pH of the solution with concentrated acetic acid until it was neutral. The mixture was concentrated to dryness, the solid collected, and washed with CH₂Cl₂. The solid was suspended in water, chilled at -20°C for 15 min, and filtered. The resulting white compound was dried on a Kugelrohr apparatus (60°C, 0.5 mm Hg). Yield 1.0 g (63%). TLC (CH₃OH/CH₂Cl₂, 5:95), R_f 0.46; UV (CH₃OH), λ_{max} 290 nm (ε 11,000); ¹H NMR (DMSO-d₆), 2.06 (m, 1H, H2''), 2.12 (s, 3H, acetyl), 3.05 (qt, 1H, H2'), 3.43 (dd, 1H, H5''), 3.59 (dd, 1H, H5'), 3.78 (m, 1H, H4'), 4.41 (br, 1H, H3'), 4.80 (br, 1H, OH), 5.22 (br, 1H, OH), 6.20 (t, 1H, H1'), 8.39 (s, 1H, H2), 10.27 (br, 1H, NH), 10.83 (br, 1H, NH); ¹³C NMR (DMSO-d₆), 23.09 (Ac), 35.58 (C2'), 62.20 (C5'), 71.10 (C3'), 81.25 (C4'), 87.47 (C1'), 110.98 (C5), 138.03 (C4), 149.61 (C2), 150.16 (C8), 150.95 (C6), 169.39 (Ac).

2'-Deoxy-7,8-dihydro adenosin-8-one. The 2'-Deoxy-7,8-dihydro adenosin-8-one was obtained by dissolving the crude per-acetylated nucleoside described above in 50 ml pyridine/CH₃OH/water (35:10:5) and treating with 10 M NaOH solution (3 ml) at room temperature. After 19 h at room temperature, the pH was adjusted to 7.0, the reaction concentrated to dryness in vacuo and then evaporated twice from toluene. A pure sample for NMR and HPLC was obtained by column chromatography on silica gel eluting with 20% CH₃OH/CH₂Cl₂. TLC (CH₃OH/CH₂Cl₂, 10:90), R_f 0.18; UV (CH₃OH), λ_{max} 270 nm (ε 10,000); ¹H NMR (CD₃OD) 2.12 (m, 1H, H2''), 2.92 (m, 1H, H2'), 3.68 (dd, 1H, H5''), 3.83 (dd, 1H, H5'), 4.02 (d, 1H, H4'), 4.55 (d, 1H, H3'), 6.37 (dd, 1H, H1'), 7.94 (s, 1H, H2); ¹³C NMR (CD₃OD), 39.14 (C2'), 64.54 (C5') 74.04 (C3'), 84.72 (C4'), 89.49 (C1'), 111.61 (C5), 148.55 (C4), 149.63 (C2), 150.00 (C6), 157.78 (C8).

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N⁶-acetyl adenosin-8-one. 2'-Deoxy-7,8-dihydro-N⁶-acetyl adenosin-8-one (0.3 g), DMTrCl (0.7 g) and DMAP (0.002 g) were suspended in pyridine (10 ml) and triethylamine (0.4 ml) under an argon atmosphere. Within 30 min, the stirred solution had changed from a red color to light brown. After 4.5 h, additional DMTrCl (0.35 g) and triethylamine (0.15 ml) were added, and the reaction continued for a further 2.5 h. The reaction was quenched by adding 40 ml saturated NaHCO₃ and 30 ml CH₂Cl₂. The organic layer was collected and the aqueous phase was back extracted with an additional 30 ml CH₂Cl₂. The combined organic layers were washed with 40 ml saturated NaCl solution, dried over Na₂SO₄, filtered, and concentrated to dryness in vacuo. The residue was purified by column chromatography (10 cm×2.5 cm) over 35 g silica gel eluted with CH₂Cl₂/CH₃OH/triethylamine (94:5:1). Column fractions containing the desired product were combined and dried. Yield 0.48 g (81%). TLC (CH₃OH/CH₂Cl₂, 3:97), R_f 0.27; UV(CH₃OH), λ_{max} 285 nm (ε 12,600); ¹H NMR(CD₃OD), 2.11 (m, 1H, H2''), 2.12 (s, 3H, acetyl), 3.04 (m, 1H, H2'), 3.14 (m, 2H, H5' and H5''), 3.70 (d, 6H, DMTr-CH₃), 3.92 (q, 1H,

H4'), 4.48 (q, 1H, H3'), 6.20 (t, 1H, H1'), 6.78 (m, 4H, DMTr), 7.18 (m, 7H,DMTr), 7.34 (d, 2H, DMTr), 8.20 (s, 1H, H2), 10.20 (br, 1H, NH), 10.82 (br, 1H, NH); ¹³C NMR (CD₃OD), 23.81 (Ac), 37.48 (C2'), 56.16 (DMT-OMe), 66.22 (C5'), 73.80 (C3'), 83.62 (C4'), 87.76 (RO-C(Ph)₃ of DMT), 88.02 (C1'), 111.55 (C5), 114.36 (Ph-OMe, C3''), 128.11 (Ph, C4''), 129.02 (Ph, C2''), 129.84 (Ph, C3''), 131.78 (Ph-OMe, C2''), 137.93 (Ph-OMe, C1''), 140.05 (C4), 147.09 (Ph, C1''), 151.82 (C2), 152.57 (C6), 153.58 (C8), 160.46 (Ph-OMe, C4''), 172.68 (Ac).

