The Substrate Specificity of MutY for Hyperoxidized Guanine Lesions in Vivo†

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ABSTRACT: The DNA damage product 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoG) is a commonly used biomarker of oxidative stress. The mutagenic potential of this DNA lesion is mitigated in *Escherichia coli* by multiple enzymes. One of these enzymes, MutY, excises an A mispaired with 8-oxoG as part of the process to restore the original G:C base pair. However, numerous studies have shown that 8-oxoG is chemically labile toward further oxidation. Here, we examine the activity of MutY on the 8-oxoG oxidation products guanidinohydantoin (Gh), two diastereomers of spiroiminodihydantoin (Sp1 and Sp2), oxaluric acid (Oa), and urea (Ur). Single-stranded viral genomes containing a site-specific lesion were constructed and replicated in *E. coli* that are either proficient in DNA repair or that lack MutY. These lesions were found previously to be potently mutagenic in repair competent bacteria, and we report here that these 8-oxoG-derived lesions are equally miscoding when replicated in *E. coli* lacking MutY; no significant change in mutation identity or frequency is observed. Interestingly, however, in the presence of MutY, Sp1 and Sp2 are more toxic than in cells lacking this repair enzyme.

Both endogenous and exogenous agents have been implicated in the oxidation of genomic DNA, and this damage is associated with aging, carcinogenesis, and neurodegenerative diseases (*1*−*6*). Of the four nucleobases, guanine is the most susceptible to oxidative damage because of its low redox potential (E_0 = 1.3, 1.4, 1.6, and 1.7 V versus normal hydrogen electrode (NHE) for G, A, C, and T, respectively) (*7*). One commonly used biomarker of oxidative stress is the DNA lesion 7,8-dihydro-8-oxo-2′-deoxyguanine (8-oxoG)[1] (Figure 1). 8-OxoG is readily detected in isolated DNA using high-performance liquid chromatography (HPLC)/electrochemical detection (*8*) or in situ using immunohistochemistry (*9*, *10*).

In vitro experiments indicate that 8-oxoG has the capacity to be a very mutagenic lesion because of its propensity to mispair with A during replication by DNA polymerases (*11*). Results obtained in vivo, in contrast, show that 8-oxoG is only mildly mutagenic, yielding <10% G → T transversions in bacterial and mammalian cells (*12*−*16*). Protection of *Escherichia coli* from the mutagenicity of 8-oxoG is achieved by the concerted efforts of three enzymes, referred to as the 8-oxoG or GO system (*17*). One enzyme, MutM, is a glycosylase that excises 8-oxoG when paired with C. MutY, also a glycosylase, removes an A misincorporated opposite 8-oxoG. Finally, MutT is a phosphatase that removes d(8-oxoGTP) from the nucleotide pool, preventing incorporation of the modified base during replication. Homologous 8-oxoG repair systems have been identified in yeast (*18*−*20*) and mammalian cells (*21*−*26*).

Interestingly, with an oxidation potential of 0.58−0.75 V vs NHE (*27*−*29*), 8-oxoG is even easier to oxidize than G. In fact, multiple groups have reported that 8-oxoG is susceptible to further oxidation. Using a host of agents, including $ONOO^-$ (*30*−*33*), $[IrCl_6]^{2-}$ (*34*, *35*), O_2[1] (*36*), and $[Cr_2O_7]^{2-}$ (*37*), numerous 8-oxoG oxidation products have been identified in vitro. These hyperoxidized products, so-called because they result from at least two sequential G oxidation events, include guanidinohydantoin (Gh), two diastereomers of spiroiminodihydantoin (Sp1 and Sp2), imidazalone (Iz), oxazalone (Oz), cyanuric acid (Ca), and oxaluric acid (Oa) (Figure 1). Additionally, in the presence of physiological levels of bicarbonate, Oa is hydrolyzed to urea (Ur) (*38*). As compared with the mildly mutagenic 8-oxoG, these hyperoxidized products are potently mutagenic, yielding predominantly G → T and/or G → C mutations both in vitro (*39*−*42*) and in vivo (*38*, *43*−*45*). Furthermore, the Sp lesion has been detected in cellular DNA, demonstrating the biological significance of 8-oxoG oxidation (*46*).

The ability of the base excision repair glycosylase MutM to excise these 8-oxoG oxidation products in duplex DNA has been explored in vitro. In contrast to 8-oxoG, which is efficiently removed only when paired with C, MutM excises Gh, Sp, Oa, and Oz when these lesions are paired with A, G, C, or T (*40*, *41*, *47*−*49*). Interestingly, Ca is not excised by MutM when paired with C (*39*, *49*); removal of this lesion paired with other nucleobases has not been explored.

