

Two clustered 8-oxo-7,8-dihydroguanine (8-oxodG) lesions increase the point mutation frequency of 8-oxodG, but do not result in double strand breaks or deletions in *Escherichia coli*

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ABSTRACT

Multiply damaged sites (MDSs) are generated in DNA by ionizing radiation. *In vitro* studies predict that base excision repair in cells will convert MDSs to lethal double strand breaks (DSBs) when two opposing base damages are situated ≥ 2 bp apart. If the lesions are situated immediately 5' or 3' to each other, repair is predicted to occur sequentially due to inhibition of the DNA glycosylase by a single strand break repair intermediate. In this study, we examined how the distance between two opposing lesions alters the mutation frequency of an 8-oxodG in an MDS, and whether repair generates DSBs and deletions in bacteria. The 8-oxodG mutation frequency declined in MutY-deficient bacteria when the opposing 8-oxodG was 6 bp away, and was similar to a single 8-oxodG when the lesions were separated by 14 bp. However, the number of deletions detected for the MDSs was equivalent to the undamaged sequence. Using a separate assay, MDSs consisting of two 8-oxodG or an 8-oxodG opposite a uracil were not converted to DSBs in the absence of DNA replication in wild-type and transcription-coupled repair-deficient bacteria. This is the first study showing that DSB-repair intermediates and deletions are not formed during repair of clustered 8-oxodGs in cells.

INTRODUCTION

Single X- and γ -ray tracks generate low energy secondary electrons that result in clusters of ionizations, which if situated near the DNA induce multiple lesions within 10–15 bp (1,2). These clustered DNA lesions or multiply damaged sites (MDSs) consist of oxidized bases, abasic (AP) sites and single strand breaks (SSBs) in the same (3,4) or opposing DNA strands (5–8), as well as double strand breaks (DSBs). Approximately 80% of MDSs induced by γ -irradiation contain

oxidized base damage, while only 20% are DSBs (5). Oxidized base damage and AP sites are repaired predominantly by base excision repair (BER) and repair initiation results in a SSB-repair intermediate [for review see (9)].

In vitro studies [for review see (10–12)] have shown that prokaryote and eukaryote repair enzymes in general can cleave at two opposing damages situated ≥ 2 bp apart in an MDS. Repair initiation of MDSs can therefore convert two opposing base damages to a SSB opposite a base damage, or a DSB. Two closely opposed uracil lesions, situated ≤ 7 bp apart within the firefly luciferase coding region of a plasmid, dramatically reduce luciferase activity and result in destruction of the plasmid, even in bacteria deficient in the major AP endonucleases (13). This suggests that the clustered uracil lesions were converted to a DSB. It is possible that there are enzymes in the cell other than the AP endonucleases, such as the AP lyases, that cleave the AP sites following removal of uracil by uracil DNA glycosylase. Therefore, although *in vitro* repair studies using purified enzymes have provided invaluable information about how and which enzymes can cleave defined MDSs, examining the outcome of repair in cells is required to ultimately determine which enzymes are involved in repair, and to determine the biological relevance of lesions in MDSs.

8-oxo-7,8-dihydroguanine (8-oxodG) is a stable lesion that is produced in DNA by ionizing radiation (14). In bacteria, the majority of 8-oxodG is removed from DNA by formamido-pyrimidine DNA glycosylase (Fpg), while endonuclease VIII (15) acts as a 'back-up' repair system. 8-oxodG is also a mutagenic lesion (16,17), since DNA polymerase can insert an adenine opposite 8-oxodG during DNA replication. 8-oxodG paired with adenine is a poor substrate for Fpg. To prevent G to T transversions during the second round of replication, bacteria and most eukaryotes have a DNA glycosylase called Mut Y, which removes the A mispaired with the 8-oxodG [for review see (18)]. We previously demonstrated that in *Escherichia coli* the mutation frequency of an 8-oxodG is enhanced by a second 8-oxodG situated immediately 5' and in the opposite DNA strand (19). This is in agreement with *in vitro* studies, which showed that repair at an 8-oxodG is inhibited by a base damage (20) or SSB-repair

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intermediate (21) situated in the opposite DNA strand and <2 bp away. In cells, repair inhibition allows time for replication to occur prior to complete repair of 8-oxodG. More recently, Pearson *et al.* (22) demonstrated a similar effect using uracil as the closely opposed lesion. These studies in cells have only demonstrated that a second lesion closely opposed to an 8-oxodG enhances the mutation frequency of 8-oxodG. The main aim of this study was to determine whether two clustered mutagenic lesions could be converted to a lethal DSB, which in a cell may contribute to death or possibly stimulate recombination and increase the production of deletions. We also hypothesized that in cells as the distance between two opposing 8-oxodGs increased, the mutation frequency of the target 8-oxodG would decrease due to the ability of BER to remove both 8-oxodGs at the same time. We have employed two assays to address these questions, both of which utilize the loss of the firefly luciferase reporter to identify the presence of mutations, misrepaired sequences or DSB formation. For the mutation assay, the plasmid carrying the MDS was allowed to replicate in bacteria, and plasmid DNA was analyzed from colonies that did not express firefly luciferase activity. To determine whether 8-oxodG-containing MDSs were converted to lethal DSBs, the plasmid was not allowed to replicate during repair and loss of firefly luciferase activity indicated that the plasmid was broken or deleted. With a combination of these two assays we have been able to determine how the distance between two opposing lesions alters the mutation frequency of an 8-oxodG in an MDS, and whether repair of clustered 8-oxodGs generates DSBs and deletions in bacteria.

MATERIALS AND METHODS

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides (oligonucleotides) were purchased from Operon Technologies Inc. (Alameda, CA). Oligonucleotides used for the mutation assay and the DNA repair assay contained 5' phosphate termini and were purified by PAGE.

The sequences of the double-stranded oligonucleotides containing MDS are shown in Tables 1 and 2. The sequences of the double-stranded oligonucleotides containing no damage or single lesions are available on line. The oligonucleotide used to sequence the mutated section of pBestluc was d(TGGATGGCTACATTCTG). The sequences of the oligonucleotides used for PCR are as follows:

Primer 1 d(GAAGACGAACACTTCTTCATAGTTGACCGCTTGAAGTCTTTAATTGTAT)

Primer 2 d(GTCATCGTCGGGAAGACCTGCCACGCC)

Primer 3 d(GAAGACGAACACTTCTTCATAGTTGACC-GCTTGAAGTCTTTAATTAATA)

Bacteria

Wild-type *E. coli* [strain BW35-Hfr KL16(PO-45) *thi-1*, *relA1*, *spoT1*, *e14*⁻, λ ⁻] and tetracycline-resistant Mut Y-deficient bacteria (*mutY*::Tn10) were obtained from Dr Susan S. Wallace (University of Vermont, Burlington, VT) and were isogenic. *E. coli* deficient in the Mfd transcription repair coupling factor (UNCNOMFD) are resistant to kanamycin (*mfd*::kanamycin) and have an AB1157 genetic background [*thr-1*, *araC14*, *leuB6*(Am), Δ (*gpt-proA*)62, *lacY1*, *tsx-33*, *qsr*⁻, *glnV44*(AS), *galK2*(Oc), λ ⁻, *Rac-0*, *hisG4*(Oc), *rfbD1*, *mgl-51*, *rpoS396*(Am), *rpsL31*(strR), *kdgK51*, *xylA5*, *mtl-1*, *argE3*(Oc), *thi-1*]. UNCNOMFD were obtained from Dr Rick Bockrath (Indiana University School of Medicine, Indianapolis, IN). *mutY*⁻ and *mfd*⁻ bacteria were grown at 10 μ g/ml tetracycline and 40 μ g/ml kanamycin, respectively, either on solid growth medium or in liquid culture during the preparation of electrocompetent bacteria. Electrocompetent bacteria were generated according to Seidman *et al.* (23).