2'-Deoxy-5'-O-(4,4'-dimethoxytrityl)-7,8-dihydro-N⁶-acetyl adenosin-8-one 3'[(2-cyanoethyl) N,N-diisopropylphosphoramidite]. The DMTr-protected nucleoside (0.07 g) prepared above was dissolved in 0.5 ml CH₃CN. Tetrazole (0.002 g in 50 μl CH₃CN) and 2-cyanoethyl N,N,N'-tetraisopropyl-phosphorodiamidite (50 μl) were added to the solution and the reaction mixture was stirred for 2 h when an additional 50 μl phosphorodiamidite was added. The mixture was stirred for an additional 1 h, when complete conversion of starting material to two spots on TLC was noted. Attempted purification of the product by silica gel chromatography invariably led to substantial degradation of the compound. Consequently the crude mixture was used directly for DNA synthesis. TLC (CH₃OH/CH₂Cl₂, 3:97), R_f 0.42; NMR ³¹P (H₃PO₄), 149.6 148.5.

Synthesis of oligonucleotides

A pentanucleotide containing a single G^{8-oxo} adduct, d(GCTA-G^{8-oxo}), was prepared by a T4 RNA ligase-mediated reaction between the tetramer d(GCTA) and 7,8-dihydro-8-oxo-2'-deoxyguanosine 3',5'-bisphosphate, by the method described in Wood *et al.* (14). An unmodified pentamer, d(GCTAG), was prepared in parallel using d(GCTA) and 2'-deoxyguanosine 3',5'-bisphosphate. An oligonucleotide of the same base sequence but containing a single A^{8-oxo} adduct [d(GCTA^{8-oxo}G)] was prepared by the phosphoramidite method of DNA synthesis using the crude A^{8-oxo}-containing monomer that was synthesized as above. Tetrazole (0.002 g in 50 μl CH₃CN) was added to the mixture immediately before addition of the mixture to the DNA synthesis resin. At this time the automated synthesis cycle was interrupted and the adducted monomer was coupled to the oligonucleotide by passing the solution containing the phosphoramidite over the resin with a syringe for 5 min. The cartridge containing the resin was refitted to the automated synthesizer and the normal cycle resumed. Following synthesis, the product was cleaved from the solid support and deprotected (60°C overnight) with concentrated NH₄OH. The solvent was removed *in vacuo* and the crude oligonucleotide was purified by anion-exchange HPLC (0.1–1.0 M KH₂PO₄ in 5% CH₃OH over 40 min at a flow rate of 1.0 ml/min) followed by reversed-phase HPLC (0–9% CH₃CN in 0.1 M ammonium acetate over 70 min at a flow rate of 1.0 ml/min). The purified oligomer was desalted by passage through a column packed with Sephadex G10 (20×2.5 cm) using water as the eluant. An unadducted pentamer, d(GCTAG), was also prepared by the phosphoramidite method of DNA synthesis. This compound was purified by reversed-phase HPLC only.

Construction of site-specifically modified genomes

Two M13 genomes, containing either a unique *Stu*I [M13+7-(*Stu*I)] or a *Nhe*I restriction site [M13+12-(*Nhe*I)], were prepared from M13mp19 DNA as previously described (14). Gapped-heteroduplex DNA (GHD) that contained a five base gap

in either the (+) or (−) strand and a non-ligatable nick in the strand opposing the gap, was obtained by hybridizing *Stu*I-linearized replicative form (RF) M13+7-(*Stu*I) DNA with *Bgl*II-linearized, 5′-dephosphorylated RF M13+12-(*Nhe*I) DNA (14). Prior to insertion of the site-specifically modified oligomers into the GHD, T4 polynucleotide kinase (30 units) was used to phosphorylate the oligonucleotides (250 ng) with ATP (1 mM) at 37°C for 20 min. At this time, the enzyme was heat-inactivated at 65°C for 15 min. In experiments designed to characterize the singly-adducted ligation products, the oligonucleotides were 5′-phosphorylated with [γ -³²P] ATP (20 μ Ci) and T4 polynucleotide kinase for 20 min at 37°C. Unlabeled ATP was then added to a final concentration of 1 mM and the solution was incubated at 37°C for an additional 20 min.

