Oxidation of 7,8-Dihydro-8-oxoguanine Affords Lesions That Are Potent Sources of Replication Errors in Vivo†

Paul T. Henderson, James C. Delaney, Feng Gu, Steven R. Tannenbaum, and John M. Essigmann*

Division of Bioengineering and Environmental Health and Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received August 2, 2001; Revised Manuscript Received November 14, 2001

ABSTRACT: Three single-stranded DNA genomes have been constructed that contain the 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) oxidation products oxaluric acid, oxazalone, and cyanuric acid. Oligonucleotides containing each lesion were synthesized by treating an oligonucleotide containing a single 8-oxodG with peroxynitrite, and the desired products were isolated by HPLC. The modified oligonucleotides were ligated into M13mp7L2 bacteriophage DNA in such a way that the lesion was situated at a known site in the *lacZ* gene fragment of the viral genome. The circular genomes were transfected into wild-type AB1157 *Escherichia coli*. The relative efficiency of lesion bypass by DNA polymerase was determined by counting the number of initial independent infections produced by each genome relative to that of an unmodified DNA control. Viral progeny were analyzed for mutation frequency and type by PCR amplification of the insert region followed by a recently developed post-labeling assay. All three secondary lesions were readily bypassed, causing G → T transversions at frequencies at least an order of magnitude higher than 8-oxodG. These data establish a model whereby the modestly mutagenic primary lesion 8-oxodG is oxidized in vivo to much more highly mutagenic secondary lesions.

The correlation between oxidative DNA damage and human disease is well established, but the relationship between specific DNA lesions and mutation has only recently begun to unfold (*1*). To achieve a full understanding of the processes of spontaneous, oxidant-induced and ionizing radiation-induced mutagenesis, the exact types and biological significance of oxidative lesions formed in DNA must be determined. Oxidative DNA damage results from exposure to both endogenous and exogenous agents and has been implicated in a variety of degenerative disorders, including diseases associated with aging and cancer (*2*, *3*). Peroxynitrite ($ONOO^-$),[1] which is formed endogenously by the reaction of nitric oxide with superoxide, is a potent oxidant that decomposes into radical species capable of oxidizing DNA and other cellular constituents including thiols, lipids, and proteins (*4*−*7*). The spectrum of $ONOO^-$-mediated DNA damage includes strand breakage and both oxidation and nitration of bases (*5*, *8*−*10*). In DNA, guanine (G) is oxidized preferentially by $ONOO^-$ because it has the lowest oxidation potential of the four nucleobases (*11*). A major product of G oxidation by $ONOO^-$ is 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG, shown in Figure 1), the presence of which is known to cause G → T transversions in prokaryotic and eukaryotic cells (*12*−*16*). The steady-state concentration of 8-oxodG increases with age and oxidative stress, making this lesion a key biomarker for monitoring diseases associated with oxidative stress (*17*).

A comprehensive repair system has evolved for 8-oxodG (*18*). In *Escherichia coli*, a bifunctional glycosylase and AP lyase, formamidopyrimidine glycosylase (Fpg), also known as MutM, removes a broad range of lesions, including 8-oxodG, from duplex DNA when they are paired with cytosine. The resulting abasic (AP) site is cleaved via a β,δ-elimination leaving a one nucleotide gap that is subsequently processed by DNA polymerase I (Pol I) and DNA ligase to afford a normal G:C pair (*19*). Another glycosylase, MutY, removes adenine (A) from an 8-oxodG:A mismatch, leaving an AP site (*20*, *21*). Ultimately, the AP site is cleaved by an AP-endonuclease to afford a nick that, when processed to form an 8-oxodG:C pair, is then repaired by the MutM pathway. To prevent incorporation of 8-oxodG into DNA during replication, an 8-oxodGTPase, MutT, removes the oxidized lesion from the dNTP pool (*22*). These related repair enzymes effectively lower the steady-state concentration of 8-oxodG in cellular DNA to reduce the endogenous mutation rate. There is a homologous repair system in yeast and mammalian cells (*23*−*31*).

On the nucleoside level, 2′-deoxyguanosine must be present at a concentration 1000-fold higher than that of 8-oxodG in order to suppress the oxidation of 8-oxodG by

† Supported by grants from the NIH (CA86489, ES07020, and CA26731).

* To whom correspondence should be addressed. Phone: (617) 253-5445. Fax: (617) 253-5445. E-mail: jessig@mit.edu.

[1] Abbreviations: AP, abasic; Ca, cyanuric acid; 8-oxodG, 8-oxo-7,8-dihydro-2′-deoxyguanine; PolI, DNA polymerase I; HPLC, high-pressure liquid chromatography; Iz, imidazalone; MALDI-TOF, matrix-assisted laser desorption ionization-time-of-flight; NHE, nickel hydrogen electrode; Oa, oxaluric acid; $ONOO^-$, peroxynitrite; Oz, oxazalone; PAGE, polyacrylamide gel electrophoresis; PNK, polynucleotide kinase; REAP, restriction endonuclease and postlabeling; ss, single-stranded; SVPD, snake venom phosphodiesterase; THF, tetrahydrofuran; TLC, thin-layer chromatography.

10.1021/bi0156355 CCC: $22.00
Published on Web 12/27/2001

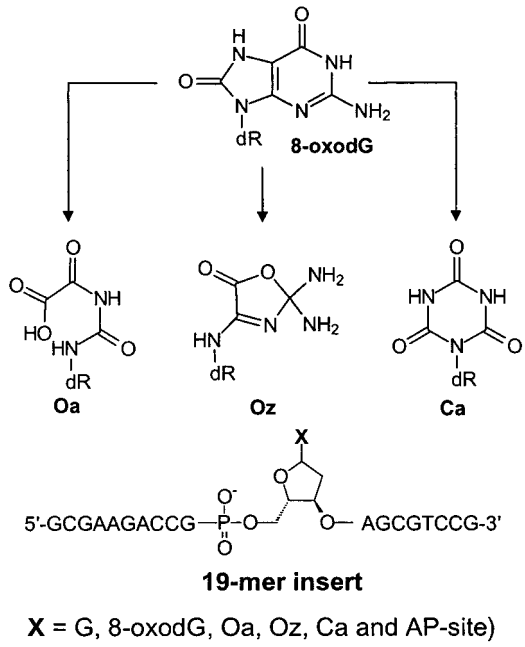

FIGURE 1: Structures of 8-oxoG, Oa, Oz, and Ca and the oligonucleotide sequence used for genome construction.

