Escherichia coli Fpg Glycosylase Is Nonredundant and Required for the Rapid Global Repair of Oxidized Purine and Pyrimidine Damage In Vivo

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Introduction

DNA damage caused by both endogenous and exogenous sources of reactive oxygen species is thought to be a primary event in the etiology of cancer and aging in humans. Reactive oxygen species induce a broad spectrum of distinct base modifications in DNA (reviewed by Wallace1). These lesions range from those that retain their coding specificities (dihydrothymine), to those that mispair during replication or transcription (8-oxoguanine), to those that block DNA and RNA polymerases and are potentially lethal (thymine glycol).2-4 In both Escherichia coli and humans, oxidized base damage is predominantly removed by the base excision repair pathway in which a DNA glycosylase cleaves the glycosidic bond to release the damaged base. The sugar phosphate backbone of the resulting apurinic or apyrimidinic (AP) site is then incised by an AP endonuclease and sometimes

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Abbreviations used: Endo III, endonuclease III; Fpg, formamidopyrimidine-N-glycosylase; AP, apurinic or apyrimidinic; Fapy-Ade, 4,6-diamino-5-formamidopyrimidine; Fapy-Gua, 2,6-diamino-4-hydroxy-5-formamidopyrimidine; 5-OHC, 5-hydroxycytosine; 5-OHU, 5-hydroxyuracil; EDTA, ethylenediaminetetraacetic acid.

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processed before a new nucleotide is polymerized and the DNA strand is religated by polymerase I and ligase (reviewed by Wallace).

To deal with the range of base modifications formed by reactive oxygen species, organisms contain a suite of evolutionarily conserved glycosylases that are involved in the initial step of base excision repair (reviewed by Krokan et al.5). In *E. coli*, exonuclease III, formamidopyrimidine-N-glycosylase (Fpg), uracil-DNA glycosylase, Endo III, Endo IV, Endo V, and Endo VIII are all involved in removing oxidized bases, while mammalian cells encode homologs—in many cases multiple homologs—for each of these glycosylases.6 In *vivo*, these glycosylases are capable of recognizing and removing a range of structurally related base modifications; in many cases, their substrate specificities overlap significantly.7–9 This, in addition to the observation that no single glycosylase mutant is hypersensitive to oxidative damage, has led to the view that redundancies are likely to exist among glycosylases to remove most major forms of oxidative base damage in the cell.7,9,10

Based on their activity in cell extracts and in purified form, Fpg and Endo III are two of the primary activities that remove common forms of oxidative purines and pyrimidines, respectively.11–14 *fpg* was initially identified as *mutM*, a gene that reduced the frequency of GC → TA transversions.15 In *vivo*, Fpg glycosylase efficiently recognizes a range of oxidized purines, including 8-oxoguanine and 4,6-diamino-5-formamidopyrimidine (Fapy-Ade) lesions, which are among the most abundant oxidized purines formed by free radical damage in *vivo*.16–18 In addition, the enzyme is also active on some prominent forms of oxidized pyrimidine base damage, including 5-hydroxyctosine (5-OHC), 5-hydroxyuracil (5-OHU), and dihydrothymine,19,20 but has little to no activity toward other prominent pyrimidines such as thymine glycols.11,19–21 Endo III was isolated as an enzyme that cleaves UV-irradiated DNA.22 Purified Endo III recognizes and removes a range of modified pyrimidines, including thymine glycols, uracil glycols, 5-OHC, 5-OHU, and dihydrothymine.7,12,23–25 Some weak activity towards the minor purine lesions Fapy-Ade, guanidinohydantoin, and spiromodihydantoin has also been reported, but it does not appear to efficiently recognize other forms of purine lesions23–25 (Fig. 1).

Much of what we know about glycosylase function has been inferred from their substrate preferences and relative activities in purified form when incubated with oxidatively damaged DNA or oligonucleotides containing defined lesions. However, in a cellular context, some glycosylases may have specialized functions that couple their activity to processes such as transcription, replication, or cell division. Glycosylases may also be regulated spatially or temporally by protein partners that effectively limit their access to lesions in the overall genome. Finally, in some cases, specific lesions are known to be efficiently recognized by multiple glycosylases in *vivo*, leading to the question of whether functional specialization or redundancy between glycosylases occurs *in vivo*.

