Loss of an apurinic/apyrimidinic site endonuclease increases the mutagenicity of N-methyl-N'-nitro-N-nitrosoguanidine to Escherichia coli

(xthA+/exonuclease III/DNA repair/alkylation damage/SOS response)

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ABSTRACT  xthA− Escherichia coli, which are missing a major cellular apurinic/apyrimidinic (AP) endonuclease, are 5- to 10-fold more sensitive than xthA+ bacteria to mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) under conditions that induce the “adaptive response.” The xthA−-dependent mutations are also dependent on SOS mutagenic processing and consist of both transversion and transition base substitutions. When MNNG-adapted xthA− bacteria are challenged with a high dose of MNNG, more xthA−-dependent SOS-dependent mutations are induced, and transversions are enhanced relative to transitions. The mutations induced by challenge are eliminated in xthA− alkA− bacteria, which are also deficient for 3-methyladenine glycosylase II activity. These data are consistent with the hypothesis that AP sites, at least some of which are produced by glycosylase activity, are mutagenic intermediates following cellular DNA alkylolation.

Apurinic and apyrimidinic (AP) sites cause errors in DNA replication in vitro and are mutagenic when present in transforming DNA (1, 2). In addition to being a probable cause of spontaneous mutations (3, 4), AP sites have been implicated as a common mutagenic lesion created by a number of DNA-damaging agents (reviewed in ref. 1). These agents include those that make bulky adducts to the DNA, such as aflatoxin B1 (5), benzo(a)pyrene (6, 7) and β-propiolactone (6, 8), as well as simple alkylating agents (6, 9). However, the mutagenic role of AP sites produced in vivo has not been well established.

The xthA gene of Escherichia coli encodes exonuclease III, a multifunctional enzyme that is the cell’s major AP endonuclease (10). xthA+ bacteria retain only 10–20% of the normal cellular AP endonuclease activity (11, 12). xthA− mutants are deficient in the repair of the AP sites created by the enzymatic removal of misincorporated uracil from DNA (13). They are also slightly sensitive to killing by methyl methanesulfonate (11, 12), an alkylating agent that produces a predominance of N-alkylated DNA bases that give rise to AP sites both spontaneously and enzymatically (3). Other phenotypes of xthA− mutants include increased sensitivity to the oxidizing agent H2O2 (14), slight sensitivity to near UV light (15), and partially defective expression of heat shock proteins (16). Since the xthA gene product is also a duplex-specific DNA 3′–5′ exonuclease (10), can remove a variety of blocking groups from 3′ termini in duplex DNA (17, 18), has RNase H activity (10), and can recognize and nick near uracil residues in DNA (19), it is not known what contribution, if any, its AP endonuclease activity may make to these other phenotypes. Despite the several phenotypes of xthA− bacteria and the many activities of exonuclease III, a role for this enzyme in spontaneous or induced mutagenesis has been difficult to establish.

Exposure of E. coli to low concentrations of alkylating agents induces the “adaptive response” (20), which includes two known DNA repair enzymes—O6-methylguanine-DNA methyltransferase (21) and 3-methyladenine–DNA glycosylase II (TAGII) (22, 23). Two other genes of known function, alkB (24) and aidB (25), also are induced. O6-methyltransferase accurately repairs O6-alkylguanine (21) and O6-alkylthymine (26), thus virtually eliminating any mutations due to these lesions. TAGII, the product of the alkA gene, removes purines alkylated at the N-3 position (22, 23) and pyrimidines alkylated at the O-2 position (26), leaving AP sites. AP sites are also produced after alkylation damage by the spontaneous loss of alkylated bases, particularly N'-alkylguanine, and by the activities of the constitutive glycosylases formamidopyrimidine-DNA glycosylase and 3-methyladenine glycosylase I (3). Thus, induction of the adaptive response by alkylating agents leads to the production of AP sites by several different pathways. Based on the distribution of alkyl lesions in DNA (27) and the known repair activities, the majority of these AP sites are expected to be due to missing purines.

In vitro studies have indicated that AP sites give rise to mutations because DNA polymerases preferentially insert purines, particularly adenosines, opposite such sites during bypass synthesis (1). Hence, depurination is expected to yield a predominance of transversion mutations while depyrimidination should yield transition mutations. In E. coli, at least some part of this postulated pathway must be dependent on SOS mutagenic processing, as mutations are not induced when SOS-deficient cells are transformed with depurinated DNA (1).