Plasmids

pACYC184 (New England Biolabs, Beverly, MA) is a low copy vector with a p15A origin of replication that encodes resistance to chloramphenicol (34 μ g/ml). pBestluc (Promega, Madison, WI) is a high copy vector with a pUC origin of

Table 1. Double-stranded oligonucleotides containing two closely opposed 8-oxodGs

Name	Position of two 8-oxodGs relative to each other		Sequence	Assay
	5' or 3'	Number of bp apart		
8oxo-3	5'	2	5' TAAATACGA <u>O</u> GGGTATCAGGTGGCCCCCGCTGAATTGGAAT 3' 3' TAATTTAT <u>O</u> CTCCCATAGTCCACCGGGGGCGACTTAACCTTAGC 5'	MA
8oxo-6	5'	5	5' TAAATACGAGGG <u>O</u> TATCAGGTGGCCCCCGCTGAATTGGAAT 3' 3' TAATTTAT <u>O</u> CTCCCATAGTCCACCGGGGGCGACTTAACCTTAGC 5'	MA
8oxo-15	5'	14	5' TAAATACGAGGGGTATCAGGT <u>O</u> GCCCCCGCTGAATTGGAAT 3' 3' TAATTTAT <u>O</u> CTCCCATAGTCCACCGGGGGCGACTTAACCTTAGC 5'	MA
8oxo-7	5'	6	5' TAAATACAAAGGATATCAGGTGGCCCCCGCT <u>O</u> AATTGGAAT 3' 3' TAATTTATGTTTCCTATAGTCCACCG <u>O</u> GGGCGACTTAACCTTAGC 5'	DR
8oxo-4	5'	3	5' TAAATACAAAGGATATCAGGTGGCCCC <u>O</u> CTGAATTGGAAT 3' 3' TAATTTATGTTTCCTATAGTCCACCG <u>O</u> GGGCGACTTAACCTTAGC 5'	DR
8oxo+2	3'	1	5' TAAATACAAAGGATATCAGGT <u>G</u> OCCCCCGCTGAATTGGAAT 3' 3' TAATTTATGTTTCCTATAGTCCACCG <u>O</u> GGGCGACTTAACCTTAGC 5'	DR
8oxo+5	3'	4	5' TAAATACAAAGGATATCAG <u>O</u> TGGCCCCCGCTGAATTGGAAT 3' 3' TAATTTATGTTTCCTATAGTCCACCG <u>O</u> GGGCGACTTAACCTTAGC 5'	DR

When the two 8-oxodGs (O) are positioned 5' to each other, the name is designated with a minus and when they are 3' to each other, the name is designated with a plus. The number in the name designates the nucleotide position of the second 8-oxodG relative to the first. Oligonucleotides were either used in the mutation assay (MA) or the DNA repair assay (DR). The 8-oxodG at the third base of codon 444 is underlined.

Table 2. Double-stranded oligonucleotides containing an 8-oxodG closely opposed to a uracil

Name	Position of the two lesions relative to each other		Sequence	Strand with 8-oxodG
	5' or 3'	Number of bp apart		
8oxo/U-11	5'	10	5' TAAATACAAAGGATATCAGGTGGCCCCCGCTGAAT U GGAAAT 3' 3' TAATTTATGTTTCCTATAGTCCACCG O GGGCGACTTAACCTTAGC 5'	Ts
8oxo/U-7	5'	6	5' TAAATACAAAGGATATCAG O TGGCCCCCGCTGAATTGGAAT 3' 3' TAATTTATGTTTCCT U ATAGTCCACCGGGGCGACTTAACCTTAGC 5'	NT
8oxo/U-5	5'	4	5' TAAATACAAAGGATATCAG O TGGCCCCCGCTGAATTGGAAT 3' 3' TAATTTATGTTTCCT U AGTCCACCGGGGCGACTTAACCTTAGC 5'	NT
8oxo/U+4	3'	3	5' TAAATACAAAGGATATCAG U GGCCCCCGCTGAATTGGAAT 3' 3' TAATTTATGTTTCCTATAGTCCACCG O GGGCGACTTAACCTTAGC 5'	Ts
8oxo/U+9	3'	8	5' TAAATACAAAGGAT U CAGGTGGCCCCCGCTGAATTGGAAT 3' 3' TAATTTATGTTTCCTATAGTCCACCG O GGGCGACTTAACCTTAGC 5'	Ts

When the 8-oxodG (**O**) and uracil (**U**) are positioned 5' to each other, the name is designated with a minus and when they are 3' to each other, the name is designated with a plus. The number in the name designates the nucleotide position of the 8-oxodG relative to the uracil. The 8-oxodG was positioned either on the transcribed strand (Ts) or on the non-transcribed strand (NT) of the plasmid.

replication that encodes resistance to ampicillin or carbenicillin (50 µg/ml). pBestluc expresses the firefly luciferase open reading frame from a tac promoter and was used in the mutation assay. At the 3' end of the luciferase open reading frame is a unique XhoI site, into which we inserted a double-stranded oligonucleotide to generate p3'luc (13), which was used in the DNA repair assay.

Mutation assay

The mutation assay utilizes the pBestluc plasmid. It is possible to identify bacteria expressing the active firefly luciferase protein by spraying the colonies with luciferase assay reagent (Promega) and detecting light emission using autoradiographic film. 8-oxodG was situated in the transcribed strand at the third base of codon 444 (Table 1). Replication synthesis passed the lesion can result in the insertion of an adenine opposite the 8-oxodG. If the adenine is not removed prior to a second round of replication, a G→T transversion is fixed at the original site of the 8-oxodG and the translation stop at codon 444 results in inactive luciferase protein. Mutations and deletions are selected on the basis of loss of luciferase activity. This assay will also detect the insertion of a guanine opposite the 8-oxodG as this also results in a translation stop codon. pBestluc was linearized with Pac I and ClaI, which removes a 45 bp fragment from the 3' region of the luciferase open reading frame. This allowed the re-insertion of double-stranded oligonucleotides containing no damage, a single 8-oxodG or an MDS between the two restriction sites. Re-ligation of the plasmid without the 45 bp insert results in a plasmid that does not express active luciferase. Preparation of linearized plasmid and the annealing of complementary oligonucleotides are described in detail by Malyarchuk *et al.* (19). A 10 µl ligation reaction was prepared containing 580 ng (~200 fmol) of linear pBestluc, 600 fmol double-stranded oligonucleotides, 1 mM rATP, 50 mM Tris-HCl, pH 7.5, 7 mM MgCl₂, 1 mM dithiothreitol and 2 U T4 DNA ligase (Stratagene, La Jolla, CA) and incubated overnight at 4°C. Salts were then removed from the DNA using the Qiaquick Nucleotide Removal kit (Qiagen Inc., Valencia, CA) and the DNA eluted using 30 µl of 10 mM Tris, pH 8.5. Electrocompetent bacteria were transformed with 5 µl (~96 ng) of the ligation reaction and the bacteria allowed to grow for 1.5 h at