Phosphorylated oligonucleotides were inserted into the GHD (1 μ g) with T4 DNA ligase using the method of Lasko *et al.* (37) (final volume = 0.1 ml). Radioactive ligation products were purified from the reaction mixture by Sepharose CL-4B chromatography (14). Non-radioactive ligation products that were to be used in bacterial transformation experiments were extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) that had been preequilibrated with 1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 8.0. The aqueous layer was removed, extracted once with butan-1-ol and the DNA precipitated with two volumes of ethanol at −80°C for 20 min. The DNA was pelleted at 15,000 g for 20 min at 4°C, washed with 3 $\times$ 1 ml washes with 80% ethanol, dried, and resuspended in 20 μ l medium salt restriction buffer (40). *Stu*I (10–20 units) was added and the solution incubated for 1.5 h at 37°C. This step was performed to relinearize any parental M13+7-(*Stu*I) DNA that had not produced GHD and had recircularized during the ligation reaction. Following digestion the solution was extracted sequentially with phenol/chloroform/isoamyl alcohol and butan-1-ol as above. The DNA was precipitated, pelleted, washed, and stored dry at 4°C until required for bacterial transformation when the DNA was resuspended in water (5 μ l) and denatured by heating at 100°C for 3.5 min followed by rapid cooling on ice. Analysis of heated samples by gel electrophoresis revealed no detectable duplex DNA in samples about to be transfected into *E. coli*.

Transformation of *E. coli* cells with site-specifically modified single-stranded genomes and mutation analysis

Denatured site-specifically modified genomes, prepared as above, were transfected into competent *E. coli* DL7 cells by the process of electroporation (14). Immediately following electroporation and addition of 1 ml SOC medium (41) to the cell/DNA suspension, a portion of the cells (infective centers) was plated in the presence of *E. coli* GW5100, X-gal, and IPTG (42). The remainder of the cells was incubated for 1.5 h at 37°C to allow for phage replication. At this time the cells were pelleted at 15,000 g and the phage-containing, cell free supernatant was transferred to a new 1.5 ml microcentrifuge tube. A portion of this supernatant (progeny phage) was plated as above and the remainder was stored at 4°C.

The frequency of mutant dark blue plaques that arose in any given transformation experiment was calculated by dividing the number of dark blue plaques observed by the total number of plaques scored in the experiment. Mutation values obtained from plating infective centers were generated from a total of 3,000–5,000 plaques/experiment. Between 15,000–20,000

plaques/experiment were scored to calculate mutation frequencies in the progeny phage population.

Dark blue plaques observed in the sets of plates obtained following transformation or after phage replication were picked and replated to ensure plaque purity. Single dark blue plates arising from these secondary plates were used to prepare single-stranded M13 DNA for sequencing (14).

RESULTS

Synthesis of an oligonucleotide containing a single A^{8-oxo} adduct

The aim of these studies was to investigate the possible mutagenicity of A^{8-oxo}, one of the frequent lesions found in oxidatively-damaged DNA. The strategy we used was to compare its mutagenicity with that of G^{8-oxo}, which had been studied earlier and shown to be mutagenic in our laboratory and others. In our previous study concerning the genetic effects of G^{8-oxo}, we prepared an oligonucleotide, d(GCTAG^{8-oxo}), by a combination of chemical and enzymatically-mediated DNA synthesis techniques (14). In order for us to assess the mutagenic properties of A^{8-oxo} in the same system, we needed to synthesize the pentanucleotide d(GCTA^{8-oxo}G). This was achieved by the phosphoramidite method of solid-phase DNA synthesis, and necessitated the prior preparation of an A^{8-oxo}-containing monomer, 2′-deoxy-5′-O-(4,4′-dimethoxytrityl)-7,8-dihydro-*N*⁶-acetyl adenosin-8-one 3′-[(2-cyanoethyl) *N,N*-diisopropylphosphoramidite]. Our method was based on the method of Guy *et al.* (39). The crucial step in the synthesis of this phosphoramidite involved acetylation of the 3′ and 5′ hydroxyl groups and the exocyclic amino (*N*⁶) functionality of 8-bromo-2′-deoxyadenosine with concomitant displacement of the bromine moiety to produce a mixture of peracetylated 8-oxo derivatives. Deacetylation with NaOH could be controlled, however, by varying the amount and molarity of base in the reaction. Treatment of the crude mixture with 0.1 M NaOH for 15 min afforded 2′-deoxy-7,8-dihydro-*N*⁶-acetyl adenosin-8-one in reasonable yield.

Although we were able to synthesize the fully protected phosphoramidite, we were unable to purify the compound successfully. Silica gel chromatography always led to degradation

Figure 1. Anion-exchange HPLC profile of the deprotected crude deoxypentanucleotide d(GCTA^{8-oxo}G). The broad peak at 18.6 min was collected. Chromatographic conditions: 0.1–1.0 M KH₂PO₄ in 5% CH₃OH over 40 min at 1.0 ml/min.

of the phosphoramidite and consequently the crude reaction product was used in DNA synthesis. This procedure was inefficient and resulted in poor coupling between the modified phosphoramidite and the 5' hydroxyl group of the adjacent 3' base, guanine (as evidenced by the low level of color observed during subsequent DMTr deprotection steps). The process did, however, permit the synthesis of enough of the site-specifically modified oligomer for characterization and genetic evaluation.