Further in vitro work has examined the activity of MutY on duplexes containing Gh:A/G or Sp:A/G base pairs (*47*, *48*). While MutY excises an A paired with 8-oxoG, it does not excise an A or G opposite Gh or Sp. However, although

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[1] Abbreviations: 8-oxoG, 7,8-dihydro-8-oxo-2′-deoxyguanine; Gh, guanidinohydantoin; Sp, spiroiminodihydantoin; Iz, imidazalone; Oz, oxazalone; Ca, cyanuric acid; Oa, oxaluric acid; Ur, urea; THF abasic site, tetrahydrofuran abasic site; PAGE, polyacrylamide gel electrophoresis.

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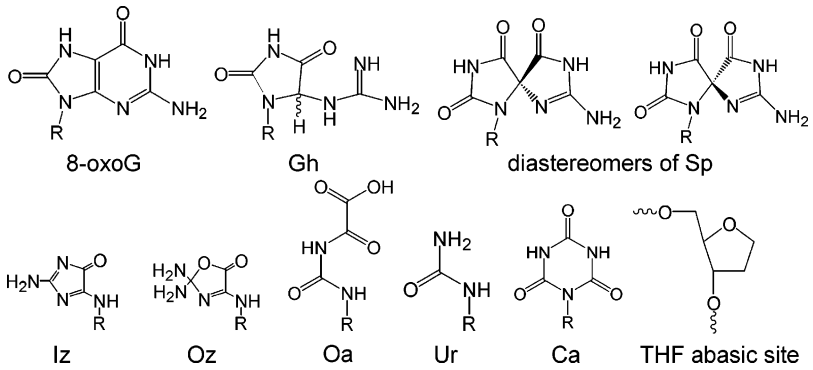

FIGURE 1: Structures of 8-oxoG and the hyperoxidized G lesions.

the enzyme does not have glycoslyase activity, MutY does have specific affinity for duplexes containing these lesions. In fact, MutY will bind to DNA containing Gh or Sp and inhibit MutM activity (*48*).

Taken together, a large literature shows that MutY is essential to protect cells against the mutagenicity, but not the toxicity, of 8-oxoG. Using a recently developed analytical system, we confirm those data as the starting point of this in vivo study. We show, however, that the hyperoxidized lesions formed from 8-oxoG, which are highly toxic and mutagenic in vivo, are not repaired to any significant extent by the MutY repair enzyme. These data fit well with the developing biochemical literature that further suggests that these hyperoxidized lesions are poorly repaired, if at all, in vivo.

EXPERIMENTAL PROCEDURES

Bacterial Strains. The *E. coli* strains used for transfection were AB1157 (wild-type) and *mutY* AB1157 (as AB1157 but *mutY*::mini-*Tn*10-*tet*). The *mutY* AB1157 partial deletion replacement mutant was constructed using P1 phage transduction (*50*) of the chromosomal Δ*mutY*::mini-*Tn*10-*tet* from *mutY* CC104 (from M. Michaels and J. H. Miller, UCLA). Transduction was confirmed by genomic polymerase chain reaction (PCR) and sequencing analysis. The strain used as the indicator bacteria was NR9050 from R. M. Schaaper (NIEHS) and that for regrowth of progeny phage was SCS110 (Stratagene).

Oligonucleotides. All DNA synthesis reagents were from Glen Research. The single-stranded 16-mer sequence was 5′-GAA GAC CTX GGC GTC C-3′ where X = G, 8-oxoG, Gh, Sp1, Sp2, Oa, Ur, or a tetrahydrofuran abasic site (THF). Oligonucleotides containing G, 8-oxoG, or a THF abasic site were synthesized using standard phosphoramidite chemistry on an ABI 391 DNA synthesizer. The oligonucleotides containing Gh (*42*), Sp1 (*42*), Sp2 (*42*), or Oa (*31*) were synthesized as previously described and purified by anion exchange HPLC on a Dionex NucleoPac PA-100 column using 10% acetonitrile (solvent A) and 1.5 M ammonium acetate, pH 8 (solvent B) (1.0 mL/min; 10−50% B over 5 min, then 50−75% B over 25 min). The purified oligonucleotides were twice desalted with NAP-10 columns (Amersham). To obtain a Ua-containing oligonucleotide, the Oa oligonucleotide was hydrolyzed by the addition of 0.1 N NaOH, incubated for 6.5 h at 37 °C, and purified by HPLC. The molecular weight of all oligonucleotides was verified with matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (Supporting Information). DNA scaffolds (5′-GGT CTT CCA CTG AAT CAT GGT CAT AGC-3′; 5′-AAA ACG ACG GCC AGT GAA TTG GAC GC-3′) were purchased from Integrated DNA Technologies.