37°C and 250 rpm, prior to growth overnight at 37°C on solid medium-containing 75 µg/ml carbenicillin, or 75 µg/ml carbenicillin and 10 µg/ml tetracycline for Mut Y-deficient bacteria. Colonies were transferred to nylon membrane (Amersham, Piscataway, NJ) and the bacteria sprayed with luciferase assay reagent. Colonies that did not express active luciferase were re-grown on solid medium and tested a second time for luciferase expression, prior to isolation of plasmid DNA. To distinguish between plasmid DNA containing either the wild-type sequence or the G to T transversion at codon 444, PCR was performed using two different sets of primers (Figure 1). Reactions were performed using 160 nM of each primer in a 25 µl reaction containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2 mM MgCl₂, 200 µM each of dGTP, dCTP, dATP and dTTP and 0.3 U of *Taq* DNA polymerase (Promega). The reactions were performed in a MyCycler™ Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) for 30 cycles with an annealing temperature of 60°C. The PCR products generated using primers 1 and 2, and primers 2 and 3 were then digested with AccI and DdeI, respectively, and subjected to electrophoresis through a 1.8% agarose gel. If the plasmid DNA contained the G to T transversion, the PCR product generated with primers 1 and 2 did not digest with AccI, while the PCR product obtained using primers 2 and 3 digested with DdeI (Figure 1). If no PCR product was obtained or if the digestion result was ambiguous, e.g. neither PCR product digested, then the DNA was analyzed by restriction enzyme analysis or by DNA sequencing.

DNA repair assay

The DNA repair assay utilizes the p3'luc plasmid, which is linearized with PacI and ClaI. This allows the re-insertion of a 45 bp double-stranded oligonucleotide carrying no damage, a single lesion or an MDS (Tables 1 and 2) into the 3' coding region of the firefly luciferase protein. Failure to insert the 45 bp oligonucleotide, or breakage or deletion of the DNA at this site results in loss of luciferase activity. Preparation of linearized plasmid and the annealing of complementary oligonucleotides are described in detail by D'souza and Harrison (13). A 30 µl ligation reaction was prepared containing 1.5 µg (~600 fmol) of linear p3'luc, 1.8 pmol double-stranded

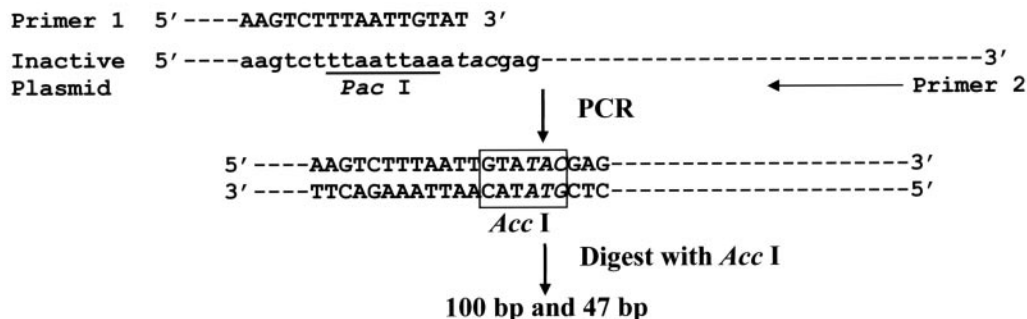
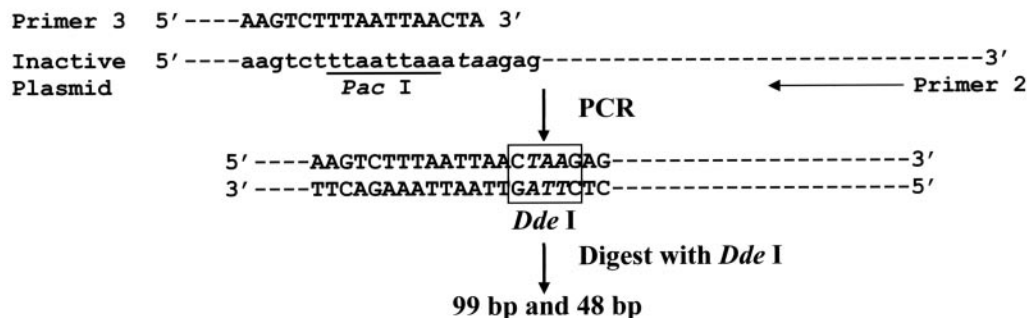
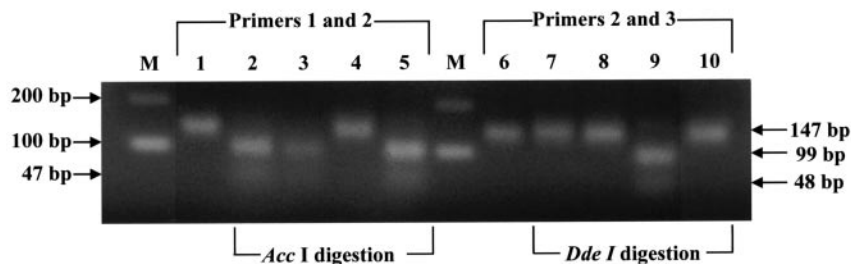
A**To detect wild type sequence at codon 444:****B****To detect a mutation at codon 444:****C**

Figure 1. PCR to detect a mutation at codon 444. Plasmid DNA was subjected to two separate PCR reactions using primers 1 and 2 to detect the wild-type sequence (A), and primers 2 and 3 to detect a C to A mutation at the third base of codon 444 (B). The wild-type (TAC) and the mutant (TAA) sequences are shown in italics. Only the wild-type sequence generates the *AccI* restriction site following PCR using primers 1 and 2 (A), and only plasmid containing the C to A mutation at the third base of codon 444 generates the *DdeI* restriction site following PCR using primers 2 and 3 (B). The sequences within the boxed regions are the restriction sites for *AccI* and *DdeI*. The PCR products were subjected to either *AccI* or *DdeI* digestion, respectively, and electrophoresed through a 1.8% agarose gel (C). M is the DNA size marker. pBestluc was amplified using either primers 1 and 2 (lanes 1 and 2) or primers 2 and 3 (lanes 6 and 7). The PCR products were examined without digestion (lane 1 and 6) and with either *AccI* (lane 2) or *DdeI* (lane 7) digestion. Samples of plasmid DNA from a previous analysis (19) known to produce inactive firefly luciferase and that had been sequenced to determine the mutation, were also amplified using the two different sets of primers. Two samples were wild-type at codon 444 (lanes 3 and 8, and lanes 5 and 10) and one sample was mutated at codon 444 (lanes 4 and 9).