approximately 50% (*32*). Tretyakova et al. recently reported that an oligonucleotide undergoes oxidative degradation by $ONOO^-$ site-specifically at 8-oxodG to afford 2′-deoxy-β-D-*erythro*-pentofuranosyl derivatives of oxaluric acid (Oa), oxazalone (Oz), and cyanuric acid (Ca) (*33*) (structures shown in Figure 1). Purified MutM excises Oa and Oz, but not Ca from lesion-containing duplex oligonucleotides (*34*). Cadet et al. showed that dAMP is preferentially incorporated opposite each of the lesions using in vitro polymerase extension assays, suggesting that these lesions are sources of G → T transversions (*35*−*37*). $ONOO^-$ treatment of plasmid DNA containing the *supF* gene, followed by transfection into mammalian or bacterial cells, induces mainly G → T, and some G → C, transversions (*38*). Reaction of $ONOO^-$ with an oligonucleotide containing a portion of the *supF* gene followed by incubation with hot piperidine, which cleaves DNA preferentially at oxidized 8-oxodG nucleotides, causes strand scission at sites coinciding with the mutational "hotspots" observed after replication in bacterial cells (*39*). Since 8-oxodG is not a known source of G → C transversions and 8-oxodG in DNA is refractory to cleavage by piperidine (*40*), these experiments suggest that the secondary DNA lesions resulting from further oxidation of 8-oxodG may contribute to $ONOO^-$ mutagenicity.

In view of the reported chemical lability of 8-oxodG to oxidation, and the observed incorporation of A opposite the oxidized lesion by purified polymerases, the mutagenic potential of Oa, Oz, and Ca was tested in vivo by inserting oligonucleotides containing each well-defined lesion into bacteriophage DNA, followed by transfection into wild-type *E. coli*. This work has demonstrated that each lesion was readily bypassed during replication in vivo. The viral progeny were analyzed for mutation frequency and specificity, and it was shown that the three secondary oxidation products were much more potently mutagenic than the parent 8-oxodG.

EXPERIMENTAL PROCEDURES

Materials. Restriction endonucleases, polynucleotide kinase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. M13mp7L2 was a gift from C. W. Lawrence (University of Rochester). The *E. coli* cell strain used for transfection was AB1157, and for plating was NR9050 from R. M. Schaaper (NIEHS). The *E. coli* strain used for regrowth of phage was SCS110 from Stratagene. 5-Bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) and isopropyl β-D-thiogalactopyranoside (IPTG) were from Gold Biotechnology (St. Louis, MO). [γ-^{32}P]ATP (6000 Ci/mmol) was from New England Nuclear, and ATP was from Pharmacia. Acetonitrile, β-mercaptoethanol, and ammonium acetate were from Aldrich, and ammonium phosphate dibasic was from Mallinckrodt (AR grade). Polyethylenimine-coated TLC plates and phosphoric acid were from J. T. Baker. Electrophoresis of constructed PCR products was carried out in horizontal agarose gels (3% agarose) prepared and run (100 V, 6 h) in 1 × TAE buffer (*41*) containing 1 μg/mL ethidium bromide. Denaturing (7 M urea) PAGE of restriction fragments was performed with 20% polyacrylamide cross-linked with bis-acrylamide (19:1) and run (550 V, 70 min) in TBE (1×). Appropriate bands were excised and eluted in 200 μL of water for 4 h at room temperature, followed by desalting with Sephadex G-25 spin columns from Boehringer Mannheim.

Oligonucleotides. DNA synthesis reagents were from Glen Research. Oligonucleotides were from Research Genetics (Huntsville, AL) or Integrated DNA Technologies (Coraville, IA) or synthesized on an Applied Biosystems model 391 DNA synthesizer. Oligonucleotides were deprotected with concentrated NH_4OH for 18 h at 55 °C. The strand containing 8-oxodG, to prevent degradation of the lesion, required 0.25 M β-mercaptoethanol in the deprotection solution. Oligonucleotides were purified by PAGE and C18 reverse-phase HPLC before use (∼99% purity). MALDI-TOF mass spectrometry verified the molecular weight of each strand after purification. The insert sequence was 5′-GCGAAGACCGX-AGCGTCCG-3′ [X = G, 8-oxodG, Oa, Oz, Ca, and a tetrahydrofuran (THF) synthetic AP site].

$ONOO^-$ Treatment of 8-OxodG in DNA. $ONOO^-$ was prepared by ozonation of sodium azide in alkaline solution (0.1 M NaOH) and stored at −80 °C (*42*). Concentrations of $ONOO^-$ were determined by spectrophotometry ($\epsilon = 1670$ L M^{-1} cm^{-1}, $\lambda = 302$ nm in 0.1 M NaOH). Reactions between $ONOO^-$ and oligonucleotides were performed in buffer containing 150 mM potassium phosphate and 25 mM sodium bicarbonate (pH 7.2). A droplet (1−5 μL) of $ONOO^-$ stock solution (∼38 mM) was placed on the underside of an Eppendorf tube lid containing the buffered oligonucleotide solution (∼50 μM, total reaction volume of 50 μL). The tube was closed, and the contents were vortexed for 1 min and incubated at room temperature for 24 h prior to analysis to allow for formation of stable products.

HPLC Purification of $ONOO^-$ Oxidation Products. The oxidized oligonucleotides were purified by HPLC using a 250 mm × 2.1 mm, 5 μm Supelcosil LC-18 column with UV detection at 260 nm. The mobile phase was 150 mM aqueous ammonium acetate (A) and 100% acetonitrile (B) with a gradient of 7−12% B over 30 min at a flow rate of 0.25 mL/min. The Ca derivative, due to slight contamination

with the starting material containing 8-oxodG, required further purification on a TSKgel DEAE-NPR (Tosoh, Tokyo, Japan) anion exchange column. The mobile phase was 1 M NaCl in 20 mM Tris-HCl (A) and 20 mM 1,3-diaminopropane adjusted to pH 10.5 with HCl (B) with a gradient of 0−100% B over 30 min at a flow rate of 1 mL/min. The quantities of each product were calculated by UV absorbance at 260 nm using the molar absorptivity of the G-containing oligomer (ϵ = 184 700 L M^{-1} cm^{-1}) for all the modified oligonucleotides. The mass of each oligonucleotide was calibrated using oligonucleotides that bracketed the molecular ion of each analyte, which allowed us to distinguish between the Oa [MW = 5843.8 (calculated), 5844.7 (observed)], Oz [MW = 5841.3 (calculated), 5841.2 (observed)], and Ca [MW = 5840.3 (calculated), 5841.0 (observed)] oligomers. HPLC chromatograms and mass spectra for each lesion-containing oligonucleotide are available as Supporting Information.