Construction of Genomes Containing a Site-Specific DNA Lesion. Single-stranded M13 genomes containing a site-specific lesion were synthesized as previously described (*51*). Briefly, the M13mp7L2 bacteriophage genome was linearized by *Eco*RI at a hairpin containing a unique restriction site. Two scaffolds, complementary to the 5′- or 3′-end of the 16-mer oligonucleotide insert and the linearized viral genome, were annealed to the linearized M13 DNA. The genome was recircularized by the addition of 5′-phosphorylated 16-mer insert and incubation with T4 DNA ligase. After ligation, the scaffolds were digested with T4 DNA polymerase. The genome constructs were phenol extracted, desalted with Sephadex G50 fine resin (Amersham), and stored at −20 °C.

Preparation of Electrocompetent Cells. LB media (150 mL) was inoculated with 1 mL of saturated wild-type or *mutY* AB1157 *E. coli* and grown on a shaker at 37 °C to an OD_{600} nm of ≈0.5. The cells were twice pelleted and resuspended in 175 mL of water. After these two washes, the cells were once again pelleted and resuspended in 1.5 mL of 10% glycerol. These electrocompetent cells were stored at 4 °C and used within 24 h.

Analysis of Mutational Spectrum. Electrocompetent cells (100 μL) and lesion-containing genomes (5 μL) were mixed and chilled on ice for 45 min prior to transfection by electroporation (2.5 kV, 129 ohms, and 2 mm gap cuvette). The number of independently transfected cells was 10^4−10^5 as determined by plating on a lawn of NR9050 *E. coli*. After electroporation, the sample was transferred to 10 mL of LB and incubated at room temperature for at least 30 min. The cultures were then grown for 4 h at 37 °C. Successful replication of the genome by *E. coli* generates progeny phage, which can be isolated by pelleting the cells and retaining the progeny-containing supernatant. To eliminate PCR amplification of nontransfected genomes, progeny (∼10^9) were regrown in SCS110 *E. coli*. Single-stranded phage DNA was isolated using a QIAPrep Spin M13 kit (Qiagen), and the sequence of interest was amplified by PCR as described previously (*51*). The REAP assay (*51*, *52*) (as outlined in the Supporting Information) was then used to determine the identity of the base at the site originally occupied by the lesion. The 61 base pair PCR product was digested with *Bbs*I, a type IIs restriction endonuclease that cleaves two bases away from its binding site at the position that originally

contained the lesion. The liberated 5′-end contained the base of interest, and following dephosphorylation with shrimp alkaline phosphatase, this 5′-end was radiolabeled using [γ-^{32}P]ATP and T4 polynucleotide kinase (USB). Restriction with *Hae*III yielded an 18-mer, which was isolated by denaturing polyacrylamide gel electrophoresis (PAGE), desalted, and digested with nuclease P1 (Roche) to provide 5′-deoxynucleotide monophosphates (5′-dNMPs). The mixture of 5′-dNMPs was spotted on a polyethyleneimine thin-layer chromatography (TLC) plate (J. T. Baker) and developed in saturated $(NH_4)_2HPO_4$ (pH 5.8). As only the base of interest is radiolabeled, quantitation of the separated 5′dNMPs by phosphorimagery provides the base composition at the lesion site from which the mutation type and frequency were determined.

Determination of Lesion Bypass Efficiency. Electroporations were performed using a mixture of lesion-containing genome with an internal standard genome, as described previously (*51*, *53*). The internal standard contains an oligonucleotide insert with a G in place of a lesion and is three nucleotides larger than the lesion-containing genomes (5′-GAA GAC CTG GTA GCG CAG G-3′). The procedure is similar to the mutational assay described above except a different primer set and PCR conditions are utilized. The PCR is performed using the primers 5′-YCAG CTA TGA CCA TGA TTC AGT GGA AGA C-3′ and 5′-YCAG GGT TTT CCC AGT CAC GAC GTT GTA A-3′ where Y is a C6 amino modifier (Integrated DNA Technologies). The PCR program is as follows: 94° for 0.5 min, followed by 30 cycles of 94° for 0.5 min, 67° for 1 min, and 72° for 1 min, with a final extension of 72° for 5 min. Following *Bbs*I digestion of the amplified product, 5′-end labeling, and *Hae*III restriction, the resultant 18-mer is quantitated with respect to the internal standard (observed as a 21-mer). This provides a measure of bypass efficiency as compared to a G-containing genome. The Student's *t* test (significance level = 0.05) was used to compare levels of bypass in wild-type and MutY-deficient *E. coli*.

RESULTS

Preparation of Bacteriophage Genomes Containing a Site Specifically Incorporated DNA Lesion. Single-stranded genomes containing a site specifically incorporated lesion were constructed from M13 viral DNA (Figure 2). Short oligonucleotides containing the lesion of interest were chemically synthesized and inserted into the single-stranded vector. Oligonucleotides containing G, 8-oxoG, or a THF abasic site were synthesized using standard phosphoramidite chemistry. The Gh and Sp oligonucleotides were produced by selective oxidation of the 8-oxoG-containing oligonucleotide with $[IrCl_6]^{2-}$. Those containing Oa were obtained by oxidation of the 8-oxoG oligonucleotide with $ONOO^-$ and subsequently hydrolyzed to obtain the Ur-containing oligonucleotide. The stability of each lesion was verified by MALDI-TOF mass spectrometry after subjecting the lesion-containing oligonucleotides to genome construction conditions.

