JOURNAL OF BACTERIOLOGY, July 1985, p. 213–220
0021-9193/85/070213-08$02.00/0

Vol. 163, No. 1

Induction of Transversion Mutations in *Escherichia coli* by *N*-Methyl-*N'*-Nitro-*N*-Nitrosoguanidine Is SOS Dependent

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Received 17 December 1984/Accepted 8 April 1985

***Escherichia coli alkA* mutants, which are deficient for an inducible DNA glycosylase, 3-methyladenine-DNA glycosylase II, are sensitive to mutagenesis by low doses of the alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). As many as 90% of the *alkA*-dependent mutations induced by MNNG are also *umuC*$^+$ dependent and thus are due to DNA lesions that are substrates for the mutagenic functions of the SOS response. A great number of these mutations are base substitutions at A · T sites, particularly A · T transversions. We discuss which DNA lesions may be responsible for these mutations. Our results show that the induction of 3-methyladenine-DNA glycosylase II, which occurs as part of the adaptive response to alkylating agents such as MNNG, significantly reduces the mutagenicity as well as the lethality of alkylation damage.**

The biological consequences of exposing bacteria to DNA-alkylating agents depend on the chemical nature of the DNA damage induced. For example, alkylation of the O^6 position of guanine creates a modified base with the coding properties of adenine (45). Thus, this lesion is highly and directly mutagenic, giving rise to G · C-to-A · T transitions (41). In contrast, alkylation of the N^3 position of adenine creates a modified base that blocks the replication fork (8; B. S. Strauss, K. Larson, D. Sagher, S. Rabkin, R. Shenkar, and J. Sahm, *in* E. Huberman, ed., *The Role of Chemicals and Radiation in the Etiology of Cancer*, in press); consequently, this lesion is lethal (24).

Exposing *Escherichia coli* to sublethal concentrations of alkylating agents induces two DNA repair activities that operate on alkylated double-stranded DNA. These activities are both regulated by the *ada* locus, and their induction is known as the adaptive response (20, 38, 42). One of these activities is O^6-methylguanine-DNA methyltransferase (MT), a product of *ada* itself (47). MT removes alkyl groups from the O^6 position of guanine and the O^4 position of thymine (major-groove adducts), restoring the bases to their original states (23, 32). Thus, cells in which MT has been induced are resistant to the mutagenic effects of agents such as *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) that predominately alkylate O^6G and O^4T. The other inducible repair activity known to be regulated by *ada* is 3-methyladenine-DNA glycosylase II (TAGII), the product of the *alkA* gene (14, 22, 52). TAGII removes purines alkylated at the N^3 and N^7 positions and pyrimidines alkylated at the O^2 position (minor-groove adducts) (22, 32). The resulting apurinic and apyrimidinic (AP) sites are presumably repaired by an error-free process involving AP endonucleases, DNA polymerase I, and DNA ligase (21, 54). Thus, cells in which TAGII has been induced are resistant to the lethal effects of DNA alkylation.

The mutagenicity of alkylating agents cannot be due entirely to directly miscoding lesions such as O^6-alkylguanine. Some alkylating agents, such as methyl methanesulfonate (MMS), are only weakly mutagenic unless the "error-prone" functions of the SOS response are active (26). The SOS response, which is induced by treatments that interfere with DNA replication, is regulated by the *recA* and *lexA* genes and includes both accurate and mutagenic DNA repair activities (for reviews, see references 30, 49, and 51). Mutagenic processing of DNA lesions is associated with the *umuDC* operon, defects in which eliminate mutagenesis by many agents without affecting the other functions of the SOS response. Presumably, nonmiscoding lesions generated by alkylating agents can under some circumstances yield mutations via a *umuDC*-dependent pathway.

In the study reported here we investigated the mutagenic potential of the alkyl lesions that are substrates for TAGII. We show that when MT activity was fully induced, thus eliminating mutations due to O^6-methylguanine (m^6G) and O^4-methylthymine, *alkA* bacteria were significantly more mutable by MNNG than *alkA*$^+$ bacteria. Most of the resulting mutations were *umuC*$^+$ dependent, i.e., were the result of SOS mutagenic processing; a large component of these *alkA-umuC*$^+$-dependent mutations were transversions at A · T base pairs.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains used in this work are listed in Table 1. Strain MS23 is *his*$^+$ *alkA* and cogenic with AB1157 except for the *his-alkA* region (53). *hisG hisD*$^+$ derivatives were constructed by transducing AB1157 (obtained from G. Walker) and MS23 (obtained from B. Sedgwick) to tetracycline resistance (Tet^r) with a P1 *vir* lysate of strain NK5526 (obtained from B. Bachmann), which contains *hisG213*::Tn*10*. This transposon is polar on *hisD*; spontaneously arising *hisD*$^+$ revertants were selected by growth on histidinol (Hol$^+$). Hol$^+$ candidates were then screened for His$^-$, Tet^s, and nonrevertability of the His$^-$ phenotype by MNNG, MMS, 4-nitroquinoline-1-oxide, and spontaneous mutations. These His$^-$ strains are presumed to have a deletion or insertion in *hisG* because of imprecise excision of the Tn*10* element, but they have not been further characterized. *umuC36* derivatives were made by P1 *vir* transduction of *zcf-289*::Tn*5* linked to the *umuC36* mutation (obtained from G. Walker) and screening kanamycin-resistant (Kan^r) isolates for lack of mutability by MMS and 4-nitroquinoline-1-oxide. Δ(*lac pro*) derivatives were made by