Construction of M13 Genomes Containing a Site-Specific Lesion. Single-stranded (ss) M13 genomes containing a unique lesion were generated by modification of a previously described method (*43*). Because of the chemical lability of Oa to heat, the genome construction procedure was modified by using shorter 18-mer scaffolds for ligation in order to facilitate their removal by thermal denaturation in the presence of a 100-fold excess of complementary "anti-scaffold", instead of *Exo*III digestion. Also, the oligonucleotide inserts were added after annealing of the scaffolds to minimize exposure of the lesions to heat. Briefly, as shown in Figure 2, 1 pmol of ss M13mp7L2 was linearized by *Eco*RI and annealed to equimolar amounts of two scaffolds (5′-GGTCTTCGCCACTGAATC-3′ and 5′-CCAGTGAATTCGGACGCT-3′), which are partially complementary to the 5′- and 3′-ends of the insert and the linearized genome. An equimolar amount of the 5′-phosphorylated 19-mer insert was added and covalently joined into the genome by incubation with T4 DNA ligase (16 °C, 1 h). The scaffolds were removed by heating (50 °C, 5 min) in the presence of a 100-fold molar excess of "anti-scaffold" complement immediately prior to transfection.

Transfection and Determination of Translesion Bypass Efficiency. Cells, grown to an OD_{600} = 0.4 in 100 mL of yeast tryptone (1X) medium (*44*), were made competent for transfection by the calcium chloride method as described previously (*45*, *46*), except that the total volume used for transfection was 100 μL. On average, the cells gave 71 transfectants per nanogram of M13mp7L2 single-stranded DNA (with a G-containing insert). Lesion bypass was determined by transfection with 5 ng of M13 DNA, and mutation frequency was determined using 50 ng of the construct. The number of independently transformed cells was determined by adding 2.5 mL of B-broth soft agar to the transfected cells and plating onto a lawn of NR9050 *E. coli*. Since cells infected with phage grow more slowly than the surrounding *E. coli*, easily visible plaques of infected cells formed around each transformed cell on the plate. The number of plaques on each plate was counted after incubation at 37 °C for 16 h. As a control to estimate the number of viable viral genomes that do not have an insert, a wild-type M13mp7L2 sample was exposed to the genome construction conditions, and the average number of resulting plaques was subtracted from the total formed by each insert-containing genomic construct. Transfections were performed in triplicate, and the bypass efficiency relative to the G control was calculated to within a 95% confidence interval of the mean for each lesion.

Mutation Frequency Determination. Cells transformed for mutation determination were added to 10 mL of LB media (*44*) and incubated on a roller drum for 5 h at 37 °C, followed by pelleting of the cells and decanting of the phage-containing supernatant into new 15 mL polypropylene tubes. To prevent *Pfu*-induced mutagenesis at the lesion site from nontransfected DNA, 100 μL of the suspension was added to 9 mL of LB and 1 mL of SCS110 *E. coli* (grown to mid-log phase from an overnight solution). The cells were incubated an additional 4 h and pelleted, and the phage-containing supernatant was decanted and stored at 4 °C. The growth and regrowth steps served to amplify the amount of progeny phage while reducing the relative amount of nontransfected DNA to a negligible amount (<0.1%). As shown in Figure 3, the mutation frequencies were determined by performing the REAP assay on a DNA duplex produced by PCR amplification of the insert region of the progeny phage. PCR was performed in 200 μL tubes in a PTC-100 thermocycler (MJ Research) with Turbo *Pfu* thermostable polymerase (Stratagene). The PCR mix included 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl (pH 8.0), 2 mM $MgCl_2$, 0.1% Triton X-100, 100 μg/mL BSA, 1.0 μM each primer, 0.2 mM dNTPs, and 0.05 unit/μL of *Pfu*. The PCR template was prepared from 700 μL of the phage supernatant using a QIAprep Spin M13 Kit (Qiagen), to afford 100 μL of purified DNA (15 μL of the solution was used for PCR amplification). The total volume of each PCR sample was 25 μL. Denaturation at 94 °C for 30 s, annealing at 58 °C, and extension at 72 °C, each for 60 s intervals, constituted the amplification cycle. After 30 cycles, samples were incubated at 72 °C for 5 min and stored at 4 °C until further use. The primers used were 5′-TTTCACACAGGAAACAGCTATGACCATG-3′ and 5′-CAGGGTTTTCCCAGTCACGACGTTGTAA-3′. The primers were modified at the 5′-end during oligonucleotide synthesis with an aminoethoxyethyl ether phosphate (5′-Amino-Modifier 5 Phosphoramidite, Glen Research) to prevent 5′-phosphorylation during subsequent postlabeling (vida infra). The presence of the PCR product was verified by agarose gel electrophoresis.

The REAP assay was performed as previously described on the 101-mer PCR product (*43*). Briefly, each sample was desalted with a Sephadex Quick Spin column (G-50, Boehringer Mannheim) and cleaved by *Bbs*I at the position that originally contained the lesion in the template vector, affording a 55-mer whose newly formed 5′-end contained the lesion site. The 55-mer was dephosphorylated with shrimp alkaline phosphatase, which exposed the lesion site at the 5′-overhang for radiolabeling with PNK and [γ-^{32}P]ATP. Incubation with *Hae*III, to yield a 19-mer, allowed PAGE purification of the radiolabeled fragment of interest. The desalted 19-mer was digested to 5′-deoxynucleotide monophosphates (5′-dNMPs) with snake venom phosphodiesterase (SVPD), followed by partitioning of the mixture on a 20 × 20 cm polyethylenimine TLC plate developed in 200 mL of saturated $(NH_4)_2HPO_4$ (adjusted to pH 6.1 with H_3PO_4). The separated radiolabeled nucleotides were quantitated by PhosphorImager analysis, thus providing the fractional base composition of each nucleotide at the lesion site, from which

the mutation frequency and type were determined. Duplicate genome constructions and transfections were performed for each sample, and the mutation frequencies were identical (within one percent) for each data set.

