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Mutagenic Frequencies of Site-Specifically Located O^6-Methylguanine in Wild-Type *Escherichia coli* and in a Strain Deficient in Ada-Methyltransferase

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The adaptive response of *Escherichia coli* involves protection of the cells against the toxic and mutagenic consequences of exposure to high doses of a methylating agent by prior exposure to low doses of the agent. Ada protein, a major repair activity for O^6-methylguanine, is activated to positively control the adaptive response; O^6-methylguanine is one of the major mutagenic lesions produced by methylating agents. We investigated the mutation frequency of wild-type *Escherichia coli* and strains containing the *ada-5* mutation in response to site-specifically synthesized O^6-methylguanine under conditions in which the adaptive response was not induced. Site-directed mutagenesis and oligonucleotide self-selection techniques were used to isolate the progeny of M13mp18 DNAs constructed to contain O^6-methylguanine at any of eight different positions. The progeny were isolated from *E. coli* strains isogeneic except for deficiency in Ada-methyltransferase repair, UvrABC excision repair, or both. The resulting O^6-methylguanine mutation levels at each position were determined by using differential oligonucleotide hybridization. We found that the wild type had up to a 2.6-fold higher mutation frequency than *ada-5* mutants. In addition, the mutation frequency varied with the position of the O^6-methylguanine in the DNA in the wild type but not in *ada-5* mutants; O^6-methylguanine lesions at the 5′ ends of runs of consecutive guanines gave the highest mutation frequencies. Determination of the mutation frequency of O^6-methylguanine in wild-type and *mutS* cells showed that mismatch repair can affect O^6-methylguanine mutation levels.

O^6-Methylguanine (O^6-meGua) is the primary premutagenic lesion of simple methylating agents, such as *N*-methyl-*N*-nitrosourea and *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (MNNG) (14, 20), and may be a product of normal cellular events (12). The mutagenic properties of O^6-meGua are caused by its preference for base pairing with T rather than C during DNA synthesis (1, 27, 28). The resulting O^6-meG · T base pair, if left unrepaired, results in a G-to-A transition mutation (13, 23).

Repair of O^6-meGua in *Escherichia coli* cells can be carried out by a number of DNA repair enzymes, including Ada-methyltransferase (12); Ogt, also known as DNA-methyltransferase II (21, 22, 26); and UvrABC excinuclease (23, 25, 32). Ada^+ protein is responsible for the adaptive response of *E. coli* to alkylating agents (reviewed in reference 12). Ada^+ protein, by transferring the methyl group from O^6-meGua to one of its own cysteine residues (Cys-321), repairs this lesion (7, 12), regenerating an undamaged guanine. Also, Ada^+ protein, upon transfer of a methyl group to Cys-69 in its N-terminal domain from a DNA methylphophotriester, becomes a strong transcriptional activator; Ada^+ protein regulates the genes *ada* (self-regulation), *alkA*, *alkB*, and *aidB* in *E. coli* (12).

The adaptive response was first noted when it was found that exposure of growing *E. coli* to low doses of MNNG adapted the cells to the toxic and mutagenic effects of higher doses by inducing resistance (24). The signal for this adaptation is production of methylphosphates in the DNA backbone. We wondered what the result of introduction of site-specifically synthesized O^6-meGua adducts into *E. coli* under conditions in which the adaptive response either could not be induced or was crippled would be. Both situations were compared; wild-type strain AB1157, which contains a low level of Ada^+ protein, was compared with an *ada-5* mutant containing a sluggish Ada-5 methyltransferase 3,000- to 4,000-fold slower in processing O^6-meGua and unable to induce gene transcription (6). In either situation, the *ada* gene was not induced, since the methylphosphate-inducing signal was not present.

More specifically, we examined mutation frequencies resulting from site-specifically synthesized O^6-meGua in several *E. coli* strains either wild type or defective in Ada-methyltransferase repair, UvrABC excision repair, or both. We used site-directed mutagenesis and oligonucleotide self-selection methods (23) to quantitate mutation frequencies. Site-directed mutagenesis allowed introduction of defined DNA lesions (O^6-meGua) without DNA-damaging agents. Oligonucleotide self-selection allowed detection and isolation of the progeny from the O^6-meGua-containing strand.

O^6-meGua was studied at each G of a G singlet, two G doublets, and a G triplet. With our system, without induction of the adaptive response, the wild-type *ada* gene resulted in higher mutation frequencies than in the *ada-5* mutant at the site of synthesized O^6-meGua. Also, O^6-meGua mutation frequencies showed sequence bias in Ada^+ cells; mutation frequencies were highest when O^6-meGua replaced the 5′-most guanine in each of the G doublets and the G triplet. The lower overall O^6-meGua mutation frequencies in the *ada-5* mutants showed no sequence bias.

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MATERIALS AND METHODS

Chemicals. 1,8-Diazabicyclo[5.4.0]undec-7-ene, β-mercaptoethanol, Ficoll, polyvinylpyrrolidone, Sephadex G-15, rATP, deoxynucleoside triphosphate (dNTPs) and dithiothreitol were obtained from Sigma (St. Louis, Mo.). T4 DNA ligase and DNA polymerase I (Klenow fragment) were obtained from Promega (Madison, Wis.). T4 DNA polynucleotide kinase was obtained from New England BioLabs (Beverly, Mass.). Tryptone, yeast extract, and Bacto-Agar were obtained from Difco (Detroit, Mich.). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside was obtained from Boehringer Mannheim (Indianapolis, Ind.). Isopropyl-β-D-thiogalactopyranoside and Tris(hydroxymethyl)aminoethane were obtained from Bethesda Research Laboratories (Gaithersburg, Md.). Bovine serum albumin pentax fraction V was obtained from ICN Biomedicals (Costa Mesa, Calif.). [γ^{32}P]ATP was obtained from New England Nuclear (Boston, Mass.). All remaining chemicals were obtained from Fisher (Raleigh, N.C.) and were reagent grade.