To ensure that the adducted oligonucleotide, d(GCTA^{8-oxo}G), obtained from DNA synthesis was purified at a level adequate for biological purposes, two modes of HPLC separation were used. Initial analysis of the crude, deprotected oligonucleotide by anion-exchange HPLC indicated that the reaction mixture consisted of a high percentage of early eluting components (3–11 min), and a smaller amount of a compound(s) that eluted as a broad peak at a retention time of 18.5 min (Fig. 1). Most of the material that eluted from the column in the first ten minutes had UV spectra similar to dG, a feature consistent with poor coupling between the adducted monomer and resin-bound dG during DNA synthesis (see above). The later eluting peak, however, appeared close to the retention time of a pentanucleotide standard (data not shown) and was consequently chosen for purification. Since the compound eluted as a broad peak from the anion-exchange column (Fig. 1), the collected material was reapplied to a reversed-phase column and subjected to an additional round of purification. Following this, the compound was desalted by Sephadex G10 chromatography. In addition to the adducted oligomer, an unmodified pentamer, d(GCTAG), was also prepared by the phosphoramidite procedure. Since this oligomer

was the predominant product obtained from DNA synthesis, it could be purified satisfactorily by reversed-phase HPLC only.

Reversed-phase HPLC profiles of the two purified pentamers are shown in Fig. 2. The adducted molecule had a retention time of 62.5 min and eluted ~3.5 min earlier than d(GCTAG). The purity of the oligonucleotides was also assessed by polyacrylamide gel electrophoresis after 5'-phosphorylation of the oligonucleotides with [γ -³²P] ATP. Phosphorimaging analysis was used to quantitate the total level of radioactively-labeled impurities in the gel. These data indicated that both oligomers were essentially pure (>99%, data not shown), although it should be noted that this analysis is valid only for radiolabeled impurities that do not comigrate with the pentamers during electrophoresis.

The base composition of the oligonucleotides was determined by reversed-phase HPLC after digestion of the compounds with bacterial alkaline phosphatase and snake venom phosphodiesterase. Peak area analysis of the chromatogram obtained from the adducted molecule, d(GCTA^{8-oxo}G) (Fig. 3), indicated that this compound contained dC, dG and dT in the same relative proportions as d(GCTAG) (Table I). Unlike the latter oligonucleotide, however, the adducted pentamer contained no dA, but a stoichiometric amount of a compound with identical retention time and UV spectrum to a dA^{8-oxo} standard (see inset, Fig. 3), which was fully characterized by ¹H- and ¹³C-NMR and GC-MS at the nucleoside and free base levels. The GC/MS using total ion scanning showed the characteristic TMS-derivatized values of m/z = 352 and 367 for the tri-TMS-derivatized A^{8-oxo} base and m/z = 540 and 556 for the tetra-TMS-derivatized dA^{8-oxo} nucleoside.

Construction and characterization of M13 genomes containing a single A^{8-oxo} adduct

An M13mp19-derived genome containing A^{8-oxo} at position 6275 ([+] strand) was synthesized by the method outlined in Fig. 4 and described in detail for another DNA lesion by Wood *et al.* (14). Prior to ligation of d(GCTAG) and d(GCTA^{8-oxo}G) into the GHD, the oligonucleotides were 5'-phosphorylated with [γ -³²P] ATP. Following ligation, the radiolabeled DNA was purified from low molecular weight components of the reaction

Figure 2. Reversed-phase HPLC profiles of the oligonucleotides d(GCTAG) (A) and d(GCTA^{8-oxo}G) (B). Chromatographic conditions: 0–9% CH₃CN in 0.1 M ammonium acetate over 70 min at 1.0 ml/min.

Figure 3. Reversed-phase HPLC chromatogram of the hydrolysate obtained after digestion of d(GCTA^{8-oxo}G) with bacterial alkaline phosphatase and snake venom phosphodiesterase. The compound that eluted at 30.8 min had the same UV spectrum and retention time as an authentic dA^{8-oxo} standard. Chromatographic conditions: 0–11.25% CH₃CN in 0.1 M ammonium acetate over 45 min at 1.0 ml/min.

and immediately analyzed by agarose gel electrophoresis. An autoradiogram of a typical gel is shown in Fig. 5. As anticipated, the adducted and unmodified radioactive ligation products comigrated with form II M13mp19 DNA, a result consistent with the presence of a non-ligatable nick in the (-) strand of the genomes.

To verify that the oligonucleotides were incorporated into the correct region of the M13 genome, the purified ligation products were incubated with *Sst*I, *Nhe*I and other restriction enzymes and then analyzed by agarose gel electrophoresis/autoradiography (Fig. 5). Both ligation products were resistant to cleavage by *Sst*I. As expected, the unmodified ligation product is linearized by *Nhe*I. However, when the A^{8-oxo} adduct was present in the *Nhe*I site, it efficiently inhibited the restriction enzyme. We observed an almost identical result when G^{8-oxo} is positioned in this recognition sequence at position 6276 of the (+) strand (14). The DNA was also analyzed by two double restriction digestions (*Cla*I/*Xba*I and *Cla*I/*Eco*RI, Fig. 5). The ³²P-labeled restriction fragments produced by these digestions should be 637 (*Cla*I/*Xba*) and 3769 (*Cla*I/*Eco*RI) base pairs in length. By

comparison with non-radioactive DNA markers in the gel, we determined that the two treatments yielded radioactive DNA fragments that corresponded to the theoretically expected sizes. These results localized the ligated oligonucleotides to a 39 base pair fragment in the M13 genome that contained the *Nhe*I restriction site.