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TABLE 1. Bacterial strains

Strain[a]	Description
AB1157[b]	.F⁻ *argE3 his-4 leuB6 proA2 thr-1 ara-14 galK2 lacY1 mtl-1 xyl-5 thi-1 rpsL31 supE44 tsx-33*
MS23[c]	...As AB1157 but His⁺ *alkA*
PF82	....As AB1157 but *umuC36 zcf-289*::Tn*5*
PF99	....As MS23 but *umuC36 zcf-239*::Tn*5*
PF352	...As AB1157 but *hisG hisD*⁺
PF353	...As MS23 but *hisG hisD*⁺
PF372	...As PF352 but *umuC36 zcf-289*::Tn*5*
PF373	...As PF353 but *umuC36 zcf-289*::Tn*5*
PF400	...As PF352 but *trpA58*
PF401	...As PF353 but *trpA58*
PF404	...As PF372 but *trpA58*
PF405	...As PF373 but *trpA58*
PF418	...As PF352 but *trpA3*
PF419	...As PF353 but *trpA3*
PF420	...As PF372 but *trpA3*
PF421	...As PF373 but *trpA3*
PF423	...As PF352 but Δ(*lac pro*) *leu*⁺(pAQ1)
PF424	...As PF353 but Δ(*lac pro*) *leu*⁺(pAQ1)
PF425	...As PF372 but Δ(*lac pro*) *leu*⁺(pAQ1)
PF426	...As PF373 but Δ(*lac pro*) *leu*⁺(pAQ1)
PF427	...As PF353 but Δ(*lac pro*) *leu*⁺ *thr*⁺ *supE*⁺(pAQ1)
PF428	...As PF373 but Δ(*lac pro*) *leu*⁺ *thr*⁺ *supE*⁺(pAQ1)

[a] Reference for strains is this paper, unless otherwise indicated.
[b] Reference 2.
[c] Reference 53.

crossing with CA7092.1 [HfrH *ara* Val^r Δ(*lac pro*)] obtained from J. Beckwith, interrupting mating after 16 min, selecting for Leu$^+$, and then screening for Lac$^-$ on 5-bromo-4-chloro-3-indolyl-β-D-galactoside–glucoseplatescontainingisopropyl-β-D-thiogalactopyranoside (33). *supE*$^+$ (Su2$^-$) derivatives were generated in the same cross by interrupting mating after 22 min and screening for the inability to suppress an amber mutation in *lacI* that is suppressed only by Su2 (amber 5; see references 10, 11, and 35). Male derivatives for M13 infections were made by crossing the strains with either F′ *lac pro* (obtained from J. Miller) or F::Tn*3* (obtained from W. Barnes). *trpA* derivatives were constructed by P1 *vir* transduction of *trpB*::Tn*10* (NK5151 obtained from E. Siegel) and screening Tet^r isolates for Trp$^-$ Ind$^-$ (inability to grow on indole). These strains were then transduced to Ind$^+$ with P1 *vir* lysates of ES579 (obtained from E. Siegel), which carries *trpA58*, or KD1089 (obtained from B. Duncan), which carries *trpA3*. All genetic manipulations were as previously described (33).

Plasmids. pAQ1 is a pBR322 derivative carrying the *hisOGD* region of *Salmonella typhimurium* and the *hisG428* ochre mutation (28). pTS46 is identical to pAQ1 but carries the *hisG46* missense mutation instead of *hisG428* (T. Standing, personal communication). pSE140 is a pSC101 derivative carrying a *umuD-lacZ* gene fusion (13). Plasmid DNA was prepared and bacterial transformations were performed as previously described (31).

Media. VB minimal medium (48) contained E salts, 0.2% glucose, 50 μg of thiamine per ml, and 50 μg of each supplemented amino acid per ml. To select for mutations, cells were added to 2 ml of top agar containing the amino acid (50 μM) for which prototrophy was being selected (arginine, histidine, or tryptophan) plus 1 mg of the other required amino acids per ml and poured on minimal VB plates. The *hisG428* mutation is partially suppressed when present on the multicopy plasmid pAQ1; thus, to select for pAQ1 revertants, no histidine (for AB1157 derivatives) or 12.5 μM histidine (for MS23 derivatives) was added to the top agar. These concentrations resulted in approximately equal numbers of spontaneous mutations in the strains. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside–glucose plates and LB medium were as previously described (33). Antibiotic concentrations in rich media were 200 μg of streptomycin, 20 μg of tetracycline, and 20 μg of ampicillin per ml; in minimal media, tetracycline and ampicillin were added at 10 μg/ml.

M13 crosses. M13hol67.18 (obtained from W. Barnes) carries the *S. typhimurium hisOGD* region with a deletion of part of *hisO* and all of *hisG* but retains homology to the *S. typhimurium his* operon on either side of the deletion (5). This phage does not recombine with the *E. coli his* operon (unpublished data; J. Fink and J. Roth, unpublished data cited in reference 18). M13 crosses were done as previously described (4) except that the recipient was PF352/F::Tn*3* and selection was for His$^+$. The results of the M13 crosses were verified in 12 cases by direct transformation of the recipient with purified plasmid DNA.