To gain a better understanding of the comparative activities of these enzymes *in vivo*, we measured the rate at which Fpg and Endo III remove their respective substrates from the genome following hydrogen-peroxide-induced DNA damage. We find that Fpg is nonredundant and required to rapidly remove its own substrates as well as a significant fraction of those recognized by Endo III, from the chromosome *in vivo*. By comparison, the absence of Endo III had only a modest effect on the global repair rate of its substrate lesions *in vivo*, and neither Endo VIII nor nucleotide excision repair contributed to the rate of lesion removal within the time frame that we examined. These observations are consistent with the known substrate specificities of Fpg and Endo III and indicate that Fpg plays a prominent role in the global repair of purine lesions and at least

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**Fig. 1.** The structure and relative abundance of some common forms of oxidized base adducts in H$_2$O$_2$-treated cultures. *A* Approximate frequency of lesions based on treatment with increasing concentrations of hydrogen peroxide.26
some pyrimidine lesions in vivo. Despite the persistence of lesions in fpg mutants, replication was not prevented from recovering after oxidative damage, suggesting that the cell contains a uniquely efficient mechanism to process oxidized lesions that may be encountered during DNA replication.

Results

Lesions recognized by Fpg and Endo III are rapidly repaired in vivo

Although the overall ranges of substrates recognized by Fpg and Endo III partially overlap, these enzymes exhibit distinct substrate preferences for oxidized purines and oxidized pyrimidines, respectively. To examine if these substrate preferences can be detected in our enzyme preparations, we treated purified high-molecular-weight E. coli DNA either with methylene blue+white light or with osmium tetroxide. Previous studies have shown that treatment with methylene blue+light generates primarily 8-oxoguanines and 2,6-diamino-4-hydroxy-5-formamidopyrimidine lesions in DNA. By contrast, treatment with osmium tetroxide almost exclusively generates thymine glycol lesions. To monitor for the presence of lesions recognized by Fpg or Endo III glycosylases, we incubated DNA samples with each glycosylase and then electrophoresed them through an alkali agarose gel. Both Fpg and Endo III contain an associated AP endonuclease activity that nicks the DNA backbone following the removal of modified bases recognized by these enzymes. Thus, the presence of lesions recognized by each glycosylase can be monitored by the loss of high-molecular-weight DNA in the glycosylase-treated samples. When Fpg glycosylase was incubated with DNA treated with methylene blue+light, the DNA was incised or nicked much more extensively than it was after incubation with Endo III (Fig. 2). The modest activity of Endo III towards DNA treated with methylene blue+light may be due to the presence of 2,6-diamino-4-hydroxy-5-formamidopyrimidine, which Endo III has been reported to weakly recognize in vitro. In contrast, when the osmium-tetroxide-treated DNA was incubated with each of the glycosylases, the situation was reversed. Endo III incubation resulted in a loss of high-molecular-weight DNA, whereas Fpg exhibited comparatively little activity towards the osmium-tetroxide-treated DNA. Importantly, incubation of mock-treated DNA with either Fpg or Endo III resulted in little or no loss of high-molecular-weight DNA, demonstrating that Fpg and Endo III activity was specific for the oxidative lesions formed by these treatments. Overall, the results are consistent with the known substrate specificities of these enzymes and demonstrate that differences in their preferred substrates can be detected using this type of assay.

To measure the repair of oxidative DNA lesions in vivo, we treated the cultures with 10 mM hydrogen peroxide for 5 min, filtered them to remove the chemical, and then resuspended them in fresh prewarmed media for recovery. Total genomic DNA was then purified from the culture at 0 min, 5 min, 10 min, 20 min, and 40 min after the hydrogen peroxide had been removed. To determine how rapidly lesions recognized by Fpg and Endo III are removed from the chromosome in vivo, we treated purified DNA from each time point with either no glycosylase, Fpg glycosylase, or Endo III, and then electrophoresed the denaturing alkali agarose gel, as described previously.