Use of a single-stranded vector indicates that translesion DNA synthesis must have occurred in order for viral progeny to be produced. DNA from a population of viral progeny was isolated and amplified by PCR to yield a duplex product. Restriction with *Bbs*I liberates a DNA fragment where the base of interest, incorporated at the site originally containing the lesion, is at the 5′ terminus. Radiolabeling at the 5′-end and digestion with *Hae*III generates an 18-mer that can be quantitated with respect to an internal standard to indicate lesion bypass efficiency. Additionally, this isolated 18-mer can be digested to dNTPs, which can be resolved by TLC. As only the base of interest is radiolabeled, quantitation of the separated dNTP's by phosphorimagery provides the base composition at the lesion site from which the mutation type and frequency are determined. The presence of a G at the site that originally contained the 8-oxoG-derived product indicates that the lesion is nonmutagenic. In contrast, if this base is A, C, or T, the lesion codes for a mutation. Importantly, the sensitivity of this assay allows for detection of all frameshift mutations that do not perturb the *Bbs*I binding site.

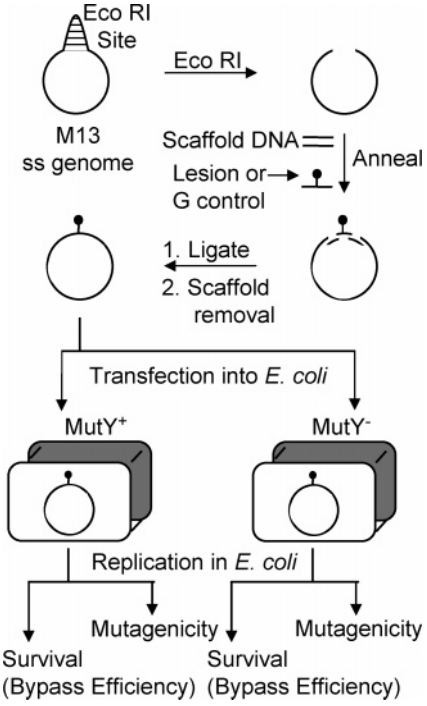

FIGURE 2: Method for constructing single-stranded genomes containing a site-specific DNA lesion. Following transfection of these genomes into bacteria, either proficient or deficient in MutY, progeny phage are analyzed to determine lesion toxicity and mutagenicity.

Frameshift Mutations. For genomes containing G, 8-oxoG, Gh, Sp1, Sp2, Oa, or Ur, replicated in either wild-type or *mutY* AB1157 *E. coli*, the uniformity of the 18-mer product isolated by PAGE following *Bbs*I and *Hae*III digestion indicates that point, rather than frameshift, mutations are formed in these experiments (Supporting Information). However, a THF abasic site does display an appreciable amount of −1 frameshifts, 13 ± 2 and 13 ± 1% in wild-type and *mutY* AB1157 *E. coli*, respectively.

Point Mutations in Repair Proficient E. coli. As shown in Figure 3, the mutational profiles of Gh, Sp1, Sp2, Oa, and Ur were determined alongside G, 8-oxoG, and THF abasic site controls. Consistent with literature reports (*12*, *13*), 8-oxoG is mildly mutagenic yielding 3% G → T transversion mutations when replicated in wild-type *E. coli*. In contrast, the hyperoxidized products are potently mutagenic with mutation frequencies greater than 98% in all cases. Gh induced a mixture of predominantly G → C and

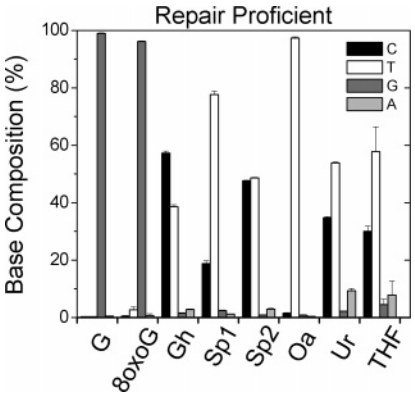

FIGURE 3: Mutation type and frequency following replication of genomes containing G, 8-oxoG, Gh, Sp1, Sp2, Oa, Ur, or a THF abasic site in repair-proficient *E. coli*. Transfections were performed in triplicate, and error bars represent one standard deviation from the mean.