Mutagenesis experiments. The adaptive response was induced by diluting overnight cultures in supplemented VB medium 1:5 into fresh medium with 0.5 to 1.0 μg of MNNG per ml and incubating them with shaking for 4 h at 32°C. Controls contained no MNNG. Cultures were then either centrifuged, suspended in E salts, and plated for mutants or divided in half with a challenge dose of MNNG added to half for 5 min at room temperature. The cultures were then centrifuged, washed, and plated. The titers of viable cells were determined on supplemented VB plates. To calculate the number of induced mutations per viable cells, we subtracted the number of spontaneous mutations (0 dose) from the total number of mutants per plate and divided the difference by the number of viable cells plated. MNNG stocks were kept frozen at 1 mg/ml in citrate buffer (pH 5.5).

Suppressor analysis. In the initial screen, His$^+$ revertants of Δ(*lac pro*) strains were gridded on histidine-minus plates, replicated onto LB plates for 18 h, and then replicated onto histidine- and proline-minus and streptomycin-plus plates spread with donor GM1 strains [Str^s Δ(*lac pro*)/F′ *lac pro*] carrying well-characterized ochre mutations in the *lacI* gene (obtained from J. Miller; 10, 11, 35). After 24 to 36 h, the mating plates were replicated onto 5-bromo-4-chloro-3-indolyl-β-D-galactoside–glucose, histidine- and proline-minus, and streptomycin-plus plates, and the suppression patterns were compared with those of strains carrying the SuB (glutamine), SuC (tyrosine), and Su5 (lysine) ochre suppressors (obtained from J. Miller; 10). Five to 10 examples of His$^+$ revertants of each suppression pattern in each strain were then single-colony purified from the original histidine-minus grids, and the suppression analysis was repeated with additional *lacI* nonsense mutations to confirm the suppressor assignments.

A suppressor was considered to be SuC if it suppressed *lacI* ochre 8 (*tyr-47*), SuB if it suppressed *lacI* ochre 10 (*gln-55*) but not ochre 8, and Su5 if it suppressed *lacI* ochre 22 (*leu-189*) but not ochre 8 or ochre 23 (*tyr-204*) (10, 11, 35, 50). In addition to phenotypes that were clearly SuB, SuC, or Su5, other suppressors were identified that gave somewhat different patterns. For example, SuB-like suppressors were found that did not suppress ochre 22, which is well suppressed by SuB. SuC-like suppressors were found that suppressed ochre 10, which is not normally suppressed by

SuC. A large class of Su5-like suppressors were found that had the correct pattern for Su5 but were relatively poor suppressors. Based on their abilities to suppress the diagnostic ochres 8, 10, and 22, these suppressors were assigned to be glutamine, tyrosine, and lysine tRNAs. In a few cases, assignments were further confirmed by testing for a noninducible phenotype with ochre 11 (*gln-78*) and reverse induction with ochre 30 (*tyr*-273) (35, 50).

Identification of ochre suppressors as lysine tRNAs was based in part on their ability to suppress an ochre mutation at a leucine residue in *lacI* (ochre 22). Thus, it seemed possible that some of the Su5-like suppressors of *hisG428* might actually be leucine tRNAs, although a leucine ochre suppressor has not been identified. This possibility was tested by comparing the Su5-like suppressors with the leucine amber suppressor, Su6. Because the Su2 amber suppressor carried in AB1157 derivatives would interfere with this analysis, we repeated the adaptation experiment with *supE*$^+$ (Su2$^-$) derivatives of the *alkA* strains (PF427 and PF428, Table 1). A total of 15 His$^+$ revertants that gave the Su5-like pattern in an initial screen were tested with several characterized *lacI* amber mutations. None of the His$^+$ revertants had a suppression pattern similar to that of the leucine amber suppressor, Su6 (data not shown).

Trp$^+$ analysis. Full and partial revertants were identified by indole glycerol phosphate accumulation and 5-methyltryptophan sensitivity (1). Fourteen *trpA58* partial revertants were also examined for linkage of the Trp$^+$ phenotype to *trpB* by transducing a *trpB*::Tn*10* strain to Ind$^+$ with a P1 *vir* lysate of the revertant; Ind$^+$ candidates were then checked for Trp$^+$ and Tet^s (16).

β-Galactosidase induction. Strains PF423 (*alkA*$^+$) and PF428 (*alkA*) carrying pSE140 were exposed to 0, 0.5, and 1.0 μg of MNNG per ml exactly as described for the mutagenesis experiments (see above). Samples were periodically withdrawn, and β-galactosidase was measured as previously described (33). For each time point, the level of constitutive β-galactosidase activity (0 MNNG) of each strain was subtracted from the MNNG-induced levels.