In wild-type cultures, mock-treated genomic DNA remained high molecular weight at all time points examined, both before and after hydrogen peroxide exposure (Fig. 3a). DNA fragments averaged greater than 40 kb in length, which was the approximate limit of resolution in our agarose gels. Incubation of the genomic DNA with Fpg glycosylase resulted in a loss of high-molecular-weight fragments at times immediately after hydrogen peroxide exposure, indicating that lesions recognized by Fpg were present in the DNA. The number
of Fpg-recognized lesions in the DNA actually increased slightly during the first 5 min after hydrogen peroxide was removed from the medium. However, within 10 min, the number of lesions recognized by Fpg in the DNA began to decrease, and lesion-free high-molecular-weight fragments began to reappear. By 40 min, more than 80% of the high-molecular-weight DNA had been restored (Fig. 3a). Repair could not be quantified at times beyond 40 min following hydrogen peroxide treatment due to the rapid recovery of DNA synthesis and accumulation of new DNA (see Fig. 5 and the text below).

Lesions recognized by Endo III were removed from the chromosome at a rate similar to that of lesions recognized by Fpg. Incubation of samples with Endo III resulted in a loss of high-molecular-weight fragments at times immediately following the removal of hydrogen peroxide. The number of enzyme-sensitive sites in the genomic DNA increased modestly during the first 5 min of the recovery period before lesion-free high-molecular-weight DNA fragments began to return. After 40 min, more than 80% of the high-molecular-weight fragments had been restored.

Importantly, no loss of high-molecular-weight DNA was observed following incubation of Fpg or Endo III with DNA from cells that were not exposed to hydrogen peroxide, indicating that glycosylase incision was due specifically to the presence of oxidized lesions recognized by these enzymes. To ensure that all potential substrates recognized by Fpg were incised, in all experiments we used the highest concentration of glycosylase that remained specific for damaged DNA (Fig. S1). The alkali buffer in the agarose gels will cleave DNA at sites containing AP sites. This, along with repair events initiated during the 5-min period in which the culture is exposed to hydrogen peroxide, is likely to account for the modest loss of high-molecular-weight DNA that averaged 30% at times immediately after exposure to hydrogen peroxide in non-glycosylase-treated samples (see Fig. 3, pretreated versus 0-min and 5-min time points). To normalize for these nicks and AP sites present in the purified DNA, we normalized the fraction of lesion-free DNA to the amount present in the mock-glycosylase-treated samples at each time point examined.

The observed increase in lesion frequency that occurs during the first 5 min of the recovery period is likely due to reactive oxygen species that continue to be generated in the cell after hydrogen peroxide has been removed. The lesion frequency at any given time, as determined based on the average DNA fragment size in the enzyme-treated samples from at least three independent experiments, never exceeded more than one Fpg substrate per ∼40-kb strand of DNA or more than one Endo III substrate per ∼20-kb strand of DNA. However, the continued induction of DNA lesions during the repair period makes it difficult to directly determine the total number of DNA lesions that were generated by this treatment. Nevertheless, the
observation that lesion-free high-molecular-weight DNA was restored within 15 min of the time at which the maximum lesion frequency was observed indicates that both classes of these lesions are rapidly repaired in vivo.

In vivo, Fpg is required for the rapid removal of its substrate lesions as well as a significant fraction of those recognized by Endo III, from the chromosome

To examine how efficiently Fpg and Endo III function in the global repair of their respective lesions in vivo, we measured the rate at which their substrates were repaired in fpg and nth mutants. We reasoned that if a redundant glycosylase is present for each of the enzyme’s substrates, then the repair rate would be unaffected when either Fpg or Endo III is absent in the cell. Alternatively, if functionally redundant activities for these substrates do not exist in vivo, then we would expect that the repair of lesions would be impaired in the absence of that glycosylase.

To this end, fpg and nth cultures were treated with hydrogen peroxide and, at the indicated times, DNA was purified and analyzed as described previously. In contrast to wild-type cultures, lesions recognized by Fpg glycosylase persisted in the genomic DNA of fpg mutants throughout the recovery period. After 40 min, less than 30% of the high-molecular-weight DNA fragments had been restored. In addition, Fpg substrates accumulated at a higher frequency in fpg mutants than in wild-type cells, based on the average size of the DNA fragments in Fpg-treated samples (Fig. 3). This observation indicates that functional redundancy does not exist to rapidly remove Fpg-recognized lesions from the overall genome in vivo.