The radioactive genomes were also used to calculate the percentage of ligation reactions that occurred at both the 5' and 3' ends of the incorporated oligonucleotides. This was done by digesting the adducted and unmodified genomes with *Pvu*II (Fig. 5, top) and analyzing the resultant radioactive 334 base pair fragments by denaturing polyacrylamide gel electrophoresis

Table 1. Relative nucleoside content of two oligonucleotides, d(GCTAG) and d(GCTA^{8-oxo}G).^a

	relative peak area	
	(GCTAG)	d(GCTA ^{8-oxo} G)
dC	1.00 (1.00) ^b	1.00 (1.00)
dG	3.21 (3.20)	3.07 (3.20)
dT	1.24 (1.19)	1.22 (1.19)
dA	2.00 (2.07)	nd ^c
dA ^{8-oxo}	nd ^c	1.39 (1.45) ^d

^a Relative nucleoside content was calculated by dividing the integrated HPLC peak area for each nucleoside by the relevant extinction coefficient at 260 nm and normalizing to dC.

^b Theoretically expected peak area ratios (relative to dC) shown in parentheses.

^c nd, not detected.

^d Value calculated assuming ϵ_{260} dA^{8-oxo} = 10,600.

Figure 4. Procedure used to prepare M13mp19-derived genomes containing either a single A^{8-oxo} or G^{8-oxo} adduct at position 6275 or 6276 of the (+) strand, respectively. T4 DNA ligase was used to insert the 5'-phosphorylated oligonucleotides d(GCTA^{8-oxo}G) and d(GCTAG^{8-oxo}G) into a unique *Nhe*I restriction site in GHD containing a five-base gap in the (+) strand and a non-ligatable nick in the (-) strand. GHD were prepared by annealing *Sst*I-linearized M13+7-(*Sst*I) DNA with *Bgl*II-linearized, 5'-dephosphorylated M13+12-(*Nhe*I) DNA. Equal quantities of a second GHD (not shown) were also formed in the annealing process. This molecule contained a five-base gap in the (-) strand but was unable to participate in ligation because the DNA strand opposite the gap was not complementary to GCTAG. CIP is calf-intestinal phosphatase.

Figure 5. Top: Partial restriction map of the genome obtained from ligation of [γ -³²P] ATP-phosphorylated d(GCTA^{8-oxo}G) into a unique *Nhe*I restriction site within M13mp19-derived GHD. Bottom: Autoradiogram of an 0.8% agarose gel to show the migration of the purified unmodified (lanes headed A) or A^{8-oxo} adducted (lanes headed A^{8-oxo}) ligation products, and after incubation with the enzymes *Sst*I, *Nhe*I, *Cla*I/*Xba*I and *Cla*I/*Eco*RI.

(14,43). Phosphorimager scanning of the gel revealed that the lanes corresponding to the digested ligation products contained three radioactive bands (data not shown). The slowest migrating band corresponded to the size expected for complete ligation of the oligonucleotide into the GHD (334 nucleotides), whereas the two faster migrating bands coincided with DNA fragments that contained an oligonucleotide ligated at either, but not both, of its 5' or 3' ends. Analysis of the gel indicated that ~60% of the ligations involving the adducted oligonucleotide occurred at both the 5' and 3' ends. The corresponding value for the unmodified oligonucleotide, 75%, is close to the 70% previously reported (14).

Comparative mutagenicity of A^{8-oxo} and G^{8-oxo} *in vivo*

An important feature of the singly-adducted genomes used in this study is the presence of an in-frame *amber* codon (5'-TA*G*-3', where the asterisked bases denote the relative positions of A^{8-oxo} and G^{8-oxo}) in the *NheI* restriction site, 5'-GCTAGC-3'. Since the *amber* codon resides in the *lacZ* gene of the virus, it causes the formation of a light blue plaque on *E. coli* GW5100, a *supE* host. Using this system, we showed that a single G^{8-oxo} adduct, positioned at the 3' end of the *amber* codon, induced a targeted G→T transversion (14). This mutation was easy to detect because it caused the formation of a distinct dark blue plaque. Since any targeted base substitution of the internal adenine in the *amber* codon will also give rise to this plaque color, we decided to use this system to determine the genetic effects of A^{8-oxo}.

Non-radioactive M13 genomes containing either a single A^{8-oxo} adduct at position 6275 of the (+) strand or a G^{8-oxo} adduct (14) at position 6276 were prepared simultaneously from the same batch of GHD. In parallel, unmodified genomes were synthesized using d(GCTAG) that had been prepared by either total synthesis (as a control for the chemically synthesized A^{8-oxo} oligonucleotide) or by the same combination of chemical synthesis and enzymatic ligation used to make the G^{8-oxo}-modified oligonucleotide (14). Heat-induced denaturation of the ligation products yielded single-stranded genomes that were

transfected into DNA repair proficient *E. coli* DL7 cells. Following electroporation, a portion of the cells was plated in the presence of *E. coli* GW5100, X-gal, and IPTG (42). The remainder of the cells was incubated at 37°C for 1.5 h at which time the cells were removed by centrifugation and a portion of the phage-containing supernatant was plated as above.