Since AP endonuclease activity is the first step in the repair of AP sites, we reasoned that if endogenous AP sites are mutagenic, the mutagenicity of alkylating agents should be enhanced by the xthA− defect under conditions that (i) maximize the formation of AP sites to saturate compensating activities; (ii) minimize the mutagenicity of other lesions such as O6-alkylguanine; and (iii) allow for SOS mutagenic processing. These conditions were achieved by exposing E. coli to a low concentration of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG), thus inducing both the adaptive response (20) and the SOS response (9). When both of these responses are active, xthA− bacteria are more mutable by MNNG than xthA+ bacteria.

MATERIALS AND METHODS

Bacterial Strains. All genetic manipulations were as described (28). The E. coli K-12 strains used for most of the manipulations were

Abbreviations: AP, apurinic/apyrimidinic; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine; TAGII, 3-methyladenine-DNA glycosylase II.
experiments reported here are derivatives of PF260 [F- ara Δ(gpt-lac-pro) thi his5 supD60]. his5 is a deletion of the entire histidine gene cluster (29). One set of experiments used PF352 and PF353 (9), which are hisG- hisD' derivatives of AB1157 (F' argE3 his- leuB6 proA2 thr-1 ara-14 galK2 lacY1 mit-1 xyl-5 thi-1 rpsL31 supE44 tsx-33; see ref. 29) and MS23 (AB1157 his- alkA'; see ref. 30), respectively. To ensure that the strains were isogenic, PF353 and an xthA- derivative of it were transduced to a Plvir lysate of NK5526 (alkA- hisG'; Tn5; obtained from B. Bachmann, Yale University). Methyl methanesulfonate resistant transductants were then made hisG- hisD+ as described (9). Results with these strains did not differ from those with PF352 and PF353.

xthA- derivatives were constructed by transducing cells with a Plvir lysate of BW9115 (Δ(xthA-ptsG) pheS1; see ref. 11; obtained from B. Weiss, The Johns Hopkins University), selecting for resistance to both 1-2 mM 6-aminonicotinamide and fluorophenylalanine (ref. 40; the effective concentrations varied among strains), and screening for methyl methanesulfonate sensitivity. umuC::Tn5 derivatives were constructed by transduction with a Plvir lysate of GW2100 (obtained from G. Walker, Massachusetts Institute of Technology) and screening kanamycin-resistant transductants for lack of UV mutability to rifampicin resistance.

F400 episomes with either the hisG46 or hisG428 mutations were mated into recipient E. coli strains using appropriate selection. The F400 episome carrying the his gene cluster of Salmonella typhimurium was obtained from R. Brent (Massachusetts General Hospital) as his01242, hisG46, gnd::Tn10, rfb+. A hisG428 construction was made by transducing a S. typhimurium strain carrying the episome as hisG- hisD' (obtained from R. Brent) to histidinol utilization (hisD') with a P22 lysate of S. typhimurium strain hisG428H, which carries the hisG428 mutation on the chromosome (obtained from D. Levin, University of California at San Francisco). Transductants were then screened for tetracycline resistance and histidine autotrophy (His+).

hisG46 is a missense mutation in the S. typhimurium hisG gene that replaces a CTC leucine with a CCC proline codon (31). Any base change at the first base and G-C to A-T or G-C to T-A changes at the second base of this codon give a His+ phenotype (31). hisG428 is a nonsense mutation in the same gene that changes a CAA glutamine codon to a TAA ochre (32). In amber suppressor strains (as here), 8 base changes as well as a small deletion will yield true revertants (ref. 32; unpublished observations). Extragenic tRNA suppressors of hisG428 are also readily induced (9, 32).

Media. Media used were as described (9). To select for His+ revertants, a limiting amount of histidine (100 nmol for hisG46 or 50 nmol for hisG428) plus 2 mg of other required amino acids were added to VB minimal medium (33) plates.

Mutagenesis Experiments. Adaptation was induced by exposing cells growing in complete minimal medium to MNNG (0.5 μg/ml) in minimal medium for 4 hr or only weakly mutagenic to wild-type E. coli. However, the mutation frequency of xthA- E. coli under these conditions was 5- to 10-fold greater than that of xthA+ bacteria. This effect was not due to differential survival although adaptation was slightly toxic to xthA- bacteria. With eight repetitions of these experiments, the absolute number of His+ revertants per plate after MNNG treatment varied from 2- to 10-fold higher for xthA- umuDC- cells than for wild-type cells. Reversion of both hisG46 and hisG428 was enhanced by the xthA- defect, although the majority of hisG428 revertants were due to the creation of extragenic suppressors (Table 1). Thus, some MNNG-induced premutagenic lesions were evidently not being repaired in xthA- bacteria.