Unexpectedly, a substantial portion of the lesions recognized by Endo III also persisted in the genome of fpg mutants (Fig. 3). Endo-III-sensitive sites persisted in the genome of fpg mutants throughout the 40-min recovery period and also accumulated to a higher frequency than in wild-type cells. Although Fpg is generally considered to repair purine-derived base damage, recent studies have shown that, in vitro, Fpg recognizes several common forms of oxidized pyrimidines as efficiently as Endo III, including 5-OHC, 5-OHU, and, to a lesser extent, dihydrothymine. We interpret this observation to indicate that although Fpg and Endo III are both capable of recognizing these lesions in vitro, Fpg is the primary enzyme that removes these substrates globally from the overall genome in vivo.

Consistent with this interpretation, a significant portion of Endo III substrates was still repaired in nth mutants that lack Endo III. Within 40 min, 50% of Endo III substrates had been removed from the genome in nth mutants, compared to 80% in wild-type cultures. The absence of Endo III had even less of an impact on the rate that Fpg-recognized lesions were repaired from the genome, consistent with in vitro studies suggesting that Endo III does not recognize the predominant Fpg substrates formed by hydrogen peroxide treatment. In nth double mutants, the repair rate of both Fpg substrates and Endo III substrates was impaired to an extent that was similar to that seen in the fpg single mutant.

The presence of Fpg did not completely complement repair in nth mutants, as a modest but detectable fraction of lesions persisted in the DNA (Fig. 3, compare Endo-III-treated samples in nth mutants to wild type). This population of lesions is likely to include thymine glycols, which represent approximately 30% of the total lesions induced by hydrogen peroxide treatment and are known to be recognized by Endo III, but not Fpg, in vitro. Taken together, these experiments indicate that Fpg glycosylase is nonredundant and necessary for the rapid global repair of its substrate lesions as well as a significant portion of the substrates that are recognized by Endo III.

Endo VIII and nucleotide excision repair do not detectably contribute to the removal of oxidized purines or pyrimidines from the overall genome

Other enzymes have also been suggested to contribute to the repair of lesions recognized by Fpg and Endo III. In vitro, the substrate specificity of Endo VIII glycosylase, encoded by nei, overlaps significantly with the substrate specificities of both Endo III and Fpg, recognizing the most abundant forms of oxidized purines and pyrimidines (8-oxoguanine and thymine glycol, respectively). In addition, UvrA, encoded by uvrA and required for nucleotide excision repair, is also capable of recognizing thymine glycols in vitro. To examine whether Endo VIII or nucleotide excision repair contributes to the removal of these lesions from the genome in vivo, we measured the repair of Fpg and Endo III substrates in nei and uvrA mutants.

DNA was purified from hydrogen-peroxide-treated nei or uvrA cultures at various times during the recovery period, and the repair of Fpg-recognized and Endo-III-recognized lesions was analyzed as described previously. In cultures lacking Endo VIII, we observed that both Fpg-recognized and Endo-III-recognized lesions were repaired at rates similar to that observed in wild-type cultures (Fig. 4). Similarly, uvrA mutants removed these lesions at rates that were comparable to wild-type cultures (Fig. 4). These data indicate that although Endo VIII and nucleotide excision repair are both capable of recognizing these lesions in vitro, they do not significantly contribute to the removal of these lesions from the overall genome.

Fpg Glycosylase is Nonredundant In Vivo

The persistence of oxidative lesions in fpg mutants does not prevent replication from resuming

More than 30% of the oxidized bases generated by hydrogen peroxide treatment consist of thymine glycols and other lesions that are known to block DNA polymerases in vitro and in vivo. Thus, one might expect that replication would be disrupted in cultures exposed to hydrogen peroxide and that the recovery of replication would be delayed in mutants where a significant amount of blocking lesions persists in the genome. Alternatively, it is possible that a repair or tolerance mechanism is specifically coupled to replication and is required to remove lesions encountered at the replication fork. Therefore, to examine whether the absence of Fpg, Endo III, Endo VIII, or nucleotide excision repair impairs the cell’s ability to replicate in the presence of oxidative damage, we monitored the recovery of DNA synthesis in these mutants following treatment with hydrogen peroxide. To this end, cultures grown in the presence of [3H]thymine were either mock treated or exposed to 10 mM hydrogen peroxide for 5 min, collected on filters, and resuspended in fresh prewarmed media supplemented with [3H]thymine. At the indicated times during and after hydrogen peroxide treatment, duplicate aliquots of cultures were lysed, and the DNA was precipitated. The amount of DNA that accumulated over time could then be followed by the amount of 3H incorporated into the DNA. In wild-type cultures, a brief inhibition of replication was observed immediately following the period of exposure to hydrogen peroxide. However, within 20 min of hydrogen peroxide removal, replication began to recover, and the accumulation of DNA returned to a rate comparable to that of unexposed cultures (Fig. 5a).