The percentages of mutant dark blue plaques that appeared amongst the population of infective centers are tabulated in Table II. Data from three separate experiments indicated that the single-stranded genome containing G^{8-oxo} gave rise to approximately four-fold higher number of dark blue plaques than the guanine containing control. Seventy dark blue plaques that were derived from the adducted genome were picked and used to prepare single-stranded DNA. Sequencing analysis showed that the mutation responsible for this phenotype in 38 of the dark blue plaques (~55% of the total) was a targeted G→T transversion at position 6276 of the (+) strand. This is the same mutation we observed previously (14), and it was likely induced by G^{8-oxo}. We note that the frequency of the G→T transversion in this study (0.2–0.5%, Table II) is lower than in our earlier report (0.5–1.0%, [14]). A possible reason for this difference was the presence of low levels of 3'→5' and 5'→3' exonucleases in the batch of *StuI* used during genome construction. Exonucleolytic degradation of linearized parental M13+7-(*StuI*) RF DNA would cause inefficient GHD formation and oligonucleotide ligation, and lead to a decrease in adduct-induced mutations. Also consistent with this hypothesis was the abundance of two deletion mutations (that gave rise to a dark blue plaque) in the *StuI* site of this vector; 5'-AGGCCT-3'→5'-AGGCT-3' or →5'-AGCCT-3'. Neither mutation was observed in our earlier study of G^{8-oxo} mutagenesis (14); however, DNA sequencing indicated that these deletions were responsible for 21 out of the 70 independent dark blue plaques that were analyzed from the plates derived from the G^{8-oxo}-derived genome. Furthermore, ~90% of mutant sequences that resulted from transfection of non-adducted genomes contained either of these two mutations (see below). The likely mechanism leading to these two mutations was the DNA ligase-mediated joining of the two sets of peeled-back DNA ends (i.e., 5'...-AGG + pCT...3' or 5'...AG + pCCT...3'). This event would probably be a highly inefficient process since the 5' and 3' nucleotides undergoing ligation in the GHD would be separated from one another by the distance occupied by six base pairs. Previous studies, however, from our laboratory have shown that DNA ligase can indeed ligate non-adjacent DNA ends in GHD molecules (44). In this earlier work the enzyme was presumably able to ligate across a four-base gap to yield genomes that eventually gave rise to progeny material containing a four base deletion at the ligation site.

The frequency of dark blue plaques that were observed following transformation with the single-stranded A^{8-oxo}-adducted genome (0.2–0.3%) was close to that obtained from the adenine-containing control (0.1–0.2%, Table II). DNA sequencing data that were generated from 58 dark blue plaques resulting from transfection of the adducted genome and from 29 derived from the control DNA revealed that the most common mutations resulting from both DNAs were the same deletions in the *StuI* site of the parental vector M13+7-(*StuI*) that had been observed in sequences derived from G^{8-oxo}-modified DNA. Together, these mutations were responsible for 42 of the 58 dark blue plaques (~70%) that resulted from transfection of the A^{8-oxo}-adducted DNA and 26 out of the 29 mutants (~90%) derived from the unadducted genome. The mutations responsible

Table 2. Mutation frequency (%) of G^{8-oxo} and A^{8-oxo}-modified M13 DNA in *E. coli*.^a

infective centers				
expt.	G ^b	G ^{8-oxo} ^c	A ^b	A ^{8-oxo} ^c
1	0.2 ^d	0.4 (0.3) ^e	0.2	0.2
2	0.2	0.9 (0.5)	0.1	0.3
3	0.1	0.6 (0.2)	0.1	0.2
progeny phage				
expt.	G ^b	G ^{8-oxo} ^c	A ^b	A ^{8-oxo} ^c
1	<0.1	0.1	0.1	0.1
2	0.1	0.2	0.1	0.1
3	0.1	0.2	<0.1	0.2

¹ Defined as number of dark blue plaques/total plaques×100.

^b Control genomes.

^c Adducted genomes.

^d Each value is the mean of two duplicate transfections.

^e Frequency of mutant genomes containing a G→T transversion position 6276 [(+) strand] shown in parentheses.

for the remaining 16 dark blue plaques obtained from transfection of the adducted DNA were a combination of single and double base substitutions within the *NheI* restriction site: *e.g.*, GCTAGC→GCTCGC, GCTAGC→GCTGGC, GCTAGC→GCTACC or GCTAGC→GCATGC. Although these mutations could conceivably have been induced by A^{8-oxo}, we note that the A→C transversion was also observed amongst mutant DNA sequences derived from the unadducted genome (2/29) and, more generally, a high proportion of the mutations were shown to be part of the spectrum of background mutations observed when this assay system was used previously to determine the mutagenic effects of a G^{8-oxo} (14). It was possible, however, that the adduct did induce a very low level of mutagenesis that had been missed because too few plaques were screened. Therefore, to obtain additional information, we decided to search for A^{8-oxo}-induced mutations amongst progeny phage DNA.