oligonucleotide, 1 mM rATP, 50 mM Tris-HCl (pH 7.5), 7 mM MgCl₂, 1 mM dithiothreitol and 6 U T4 DNA ligase (Stratagene) and incubated overnight at 4°C. Salts were removed from the DNA using the Qiaquick Nucleotide Removal kit (Qiagen Inc.) and the DNA eluted using 30 µl of 10 mM Tris, pH 8.5. Electrocompetent bacteria were co-transformed with 4 µl (~200 ng) of the ligation reaction and 0.1–2 ng of pACYC184 at 2.5 kV, 200 Ω, 25 µF. Following transformation, the bacteria were incubated at 37°C and

250 rpm for 4 h in 1.5 ml Luria-Bertani medium (LB) containing 80–100 µM novobiocin (Sigma-Aldrich, St. Louis, MO) and 1 mM isopropyl-β-D-thiogalactopyranoside (Sigma-Aldrich). After 4 h, the culture was grown on triplicate plates of solid medium containing chloramphenicol (34 µg/ml), or kanamycin (40 µg/ml) and chloramphenicol (34 µg/ml) for Mfd-deficient bacteria, to determine the level of transformation of each sample. To measure luciferase activity, cell-free extracts were prepared from 1 ml of bacterial culture and 20 µl

of extract was mixed with luciferase assay reagent (100 μ l; Promega) in a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). The relative light units/chloramphenicol resistant colony was calculated. This activity for each transformation was expressed as a percentage of the activity of the undamaged sequence.

Statistical analysis

To determine whether the frequency of mutations and deletions was significantly altered by the presence of a second 8-oxodG, the Statistical Analysis Software (SAS 9.0, Cary, NC) system was used to perform the Chi square and Fisher's exact tests. A *P*-value of ≤ 0.05 was considered significant. The *P*-values in the text are for the Chi square test. The data for MDS-1, 8oxo-3, 8oxo-6 and 8oxo-15 were also examined using the Cochran-Armitage trend test. A two sided *P*-value was used to determine whether there was a trend to increase or decrease the mutation or deletion frequency as the two 8-oxodGs were separated by greater distances. The data from the DNA repair assay was analyzed using the Instat3 program and the unpaired *t*-test, to determine whether the MDS differed significantly from the corresponding single lesion.

RESULTS

Modification to the mutation assay

The predominant mutation following by-pass of an 8-oxodG is a G to T transversion (16,17). Previously, we described an assay using the firefly luciferase reporter to determine the mutation frequency of an 8-oxodG situated in the transcribed DNA strand, immediately 5' to a second 8-oxodG in the non-transcribed strand (19). Insertion of adenine opposite an 8-oxodG situated at the third base of codon 444 on the transcribed strand, followed by a second round of replication in which a thymine is placed opposite the adenine, produces a translational stop codon (TAA) and a truncated inactive protein. A mutation is therefore identified by loss of luciferase activity. The screen for bacteria expressing active luciferase enzyme involves spraying the colonies with luciferase substrate and capturing an image on autoradiographic film after an exposure time of ~ 5 s. It is therefore very simple and quick. Since the MDS is introduced into the pBestluc plasmid using synthetic oligonucleotides, colonies are identified by the screen that contain mutations due to errors in the oligonucleotide synthesis. A disadvantage of the assay was the need to identify the colonies containing the G to T transversion by DNA sequencing. We have therefore modified the assay to allow for the identification of the wild-type (TAC) and the mutated (TAA) sequences at codon 444 by performing two separate PCR reactions (Figure 1). Oligonucleotides (primers 1 and 3) were designed to the sequence 5' to codon 444. Primer 1 is a 49mer, the 3' terminus of which ends at the first base of codon 444. Therefore during amplification the last two bases of codon 444 are replicated from the plasmid DNA. If the plasmid does not express luciferase due to an oligonucleotide synthesis error, the wild-type sequence (TAC) will exist at codon 444. Primer 1 contains two mismatches (GT instead of AA) compared to the sequence of the plasmid, at positions 3 and 4 from the 3' terminus (Figure 1A). If the PCR product

contains the TAC sequence at codon 444, an AccI restriction site is generated, and digestion results in fragments of 100 and 47 bp. If the PCR product contains the mutated (TAA) sequence at codon 444, the AccI restriction site is not present and the PCR product is not cleaved by the enzyme. To further confirm the sequence at codon 444, primer 3 was designed, the 3' terminus of which ends at the second base of codon 444 (Figure 1B). This primer contains one mismatch (C instead of A) at position 3 from the 3' terminus of the primer. If the PCR product contains TAA at codon 444, a DdeI restriction site is generated and digestion produces fragments of 99 and 48 bp. If TAC is present, the PCR product will not digest with DdeI. To confirm that the two PCR reactions could identify the wild-type and the mutated sequences, PCR was performed using pBestluc and some samples from our previous study (19). The mutations in these latter samples had been determined by DNA sequencing. Amplification of the plasmid DNA produced a PCR product of 147 bp (Figure 1C, lane 1 and 6 for pBestluc). PCR products were digested with AccI, if amplified by primers 1 and 2, or DdeI, if amplified by primers 2 and 3. As can be seen in Figure 1C, AccI digested the product obtained from pBestluc (lane 2) and from two samples (lane 3 and 5) that had previously been determined to be wild-type at codon 444. DdeI did not digest the PCR product from these samples (lanes 7, 8 and 10). The PCR products from one sample known to contain TAA at codon 444, digested with DdeI (lane 9) and not AccI (lane 4). For further confirmation of the assay, 20 samples were sequenced from the experiment using wild-type bacteria and the MDS containing two 8-oxodGs separated by 2 bp (Table 3, 8oxo-3). The 20 samples were initially predicted to be mutations at codon 444 by PCR analysis and all 20 were confirmed to contain TAA at codon 444 by DNA sequencing.

Mutation frequency of 8-oxodG within an MDS

The MDSs we examined contained two 8-oxodG: one situated on the transcribed strand at the third base of codon 444 and one situated on the non-transcribed strand. We have positioned the second 8-oxodG of the MDS 3, 6 and 15 bp away from the 8-oxodG in the transcribed strand (Table 1). The 8-oxodG in the non-transcribed strand was therefore positioned at the third base of codon 445, the third base of codon 446, or the third base of codon 449. We have previously shown that the introduction of a G to T transversion at the third base of codon 445 and the third base of codon 446 does not eliminate luciferase activity (19). A G to T transversion at the third base of codon 449 does not result in a change of the amino acid and therefore was not expected to alter luciferase activity. This study therefore only determined the mutation frequency of the 8-oxodG in the MDS situated at the third base of codon 444 in the transcribed strand.

Oligonucleotides containing a single 8-oxodG or the MDS (Table 1) were ligated into linearized pBestluc and transformed into wild-type or Mut Y-deficient bacteria. Growth of the bacteria and selection of colonies expressing inactive luciferase were performed as described above. A separate annealing reaction was performed for each ligation reaction and two ligation reactions were used to generate the data for the MDS-containing oligonucleotides, while one ligation was used for the single 8-oxodG-containing substrates.

A single 8-oxodG situated in the non-transcribed strand at codon 445, 446 or 449 was not expected to result in the introduction of a translational stop at codon 444. As can be seen from Tables 3 and 4, no translational stop codons at 444 were detected for two of the single 8-oxodGs on the non-transcribed strand (8-oxodG₁₀ and 8-oxodG₁₃). A very low level of mutation at codon 444 was found for the oligonucleotide 8-oxodG₂₂ in both wild-type (0.03%) and Mut Y-deficient bacteria (0.04%). Since the frequencies are very similar for the two types of bacteria, it is likely that this was due to the synthesis of the oligonucleotide.