When we examined replication in fpg mutants, we observed that DNA replication resumed within 20 min of hydrogen peroxide removal, and DNA began to accumulate at a rate that was comparable to wild-type cells. The resumption of replication was somewhat unexpected, considering that the oxidative lesions persist in the genome of these mutants (including those recognized by Endo III), and suggests that the cells may contain an efficient mechanism to either couple the repair to replication or tolerate these lesions.

When we examined the replication that occurred following exposure to hydrogen peroxide in cultures of nth, nei, or uvrA mutants, we observed that it was inhibited to a similar extent and to a similar duration as in wild-type cultures. In each case, replication resumed at a time similar to that seen in wild-type cultures. Consistent with this observation and with previous reports, the absence of Fpg, Endo III, Endo VIII, or UvrA did not render cells hypersensitive to oxidative damage generated by hydrogen peroxide (Fig. 5b). By contrast and for purposes of control, the viability of recA cultures was reduced by approximately 4 orders of magnitude following a 5-min exposure to 10 mM hydrogen peroxide. Taken together, the observations indicate that the absence of these repair enzymes does not prevent replication from resuming following the induction of oxidative DNA damage.

Discussion

Fpg and Endo III have sometimes been generalized as glycosylases that predominantly repair oxidized purine and pyrimidine damage, respectively. However, recent biochemical studies have demonstrated that Fpg is also capable...
Fig. 5. The absence of Fpg, Endo III, Endo VIII, or nucleotide excision repair does not prevent DNA synthesis from following hydrogen-peroxide-induced DNA damage. (a) Cultures grown in the presence of $[^{3}H]$ thymine were exposed to hydrogen peroxide for 5 min (open circles) or mock treated (filled triangles), resuspended in fresh medium at 37 °C, and allowed to recover. The relative amount of $[^{3}H]$ thymine incorporated into the DNA is plotted over time. All plots represent an average of two independent experiments. Error bars represent the standard error of the mean. The amount of $[^{3}H]$ in each sample at time 0 was between 7000 cpm and 13,000 cpm for all experiments. (b) Cells lacking DNA glycosylases or nucleotide excision repair are not hypersensitive to hydrogen peroxide. The survival of each strain following exposure to 10 mM hydrogen peroxide for the indicated time period is plotted. Strains: wild-type (open square), recA (CL21; filled square), $n$th (CL1006; filled triangle), $n$th (CL1005; filled circle), $n$th (CL1005; filled diamond), $n$th (CL1006; filled triangle), $n$uvrA (CL21; filled square), and $n$uvrA (CL1002; open circle). Plots represent an average of three independent experiments. Error bars represent the standard error of the mean.

of recognizing some commonly formed oxidized pyrimidines, including 5-OHC and 5-OHU, as efficiently as Endo III. Similarly, Endo III has been reported to recognize at least one minor form of a purine-derived lesion, Fapy-Ade. While the substrates and efficiencies of these glycosylases have been characterized extensively in vitro, the relative contribution of each enzyme to repairing oxidative damage in vivo has not been examined directly.