In vitro and in vivo evidence indicates that AP sites are only mutagenic if the bacterial "SOS response" is induced (1). As the functions of the umuDC operon are necessary for SOS mutagenic processing (35), xthA- dependent mutations should also be umuDC- dependent if they are due to AP sites. As predicted, the enhanced mutagenicity of xthA- bacteria was greatly reduced by a defect in umuC (Table 1).

When bacteria were exposed to a high concentration of MNNG (10 μg/ml for 5 min), the xthA- defect appeared to have little effect on the mutational frequency (Table 2). Thus, the xthA- mutagenic phenotype is obscured under normal conditions of MNNG mutagenesis, presumably because most mutations are due to the directly miscoding SOS-independent lesion, O6-methylguanine (3, 35). However, if the bacteria were first adapted by exposure to a low concentration of MNNG, as in the previous experiments, and then challenged with MNNG-exposed cells to give the number of MNNG-induced mutations per plate. This difference was then divided by the number of cells plated. In all cases except xthA+ and umuC::Tn5/F hisG428 strains (see Table 1), the induced mutations per plate were greater than twice the spontaneous background; for all experiments with xthA- alkA+ umuDC- hisG428 strains, the induced mutations per plate were 20-40 times the spontaneous background.

Analysis of Mutations. Revertants of hisG46 were single colony purified and infected with phage HfrH (34). To select for recombinant phage donor, a His+ recipient (PF665 = P90C recA13 hisG- hisD'/F::Tn3) was infected with selection for the His+ phenotype. Single-stranded DNA was prepared and sequenced by standard dideoxy methods with a synthetic oligonucleotide priming close to the hisG46 region. In a few cases, M13 crosses were repeated selecting for histidinol utilization in WB351 (34) to ensure that spontaneous mutations were not being generated during the phage crosses; no differences in the sequences were found. True revertants of hisG428 were identified by their ability to transfer the His+ phenotype by mating. To determine the percentage of true revertants of hisG428 (Table 1), 291 revertants from MNNG-treated cultures and 100 revertants from nontreated cultures of the xthA- umuDC- strain and 50 revertants from both treated and nontreated cultures of each of the other strains were analyzed. The different classes of hisG428 revertants in our strains could not be identified using thiazole alanine sensitivity as described for S. typhimurium (32).

To calculate the MNNG-induced frequency of mutational events, the fraction of the total analyzed represented by a given class of mutation was multiplied by the total number of colonies on the plate from which the revertants were taken. The number of spontaneous occurrences of that mutation, calculated in the same way, was then subtracted and the difference was divided by the number of cells plated.

RESULTS

The xthA- Defect Increases the Mutagenicity of MNNG. As shown in Table 1, exposure to a low concentration of MNNG (0.5 μg/ml) in minimal medium for 4 hr was only weakly mutagenic to wild-type E. coli. However, the mutation frequency of xthA- E. coli under these conditions was 5- to 10-fold greater than that of xthA+ bacteria. This effect was not due to differential survival although adaptation was slightly toxic to xthA- bacteria. With eight repetitions of these experiments, the absolute number of His+ revertants per plate after MNNG treatment varied from 2- to 10-fold higher for xthA- umuDC- cells than for wild-type cells. Reversion of both hisG46 and hisG428 was enhanced by the xthA- defect, although the majority of hisG428 revertants were due to the creation of extragenic suppressors (Table 1). Thus, some MNNG-induced premutagenic lesions were evidently not being repaired in xthA- bacteria.
by a high concentration of MNNG, >90% of the mutations induced in unadapted wild-type bacteria were eliminated. Under these conditions, when O6-methylguanine lesions are repaired by high levels of O6-methyltransferase activity (21), xthA- bacteria were again 10-fold more mutable than wild-type bacteria. This increased mutagenesis was eliminated by the umuC::Tn5 defect (Table 2). As above, the xthA- effect was not due to differential survival—although fewer cells were plated, the absolute number of His+ revertants per plate for challenged xthA- umuC+ bacteria was 1.5 to 5 times that of the wild-type bacteria.

The Specificity of xthA-Dependent Mutations. The bias of DNA polymerases for inserting purines opposite AP sites predicts that in SOS-proficient bacteria, depurination should yield transversion mutations, while depyrimidination should yield transition mutations. Transitions could also result from misincorporation by unrepaired O6-methylguanine lesions, but these mutations would also occur in SOS-deficient bacteria (3, 35). Thus, it was of interest to determine the specificity of the xthA- dependent changes. Presented in Table 3 are the sequenced base changes of 126 hisG46 revertants from MNNG-exposed cultures and 45 spontaneous hisG46 revertants isolated from the experiments given in Tables 1 and 2.