Previously, we showed that the mutation frequency of the single 8-oxodG on the transcribed strand at codon 444 was 0.15% in wild-type bacteria and that this increased to 0.26% when a second 8-oxodG was situated immediately 5' in the non-transcribed strand (MDS-1, (19)). We performed the

MDS-1 experiment again with two ligations and found that the mutation frequency was 0.47% (Table 3). This higher frequency may have been due to the detection frequency of the inactive colonies, since originally we detected only 1.34% inactive colonies as compared to 1.92% found during this experiment. The majority of the mutations identified were G→T transversions, generated by the insertion of adenine opposite the 8-oxodG. For some of the lesions studied, a small percentage of the mutations (1–5%) were G→C changes. This insertion of guanine opposite the 8-oxodG was found for the single lesion (transcribed strand) as well as certain MDSs. The number of the G→C mutations was very low and there was no evidence of the MDS causing a change in the mutation spectrum of 8-oxodG. In both wild-type (Table 3) and Mut Y-deficient bacteria (Table 4), the presence of a second opposing 8-oxodG in the non-transcribed strand 1

Table 3. Mutations detected with wild-type bacteria (BW35)

Inserted DNA	Number of colonies screened	Translation stop codon at 444		Deletion of damaged sequence		Size (kb)
		Number of mutants	% mutants	Number of deletions	% deleted	
Undamaged	7165*	0*	0*	2	0.028	2
8-oxodG (transcribed strand)	9326*	14*	0.15*	1	0.011	1.6
8oxo ₁₀	6441	0	0	0	0	—
8oxo ₁₃	6823	0	0	5	0.073	0.4–2
8oxo ₂₂	7515	2	0.03	4	0.053	0.3–1.8
MDS-1	4230	20	0.47 ^a	2	0.047	0.8–1.8
8oxo-3	13974	78	0.56 ^a	4	0.029	0.8–2
8oxo-6	12689	49	0.39 ^a	2	0.016	0.3–1
8oxo-15	9281	16	0.17 ^b	1	0.011	1.4

The double-stranded oligonucleotides containing a single 8-oxodG in the non-transcribed strand were 8oxo₁₀, 8oxo₁₃ and 8oxo₂₂. These single lesions formed part of the 8oxo-3, 8oxo-6 and 8oxo-15 MDS, respectively. Two ligation reactions, prepared from separate batches of annealed oligonucleotides, were used in separate transformations to generate the total number of colonies screened, except for 8-oxodG₁₀, 8-oxodG₁₃ and 8-oxodG₂₂ where one ligation and transformation was performed. Plasmid DNA was isolated from colonies that did not express firefly luciferase. Asterisks represent our previously published results (19) that were screened by sequencing. The samples for which we did not previously obtain sequence information were reanalyzed by restriction digest for the presence of deletions. The samples from the new experiments were screened by PCR to identify a mutation at codon 444. Samples were restriction digested and/or sequenced if an ambiguous result was obtained by PCR. Deletions of single base pairs were not included in the analysis as these were likely due to oligonucleotide synthesis. MDS-1 is an oligonucleotide that contains two 8-oxodGs situated immediately 5' to each other in opposing DNA strands. 8oxo-3, 8oxo-6 and 8oxo-15 are oligonucleotides that contain two 8-oxodGs situated 5' to each other in opposing DNA strands separated by 2, 5 and 14 bp, respectively. The point mutation frequency of the MDSs were statistically compared with the results for the 8-oxodG in the transcribed strand (^a*P* < 0.05) or for MDS-1 (^b*P* < 0.05).

Table 4. Mutations detected with Mut Y-deficient bacteria

Inserted DNA	Number of colonies screened	Translation stop codon at 444		Deletion of damaged sequence		Size (kb)
		Number of mutants	% mutants	Number of deletions	% deleted	
Undamaged	3954*	0*	0*	2	0.051	2
8-oxodG (transcribed strand)	12022*	59*	0.49*	0	0	—
8oxo ₁₀	7318	0	0	0	0	—
8oxo ₁₃	5996	0	0	1	0.017	0.5
8oxo ₂₂	5787	2	0.04	2	0.035	0.8–1.6
MDS-1	4137*	160*	3.9* ^a	2	0.048	0.5–0.6
8oxo-3	5351	228	4.26 ^a	4	0.075	0.4–1.6
8oxo-6	7016	176	2.5 ^{a, b}	3	0.043	0.8–1.8
8oxo-15	12221	143	1.17 ^{a, b}	2	0.016	0.3–1.8

The double-stranded oligonucleotides containing a single 8-oxodG in the non-transcribed strand were 8oxo₁₀, 8oxo₁₃ and 8oxo₂₂. These single lesions formed part of the 8oxo-3, 8oxo-6 and 8oxo-15 MDS, respectively. Two ligation reactions, prepared from separate batches of annealed oligonucleotides, were used in separate transformations to generate the total number of colonies screened, except for undamaged, 8-oxodG₁₀, 8-oxodG₁₃ and 8-oxodG₂₂ where one ligation and transformation was performed. Plasmid DNA was isolated from colonies that did not express firefly luciferase. Asterisks represent our previously published results (19) that were screened by sequencing. The samples for which we did not previously obtain sequence information were reanalyzed by restriction digest for the presence of deletions. The samples from the new experiments were screened by PCR to identify a mutation at codon 444. Samples were restriction digested and/or sequenced if an ambiguous result was obtained by PCR. Deletions of single base pairs were not included in the analysis as these were likely due to oligonucleotide synthesis. MDS-1 is an oligonucleotide that contains two 8-oxodGs situated immediately 5' to each other in opposing DNA strands. 8oxo-3, 8oxo-6 and 8oxo-15 are oligonucleotides that contain two 8-oxodGs situated 5' to each other in opposing DNA strands separated by 2, 5 and 14 bp, respectively. The point mutation frequency of the MDSs were statistically compared with the results for the 8-oxodG in the transcribed strand (^a*P* < 0.05) or for MDS-1 (^b*P* < 0.05).

(MDS-1), 3 (8oxo-3) and 6 (8oxo-6) bp away enhanced the mutation frequency of the single 8-oxodG in the transcribed strand ($P \leq 0.0012$). When the two 8-oxodGs were separated by 14 bp (8oxo-15), the mutation frequency at codon 444 was only significantly higher than the single lesion (transcribed strand) in Mut Y-deficient bacteria ($P < 0.0001$). Upon examination of the results for all the MDSs using the Cochran-Armitage trend test, the mutation frequency decreased as the distance between the lesions (0-14 bp) increased in both bacterial strains. However, in wild-type bacteria no significant change was detected on comparison of 8oxo-3 or 8oxo-6 with MDS-1 (Table 3), while there was a significant decrease in the mutation frequency in Mut Y-deficient bacteria (Table 4) when the two 8-oxodGs were situated 5 bp apart (8oxo-6, $P < 0.0001$) as compared to 2 bp apart (8oxo-3).