Here, we examined the efficiency with which Fpg and Endo III remove their respective substrate lesions from the chromosome. Our results indicate that Fpg is required for the rapid global repair of its substrates, as well as a significant portion of the substrates recognized by Endo III. Endo III had a detectable but minor contribution to the repair of its own substrate lesions in vivo and did not significantly contribute to the repair of Fpg-recognized lesions. Neither Endo VIII nor nucleotide excision repair, which both have overlapping substrate specificities in vitro, detectably contributed to the repair of Fpg-recognized or Endo-III-recognized substrates in vivo. The persistence of Endo III substrates in fpg mutants, but less so in nth mutants, indicates that even though both Fpg and Endo III are equally capable of recognizing pyrimidine-derived lesions such as 5-OHC and 5-OHU in vitro, Fpg is the predominant activity that removes these lesions globally from the genome in vivo. Considering that Fapy-Ade is a relatively minor oxidative product and that Endo III has not been reported to recognize any other purine-derived lesions, the observation that Endo III does not contribute to the repair of Fpg substrates in vivo would be consistent with its known substrate preferences in vitro. We would speculate that the modest portion of lesions that depend on Endo III for repair is likely to be made up of thymine glycol lesions, at least in part. Thymine glycols represent approximately 30% of the lesions generated by hydrogen peroxide treatment and are known substrates for Endo III, but not Fpg glycosylase.

In vitro, the substrate specificities of oxidative glycosylases overlap significantly, and no single glycosylase mutant is hypersensitive to oxidative challenges. In combination, these observations have sometimes been interpreted to suggest that significant redundancies exist among glycosylases to repair oxidative lesions. The observed persistence of lesions in fpg mutants implies that significant redundancies are not present in this case at the level of glycosylase function. The rapid recovery of replication in these mutants, despite the persistence of the lesions, implies that survival in these cells may depend on an efficient mechanism for repairing or tolerating oxidative lesions that is coupled to replication. It remains possible that a specific glycosylase and/or translesion DNA polymerase efficiently removes or bypasses lesions encountered by the replication machinery.

Elucidating which glycosylases, translesion DNA polymerases, or alternative enzymes allow replication to occur in the presence of oxidative damage is likely to require a detailed kinetic analysis of candidate mutants, both singly and in combination. Analogously, the mechanism by which replication recovers after UV-induced damage required an examination of multiple mutants in combination. In this case, it has been shown that replication forks blocked by UV-induced damage are processed by RecJ and nucleotide excision repair. However, in the absence of any of these gene products, the recovery of synthesis still occurs but becomes dependent on translesion synthesis by DNA polymerase V. Under these conditions, although DNA polymerase V prevents lethality and allows replication to
Table 1. Bacterial strains used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Source</th>
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<tr>
<td>SR108</td>
<td>Wild type</td>
<td>Mellon and Hanawalt</td>
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<tr>
<td>CL002</td>
<td>recA::Tn10</td>
<td>Courcelle et al.</td>
</tr>
<tr>
<td>CL021</td>
<td>uvrA::Tn10</td>
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<td>CL1005</td>
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<td></td>
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<td>into SR108</td>
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<td>CL1006</td>
<td>nth::kan</td>
<td>P1 from BW402</td>
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<td></td>
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<td>CL1009</td>
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<td>P1 from SW2-F</td>
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<td></td>
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<td>DY351</td>
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<td>PCR</td>
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<td>into CL1006</td>
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Resume, it occurs with slower kinetics and is associated with an elevated frequency of mutagenesis.49-51 In support of this idea, eukaryotic DNA polymerase η is capable of bypassing oxidative damage with limited efficiency, and some evidence suggests that translesion DNA polymerases also participate in the bypass of oxidative lesions in E. coli.52-54 Similarly, although no single glycosylase mutant is hypersensitive to oxidative damage, many double mutants such as nth nei exhibit high levels of mutagenesis,8,10 perhaps suggesting that secondary mechanisms operate to allow recovery to occur under these conditions.

Materials and Methods

Bacterial strains

Table used in this paper are summarized in Table 1. All strains are derived from SR108, a thyA36 deoC2 derivative of W3110.85 CL1005 (SR108 nei::cam) was constructed via P1 transduction of the nei::cam allele from SW2-8 into SR108.86 CL1006 (SR108 nth::kan) and CL1009 (SR108 fpg::::amp) were constructed by P1 transduction of nth::kan and fpg::::amp alleles from BW402 and SW2-F, respectively, into SR108.8 The presence of the fpg, nth, and nei alleles was confirmed by PCR. CL002 (SR108 recA::Tn10) and CL21 (uvrA::Tn10) have been previously described.53,54 CL1775 (DY351 fpg::::tet) was constructed by gene replacement using the recombinaseering strain DY351.56 The tetracycline resistance cassette was amplified from Tn10 using the fpgF primer 5’TAGCGGTCGACGCGGGGCGAAGATATCTCTGCTGCAGCTGACATCTTGGTTACCG and the fpgR primer 5’CTTTAACATCCACGCTAACAATTCAACTCAGCTGACGCGACAAAGGGTCATTATATTTTCG. The PCR product was transformed into DY351 to generate CL1775, selecting for tetracycline resistance. The gene replacement was then moved into SR108 by standard P1 transduction, generating strain CL1778 (SR108 nth::kan fpg::::tet).