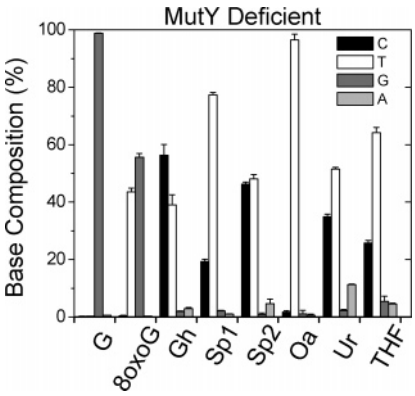

FIGURE 4: Mutation type and frequency following replication of genomes containing G, 8-oxoG, Gh, Sp1, Sp2, Oa, Ur, or a THF abasic site in MutY-deficient *E. coli*. Transfections were performed in triplicate, and error bars represent one standard deviation from the mean.

G → T mutations (57 and 40%, respectively) with a small amount of G → A mutation (3%). Sp1 and Sp2, the diastereomers of Sp named for their relative retention times on an anion exchange HPLC column, both yielded largely G → C and G → T mutations, although in different proportions. For Sp1, we observed 19% G → C and 78% G → T; Sp2 generated 48% G → C and 49% G → T mutations. Additionally, both Sp1 and Sp2 caused small amounts of G → A transitions (1 and 3%, respectively). The Oa lesion yielded almost exclusively G → T transversions (97%) with low levels of G → C (2%) mutations. When Oa is hydrolyzed to generate Ur, a mixture of 35% G → C, 54% G → T, and 9% G → A mutations is observed. The THF abasic site is also significantly mutagenic (30% G → C, 58% G → T, and 8% G → A mutations).

Point Mutations in E. coli Lacking the Base Excision Repair Glycosylase MutY. The identity and frequency of mutations caused by this family of oxidized guanine lesions were also determined in *E. coli* lacking the base excision repair glycosylase MutY (Figure 4). Given that MutY is only active on DNA duplexes, replication of the single-stranded genome must occur to generate a potential substrate for the enzyme. In contrast to the 3% G → T transversions observed in repair-proficient *E. coli*, 8-oxoG yields 44% G → T mutations following replication in *mutY* AB1157 *E. coli*. Duplexes containing 8-oxoG:A base pairs are known substrates for MutY, and this increase in mutagencity in a strain of *E. coli* deficient in MutY is consistent with literature reports (*54*, *55*). However, in contrast to this increase in mutagenicity for 8-oxoG, no significant changes in mutation type or frequency were observed following replication of Gh, Sp1, Sp2, Oa, or Ur genomes in *mutY* AB1157 *E. coli*. The mutation type and frequency observed for these lesions are identical in wild-type *E. coli* and an isogenic strain lacking MutY. These data show how quantitatively powerful MutY is as protection against misreplicated 8-oxoG:A base pairs. They also show, however, that MutY is unable to protect against the hyperoxidized G lesions, some of which have now been seen in vivo, that form by the facile oxidation of 8-oxoG.

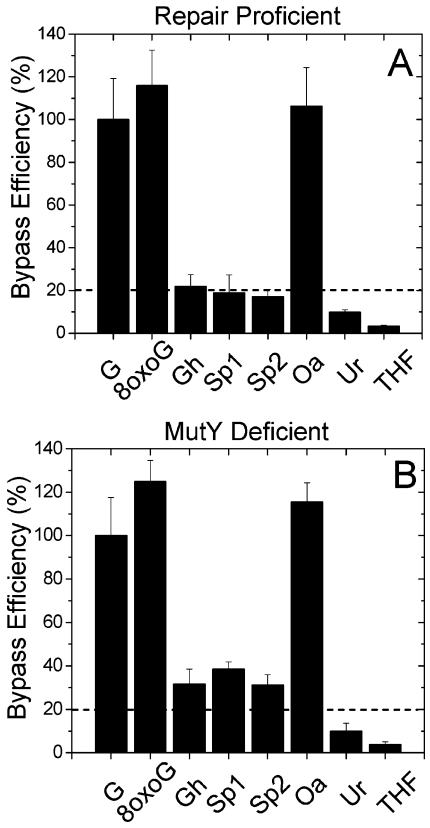

FIGURE 5: Lesion bypass efficiency, as compared to G, for 8-oxoG, Gh, Sp1, Sp2, Oa, Ur, and a THF abasic site in (A) wild-type *E. coli* and (B) *mutY* AB1157 *E. coli*. Transfections were performed in triplicate, and error bars represent one standard deviation from the mean.