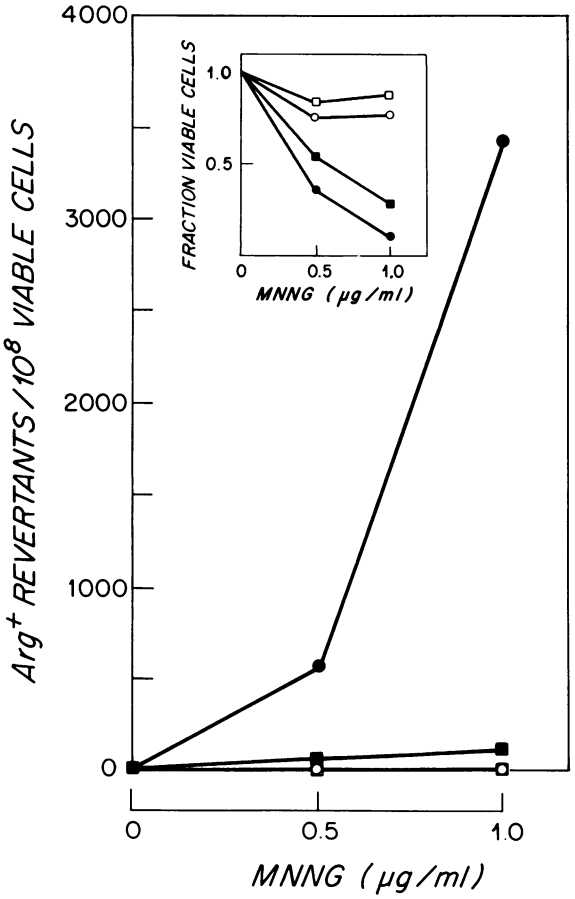

FIG. 1. MNNG-induced reversion of the *argE3* ochre mutation in *alkA*$^+$ and *alkA* bacteria under adapting conditions. Inset, Fraction of viable cells. Symbols: ○, AB1157 (*alkA*$^+$ *umuC*$^+$); □, PF82 (*alkA*$^+$ *umuC36*); ●, MS23 (*alkA umuC*$^+$); ■, PF99 (*alkA umuC36*).

TABLE 2. Adaptation of *alkA*$^+$ (AB1157) and *alkA* (MS23) bacteria to a challenge dose (10 μg/ml) of MNNG

Adapting dose of MNNG (μg/ml)	No. of Arg$^+$ revertants/10^8 viable cells			
	Mutations induced by adaptation		Mutations induced by challenge[a]	
	alkA$^+$	*alkA*	*alkA*$^+$	*alkA*
0			1,495	2,707
0.5	38	743	0	83
1.0	80	1,601	1	14

[a] Mutations induced by adaptation were subtracted.

RESULTS

Induction of *umuC*$^+$-dependent mutations by MNNG in *alkA E. coli*. In wild-type bacteria the majority of MNNG-induced mutations are not *umuC*$^+$ dependent, presumably because MNNG produces a predominance of directly miscoding lesions (40). To evaluate the mutagenic potential of the alkyl lesions that are substrates for TAGII, we sought to eliminate both TAGII activity and the mutagenic contributions of m^{6}G and O^4-methylthymine. We exposed *alkA* bacteria to conditions that fully induce MT and asked whether the resulting mutations were dependent on *umuC*$^+$ activity. The results presented in Fig. 1 show that, whereas exposure to 0.5 to 1.0 μg of MNNG per ml for 4 h (adaptation) was not mutagenic to *alkA*$^+$ bacteria, such exposure was highly mutagenic to *alkA* bacteria. Furthermore, 90 to 97% of the mutations induced in the *alkA* strain under adapting conditions were eliminated in a *umuC* derivative. Yet the *umuC36* mutation had no effect on the sensitivity of either *alkA*$^+$ or *alkA* bacteria to the toxic effects of MNNG (Fig. 1).

The large difference in mutability between the *alkA*$^+$ and *alkA* strains was not due to a difference in their ability to induce MT. The data in Table 2 show that after adaptation, *alk* bacteria were 30- to 100-fold more resistant than were unadapted bacteria to the mutagenic effects of a subsequent challenge dose (10 μg/ml) of MNNG. Thus, the *umuC*$^+$-dependent mutations induced in *alkA* bacteria most likely were due solely to the lack of TAGII activity, suggesting that one or more of the substrates for TAGII may be mutagenic via SOS processing. An alternative explanation for these results is that one or more of the substrates for TAGII, by persisting in the DNA, may induce the SOS response more fully in *alkA* than in *alkA*$^+$ bacteria. Mutations could then result from the mutagenic processing of other alkylation-induced lesions, not just the substrates for TAGII (E. Seeberg, personal communication). A third possibility is that some combination of these explanations obtains. To evalu-

TABLE 3. MNNG-induced reversion of the *hisG46* and *hisG428* mutations in *alkA*$^+$ and *alkA* bacteria under adapting conditions[a]

Strain	No. of His$^+$ revertants/10^8 viable cells		
	pTS46 *hisG46*	pAQ1 *hisG428*[b]	pAQ1[b] plasmid revertants[c]
PF352 *alkA*$^+$ *umuC*$^+$	55	18	12
PF372 *alkA*$^+$ *umuC36*	72	52	21
PF353 *alkA* *umuC*$^+$	591	2,628	268
PF373 *alkA* *umuC36*	230	192	72

[a] MNNG dose, 0.5 μg/ml. Approximately 50 pAQ1 revertants of each strain were tested for transfer of the His$^+$ phenotype by M13 crosses or by transformation with plasmid DNA (see the text).
[b] Average of two experiments.
[c] His$^+$ phenotype was transferable by M13 crosses or transformation.

ate these hypotheses, we investigated the nature of the *alkA-umuC*$^+$-dependent mutations generated by MNNG.