The insert was positioned in-frame in the M13 polylinker region that interrupts the *lacZ* gene, which codes for the α-fragment of *β*-galactosidase, enabling verification of G → T transversions by an α-complementation assay in which plaques of different shades of blue were counted. In the assay, lawns of NR9050 *E. coli*, which contain the F′ episome for M13 infection and express the *β*-galactosidase *ω* peptide, were infected with progeny phage that caused the formation of plaques. The parental genomes possessed the insert sequence 5′-**X**AG-3′, which was either repaired, non-mutagenically bypassed, or mutated to afford progeny phage with in-frame 5′-**G**AG-3′ (no mutation) or 5′-**T**AG-3′ (mutation) codons in the insert sequence. Phage with the 5′-**G**AG-3′ sequence formed dark blue plaques on IPTG/X-gal indicator plaques because *β*-galactosidase gene activity was restored through α-complementation with the *ω* fragment, thus allowing hydrolysis of the chromogenic substrate X-gal to yield the deep blue dye 5-bromo-4-chloroindol (*47*). Phage with the mutant 5′-**T**AG-3′ sequence (a stop codon in *E. coli*) formed light blue plaques because the translation of *lacZ* mRNA was almost completely abrogated. Briefly, mutation frequencies determined by the REAP assay were verified by addition of X-gal and IPTG to a solution of progeny phage and plating onto a lawn of NR9050 *E. coli* cells, as previously described by Delaney and Essigmann (*43*).

RESULTS

The purpose of this study was to evaluate the mutagenic potential of the lesions Oa, Oz, and Ca by using a ss DNA system that served as a model for replication past an oxidized guanine lesion.

Genome Construction. The viral genomes for this study were constructed from M13mp7L2 ss DNA as shown in Figure 2. The 8-oxodG-containing oligonucleotide was chemically synthesized using solid-phase phosphoramidite methodology. The Oa, Oz, and Ca oligonucleotides were produced by selective oxidation of the corresponding 8-oxodG-containing oligonucleotide by $ONOO^-$. The identities of the lesions in DNA were assigned based on molecular mass as determined using matrix-assisted laser desorption ionization-time-of-flight spectroscopy (MALDI-TOF). In each case, the modified base was situated at the cleavage site of a unique *Bbs*I recognition domain, which allowed for interrogation of the base at the lesion site. Each oligonucleotide insert was stable as verified by MALDI-TOF analysis after exposure of the lesions to the exact conditions of genome construction.

Determination of Polymerase Blockage by Measuring M13 Survival. The original insert-containing phage vectors were ss DNA, so translesion synthesis must have occurred in order for progeny phage to be produced. Since lesions in the DNA may block or inhibit replication, the relative number of progeny phage when plated on a lawn of indicator bacteria immediately after transfection gives a measure of the efficiency of translesion synthesis, as described in Figure 2. Comparison of the number of plaques obtained after replication of the damaged phage genome with those from an identically constructed vector that did not carry any oxidative

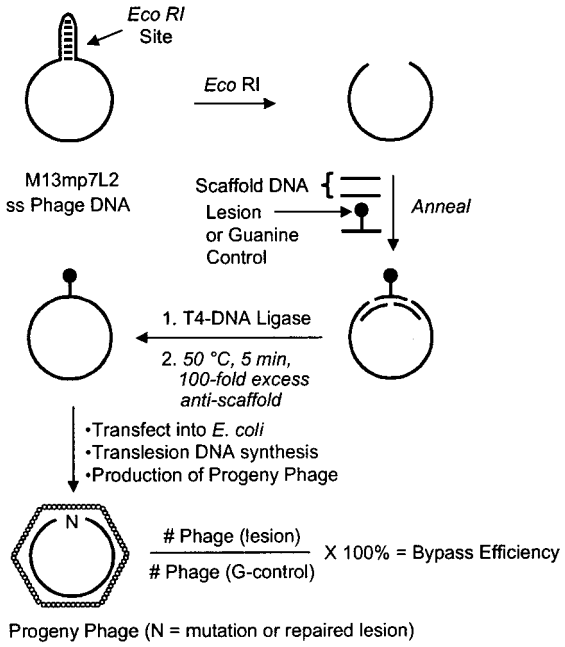

FIGURE 2: Method for inserting lesion-containing oligonucleotides into the single-stranded M13mp7L2 vector. Plating of the progeny phage onto a lawn of generic bacteria immediately after transfection caused formation of plaques, whose number allowed calculation of the efficiency of polymerase bypass for each lesion relative to a guanine control.

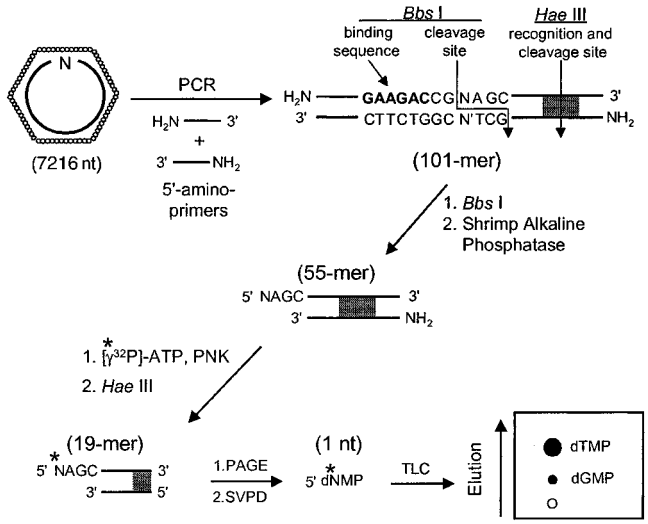

FIGURE 3: Detection of mutations using the REAP assay. An adducted genome was transfected into a repair-proficient *E. coli* cell line to afford a population of progeny phage. The region flanking the insert was amplified by PCR using 5-amino-terminated primers to afford a 101 bp fragment whose 5′-termini were refractory to labeling by PNK. The DNA was cleaved at the position that had contained the lesion in the parental vector using the type IIs restriction endonuclease *Bbs*I. The 5′-ends formed by *Bbs*I digestion were radiolabeled, and treatment with *Hae*III liberated a 19-mer, of which the base composition at the 5′-end provided the mutation frequency. The 19-mer was digested to 5′-dNMPs. Separation of the radioactive 5′-dNMPs on a TLC plate, followed by PhosphorImager analysis, provided the mutation frequency and type at the lesion site.