Deletion of damaged sequences during repair and replication of the MDSs

Samples had previously been sequenced to determine the mutation frequency for the undamaged sequence, the single 8-oxodG on the transcribed strand and the MDS containing two 8-oxodG situated immediately 5' to each other [MDS-1, (19)]. The sequences for these samples were re-examined for the presence of deletions and those that could not originally be sequenced using the primer near the PacI site were analyzed for deletions by restriction enzyme analysis. As described above, the mutation frequency for the 8-oxodG in the transcribed strand was determined by PCR for the single and MDS lesions. Samples that did not produce a PCR product, or a product that could not be digested by one of the enzymes AccI or DdeI, were analyzed by restriction digestion or by sequencing to determine the deletion frequency. A proportion of the colonies (0-0.1% for wild-type and 0.02-0.11% for mutY⁻) produced from the undamaged, single and MDS transformations contained plasmid DNA that lacked the PacI and ClaI sequence. These alterations were likely generated by re-ligation of the linear plasmid without insertion of the oligonucleotide during the *in vitro* ligation reaction and were therefore not included in the analysis. As can be seen from Tables 3 and 4, deletions were found in the undamaged and certain single lesions as well as the MDSs. Statistical analysis indicated that the number of deletions detected for the MDSs was not significantly different from that of the undamaged sequence, and the Cochran-Armitage trend test comparing the data for the MDSs did not detect a trend (increased or decreased) in the deletion frequency as the opposing lesions were situated further apart. The same types of deletions were also found in the single 8-oxodG substrates and the MDS-containing substrates. The data therefore suggests that the deletions were not due to the repair of the MDS and that the two closely opposed 8-oxodGs were not converted to a DSB-repair intermediate. It was possible that deletions were not detected due to destruction of linearized plasmid. To examine this, two ligations were prepared for the undamaged sequence, two single lesions (8oxo₁₀ and 8oxo₁₃) and each of the MDSs containing two closely opposed 8-oxodGs. Each ligation was co-transformed into BW35 with 0.1 ng pACYC184, which was used to normalize the transformation efficiency, and after 1.5 h growth at 37°C and 250 rpm, bacteria were grown on either carbenicillin or

chloramphenicol containing solid medium in triplicate. After overnight growth, colonies were counted and a ratio of the carbenicillin/chloramphenicol colonies calculated. On comparison with the undamaged sequence (100%) no difference was found for plasmids containing a single 8-oxodG (8oxo₁₀ = 82%, 8oxo₁₃ = 81%), two 8-oxodG situated immediately 5' to each other (88%), or separated by 2 (8oxo-3 = 97%), 5 (8oxo-6 = 74%) or 14 (8oxo-15 = 98%) bp, indicating that the pBestluc plasmid had not been destroyed.

Repair of an 8-oxodG-containing MDS in wild-type bacteria in the absence of DNA replication

Results from the mutation assay suggested that DSB-repair intermediates were not formed during the repair of two closely opposed 8-oxodG lesions. We decided to confirm this using a DNA repair assay. The assay utilizes a plasmid, p3'luc, where the MDS is situated in the 3' end of the luciferase coding region (13). A double-stranded oligonucleotide is ligated into the PacI and ClaI sites, the ligation reaction is transformed into bacteria and luciferase activity is measured after a 4 h incubation in the presence of novobiocin. Novobiocin inhibits DNA gyrase and has previously been shown to inhibit bacterial and plasmid replication (24,25). We assume that a decrease of luciferase activity is due to the conversion of the MDS to a DSB. Using this assay, we have previously shown that luciferase activity is decreased when uracil DNA glycosylase (Ung) initiates repair of two closely opposed uracils ≤ 7 bp apart (13). Since 8-oxodG has been shown to undergo transcriptional mutagenesis in *E.coli* (26), it was necessary to place the 8-oxodG in the transcribed strand at the third base of codon 450 (the corresponding single lesion is 8oxo₂₅). This codon (GCC) translates into alanine. During transcription adenine can be inserted opposite 8-oxodG by RNA polymerase (26). The resulting GCA codon in our system will still translate into an alanine at codon 450 and should not therefore alter luciferase activity. However, it was still important to determine the effect of a single 8-oxodG on luciferase activity, as Brégeon *et al.* (26) also showed that transcripts could be generated with a single deletion opposite the site of an 8-oxodG. As can be seen from Figure 2A, a single 8-oxodG situated in either the transcribed or non-transcribed strand did not alter luciferase activity in our system. MDSs containing either two 8-oxodGs or an 8-oxodG opposite a uracil were examined (Tables 1 and 2). The two 8-oxodG in opposite DNA strands were situated either 3' to each other and separated by 1 and 4 bp, or situated 5' to each other and separated by 3 and 6 bp. MDSs containing an 8-oxodG and a uracil were situated in opposite strands and either 3' to each other and separated by 3 and 8 bp, or 5' to each other and separated by 4, 6 and 10 bp. None of these lesions decreased luciferase activity (Figure 2B and C). The results for the MDSs consisting of two 8-oxodGs were compared to those obtained for the corresponding single lesions and were found not to be significantly different from at least one of the corresponding single lesions. 8oxo/U-5 was the only MDS consisting of an 8-oxodG and a uracil that was significantly different from the corresponding single 8-oxodG. This MDS however, was not significantly different from the corresponding single uracil lesion [U₁₅, (13)]. This therefore suggests that the MDSs were not converted to DSBs and is in agreement with the lack of deletions detected in the mutation