Lesion Bypass Efficiency in Wild-Type and MutY E. coli. Using a recently developed assay (*51*, *53*), bypass efficiency of each hyperoxidized product was determined in wild-type and MutY-deficient *E. coli* (Figure 5). All values are relative to the bypass efficiency of G, which is set at 100%. As seen in Figure 5A, two distinct sets of lesions are defined by the observed levels of bypass in wild-type cells. Well-bypassed DNA bases, those with efficiencies comparable to G, include 8-oxoG and Oa. Poorly bypassed lesions include Gh, Sp1, Sp2, Ur, and a THF abasic site, all with efficiencies ≤20%. Similarly, these two distinct classes are observed in *mutY* AB1157 (Figure 5B). 8-OxoG and Oa are well-bypassed, whereas Gh, Sp1, Sp2, Ur, and the THF abasic site display significantly lower levels of bypass. However, closer inspection reveals that bypass efficiencies are not identical in the two cell strains. In particular, Sp1 and Sp2 display statisti-

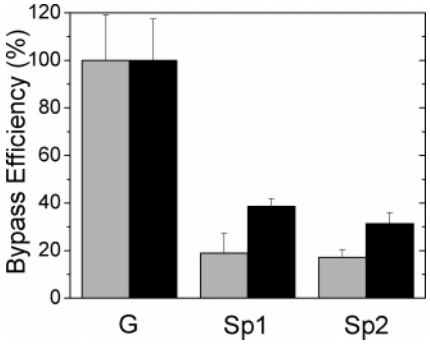

FIGURE 6: Lesion bypass efficiency, as compared to G, for Sp1 and Sp2 in wild-type *E. coli* (gray) and *mutY* AB1157 *E. coli* (black).

cally significant increases in bypass efficiency in *mutY* AB1157 *E. coli*. As compared to wild-type *E. coli*, Sp1 and Sp2 are replicated 3- and 2.5-fold ($p = 0.01$ for both) more efficiently in a strain lacking MutY (Figure 6). The increased levels of bypass efficiency observed for the other lesions, including 8-oxoG, are not statistically significant.

DISCUSSION

Reactive radicals generated normally during metabolism or as part of the immune response are capable of oxidizing cellular DNA (*56*−*58*). One lesion detected following oxidative stress is 8-oxoG. However, because it possesses an oxidation potential considerably lower than the canonical bases, 8-oxoG is susceptible to further oxidation (*27*−*29*). In fact, numerous oxidation products of 8-oxoG have been identified both in vitro and in vivo (*30*−*37*, *46*). Here, we explore the mutagenic potential of some of these products in repair-proficient and MutY-deficient *E. coli*.

Incorporation of a lesion into a single-stranded bacteriophage genome, as opposed to a double-stranded genome, has several benefits that are exploited in these experiments. First, single-stranded DNA is not subject to many of the degradation pathways utilized by *E. coli* to destroy invading genetic material. Second, the lack of a complementary strand allows any stable lesion, even those with very bulky or helix-distorting substituents, to be incorporated into the vector. Third, the single-stranded nature of the vector imposes translesion synthesis as the only pathway to give rise to viral progeny. Furthermore, translesion synthesis by the *E. coli* replication machinery elucidates the coding properties of an individual lesion. Importantly, this strategy allows us to examine postreplicative DNA repair, as practiced by MutY. Enzymatic activity on the complement, rather than the lesion-containing strand, can be readily visualized in our assay as a change in mutation identity or frequency. In contrast, removal of the lesion itself following replication would not change either of these properties, as determined in this assay, since the coding properties of the lesion are defined by incorporation of the opposing base. Therefore, vectors of this type are not ideal for the study of, for instance, MutM activity on a particular lesion.

Using an earlier version of this single-stranded vector assay, and a different lesion sequence context, previous experiments examined the mutagenic potential and toxicity of these 8-oxoG-derived lesions in repair-proficient AB1157 *E. coli* (*38*, *43*, *44*). We can verify here that these hyperoxidized products are much more significantly mutagenic than 8-oxoG and represent a major threat to genomic integrity if generated within cells. The mutational spectra, obtained in this work, of 8-oxoG, Oa, and Sp2 replicated in wild-type *E. coli* are consistent with those published previously (*43*, *44*), although Oa was replicated twice as efficiently here than in earlier work (*43*). The latter two lesions are nearly 100% miscoding with Oa yielding G → T transversions and Sp2 a mixture of G → T and G → C. For Gh, Sp1, and Ur, we see some quantitative differences from earlier literature reports. Gh was previously found to code almost exclusively for a G → C transversion (*44*) whereas in this work the lesion yields a mixture of G → C and G → T mutations. In the current experiment, Gh was also much more replication-inhibiting (20 vs 80%) than observed previously. Furthermore, in literature reports, Sp1 generated G → C and G → T mutations in a 3:1 ratio (*44*); in the current work, the same mutations are seen but this ratio is reversed. Ur was previously found to code exclusively for G → T mutations (*38*) whereas in this work the lesion is more degenerate yielding G → T > G → C ≫ G → A mutations.