damage allowed calculation of the bypass efficiency for each lesion. As shown in Figure 4, 8-oxodG, Oa, Oz, and Ca were all readily bypassed relative to the G control. Vector survival for 8-oxodG was 88 ± 11% with respect to the untreated control, which clearly demonstrates that 8-oxodG only

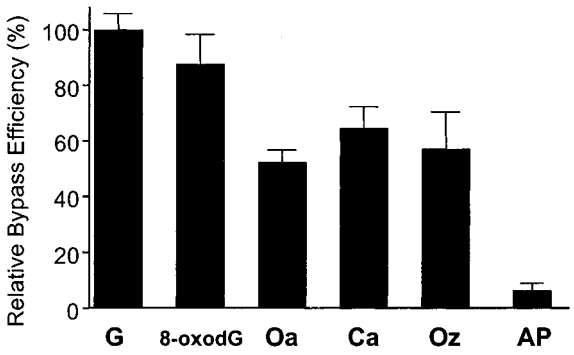

FIGURE 4: Replication efficiency, as a percent of the G control, of vectors carrying a single 8-oxodG, Oa, Ca, Oz, or AP site. Percentages reported are the average of three experiments. The error bars represent a 95% confidence interval of the mean. Data exclude progeny of uncut and religated M13mp7L2. Such phage, as determined by a no-insert control, constitute approximately 10% of all constructs.

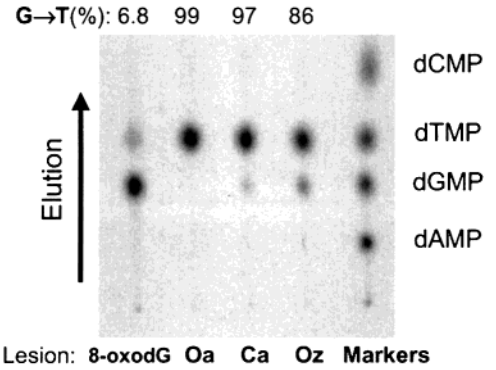

FIGURE 5: TLC showing mutation frequency analysis by the REAP assay for 8-oxodG, Oa, Ca, and Oz. The 5′-dNMP markers were derived from SVPD digestion of a 13-mer oligonucleotide that contained a radiolabeled degenerate 5′-end.

weakly inhibited plasmid replication. This result agrees, within experimental error, with a value of 80% reported previously for replication in COS7 monkey cells past a pS189 ss vector containing a single 8-oxodG (*48*). For the secondary oxidative lesions Oa, Oz, and Ca, synthesis past each lesion was effective for 52 ± 5, 57 ± 13, and 65 ± 8% of the vectors, respectively. As a control demonstrating DNA replication blockage, lesion survival for a synthetic THF AP site was 6 ± 3%. Interestingly, in vivo DNA replication past a THF AP site has not been reported, and the relative bypass is somewhat higher than that reported for the natural aldehydic AP site placed site-specifically in a nearly identical bacteriophage system (*49*).

Mutation Frequency and Type. The ligated genomes were transfected into wild-type AB1157 *E. coli* cells, which afforded progeny phage (Figure 2). Mutation frequencies were determined by PCR amplification of the region containing the lesion site from the progeny phage to yield a duplex substrate with recognition sites for the endonucleases *Bbs*I and *Hae*III (Figure 3). The 101 bp PCR product consisted of a population of duplexes that varied only by the composition of the base at the position that originally contained the lesion in the constructed genome. Briefly, the REAP assay was performed by completely digesting the 101-mer with *Bbs*I, 5′-phosphorylation of the liberated 55-mer with PNK and [γ-^{32}P]ATP, and incubation with *Hae*III to afford a 19-mer, whose radiolabeled 5′-end gave the mutation frequency and type. The purified and desalted 19-mer was digested to 5′-[^{32}P]-dNMPs, which were resolved by TLC, and the radioactivity and migration of each spot were used to calculate the mutation frequency and type. Additionally, the G → T mutation frequencies were determined independently by a β-galactosidase α-complemention assay, and matched the REAP assay results within a 95% confidence interval of the mean for each lesion (data not shown). Frameshifts and small deletions or insertions are assumed to be negligible in this work, since there was no detectable variation in the size of the *Bbs*I/*Hae*III restricion fragment. Deletions that encompass the *Bbs*I site or either of the PCR primer sites would not be detected by the REAP assay.

As shown in Figure 5, the mutation frequencies were determined alongside a control of 8-oxodG, and with radiolabeled 5′-dNMPs as markers for the relative migration of each nucleotide. The mutation frequency of 8-oxodG was modest (7%). The predominant mutation observed was a G → T transversion, although low levels of G → C and G → A mutations were also observed for all lesions (<1% in either case). In contrast to the low mutation frequency of 8-oxodG, the frequencies of G → T transversion for Oa, Ca, and Oz were strikingly high (97%, 86%, and 95%, respectively). Thus, the oxidation of guanine results in the formation of 8-oxodG, which is weakly mutagenic. However, if 8-oxodG in DNA suffers additional oxidation, which previously has been shown to be a favorable event, the weakly mutagenic lesion is converted into several powerfully mutagenic secondary lesions that, coincidentally, have the same mutational specificity as 8-oxodG.

DISCUSSION

8-OxodG is one of the most common oxidative DNA base lesions owing to the low oxidation potential of guanine [$E°$ = 1.29 V vs a nickel hydrogen electrode (NHE) for 2′-deoxyguanosine, the lowest of the four major nucleosides] (*11*). The seemingly remote chance of a second oxidation event is offset by the inherent ease of oxidation of 8-oxodG ($E°$ = 0.58−0.75 V vs NHE for the 8-oxodG nucleoside) (*50*−*52*). Furthermore, the ability of a radical cation (the initial product of one electron oxidation of a base) to migrate over long distances (>55 bp) in duplex DNA (*53*, *54*), and then become trapped preferentially at 8-oxodG (*55*, *56*), suggests that secondary oxidation products could be formed at substantial levels in vivo. Oxidation of 8-oxodG results in the formation of a variety of products such as Ca, Oa, a 2,5-diaminoimidazalone (Iz), which undergoes spontaneous hydrolysis to form Oz, as well as several hydantoin derivatives (*33*, *57*−*62*).