Fig. 1 shows the frequencies of each base change at the hisG46 codon induced during adaptation to MNNG in the xthA+, xthA-, umuC+, and umuC::Tn5 strains. The major effect of the xthA- defect was to enhance both G:C to T:A transversions and G:C to A:T transitions at the second base of the hisG46 codon. The bias for the occurrence of xthA- dependent mutations at the second base of the hisG46 codon could reflect either the distribution of DNA lesions or a preference for accurate repair at the first base.

The umuC::Tn5 defect reduced the frequency of all xthA- enhanced mutations but had a larger effect in reducing transversions than transitions. That the frequency of transitions at the first base differed little among the four strains suggests that these mutations, which were neither xthA- nor umuDC+ dependent, were probably due to O6-methylguanine lesions. In contrast, umuDC- dependent transitions at the second base, which were enhanced by the xthA- defect, were unlikely to be due to a simple miscoding lesion.

Fig. 2 presents the frequencies of each base change induced by a high dose of MNNG given to previously adapted xthA- umuDC+ bacteria. To illustrate the magnitude of the effect, the mutations induced during adaptation have not been subtracted from the total but are given separately. Relative to adaptation, the challenge dose of MNNG enhanced the occurrence of all mutational events 2- to 4-fold except transitions at the second base.

The Mutations Induced in xthA- Bacteria by a Challenge Dose of MNNG Are Dependent on alkA+ Activity. One explanation for the specificity of the xthA- dependent mutations induced by MNNG is that it reflects the distribution of AP sites left by the removal of 3-methylguanine and O6-methylcytosine by the activity of TAGII, the product of Table 2. Sequenced hisG46 revertants

<table>
<thead>
<tr>
<th>Strain</th>
<th>His* revertants per 10^6 cells</th>
<th><em>Nonadapted cells</em></th>
<th><em>Adapted cells</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>xthA+ umuC+</td>
<td>78</td>
<td>820</td>
<td>602</td>
</tr>
<tr>
<td>xthA+ umuC+</td>
<td>72</td>
<td>989</td>
<td>714</td>
</tr>
<tr>
<td>xthA+ umuC-</td>
<td>88</td>
<td>682</td>
<td>704</td>
</tr>
<tr>
<td>xthA- umuC-</td>
<td>65</td>
<td>691</td>
<td>585</td>
</tr>
<tr>
<td>Nonadapted cells</td>
<td>74</td>
<td>15</td>
<td>&lt;1</td>
</tr>
<tr>
<td>xthA+ umuC+</td>
<td>43</td>
<td>164</td>
<td>125</td>
</tr>
<tr>
<td>xthA+ umuC-</td>
<td>61</td>
<td>&lt;1</td>
<td>2</td>
</tr>
<tr>
<td>xthA- umuC-</td>
<td>60</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
</tbody>
</table>

Adapted and nonadapted cells were exposed to MNNG at 10 \( \mu \text{g/ml} \) for 5 min.

*Average of the experiments with both hisG46 and hisG428 in each genetic background.

†For adapted cells, the number of His* revertants has been corrected for the mutants induced during adaptation.

‡Average of two experiments.
the alkA+ gene. Insertion of adenines opposite these missing bases would yield both G-C to T-A transversions and G-C to A-T transitions. We tested this hypothesis with isogenic xthA+, alkA+, and xthA- alkA- strains carrying the hisG46 episome. It was not possible to make these constructions in the genetic background used in the previous experiments. In the strains used, derived from AB1157 (29) and MS23 (AB1157 alkA; ref. 30), induced and spontaneous mutation rates were more variable and the relative mutagenic effect of xthA- was less. In addition, the alkA- defect increases both the toxicity and mutagenicity of MNNG, although the mutagenic effect at the hisG46 locus is slight (9).

As shown in Table 4, the frequencies of hisG46 revertants induced during adaptation to MNNG were approximately the same for the xthA- alkA- double mutant as for the xthA- alkA- strain. However, the enhanced mutagenicity of a challenge dose of MNNG given to previously adapted xthA- bacteria was reduced by a factor of 5 by the alkA- defect. Since the mutations induced under these conditions are mainly transversions (Fig. 2), these results suggest that the alkA+ gene. Insertion of adenines opposite these missing bases would yield both G-C to T-A transversions and G-C to A-T transitions. We tested this hypothesis with isogenic xthA+, alkA+, and xthA- alkA- strains carrying the hisG46 episome. It was not possible to make these constructions in the genetic background used in the previous experiments. In the strains used, derived from AB1157 (29) and MS23 (AB1157 alkA; ref. 30), induced and spontaneous mutation rates were more variable and the relative mutagenic effect of xthA- was less. In addition, the alkA- defect increases both the toxicity and mutagenicity of MNNG, although the mutagenic effect at the hisG46 locus is slight (9).