The frequencies of dark blue plaques in the progeny phage are shown at the bottom of Table II. It was immediately apparent that the proportion of mutant phage in the progeny was lower than that observed in the population of infective centers (Table II, top). This difference was especially marked in the data concerning G^{8-oxo} mutagenesis, where the average frequency of mutant phage appeared to drop by three-fold between the two platings (from 0.6% to 0.2%). The frequency of the G^{8-oxo}-induced G→T transversion would be expected to drop by an equal amount (to ~0.1% in the progeny). This reduction probably reflects a bias in the assay when mutation frequencies are determined from infective centers because mutant dark blue plaques in this population are likely to be a composite of both mutant and wild type phage, and this would lead to an overestimation of the true mutant fraction. To ascertain whether this was a general phenomenon, we decided to analyze the phage stock that was generated in our earlier study on the genetic effects of G^{8-oxo} (14). We originally showed that transformation with the single-stranded G^{8-oxo}-adducted genome gave rise to dark blue plaques at a frequency of ~1.6% among the infective centers. A targeted G^{8-oxo}→T transversion was responsible for 40% of the mutations that resulted in this phenotype, and this corresponded to a mutation frequency of 0.7% for the adduct. Analysis of the progeny phage stock showed that the average frequency of dark blue plaques was indeed reduced, from 1.6% to 1.0% (data not shown). This value is higher than the equivalent mutation frequency of 0.2% obtained from the data in the present study (Table II); however, we believe that this latter value is a low estimate, possibly owing to inefficient ligation of the oligonucleotide into the GHD that resulted from exonuclease-mediated degradation of the linearized parental genome M13+7-(*StuI*) (vide supra).

A further 114 dark blue plaques that were derived from the single-stranded A^{8-oxo}-adducted genome and 112 plaques from the adenine-containing control were picked and the DNA sequenced. As anticipated, the majority (76) of the mutant DNAs obtained from the adducted DNA contained one or other of the -1 deletion mutations in the *StuI* site of M13+7-(*StuI*). The remainder of the mutations again consisted primarily (30/38) of single or double base pair substitutions in the *NheI* recognition sequence of the M13+12-(*NheI*) genome (large scrambled mutations in and around the site of ligation accounted for the other eight mutations). Over two-thirds of these substitutions (23/30) were common to mutations obtained from the control experiment, and these consisted of (i) T→C, T→G or T→A substitutions at position 6274, (ii) A→C or A→T transversions

at position 6275, and (iii) a G→C transversion and a T→C transition at positions 6272 and 6274, respectively.

DISCUSSION

Endogenously and exogenously mediated oxidative DNA damage is likely to play a significant role in the aging process and associated diseases (1–4). Therefore, it is of primary importance to obtain information on the cellular events that might initiate these processes. Carcinogenesis in humans and experimental animals appears to occur by discrete, step-wise changes, some of which are characterized by the acquisition of heritable chromosomal changes (45). Consequently, because of the high levels of DNA damage caused by reactive oxygen species, a considerable body of research has been devoted to studying the genetic effects of this damage. This is a complex task, since many types of DNA lesions are produced by these predominantly free radical-initiated reactions (9). To circumvent these problems, we have carried out a number of detailed studies designed to assess and compare the individual genetic effects of DNA adducts produced after reaction of reactive oxygen species and DNA. These studies have utilized a site-specific mutagenesis system with which we were able to show that a single G^{8-oxo} adduct, the major lesion produced in DNA after cellular exposure to many oxygen radical sources, was mutagenic and induced targeted G→T transversions. Since this adduct is so highly prevalent in DNA we decided that the potential mutagenicity of other radiation- and oxidant-induced DNA adducts would be most usefully studied if these experiments were carried out using G^{8-oxo} as a reference point. In the work reported here, we have compared the genetic effects of A^{8-oxo} and G^{8-oxo}. Each lesion was inserted into oligonucleotides of the same base sequence (5'-GCTA*G*-3'; the asterisked bases, A* and G*, denote the positions of either A^{8-oxo} or G^{8-oxo}, respectively). To determine the relative genetic effects of A^{8-oxo} and G^{8-oxo} *in vivo*, bacteriophage genomes containing either of the two lesions were constructed and used to transform a DNA repair proficient strain of *E. coli*. Mutant progeny were scored and the nature of the mutations determined by DNA sequencing.

Phage DNA from a total of 172 mutant dark blue plaques derived from the single stranded A^{8-oxo}-adducted genome and 141 mutant sequences from unmodified control DNA were analyzed. No evidence was obtained to indicate that the adduct induced any targeted base substitution mutation, nor any other type of mutation that would result in the formation of a dark blue plaque. We estimate that A^{8-oxo} is at least an order of magnitude less mutagenic than G^{8-oxo}, since the latter lesion induced a targeted G→T transversion at a frequency of ~0.3% in the infective center population (Table II). It is conceivable that A^{8-oxo} could induce mutations that do not lead to a dark blue plaque (*e.g.*, addition or deletion mutations that would lead to a colorless plaque). Although this mutagenesis system can be used to detect any mutation that occurs within the recognition sequence of *NheI*, these mutations would have been missed with the direct screening method used here. On the basis of the known structural and electronic similarities between A^{8-oxo} and G^{8-oxo} adducts (26–28), however, mutagenesis by A^{8-oxo} might be expected to result via the formation of targeted mispairings during DNA replication. Mismatching of A^{8-oxo} with any base other than thymine would result in a dark blue plaque in our experimental system.