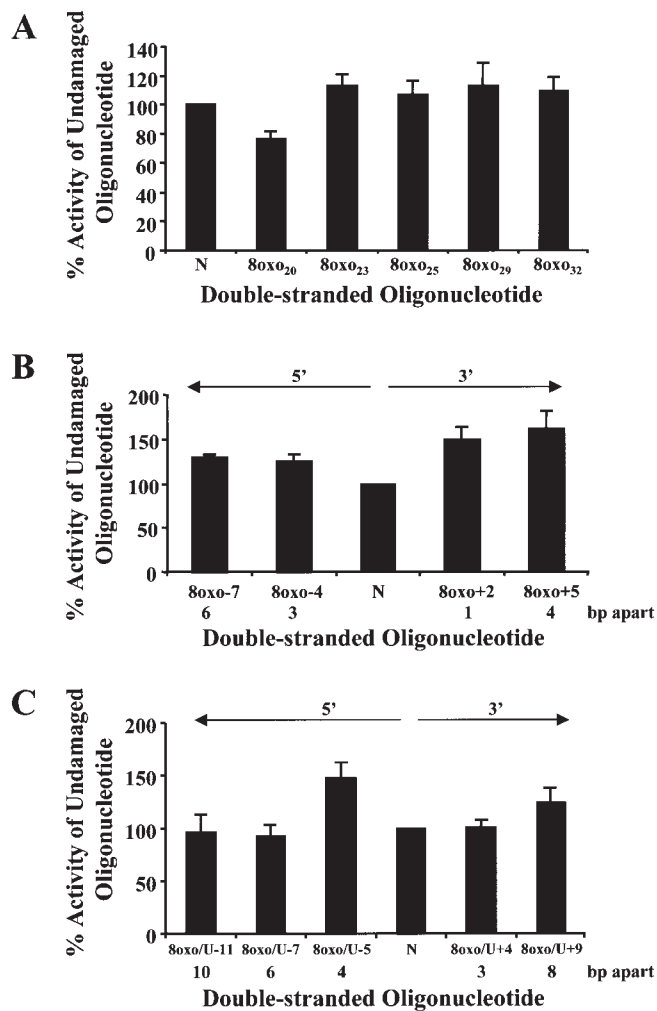


Figure 2. MDS containing two closely opposed 8-oxodGs, or an 8-oxodG opposed to a uracil, are not converted to a double-strand break in wild-type *E. coli*. Double-stranded oligonucleotides containing a single 8-oxodG (A), two closely opposed 8-oxodG (B), an 8-oxodG closely opposed to a uracil (C) or no damage (represented as N on the graphs) were ligated into p3'luc and the ligation reaction co-transformed with pACYC184 into wild-type bacteria. After a 4 h incubation, the luciferase activity was measured in a cell-free extract and the activity normalized to the number of chloramphenicol-resistant colonies obtained after overnight growth on solid medium. These results were used to determine the percentage of activity measured for each sample compared to the undamaged control sequence. At least two ligations and five transformations were performed. The average and the standard error are shown graphically. For substrates containing two 8-oxodG (B) or an 8-oxodG closely opposed to a uracil (C), the number of base pairs (bp) separating the two lesions, as well as the orientation (5' or 3') of the damage with respect to each other, is indicated. 8oxo₂₀, 8oxo₂₃, 8oxo₂₅, 8oxo₂₉ and 8oxo₃₂ are double-stranded oligonucleotides containing a single 8-oxodG. The single lesion in 8oxo₂₅ was situated in the transcribed strand and formed one lesion of the 8oxo-7, 8oxo-4, 8oxo+2, 8oxo+5, 8oxo/U-11, 8oxo/U+4 and 8oxo/U+9 MDSs. The single lesion in 8oxo₂₀ was situated in the non-transcribed strand and formed one lesion of the 8oxo+5, 8oxo/U-7 and 8oxo/U-5 MDSs, and the single lesions in 8oxo₂₃, 8oxo₂₉ and 8oxo₃₂ were also situated in the non-transcribed strand and formed one lesion of the 8oxo+2, 8oxo-4 and 8oxo-7 MDSs, respectively.

assay (Table 3). The sequence containing the MDSs is GC rich and would be expected to have a high degree of hydrogen bonding, possibly preventing the formation of a DSB from two SSB-repair intermediates. However, this is unlikely since the 8oxo/U-5 MDS (Table 2) is situated in the same sequence as

a previously examined MDS (U-6), which consisted of two uracils and decreased luciferase activity to 16% of the undamaged sequence (13). To confirm that plasmid DNA did not contain deletions following DNA repair, transformations for the undamaged sequence, two single lesions (8oxo₂₀ and 8oxo₂₅) and each type of MDS ligation, were repeated using 1 μ l of the ligation reaction. After the 4 h incubation in the presence of novobiocin, the cultures were grown on solid medium containing carbenicillin (50 μ g/ml). One hundred carbenicillin-resistant colonies from each ligation were screened for luciferase activity. Ninety-three percent of the colonies obtained from the ligation with the undamaged sequence, and >90% (91–97%) of the colonies from the other ligations, expressed active firefly luciferase. Plasmid DNA was sequenced from colonies that did not express luciferase activity. Thirty out of fifty three plasmids did not contain the PacI and ClaI sequence (45–56 bp) as well as a few base pairs on either side of the insert. This type of sample was derived from every ligation examined, including undamaged, 8oxo₂₀ and 8oxo₂₅, which indicates that the deletion was not due to the repair of the MDS and was likely generated during the *in vitro* ligation reaction. No large deletions were found and the point deletions/insertions/mutations present in the remaining samples were not at sites of DNA damage.

Repair of an 8-oxodG-containing MDS in transcription-coupled repair-deficient bacteria in the absence of DNA replication

It has recently been shown that transcription-coupled repair (TCR) of 8-oxodG does occur in *E. coli*, and that the Mfd protein, as well as Fpg, is involved in the removal of 8-oxodG from the transcribed strand (26). Our DNA repair assay examines repair of MDSs consisting of one lesion in the transcribed strand and one lesion in the non-transcribed strand. It was therefore possible that DSBs did not form during the repair of two closely opposed 8-oxodG because of preferential and complete repair of the lesion in the transcribed strand by TCR, before BER could be initiated at the lesion in the non-transcribed strand. We therefore examined the repair of 8-oxodG-containing MDSs in bacteria deficient in Mfd. Initially, we tested the single 8-oxodG lesions and determined that a single 8-oxodG did not decrease luciferase activity (data not shown). A similar result was found for MDSs consisting of two 8-oxodG, or an 8-oxodG and a uracil (Figure 3). The Mfd-deficient bacterial strain is isogenic with the AB1157 wild-type strain, and we found no decrease in luciferase activity with MDSs consisting of two 8-oxodG in AB1157 (data not shown). This therefore indicates that TCR is not preferentially repairing the lesion in the transcribed strand, preventing the conversion of the MDS into a DSB in our system.

DISCUSSION

We have utilized a mutation assay in which MDSs containing two opposing 8-oxodG are positioned within the firefly luciferase reporter. This study extends our previous work which demonstrated that the mutation frequency of 8-oxodG is enhanced by an 8-oxodG situated immediately 5' in the opposite strand [MDS-1, (19)]. We now find a similar level of enhancement in wild-type bacteria even when the

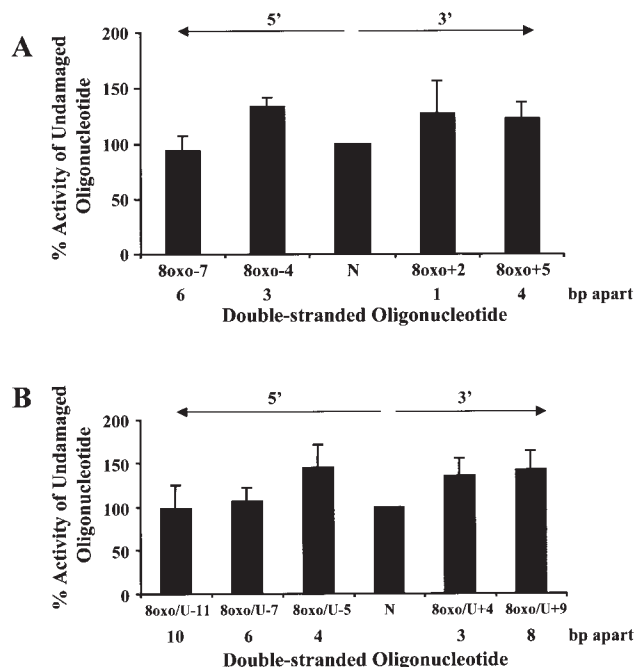


Figure 3. Bacteria deficient in the transcription-repair coupling factor, Mfd, do not convert MDS containing 8-oxodG into a double strand break. Double-stranded oligonucleotides containing two closely opposed 8-oxodG (A), an 8-oxodG closely opposed to a uracil (B) or no damage (represented as N on the graphs) were ligated into p3'luc and the ligation reaction co-transformed with pACYC184 into *mfd*⁻ bacteria. After a 4 hr incubation, the luciferase activity was measured in a cell-free extract and the activity normalized to the number of chloramphenicol-resistant colonies obtained after overnight growth on solid medium. These results were used to determine the percentage of activity measured for each sample compared to the undamaged control sequence. Triplicate transformations were performed for each ligation reaction and two ligations were examined. The average and the standard error are shown graphically. The number of base pairs (bp) separating the two lesions in the MDS, as well as the orientation (5' or 3') of the lesions with respect to each other, is indicated.