CL1938 (SR108 nth::kan fpg::::tet) was constructed by moving fpg::::tet from CL1778 into CL1006 (SR108 nth::kan). Strains CL1778 and CL1938 were confirmed by selecting for tetracycline resistance and by PCR.

Preparation of 8-oxoguanine and thymine glycol containing DNA

Five micrograms of high-molecular-weight E. coli DNA was treated with 1 μg/ml methylene blue (Acros Organics) and placed under a 100-W GE white light bulb for 5 min on ice to generate oxidized purine DNA.27 Previous studies have shown that this treatment generates 8-oxoguanine and formamidopyrimidine lesions in a ratio of approximately 20:1. The DNA was then gently precipitated with an equal volume of isopropanol, washed in 70% ethanol, and resuspended in TE [10 mM Tris and 1 mM ethylenediaminetetraacetic acid (EDTA; pH 8.0)]. To generate oxidized pyrimidine DNA, we incubated 5 μg of high-molecular-weight E. coli DNA in 0.4% osmium tetroxide (Acros Organics) for 1 h at 30 °C (adapted from Dizdaroglu et al.28). The treated DNA was then extracted four times with chloroform to remove osmium tetroxide and dialyzed against 200 ml of TE for 1 h.

Hydrogen peroxide survival

Fresh overnight cultures were diluted 1:100, grown to an OD 600 of 0.4 (approximately 6 × 10^8 cells/ml), and then treated with 10 mM hydrogen peroxide (Fisher Scientific). At the times indicated, 0.1-ml aliquots of each culture were removed and serially diluted in 10-fold increments. Triplicate 10-μl aliquots of each dilution were then spotted on Luria–Bertani (LB) plates supplemented with 10 μg/ml thymine (LBthy). Viable colonies were counted following overnight incubation at 37 °C.

DNA accumulation

Overnight cultures were diluted 1:100 and grown in LB medium supplemented with 0.1 μCi/ml [3H]thymine and 10 μg/ml thymine to an OD 600 of 0.3, at which time half of the culture was treated with 10 mM hydrogen peroxide for 5 min while the other half was mock treated. The cells in each culture were then collected on Fisher 0.45-μm general membrane filters and resuspended in prewarmed LB medium supplemented with 0.1 μCi/ml [3H]thymine and 10 μg/ml thymine. At the times indicated, duplicate 0.5-ml aliquots of culture were lysed, and the DNA was precipitated in cold 5% trichloroacetic acid and filtered onto Millipore glass fiber filters. The amount of [3H]on each filter was determined by scintillation counting.

Glycosylase repair assay

Overnight cultures were diluted 1:100 and grown in LBthy medium to an OD 600 of 0.3. At this time, the cultures were treated with 10 mM hydrogen peroxide for 5 min. The cells were then filtered onto a 0.45-μm membrane and resuspended in prewarmed LBthy medium. At the times indicated, a 0.75-ml aliquot of culture was transferred to an equal volume of ice-cold NET.
Quantification of lesion-free DNA fragments

The intensity of each high-molecular-weight band was determined using ImageQuant software (GE Healthcare). The fraction of lesion-free DNA fragments was quantified as the ratio of high-molecular-weight DNA in the glycosylase-treated band to the band with no glycosylase as the ratio of high-molecular-weight DNA in the DNA at time T. Enzyme preparations were titrated using purified undamaged genomic DNA as template. For Fpg, the highest enzyme concentration that did not exhibit nonspecific activity on the undamaged DNA (corresponding to 0.53 μM) was used. In the case of Endo III, nonspecific activity was not observed even at the highest concentration examined (Fig. S1).

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