The noteworthy difference between previous experiments (*38*, *43*, *44*) on Gh, Sp1, Sp2, Oa, and Ur and those presented here is the sequence context of the DNA lesion. The work presented here utilized a 5′-TXG-3′ sequence (where X represents the lesion) whereas in previous experiments the lesion was present in a 5′-GXA-3′ context. A sequence context effect is observed in primer extension assays examining the in vitro bypass and miscoding potential of Gh and Sp (*42*). For instance, Gh is more readily bypassed by *E. coli* Klenow fragment when the lesion is present 5′ to G. The authors suggest that primer misalignment may account for these differences. Furthermore, the surrounding sequence was found to affect the proofreading of a terminal Gh or Sp lesion by Klenow fragment (*59*). Indeed, translesion synthesis via both nonslipped and slipped primer/template pathways has been proposed (*60*). In the former, incorporation of an incorrect nucleotide opposite the lesion results in a point mutation, as observed for Gh, Sp1, Sp2, Oa, and Ur. However, some nucleotide sequences allow the primer−template complex to reorganize or slip during replication. This slippage can give rise to frameshift mutations, as observed for the THF abasic site. This frameshift mutation was observed previously for the THF lesion in a 5′-TXG-3′ context, and even higher amounts of this −1 product were obtained when the lesion was 3′ to C (*61*). Furthermore, recent work from Kroeger and co-workers has shown that replication of a C2′ oxidized abasic site in *E. coli* relies on a dNTP-stablized primer/template misalignment mechanism to read the bases both upstream and downstream from the abasic site (*62*). This underscores the importance of the nucleotides surrounding a lesion in determining the outcome of translesion synthesis. Clearly, further experiments examining the mutational frequency of these 8-oxoG-derived lesions in different sequence contexts are necessary to appreciate fully their miscoding potential.

Presented here is the first in vivo examination of the ability of MutY to alleviate the mutagenicity or alter the coding specificity of the hyperoxidized lesions Gh, Sp1, Sp2, Oa, and Ur. Specifically, the mutation type and frequency of site-specifically incorporated lesions following replication in wild-type or MutY deficient *E. coli* were determined. As these lesions are nearly 100% mutagenic in wild-type

bacteria, increases in mutational frequency cannot be observed. However, changes in the distribution of mutations can be observed (i.e., the relative numbers of G → T, G → C, and G → A mutations). More importantly, the toxicity of a particular lesion, determined as bypass by the replication machinery relative to a G-containing genome, can be observed in bacteria proficient or deficient in MutY activity.

Mutational analyses can be very informative if one wishes to detect glycosylase activity of a particular enzyme in vivo. For example, polymerases misread 8-oxoG and incorporate A a portion of the time, as opposed to the correct base C (*11*). Both bases in an 8-oxoG:A pair are mutagenic and must be replaced to restore the original G:C pair. MutY excises the A, effectively giving the cell a second chance to incorporate a nonmutagenic C and avoid a G:C → T:A mutation. Therefore, bacteria lacking this glycosylase display increased levels of G → T mutations. Indeed, as observed here, the level of G → T transversions increases ~15-fold from 3% in wild-type *E. coli* to 44% in an isogenic strain lacking MutY. This change in mutational profile indicates not only that MutY has glycosylase activity toward a double-stranded product generated in vivo but also identifies the specific target of the enzyme. In this case, the increase in G → T mutations at the lesion site indicates that MutY is responsible for excising a misincorporated A and the 15-fold enhancement provides a biologically valuable metric of the extent of protection afforded the cell by the MutY glycosylase.

In contrast to these changes observed for 8-oxoG, the mutation type and frequency observed for Gh, Sp1, Sp2, Oa, and Ur were identical in wild-type and MutY-deficient bacteria. Results obtained in repair-proficient *E. coli* indicate that both G and A are incorporated opposite Gh, Sp1, and Sp2 to generate Gh:G/A, Sp1:G/A, and Sp2:G/A pairs inside cells. However, given that the level and type of mutations are identical in cells having or lacking MutY, MutY likely does not remove A or G when paired with Gh or either of the Sp diastereomers in wild-type *E. coli*. Similarly, MutY does not remove the A incorporated opposite Oa. A lack of activity would be in agreement with in vitro experiments (*47*, *48*).

The inability of MutY to act on DNA containing these hyperoxidized lesions can be rationalized by examining the structure of a MutY−DNA lesion−recognition complex. A stalled 8-oxoG:A recognition complex was characterized by X-ray crystallography and reveals the structural basis by which this base pair is identified among a vast excess of canonical T:A sites (*63*). In this structure, the substrate A is extruded from the DNA helix into a pocket of the enzyme's catalytic domain. MutY recognizes 8-oxoG in the opposing strand through an extensive network of hydrogen bonds. Interactions with the Watson−Crick, minor groove, and Hoogsteen faces are suited for identification of 8-oxoG, but not T. Given that the structures and hydrogen-bonding abilities of Gh, Sp, Oa, and Ur are strikingly different from 8-oxoG, this multitude of interactions is likely not possible.