opposing 8-oxodG is situated 3 (8oxo-3, Table 3) or 6 (8oxo-6) nt away from the target 8-oxodG. In fact, the point mutation frequency of the 8-oxodG at codon 444 was equivalent for the MDS-1, 8oxo-3 and 8oxo-6 lesions, but approached that of the single 8-oxodG when the two 8-oxodGs were 14 bp apart (8oxo-15, Table 3). As previously discussed (19), the enhancement of the mutation frequency is likely due to the conversion of the 8-oxodG in the non-transcribed strand to a SSB-repair intermediate, which inhibits the removal of the target 8-oxodG in the transcribed strand, resulting in a higher probability that DNA replication will occur prior to repair. In Mut Y-deficient bacteria, a decline in the mutation frequency of the 8-oxodG at codon 444 was detected when the two 8-oxodGs were separated by 5 bp (8oxo-6, Table 4). The mutation assay is more sensitive in *mutY*⁻ bacteria, since the adenine inserted opposite the 8-oxodG during replication is not removed. The decline in mutation frequency can be explained two ways: first, a decrease in Fpg inhibition due to an increase in distance between the SSB-repair intermediate and 8-oxodG, and second, complete repair of the SSB in the non-transcribed strand, allowing Fpg to cleave the lesion in the transcribed strand. Work *in vitro* has shown that Fpg cleavage does increase as the lesions in the MDS are separated

by ≥ 2 bp (20,21), and even when all the enzymes are present *in vitro* to complete repair of the MDS, cleavage of the 8-oxodG opposite a SSB results in the formation of a DSB (21). Therefore the first scenario, where 8-oxodG is removed even when there is an opposing SSB, should cause an increase in deletion frequency and/or loss of plasmid DNA as the 8-oxodG-mutation frequency declines in bacteria. In our study, we determined that as the distance between the two 8-oxodGs increased, the deletion frequency did not increase (Tables 3 and 4) and the number of carbenicillin-resistant colonies following transformation into wild-type bacteria was similar for each type of ligation. Using a DNA repair assay, we did not detect a decrease in luciferase activity (Figure 2) or the introduction of deletions when two 8-oxodGs were situated 3' to each other and separated by 1 and 4 bp, and 5' to each other and separated by 3 and 6 bp, which suggests that DSBs were not formed. The lack of DSB and deletion formation in our system was not due to preferential repair of the 8-oxodG in the transcribed strand by TCR, since we did not detect a decrease in luciferase activity during the repair of the MDS in *Mfd*-deficient bacteria. Our results therefore suggest that the mutation frequency of the 8-oxodG in the transcribed strand declined because Fpg initiated repair of the target 8-oxodG after complete repair of the SSB in the non-transcribed strand. This explains why DSB-repair intermediates were not detected. Even though the *in vitro* studies predict that the inhibition of 8-oxodG repair, and hence the mutation frequency, should decline when the lesions are 2 bp apart, we only detected a decrease in mutation frequency in Mut Y-deficient bacteria when the lesions were separated by 5 bp. This infers that the inhibition of Fpg by the closely opposed SSB occurs over a greater distance in bacteria than demonstrated *in vitro*, preventing DSB formation. Interestingly, bacteria deficient in Fpg accumulate fewer DSBs than wild-type when given time to repair ionizing radiation damage in the absence of replication (27). Our work suggests that Fpg in irradiated wild-type bacteria does not convert two opposing 8-oxodG to a DSB, but may cleave at 8-oxodG situated in close opposition to a base damage or AP site that can also be converted to a SSB during repair.

Pearson *et al.* (22) also found a decline in the mutation frequency when an 8-oxodG and opposing uracil were 5' to each other and 4 bp apart in wild-type bacteria, which is similar to our results, and 2 bp apart in Mut Y-deficient bacteria. They were unable to measure the deletion frequency using their assay, but we have determined that MDSs containing an 8-oxodG and a uracil did not reduce luciferase activity or result in the production of deletions when repair was allowed to occur in the absence of DNA replication (Figure 2). Again, this suggests the scenario where complete repair of the uracil and the SSB-repair intermediate in the opposing strand occurs prior to removal of the 8-oxodG. If 8-oxodG was removed from the MDS before uracil, our previous study examining clustered uracil lesions (13) predicts that the MDS would be converted to a DSB. Uracil is predominantly removed from DNA by (Ung) in bacteria. In an *E.coli* cell, the number of molecules of Fpg [~ 400 , (9)] and Ung [~ 300 , (28)] are similar. The kinetic parameters for these two enzymes have not been compared using uracil or 8-oxodG substrates with similar sequence context and reaction conditions. However, the K_{cat}/K_m ratios available

[Fpg = $4.8 \times 10^7 \text{ M}^{-1} \text{ min}^{-1}$, (20); Ung = $2 \times 10^{10} \text{ M}^{-1} \text{ min}^{-1}$, (29)] predict that Ung would remove uracil from the DNA before Fpg cleaved at the 8-oxodG.

The biological outcome of repair of MDSs containing 8-oxodG is very different from those containing two uracil lesions, which appear to be converted to DSBs (13). The difference seems to be whether the repair enzyme is inhibited by a closely opposed SSB-repair intermediate. After removal of the uracil, the resulting AP sites are cleaved by either exonuclease III or endonuclease IV, and *in vitro* studies have shown that exonuclease III can cleave at two opposing AP sites (30). Only one substrate tested (two opposing AP sites immediately 5' to each other) was resistant to exonuclease III cleavage at the second AP site, once the first was converted to a SSB-repair intermediate. Our previous work in bacteria (13) suggests that either physiological levels of exonuclease III can cleave this type of substrate in the cell, or that another enzyme is able to convert two AP sites situated immediately 5' to each other into a DSB. Work is underway in this laboratory to determine which enzymes in bacteria cleave at the AP sites.

In summary, the mutation frequency of an 8-oxodG was enhanced when it was positioned in the opposite strand and ≤ 5 bp apart from a second 8-oxodG, and MDSs containing two 8-oxodG or an 8-oxodG and a uracil did not reduce luciferase activity, or result in the formation of deletions during DNA repair in bacteria, which is consistent with a lack of DSB formation. This indicates that repair of 8-oxodG is inhibited by an opposing SSB-repair intermediate situated ≤ 6 nucleotides away, which is a greater distance than that predicted by the *in vitro* studies. We have also demonstrated that transcription-coupled repair of 8-oxodG does not alter the biological outcome of MDSs containing an 8-oxodG on the transcribed strand in our system. Brégeon *et al.* (26) did suggest that BER and TCR are in competition to repair the transcribed strand. It is possible that TCR only repairs a minor amount of 8-oxodG damages when BER is not limiting. This work does demonstrate the importance of examining the repair of MDSs in cells, as *in vitro* studies would have predicted the generation of DSBs from the MDSs examined in this study. The types of lesions as well as the spatial distribution within the MDS is therefore critical to the biological relevance of the MDS.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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