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**Fig. 2.** Base changes induced at the hisG46 codon of xthA- umuDC+ bacteria during adaptation and by a challenge dose of MNNG. Frequencies were computed from the data in Tables 2 and 3 and have been corrected for spontaneously occurring mutations of each class. A, Mutations induced during adaptation; B, mutations induced by challenge of adapted cells.

**Table 4.** Effect of the alkA- defect on the induction of hisG46 revertants by MNNG in xthA- and xthA+ bacteria

<table>
<thead>
<tr>
<th>Strain</th>
<th>Number of determinations</th>
<th>% viable cells</th>
<th>His+ revertants per 10^6 cells*</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>xthA- alkA+</td>
<td></td>
<td></td>
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<tr>
<td>xthA- alkA+</td>
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<tr>
<td>xthA- alkA-</td>
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<tr>
<td>xthA- alkA-</td>
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</tbody>
</table>

Values are averages ± SD.

*For challenged cells, the values have been corrected for the mutations induced during adaptation.

**DISCUSSION**

The results presented here provide strong evidence that AP sites produced in vivo are mutagenic. Furthermore, these lesions are intermediates in the induction of mutations by MNNG and, by extension, other mutagens that produce AP sites. These conclusions are from the following observations: (i) xthA- bacteria, deficient in the AP endonuclease, exonuclease III, were more mutable by MNNG under conditions that maximize the production of AP sites and minimize the occurrence of mutations due to other lesions; (ii) the majority of the xthA- dependent mutations induced by MNNG were also dependent on SOS mutagenic processing, as expected for mutations due to AP sites; and (iii) the xthA- mutagenic effect was decreased by a deficiency in TAGII, thus demonstrating that production of AP sites by glycosylase activity is mutagenic in the absence of exonuclease III. The simplest explanation for these results is that the AP endonuclease activity of exonuclease III participates with TAGII in an error-free DNA repair pathway active on alkylated-damaged bases. The activities of other glycosylases and the spontaneous loss of alkylated bases also may produce substrates for the AP endonuclease activity of exonuclease III.

The occurrence of SOS-dependent mutations requires two conditions—the presence of a lesion in the DNA and the induction of the SOS response (36). Hence, it is possible that lesions persisting in the DNA as the result of the loss of a DNA repair activity, such as exonuclease III, may serve as inducers for SOS mutagenic processing that may act elsewhere in the DNA (e.g., see ref. 9). However, there are several reasons why this explanation does not pertain to our results. As measured by the induction of a umuD-lacZ gene fusion, the xthA- defect enhanced the induction of the SOS response by MNNG only 2-fold (data not shown), an effect unlikely to produce the differences we observed. In contrast, induction of the SOS response by MNNG is enhanced in alkA- bacteria 8-fold, but the mutagenic effect of the alkA- defect differs from that of xthA- (ref. 9; Table 4). In addition, induction of the SOS response by thermal shift of isogenic recA441 (tif-1) strains did not increase the mutagenicity of MNNG under our experimental conditions (data not shown). Finally, G-C to A-T transitions at the second base of the hisG46 codon, although SOS dependent, were not induced by a challenge dose of MNNG (Figs. 1 and 2). Thus, simple induction of the SOS response cannot account for the mutations we observed.

The mutagenic phenotype of xthA mutants has been difficult to demonstrate (11), presumably because of the
The results presented here provide direct evidence for the mutagenic role of AP sites produced in vivo by spontaneous loss of damaged bases or as intermediates in the repair of other DNA lesions. The high rate of spontaneous depurination (10³ per mammalian cell per day; ref. 38) plus the existence of multiple pathways for the repair of AP sites, argues for the potential importance of these lesions. When the AP site repair pathways are saturated by exposure to a DNA damaging agent, even a relatively low rate of base loss could have severe mutagenic and carcinogenic consequences.

Note. It has recently been demonstrated that a strain defective in both xthA and nfo, the gene that encodes the AP endonuclease, endonuclease IV, is more mutable by methyl methanesulfonate than either single mutant strain (39).

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