We investigated the repair and mutagenic potential of the three major products of peroxynitrite-oxidized 8-oxodG in DNA: Oa, Oz, and Ca. Each lesion was placed site-specifically in a bacteriophage genome and assayed for bypass efficiency and mutation type following transfection into *E. coli*. The substantial polymerase bypass across each lesion is presumably due to the DNA polymerase III holoenzyme, which is known to replicate M13 DNA in *E. coli* (*63*). SOS induction of the AB1157 cells prior to transfection resulted in no significant change in bypass or mutation (data not shown), which indicates DNA polymerase V (UmuD′2) is not involved in polymerase extension across the lesions (*64*).

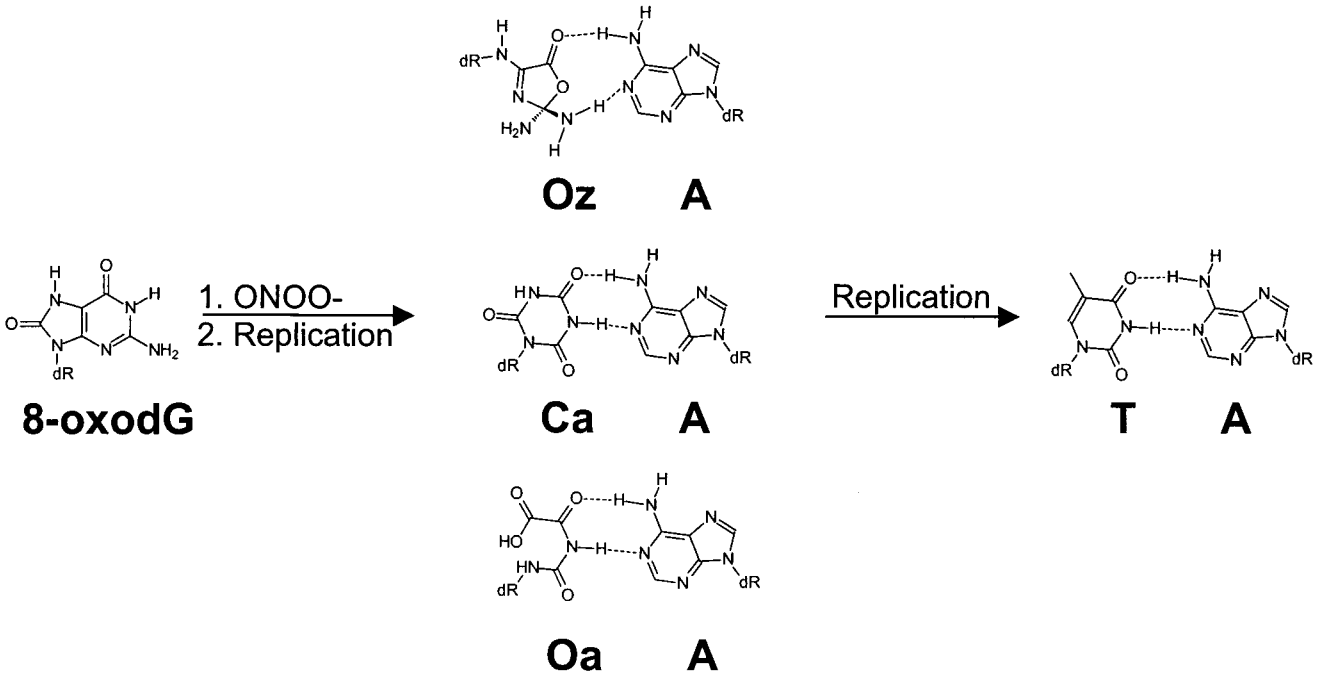

FIGURE 6: Proposed base-pairing scheme for G → T transversion mutations caused by Oz, Ca, and Oa.

A modest mutation frequency (7%) was observed for 8-oxodG, as expected based upon literature values (*15*, *65*, *66*). The predominant mutation for this lesion was G → T. It is known that 8-oxodG, placed site-specifically in a ss DNA vector, is subject to repair in *E. coli*, since the mutation frequency increases approximately 10-fold in cells deficient in MutM and MutY (*67*). Presumably, the repair occurs after the first round of replication, since both MutM and MutY require duplex substrates. The secondary lesions Oa, Oz, and Ca are not well repaired compared to 8-oxodG, yet they are bypassed. This combination of properties apparently contributes to their potent mutagenicity.

As shown in Figure 6, the extremely high mutation frequencies observed for Oa, Oz, and Ca might result from the presence of a genetically instructive carbonyl moiety. It is reasoned that this functional group defines the base-pairing preference of the lesions for adenine, since the oxygen atom can act as a hydrogen bond acceptor analogous to that of O^4 in thymine. Importantly, none of the lesions possess a hydrogen-bonding surface that is complementary to C, unlike 8-oxodG. The 86−97% mutation frequencies observed may implicate these lesions collectively as the primary source for the frequently observed G → T transversions caused by $ONOO^-$ oxidation of DNA.

The adverse role that 8-oxodG-derived lesions play in mammalian cells remains to be seen. Juedes and Wogan investigated the mutagenicity of peroxynitrite using the *supF* gene of the pSP189 shuttle vector as a mutation target in bacterial and mammalian cells (*38*). Exposure to $ONOO^-$ caused the mutation frequency to increase 21-fold in the vector when replicated in *E. coli* MBL50 cells, and 9-fold upon replication in human Ad293 cells compared to untreated vector. In both systems, most mutations occurred at G:C base pairs, predominantly involving G → T transversions (65% when replication was in bacteria and 63% when in human cells). G → C transversions were observed at lower frequency (28% in MBL50 and 11% in Ad293 cells). The work presented here provides an explanation for the G → T component of the mutation spectrum observed in the *supF* work.