The differences in mutagenicity between A^{8-oxo} and G^{8-oxo}

in vivo are in agreement with studies performed *in vitro* when oligonucleotides containing either of the two lesions are used as templates for replication by bacterial or mammalian DNA polymerases (18,46,47). In the case of A^{8-oxo}, it has been shown that efficient extension of the newly replicated strand occurs only when thymine is paired with the adduct (46,47). The mammalian polymerases α and β also incorporate guanine opposite A^{8-oxo} but this mispair is not extended (46). The Klenow fragment of DNA polymerase I will only insert thymine opposite the lesion and A^{8-oxo}-mispairs are substrates for the 3'→5' exonuclease activity of this enzyme (46). These data are in contrast to those obtained with oligonucleotides containing G^{8-oxo}. Here, both adenine and cytosine are inserted opposite the adduct when either DNA polymerase α or DNA polymerase I (Klenow fragment) are used as the replicating enzymes (18). Neither pairing is a substrate of the 3'→5' exonuclease activity of the bacterial polymerase and, interestingly, chain extension occurs more efficiently from the G^{8-oxo}-mispair than from the non mutagenic G^{8-oxo}-base pair (18). Presumably, the G^{8-oxo}-pairs formed during DNA replication *in vivo* lead to the G^{8-oxo}→T transversion mutations induced by this adduct (14–16).

Structural studies on G^{8-oxo}- and A^{8-oxo}-related compounds have shed light on the possible basis of the differing genetic effects of the two adducts. Evans and coworkers have performed extensive spectroscopic measurements on the nucleoside derivatives of G^{8-oxo} and A^{8-oxo} and have shown that there are strong structural similarities between the two adducts (26–28). UV and NMR spectroscopies have been used to show that the 6,8-diketo tautomers of both lesions predominate at physiological pH. Moreover, the G^{8-oxo} and A^{8-oxo} moieties of the free nucleosides appear to adopt the syn conformation about the glycosyl bond. NMR analyses of duplex oligonucleotides that contain G^{8-oxo} have shown, however, that this adduct adopts the syn conformation only when it is mispaired with adenine (29). When G^{8-oxo} is base paired with cytosine, the lesion assumes the anti conformation (30). Both the G^{8-oxo}(syn)·A(anti) and G^{8-oxo}(anti)·C(anti) base pairs appear to cause little distortion of the DNA helix (29,30) and these findings may help explain why G^{8-oxo} directs the insertion of both cytosine and adenine during DNA replication *in vivo* and *in vitro*. Although less comprehensive, NMR studies on duplexes containing A^{8-oxo} have shown that this adduct, like G^{8-oxo}, adopts an anti conformation when it is opposed to its normal Watson–Crick partner, thymine (47). In this case, the lesion provokes minimal distortion of the DNA helix. To date, no NMR studies have been carried out to determine the effects of pairing A^{8-oxo} with any other base. These experiments are warranted because they may provide a structural rationale for the non-mutagenicity of this adduct.

A possible conclusion from this study is that A^{8-oxo} does not contribute significantly to the spectrum of mutations that occur at A·T base pairs in oxidatively-damaged DNA. It is important to note, however, that under an alternative set of experimental conditions, different results may be obtained. For example, although unlikely, it is conceivable that the A^{8-oxo} adduct may have been removed from the single-stranded genome by some unknown chemical mechanism, possible during the electroporation step. A more realistic reason for the lack of mutagenicity of this lesion is that these studies were performed into *E.coli* DL7, a cell line that is proficient for DNA repair. It is conceivable that the A^{8-oxo} adduct was removed from the

M13 genome by an *E.coli* repair enzyme(s) prior to DNA replication and mutation fixation. To our knowledge no repair activity for A^{8-oxo} has yet been found, although it may be that in the absence of repair (*e.g.*, in an *E.coli* strain deficient in UVR nucleotide excision repair), the adduct may be mutagenic. Another possibility is that A^{8-oxo} may be one of many lesions that is mutagenic only in bacterial cells that are expressing the SOS response. The *E.coli* cells used in this study were SOS uninduced. By carrying out experiments that are designed to test these hypotheses, we shall be able to evaluate more fully the genetic consequences of this adduct in DNA.

ACKNOWLEDGEMENTS

We would like to thank Jennifer Wilson and Sophia Wang for expert technical assistance. This work was supported by Grant CA52127 and ES07020 from the National Institutes of Health. MLW and AE were supported by Fellowships from the Procter and Gamble Co. and the Consejo Superior de Investigaciones Científicas (CSIC) of Spain, respectively.

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