Identification of the *alkA-umuC*$^+$-dependent mutations: reversion of two *hisG* mutations. To determine the specificity of the *alkA-umuC*$^+$-dependent mutations, we compared the MNNG-induced reversion of two characterized *hisG* mutations from *S. typhimurium* (*hisG46*, a CCC [proline] missense codon, and *hisG428*, a TAA [ochre] nonsense codon), both of which can revert via several different base substitutions (29, 30; W. Barnes, E. Tuley, and E. Eisenstadt, Program Abstr. 13th Annu. Meet. Environ. Mutagen Soc., abstr. no. Aa-1, p. 59, 1982). *E. coli* strains with nonrevertable *hisG* mutations on the chromosome and carrying the *hisG46* or *hisG428* mutations on multicopy plasmids were exposed to adapting doses of MNNG. Reversion of the *hisG46* mutation was increased about 10-fold in the *alkA* strain, and about 60% of these mutations were *umuC*$^+$ dependent (Table 3). In contrast, reversion of the *hisG428* mutation was increased over 100-fold in the *alkA* strain, and 90% of the *alkA*-dependent mutations were *umuC*$^+$ dependent. However, most of the *alkA-umuC*$^+$-dependent revertants of *hisG428* were extragenic; only 10% of these revertants had His$^+$ phenotypes that could be transferred either by M13 crosses or by transformation with plasmid DNA (see above). Thus, a major component of the *alkA-umuC*$^+$-dependent revertants of *hisG428* appeared likely to be tRNA ochre suppressors. Because the base substitutions giving rise to tRNA suppressors are defined, the spectrum of mutations generated by MNNG could be determined by identifying the induced ochre suppressors.

Analysis of the MNNG-induced extragenic suppressors of *hisG428*. To identify the ochre suppressors induced by MNNG, we introduced a chromosomal deletion covering the *lac-pro* region into the pAQ1-carrying strains. After MNNG mutagenesis, F′ *lac pro* episomes with well-characterized nonsense mutations in the *lacI* gene were mated into the His$^+$ revertants (see above). The ability of these revertants to suppress the *lacI* nonsense mutations was compared with the ochre suppressors SuB (glutamine), SuC (tyrosine), and Su5 (lysine), which are known to suppress *hisG428* in *S. typhimurium* (D. Levin, personal communication). Approximately 40% of the extragenic His$^+$ revertants were identifiable by this method (Table 4). (The remaining extragenic revertants, many of which appeared as His$^+$ colonies only after 4 days of incubation, may have consisted of a class of inefficient ochre suppressors, previously identified in *S. typhimurium*, that suppress *hisG428* on a multicopy plasmid but not on the chromosome [D. Levin, personal communication].)

The results of the suppression analysis are presented in Table 4 as the MNNG-induced frequencies of each class of suppressor (i.e., spontaneously occurring suppressors of each class have been subtracted). The largest class of suppressors induced in the *alkA umuC*$^+$ strain was lysine tRNAs, which represented 94% of the total identifiable suppressor mutations and at least 30% of all the His$^+$ revertants. Lysine ochre suppressors are created only by A · T-to-T · A transversions (36); thus, this mutation, which occurred over 100-fold above background, appears to be a major *alkA-umuC*$^+$-dependent base substitution induced by MNNG. The frequency of glutamine tRNA ochre suppressors, which arise only by G · C-to-A · T transitions (36), was elevated approximately 10-fold in the *alkA* strain, but less than 50% of these mutations were *umuC*$^+$ dependent. Tyrosine tRNA ochre suppressors, which are created only by G · C-to-T · A transversions (19, 36), were slightly elevated in both *alkA* strains, but the frequencies for tyrosine given in Table 4 are just at the level of detection.

Confirmation of the specificity of the *alkA-umuC*$^+$-dependent mutations by using two *trpA* alleles. Because suppression analysis identified base substitutions at A · T base pairs as major MNNG-induced mutations in *alkA umuC*$^+$ bacteria, we tested the ability of MNNG to revert two *trpA* alleles (*trpA3*, which reverts by an A · T-to-T · A transversion, and *trpA58*, which reverts by both an A · T-to-G · C transition and an A · T-to-C · G transversion at the same base pair [12, 55]). We compared the effects of the *alkA* and *umuC* genotypes on the induction of these mutations under adapting conditions, by a challenge dose of MNNG and by a challenge dose given to adapted cells (Table 5).

Both *trpA3* and *trpA58* are also reverted by the creation of missense suppressors (55). These extragenic revertants are easily identified by their slow growth and high levels of indole glycerol phosphate accumulation (17; see above). They were induced by challenge doses of MNNG in the *alkA umuC*$^+$ *trpA3* strain and in both *alkA trpA58* strains. Because the mutations giving rise to missense suppressors are uncharacterized, they were not included in this analysis.

Reversion of *trpA3* by A · T-to-T · A transversions was elevated in the *alkA* strain over 100-fold by the adapting

TABLE 4. Frequencies of tRNA ochre suppressors induced by MNNG in *alkA*$^+$ and *alkA* bacteria under adapting conditions[a]

Class of revertant	No. of His$^+$ revertants/10^8 viable cells				Inferred base change
	PF423 *alkA*$^+$ *umuC*$^+$	PF425 *alkA*$^+$ *umuC36*	PF424 *alkA* *umuC*$^+$	PF426 *alkA* *umuC36*	
Total suppressors	20	18	4,448	224	
Su5	<1	3	2,550	<56	
Other Lys	<1	15	1,622	56	
Total Lys	<1	18	4,172	56	A · T to T · A
SuB	1	<8	192	56	
Other Gln	15	<8	20	56	
Total Gln	16	<8	212	112	G · C to A · T
SuC	<1	<3	<64	<56	
Other Tyr	3	<3	64	56	
Total Tyr	3	<3	64	56	G · C to T · A

[a] MNNG dose, 0.5 μg/ml. Approximately 30 His$^+$ revertants of each strain were tested for suppression except for PF424, of which 100 revertants were tested (see the text).

exposure to MNNG, and 91% of these mutations were *umuC*$^+$ dependent (Table 5). A similar level of *alkA-umuC*$^+$-dependent *trpA3* revertants was induced by a high dose of MNNG, and prior adaptation increased the frequency of this event approximately twofold. Thus, under all conditions of MNNG exposure the induction of A · T-to-T · A transversions was strictly *alkA-umuC*$^+$ dependent.