Although MutY does not appear to be active on substrates containing these 8-oxoG-deived lesions, MutM will excise Gh, Sp, Oa, and Oz in vitro (*40*, *41*, *47*−*49*). The broader substrate specificity of MutM is rationalized by inspection of two crystal structures of the enzyme bound to lesion-containing DNA (*64*). A recognition competent but catalytically inactive form of MutM recognizes 8-oxoG over G by exploiting the difference in protonation state at N7. In the crystal structure, a main chain carbonyl group of MutM lies in close hydrogen-bonding contact with the N7 proton of 8-oxoG, an interaction that would be repulsed by the lone pair on the N7 of G. In a manner similar to that observed for hOgg1 (*65*), the functionally analogous human enzyme, MutM is proposed to detect indirectly oxidation at C8 by recognizing the change in protonation state at N7. Further insight into lesion recognition is gained from a crystal structure of this same catalytically inactive form of MutM with DNA containing 5,6-dihydrouracil (*64*). The two carbonyl groups of this lesion reside in locations almost identical to the C6 and C8 carbonyl groups of 8-oxoG. Furthermore, the protonated N3 of dihydrouracil is in the same position as the protonated N7 of 8-oxoG. The authors propose that the primary structural requirements for MutM recognition of a lesion are a hydrogen bond donor and acceptor positioned approximately where N7 and O6 of 8-oxoG are located in the 8-oxoG recognition complex. These requirements are met by Gh, Sp, Oa, and Oz, all of which are excised by MutM in vitro. However, using this same rationale, it is not obvious why Ca would not be a substrate for MutM since this lesion also meets these requirements. Therefore, there may be an additional factor-(s) that contributes to MutM recognition and/or catalysis.

Interestingly, Hazra and co-workers have reported that despite a lack of glycosylase activity toward these substrates, MutY does bind to and form stable complexes with Gh:A/G, Sp:A/G, and to a lesser extent Gh:C and Sp:C base pairs, as observed by gel mobility shift (*48*). In fact, this binding of MutY inhibits the ability of MutM to excise Gh and Sp when these lesions are paired with G. Furthermore, binding of MutY to Sp:A/G base pairs without accompanying glycosylase activity may also limit the propagation of mutations. As shown here, decreased levels of translesion synthesis are observed for Sp1 and Sp2 in bacteria with active MutY. Higher levels of lesion bypass and cell survival are observed when MutY is absent. In vitro lesion bypass studies would help explore the hypothesis that MutY prevents the propagation of mutations by decreasing the efficiency of DNA replication. This decrease in lesion bypass in the presence of MutY could also be explained by a futile cycling of excision of a nucleobase and repair to reinsert the same nucleotide.

Although MutY does not appear to have glycosylase activity on the DNA lesions studied here, Hailer and co-workers reported that the base excision repair enzyme Nei plays a role in reducing the genomic levels of the Sp lesion resulting from $[Cr_2O_7]^{2-}$ exposure (*46*). This DNA damage product was identified in Nei-deficient *E. coli* at levels 20-fold higher than in wild-type bacteria. Interestingly, Sp did not accumulate in a double mutant lacking both MutM and MutY whereas 8-oxoG was present at levels 20-fold higher than in a wild-type strain. However, the wild-type strains used in these two experiments are not isogenic and, in fact, display different sensitivities to $[Cr_2O_7]^{2-}$, precluding any direct comparisons between the Nei and the MutM/MutY mutant strains.

Given that 8-oxoG is formed inside cells, that the lesion is chemically labile toward further oxidation, and that one of these oxidation products, Sp, has been detected in vivo,

the biological consequences of 8-oxoG-derived lesions must be considered. The bypass of these DNA lesions by the cellular replication machinery and the adducts' potent mutagenicity pose a significant threat to the informational integrity of the genome. Furthermore, as demonstrated here, MutY, one of the base excision enzymes responsible for countering the mutagenic potential of 8-oxoG, does not alleviate the potent mutagenicity or alter the coding specificity of the hyperoxidized products. However, MutY may play a protective role by limiting the survival of cells that harbor these mutagenic lesions (Figure 5). Thus, in conjunction with other repair enzymes such as Nei, MutY may contribute to lowering the genotoxic effects of these 8-oxoG-derived lesions. Additional work is necessary to identify other potential repair pathways that cells may utilize to counter the miscoding potential of these DNA damage products.

SUPPORTING INFORMATION AVAILABLE

MALDI-TOF-MS of the lesion-containing oligonucleotides, an outline of the mutational frequency and lesion bypass assays, and PAGE showing the 18-mer isolated for mutational analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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