The G → C component of the mutations of $ONOO^-$ has yet to be explained. However, it should be noted that Oa is temperature-sensitive, and its major hydrolysis product is a urea lesion, which we have found may be responsible, in part, for the previously reported G → C transversions (unpublished results). Additionally, the precursor to Oz is the 2-amino-5-[(2′-deoxy-β-D-*erythro*-pentofuranosyl)amino]-4*H*-imidazol-4-one derivative (Iz) (*68*), which is hydrolyzed to Oz with a half-life of 20 h at 37 °C in duplex DNA (*69*). Iz is known to pair preferentially with G during primer extension with Pol I, and therefore may be a transient contributor to G → C transversions in vivo (*70*).

Kreutzer and Essigmann reported the secondary cytosine oxidation products derived from 5-hydroxycytosine (5-OH-C) as being particularly mutagenic (*71*). The initial lesion 5-OH-C, like 8-oxodG, either is well repaired or is weakly mutagenic (0.05% mutation frequency), but the secondary products uracil glycol and 5-hydroxyuracil are highly mutagenic (83% and 80% C → T transitions, respectively). Therefore, the high mutagenic potential of secondary products of DNA oxidation may be a general phenomenon, which is not restricted to guanine chemistry.

This work demonstrates that secondary lesions arising from facile oxidation of 8-oxodG by $ONOO^-$ are likely the main cause of G → T transversions upon replication of $ONOO^-$-treated DNA in *E. coli*, and calls attention to the potential importance of secondary DNA oxidation products in other species. If mammalian polymerases misincorporate bases opposite Ca, Oa, and Oz with the frequency and efficiency observed here, then these lesions pose a serious threat to mammalian genomic integrity.

SUPPORTING INFORMATION AVAILABLE

HPLC chromatograms and mass spectra for each lesion-containing oligonucleotide. This material is available free of charge via the Internet at http://pubs.acs.org.