Because both a transition and a transversion at the same base pair revert *trpA58*, it was possible to determine at this site whether the *alkA* and *umuC* genotypes differentially influenced the induction of transitions and transversions by MNNG. The A · T-to-C · G transversion at the *trpA58* locus was induced in *alkA umuC*$^+$ bacteria under all conditions of MNNG exposure but was not induced to an appreciable level in any other strain (Table 5). Prior adaptation had little effect on the frequency of this event. Under adapting conditions, the A · T-to-G · C transition at this same base pair was induced about a third as well as the transversion. In contrast to both the A · T-to-C · G transversion at this site and the A · T-to-T · A transversion at the *trpA3* locus, the A · T-to-G · C transition was induced by a high dose of MNNG even in *alkA*$^+$ bacteria; approximately 40% of these transitions were *umuC*$^+$ independent. Also in contrast to the transversions, the frequency of A · T-to-G · C transitions was reduced by prior adaptation. Thus, whereas the induction of A · T transversions occurred only via an *alkA-umuC*$^+$-dependent pathway, there appeared to be several pathways by which MNNG could induce A · T-to-G · C transitions.

Enhanced induction of the SOS response in *alkA* bacteria by MNNG. Recent evidence has suggested that alkylation damage may induce the SOS response more readily and to a higher level in *alkA* than in *alkA*$^+$ bacteria because of the persistence in the DNA of one or more of the substrates for TAGII (8; E. Seeberg, personal communication). To test this hypothesis, we compared the induction of β-galactosidase by adapting doses of MNNG in *alkA*$^+$ and *alkA* strains carrying pSE140, a low-copy plasmid with a *umuD-lacZ* gene fusion (13). Induction of β-galactosidase was 5- to 10-fold higher in the *alkA* than in the *alkA*$^+$ bacteria (Fig. 2). We obtained the same results with *alkA* derivatives of strains with a chromosomal *umuC*::Mu d(Ap^r *lac*) fusion (3) and a chromosomal *dinD*::Mu d(Ap^r *lac*) fusion (25) (data not shown). Because each of these genes is induced as part of the SOS response (3, 13, 25), our results confirmed that *alkA* bacteria are more sensitive to SOS induction by MNNG exposure. Thus, one or more of the substrates for TAGII appears to be an SOS-inducing lesion.

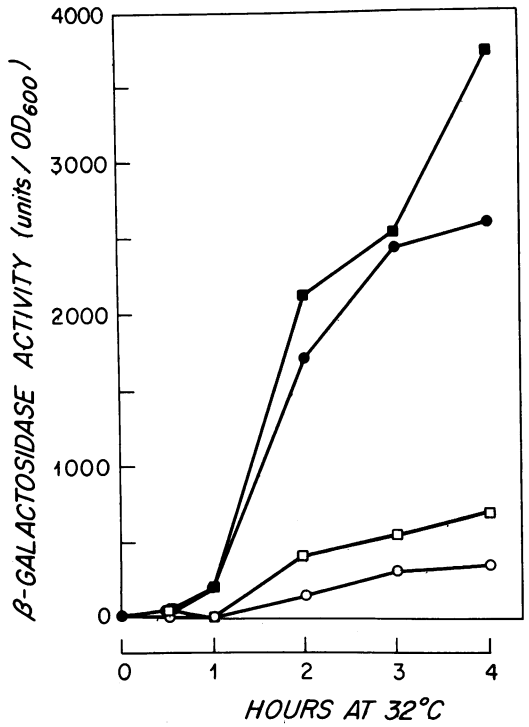

FIG. 2. Kinetics of MNNG induction of β-galactosidase activity in *alkA*$^+$ and *alkA* bacteria with a *umuD-lacZ* fusion on a low-copy plasmid. Open symbols, PF423(pSE140) (*alkA*$^+$); closed symbols, PF424(pSE140) (*alkA*); ○ and ●, 0.5 μg of MNNG per ml; □ and ■, 1.0 μg of MNNG per ml.

TABLE 5. MNNG-induced reversion of two *trpA* alleles in *alkA*$^+$ and *alkA* bacteria[a]

Strain	No. of Trp$^+$ revertants/10^8 viable cells		
	Mutations induced by adaptation	Mutations induced by challenge	Mutations induced by challenge of adapted cells[b]
trpA3: A · T to T · A			
PF418 *alkA*$^+$ *umuC*$^+$	0.6	3.7	0.9
PF420 *alkA*$^+$ *umuC36*	<0.9	0.5	<0.9
PF419 *alkA umuC*$^+$	106	128	226
PF421 *alkA umuC36*	9.6	0.5	<1.0
trpA58: A · T to C · G			
PF400 *alkA*$^+$ *umuC*$^+$	<0.1	<4	<1
PF404 *alkA*$^+$ *umuC36*	<0.1	2	0.6
PF401 *alkA umuC*$^+$	90	21	31
PF405 *alkA umuC36*	6	1	1
trpA58: A · T to G · C			
PF400 *alkA*$^+$ *umuC*$^+$	0.4	84	29
PF404 *alkA*$^+$ *umuC36*	0.4	31	2
PF401 *alkA umuC*$^+$	33	62	35
PF405 *alkA umuC36*	5	23	17

[a] Adapting dose, 1 μg of MNNG per ml; challenge dose, 40 μg of MNNG per ml. The phenotypes of approximately 20 revertants of each strain and treatment were analyzed (see the text).