REFERENCES

1. Wang, D., Kreutzer, D. A., and Essigmann, J. M. (1998) *Mutat. Res.*, 84−87.
2. Halliwell, B., and Gutteridge, J. M. C. (1989) *Free radicals in biology and medicine*, Clarendon Press, Oxford, U.K.
3. Ames, B. N., Shigenaga, M. K., and Hagen, T. M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 7915−7922.
4. Huie, R. E., and Padmaja, S. (1993) *Free Radical Res. Commun. 18*, 195−199.
5. Salgo, M. G., Stone, K., Squadrito, G. L., Battista, J. R., and Pryor, W. A. (1995) *Biochem. Biophys. Res. Commun. 210* (3), 1025−1030.
6. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) *Biochemistry 35*, 7855−7861.
7. Szabo, C., Zingarelli, B., O'Connor, M., and Salzman, A. L. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 1753−1758.
8. Yermilov, V., Yoshie, Y., Rubio, J., and Ohshima, H. (1996) *FEBS Lett. 399*, 67−70.
9. Kennedy, L. J., Moore, K. J., Caulfield, J. L., Tannenbaum, S. R., and Dedon, P. C. (1997) *Chem. Res. Toxicol. 10*, 386−392.
10. Yermilov, V., Rubio, J., Becchi, M., Friesen, M. D., Pignatelli, B., and Ohshima, H. (1995) *Carcinogenesis 16*, 2045−2050.
11. Steenken, S., and Jovanovic, S. V. (1997) *J. Am. Chem. Soc. 119*, 617−618.
12. Wood, M. L., Dizdaroglu, M., Gajewski, E., and Essigmann, J. M. (1990) *Biochemistry 29*, 7024−7032.
13. Cheng, K. C., Cahill, D. S., Kasai, H., Nishimura, S., and Loeb, L. A. (1992) *J. Biol. Chem. 267*, 166−172.
14. Moriya, M., Ou, C., Bodepudi, V., Johnson, F., Takeshita, M., and Grollman, A. P. (1991) *Mutat. Res. 254*, 281−288.
15. Klein, J. C., Bleeker, M. J., Lutgerink, J. T., van Dijk, W. J., Brugghe, H. F., van den Elst, H., van der Marel, G. A., Westra, J. G., and Berns, A. J. (1990) *Nucleic Acids Res. 18*, 4131−4137.
16. Klein, J. C., Bleeker, M. J., Saris, C. P., Roelen, H. C. P. F., Brugghe, H. F., van den Elst, H., van der Marel, G. A., van Boom, J. H., Westra, J. G., Kriek, E., and Berns, A. J. M. (1992) *Nucleic Acids Res. 20*, 4437−4443.
17. Helbock, H. J., Beckman, K. B., Shigenaga, M. K., Walter, P. B., Woodall, A. A., Yeo, H. C., and Ames, B. N. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 288−293.
18. Michaels, M. L., and Miller, J. H. (1992) *J. Bacteriol. 174*, 6321−6325.
19. Boiteux, S., O'Connor, T. R., Lederer, F., Gouyette, A., and Laval, J. (1990) *J. Biol. Chem. 265*, 3916−3922.
20. Michaels, M. L., Tchou, J., Grollman, A. P., and Miller, J. H. (1992) *Biochemistry 31*, 10964−10968.
21. Michaels, M. L., Cruz, C., Grollman, A. P., and Miller, J. H. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 8778−8783.
22. Maki, H., and Sekiguchi, M. (1992) *Nature 355*, 273−275.
23. Nash, H. M., Bruner, S. D., Schaeer, O. D., Kawate, T., Addona, T. A., Spooner, E., Lane, W. S., and Verdine, G. L. (1996) *Curr. Biol. 6*, 968−980.
24. van der Kemp, P. A., Thomas, D., Barbey, R., de Oliveira, R., and Boiteux, S. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 5197−5202.
25. Radicella, J. P., Dherin, C., Desmaze, C., Fox, M. S., and Boiteux, S. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8010−8015.
26. Rosenquist, T. A., Zharkov, D. O., and Grollman, A. P. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 7429−7434.
27. Roldan-Arjona, T., Wei, Y. F., Carter, K. C., Klungland, A., Anselmino, C., Wang, R. P., Augustus, M., and Lindahl, T. (1997) *Proc. Natl. Acad. Sci. U.S.A. 94*, 8016−8020.
28. McGoldrick, J. P., Yeh, Y. C., Solomon, M., Essigmann, J. M., and Lu, A. L. (1995) *Mol. Cell. Biol. 15*, 989−996.
29. Slupska, M. M., Baikalov, C., Luther, W. M., Chiang, J. H., Wei, Y. F., and Miller, J. H. (1996) *J. Bacteriol. 178*, 3885−3892.
30. Sakumi, K., Furuichi, M., Tsuzuki, T., Kakuma, T., Kawabata, S., Maki, H., and Sekiguchi, M. (1993) *J. Biol. Chem. 268*, 23524−23530.
31. Kakuma, T., Nishida, J., Tsuzuki, T., and Sekiguchi, M. (1995) *J. Biol. Chem. 270*, 25942−25948.
32. Uppu, R. M., Cueto, R., Squadrito, G. L., Salgo, M. G., and Pryor, W. A. (1996) *Free Radical Biol. Med. 21*, 407−411.
33. Tretyakova, N. Y., Niles, J. C., Burney, S., Wishnok, J. S., and Tannenbaum, S. R. (1999) *Chem. Res. Toxicol. 12*, 459−466.
34. Tretyakova, N. Y., Wishnok, J. S., and Tannenbaum, S. R. (2000) *Chem. Res. Toxicol. 13*, 658−664.
35. Gasparutto, D., Da Cruz, S., Bourdat, A. G., Jaquinod, M., and Cadet, J. (1999) *Chem. Res. Toxicol. 12*, 630−638.
36. Duarte, V., Gasparutto, D., Jaquinod, M., and Cadet, J. (2000) *Nucleic Acids Res. 28*, 1555−1563.
37. Duarte, V., Gasparutto, D., Jaquinod, M., Ravanat, J., and Cadet, J. (2001) *Chem. Res. Toxicol. 14*, 46−53.
38. Juedes, M. J., and Wogan, G. N. (1996) *Mutat. Res. 349*, 51−61.
39. Tretyakova, N. Y., Burney, S., Pamir, B., Wishnok, J. S., Dedon, P. C., Wogan, G. N., and Tannenbaum, S. R. (2000) *Mutat. Res. 447*, 287−303.
40. Muller, J. G., Duarte, V., Hickerson, R. P., and Burrows, C. J. (1998) *Nucleic Acids Res. 26*, 2247−2249.
41. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning*: *A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
42. Pryor, W. A., Cueto, R., Jin, X., Koppenol, W. H., Ngu-Schwemlein, M., Squadrito, G. L., Uppu, P. L., and Uppu, R. M. (1995) *Free Radical Biol. Med. 18* (1), 75−83.
43. Delaney, J. C., and Essigmann, J. M. (1999) *Chem. Biol. 6*, 743−753.
44. Miller, J. H. (1992) *A short course in bacterial genetics*: *a laboratory manual and handbook for Escherichia coli and related bacteria*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
45. Hayes, R. C., and LeClerc, J. E. (1983) *Gene 21*, 1−8.
46. Banerjee, S. K., Borden, A., Christensen, R. B., LeClerc, J. E., and Lawrence, C. W. (1990) *J. Bacteriol. 172*, 2105−2112.
47. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning*: *a laboratory manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
48. Le Page, F., Margot, A., Grollman, A. P., Sarasin, A., and Gentil, A. (1995) *Carcinogenesis 16*, 2779−2784.
49. Lawrence, C. W., Borden, A., Banerjee, S. K., and LeClerc, J. E. (1990) *Nucleic Acids Res. 18*, 2153−2157.
50. Berger, M., Anselmino, C., Mouret, J.-F., and Cadet, J. (1990) *J. Liq. Chromatogr. 13*, 929−932.
51. Goyal, R. N., and Dryhurst, G. (1982) *J. Electroanal. Chem. 135*, 75−91.
52. Yanagawa, H., Ogawa, Y., and Ueno, M. (1992) *J. Biol. Chem. 267*, 13320−13326.
53. Henderson, P. T., Jones, D. J., Hampikian, G., Kan, Y., and Schuster, G. B. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 8353−8358.
54. Nunez, M. E., Hall, D. B., and Barton, J. K. (1999) *Chem. Biol. 6*, 85−97.
55. Gasper, S. M., and Schuster, G. B. (1998) *J. Am. Chem. Soc. 119*, 12762−12771.
56. Ly, D., Sanii, L., and Schuster, G. B. (1999) *J. Am. Chem. Soc. 121*, 9400−9410.
57. Sheu, C., and Foote, C. S. (1995) *J. Org. Chem. 60*, 4498−4503.
58. Luo, W., Muller, J. G., Rachlin, E. M., and Burrows, C. J. (2001) *Chem. Res. Toxicol. 14*, 927−938.
59. Luo, W., Muller, J. G., Rachlin, E. M., and Burrows, C. J. (2000) *Org. Lett. 2*, 613−616.
60. Raoul, S., and Cadet, J. (1996) *J. Am. Chem. Soc. 118*, 1892−1898.
61. Sheu, C., and Foote, C. S. (1995) *J. Am. Chem. Soc. 117*, 6439−6442.
62. Adam, W., Saha-Moeller, C., Schoenberger, A., Berger, M., and Cadet, J. (1995) *Photochem. Photobiol. 62*, 231−238.

63. Geider, K., and Kornberg, A. (1974) *J. Biol. Chem. 249*, 3999−4005.
64. Bagg, A., Kenyon, C. J., and Walker, G. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 5749−5753.
65. Wood, M. L., Dizdaroglu, M., Gajewski, E., and Essigmann, J. M. (1990) *Biochemistry 29*, 7024−7032.
66. Moriya, M. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 1122−1126.
67. Moriya, M., and Grollman, A. P. (1993) *Mol. Gen. Genet. 239*, 72−76.
68. Cadet, J., Berger, M., Buchko, G. W., Joshi, P. C., Raoul, S., and Ravanat, J. L. (1994) *J. Am. Chem. Soc. 116*, 7403−7404.
69. Ikeda, H., and Saito, I. (1999) *J. Am. Chem. Soc. 121*, 10836−10837.
70. Kino, K., and Sugiyama, H. (2001) *Chem. Biol. 8*, 369−378.
71. Kreutzer, D. A., and Essigmann, J. M. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3578−3582.

BI0156355