[b] Mutations induced by adaptation were subtracted.

DISCUSSION

Although mutagenesis by MNNG is largely *umuC*$^+$ independent (40), our results show that a significant class of *umuC*$^+$-dependent mutations can also be induced by MNNG but is normally masked by the dominant *umuC*$^+$-independent mutations due to m^{6}G. In the experiments reported here, the adapting treatment increased the relative frequency of the *umuC*$^+$-dependent mutations by (i) inducing MT and thus minimizing the mutations due to m^{6}G and O^4-methylthymine and (ii) inducing TAGII in the *alkA*$^+$ strains and thus maximizing any effect on mutagenesis due to the lack of this glycosylase in the *alkA* strains. Under these conditions, *alkA* bacteria are as much as 100-fold more mutable by MNNG than are *alkA*$^+$ bacteria, and over 90% of these additional mutations are *umuC*$^+$ dependent.

The *alkA-umuC*$^+$-dependent mutations induced by MNNG appeared to be dominated by base substitutions at A · T base pairs. These mutations consisted of all three possible base changes, apparently in the order A · T to T · A > A · T to C · G > A · T to G · C. However, the distribution

of mutations in DNA can be extremely nonrandom (6, 15, 34), and caution is necessary when comparing mutation frequencies at a limited number of sites.

That A · T-to-T · A transversions were strongly induced by MNNG in *alkA umuC*$^+$ bacteria is suggested by the frequencies at which MNNG induced lysine ochre suppressors of *hisG428* (Table 4) and true revertants of *trpA3* (Table 5). Prior adaptation did not decrease, and may even have enhanced, the induction of this mutation. Thus, in the absence of a functional *alkA* gene, the premutagenic lesions giving rise to A · T-to-T · A transversions appear not to be repaired by any other activity of the adaptive response and can produce mutations only via SOS mutagenic processing. The direct comparison of the frequencies of A · T-to-C · G transversions and A · T-to-G · C transitions at the *trpA58* locus (Table 5) is complicated by the fact that there appear to be at least three pathways by which MNNG can induce A · T transitions—one independent of both *alkA* and *umuC*, one independent of *alkA* but *umuC*$^+$ dependent, and one both *alkA* and *umuC*$^+$ dependent. *alkA*-dependent A · T-to-G · C transitions at the *trpA58* locus were induced only by adapting doses of MNNG and at about one-third the frequency at which A · T-to-C · G transversions were induced by the same conditions. Thus, among the *alkA-umuC*$^+$-dependent mutations induced by MNNG, A · T transversions appeared to dominate over A · T transitions.

umuC$^+$-dependent revertants of *hisG46*, a CCC codon, were elevated approximately twofold in *alkA umuC*$^+$ bacteria (Table 3); thus, MNNG can also induce *alkA-umuC*$^+$-dependent mutations at G · C sites. In addition, some of the weak ochre suppressors of *hisG428* and the missense suppressors of the *trpA* alleles may have been due to mutations at G · C base pairs. However, strong suppressors of *hisG428* arising via G · C-to-A · T transitions (at glutamine tRNAs) or G · C-to-T · A transversions (at tyrosine tRNAs) were poorly induced by a low dose of MNNG in the *alkA* strains (Table 4), although they are well induced by other mutagens (D. Levin, personal communication). Glutamine tRNA suppressors are normally well induced by MNNG, representing 93% of the revertants of the *argE* ochre mutation found after exposure of wild-type bacteria to high doses of MNNG (43). Thus, our finding that in *alkA* bacteria 94% of the strong ochre suppressors induced by a low dose of MNNG were lysine tRNAs demonstrates a major shift in the mutagenic specificity of MNNG from G · C transitions to A · T transversions. Because the same mutations, A · T-to-T · A transversions, that give rise to lysine tRNA suppressors can directly revert *hisG428* (29), it appears that lysine tRNAs represent hot spots for either a specific alkylation-induced DNA lesion or an *alkA*$^+$-mediated repair. The fact that this same base change was strongly induced at a nonsuppressor site (*trpA3*) under the same conditions argues, however, that its induction was not dependent on the peculiarities of suppressor tRNA loci.

Yamamoto and Sekiguchi (54) previously showed that *alkA* bacteria are more sensitive than *alk*$^+$ bacteria to both the lethal and mutagenic effects of MMS; lethality was increased but mutagenesis was eliminated in an *alkA recA* strain. We have shown that the *alkA*-dependent mutations induced by MMS are also *umuC*$^+$ dependent (unpublished data); because *recA* bacteria cannot induce the *umuDC* operon, these mutations are likely to be the same as those observed by Yamamota and Sekiguchi (54). MMS produces little m^{6}G but a relatively higher level of other alkylation products in DNA (7). We predict that a major component of the *alkA*-dependent mutations induced by MMS is due to the same DNA lesions as those induced by MNNG and consists of the same base changes.

Previous studies of the base substitution mutations induced by MNNG have shown that transversions are extremely rare under most conditions of MNNG mutagenesis (11, 43). Mutations at A · T sites are also infrequent and consist of transitions (11). Thus, it is of interest to identify which alkylation-induced DNA lesions are responsible for the *umuC*$^+$-dependent transversions at A · T base pairs that we observed. The simplest interpretation of our results is that one or more of the substrates for TAGII, the product of the *alkA* gene, persisted in the DNA in *alkA* bacteria after MNNG exposure and produced mutations when acted upon by the mutagenic functions of the SOS response. However, it is clear from the results presented in Fig. 2 that one or more of the substrates for TAGII was a good SOS-inducing lesion. Thus, another interpretation of our results is that the increased mutagenesis observed in *alkA* bacteria was due to DNA lesions that are not substrates for TAGII, are mutagenic when the SOS response is induced, but are themselves poor inducers of SOS.

There are three substrates for TAGII at A · T sites: 7-methyladenine, O^2-methylthymine, and 3-methyladenine (m3A). When the mutagenic potential of these lesions is considered, only the latter appears likely to give rise to the class of mutations we observed. Adaptation does not enhance the removal of 7-methyladenine from DNA (22); thus, this lesion could not account for the large difference in mutagenesis between the *alkA*$^+$ and *alkA* strains. In vitro studies of O^2-methylthymine have suggested that it miscodes as cytosine (44), which would result in A · T-to-G · C transitions. Such mutations would probably not be *umuC*$^+$ dependent (the O^2-methylpyrimidine residues may, in fact, be responsible for the increase in *umuC*$^+$-independent mutations in the *alkA* strains), and it is difficult to see how O^2-methylthymine could produce transversions, which would require the damaged thymine to pair with another pyrimidine. Thus, among the substrates for TAGII, the most likely candidate for an *alkA-umuC*$^+$-dependent premutagenic lesion at A · T sites is m3A. An analogous argument would identify 3-methylguanine, also a substrate for TAGII, as potentially an *alkA-umuC*$^+$-dependent premutagenic lesion at G · C sites. Although known to be highly lethal (22, 24), 3-methylpurines have not been shown to be mutagenic; m3A, however, has been shown to be an inducing lesion for the SOS response (8).

In addition to being a substrate for TAGII, m3A is excised from DNA by a constitutively expressed DNA glycosylase, 3-methyladenine-DNA glycosylase I (TAGI) (37). TAGI is the product of the *tagA* gene (24) and is specific for m3A (37). E. Seeberg (personal communication) has suggested that m3A is not itself mutagenic but rather serves only to induce the SOS response, which then produces mutations by acting on the apurinic sites that result from TAGI activity. In support of this hypothesis, he has shown that although the mutagenicity of MMS is increased in either *tagA* or *alkA* strains, it is decreased in bacteria with both defects. Thus, the absence of either glycosylase may leave SOS-inducing lesions in the DNA, while the presence of either glycosylase creates AP sites that may be mutagenic if subject to SOS processing.

Our results are consistent with this second hypothesis because they show that (i) SOS-induction is enhanced in *alkA* bacteria under adapting conditions with MNNG (Fig. 2), (ii) the mutations induced by MNNG under these conditions appear to be predominately at A · T sites and thus

could be the result of TAGI activity, and (iii) the most frequent mutations appear to be A · T transversions, the base substitutions that would be expected if the premutagenic lesions are apurinic sites left by the removal of damaged adenines. Apurinic sites give rise to transversions via an SOS-dependent pathway, apparently because purines, particularly adenines, are preferentially inserted opposite such sites during bypass synthesis (27, 39, 46). The specificity of the *alkA-umuC*$^+$-dependent mutations that we observed would thus be accounted for by the frequent insertion of adenine, yielding A · T-to-T · A transversions, the less frequent insertion of guanine, yielding A · T-to-C · G transversions, and the rarer insertion of cytosine, yielding A · T-to-G · C transitions.

Because TAGI is specific for m3A, its activity cannot account for the *alkA-umuC*$^+$-dependent mutations occurring at G · C sites (Table 3). But once the SOS response is induced, other lesions could give rise to these mutations. For example, spontaneous depurination of 7-methylguanine or the enzymatic removal of methylformamidopyrimidine, a product of 7-methylguanine (9), would create AP sites at guanines. However, our data also are consistent with the hypothesis that m3A is mutagenic because it is functionally equivalent to an apurinic site and thus gives rise to A · T transversions directly. Analogously, 3-methylguanine could lead to G · C transversions. Thus, the identity of the *alkA-umuC*$^+$-dependent premutagenic lesion remains ambiguous.

By removing premutagenic lesions, SOS-inducing lesions, or both, TAGII activity clearly can prevent a significant amount of the mutations potentially induced by DNA alkylation. After exposure of bacteria to an alkylating agent, the level of induction and the degree of saturation of this enzyme will strongly influence the contribution that the SOS functions make to the overall frequency of mutagenesis. Thus, in addition to its role in reducing the lethality of alkylating agents, TAGII contributes to the resistance to alkylation-induced mutagenesis that is part of the adaptive response.

ACKNOWLEDGMENTS

We thank the many people mentioned above for providing bacterial strains, plasmids, and phage stocks, especially T. Standing, who constructed plasmid pTS46. We thank G. Walker and B. Demple for helpful discussions, D. Levin, J. Laval, and E. Seeberg for communicating and discussing their results before publication, and J. Miller and R. Brent for critically reading this manuscript.

This work was supported by Public Health Service grants ES03182 and ES02021 awarded to E.E. by the National Institutes of Health and CA37880 awarded to P.L.F. by the National Cancer Institute. P.L.F. was also supported in part by a postdoctoral fellowship from the American Cancer Society, Massachusetts Division.

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