Bacterial strains. For studies of the effects of *ada* and *uvr* mutations on O^6-meGua mutagenesis, strains AB1157 (wild type) (*ada*$^+$ *uvrA*$^+$), BS24 (*ada-5 uvrA*$^+$), UNC1158 (*ada*$^+$ *uvrA*::Tn*10*), and UNC25 (*ada-5 uvrA*::Tn*10*) were obtained from Aziz Sancar (30). All of these bacterial strains were isogeneic except for the genetic differences indicated; they were all derived from AB1157. For studies of the effect of mismatch repair on O^6-meGua mutagenesis, KMBL3752 (wild type) and KMBL3775 (*mutS*) were obtained from Barry Glickman (9). All of the bacterial strains displayed the appropriate phenotypes upon treatment with UV light and MNNG or DNA containing a mismatch.

Oligonucleotide synthesis and M13-pras construction. Oligonucleotides were synthesized on an Applied Biosystems DNA Synthesizer (Applied Biosystems, Foster City, Calif.). Oligonucleotides were detritylated by treatment with 80% acetic acid as previously described (31).

Primers (33-mers) were deprotected by treatment with 1,8-diazabicyclo[5.4.0]undec-7-ene and subsequently gel purified on a 20% denaturing polyacrylamide gel as previously described (31). Oligonucleotide probes (12-mers) were detritylated and deprotected as described above but were not gel purified.

Primers were prepared with O^6-meGua synthesized singly at eight different sites (Fig. 1). Representative O^6-meGua-containing oligonucleotides were hydrolyzed, and their compositions were determined in triplicate by high-pressure liquid chromatography as described elsewhere (31). The results indicated that the oligonucleotides contained the expected composition (±0.1 base) and a single (±0.1) O^6-meGua. A probe was synthesized for each primer (Fig. 1). Probes were synthesized to detect mutations in the progeny produced from the O^6-meGua-containing primer strand. All probes were synthesized with five bases 5′ and six bases 3′ to the expected mutation site. M13-pras is an M13 construct engineered, as described elsewhere (23), to contain 28 bases of the H-*ras* sequence and a TAG stop codon in the N-terminal portion of the α-complementing peptide.

Formation of O^6-meGua-containing double-stranded M13 DNA. O^6-meGua was synthesized into oligonucleotide primers at the unique sites described in Fig. 1. The primers were phosphorylated by incubation for 60 min at 37°C with 2 U of T4 polynucleotide kinase in 1 mM rATP–25 mM Tris–5 mM $MgCl_2$–2.5 μM dithiothreitol–50 μM EDTA (pH 7.6) in a total volume of 40 μl. The primer was then annealed to the M13 construct (M13-pras) as follows. An M13-pras single-

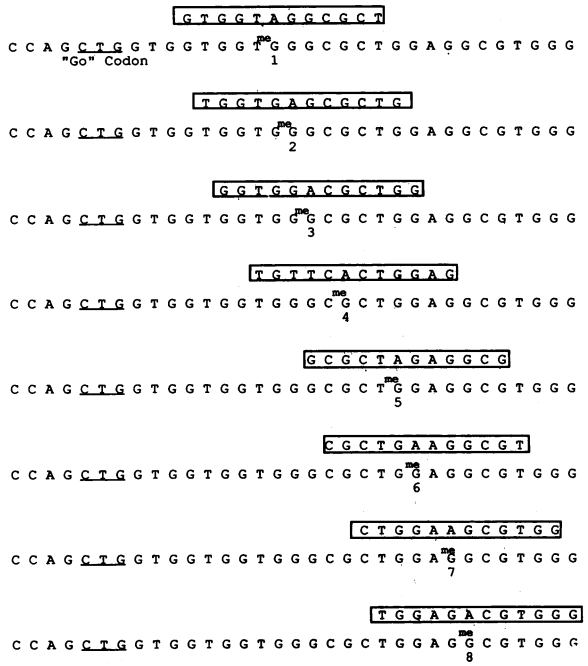

FIG. 1. Oligonucleotides synthesized and used as primers and probes. The probes are shown as boxed sequences above their primer partners. Primers contained O^6-meGua at eight separate sites shown as ^{me}G. Each primer contained a CTG, which resulted in a CAG codon in the M13-pras progeny to give functional α complementation. Probes were synthesized to detect mutations in progeny from polymerized O^6-meGua-containing primer strands.

stranded template (1.2 pmol) was mixed with 26 pmol of phosphorylated 33-mer primer (primer in about 20-fold excess) in 7 mM Tris–3 mM $MgCl_2$–30 μM β-mercaptoethanol–33 mM NaCl (pH 7.6) in a final volume of 5 μl. The mixture was heated at 70°C for 10 min and then slowly cooled the 25°C over 1 h. The annealed M13-pras–33-mer complex was then polymerized and ligated to a closed double-stranded form as follows. rATP and dNTPs were added to the annealed complex to give final concentrations of 165 and 135 μM, respectively. DNA polymerase fragment I (Klenow; 7 U) and T4 DNA ligase (0.3 U) were added, and the mixture was incubated at 25°C for 15 min. A chase mixture of dNTPs and rATP was added to give final concentrations of 800 and 950 μM, respectively. Finally, 4 U of T4 DNA ligase was added to give a final volume of 15 μl before overnight incubation at 25°C. The incubation mixture was desalted on a Sephadex G-15 spin column (5 by 50 mm) equilibrated with 10 mM Tris (pH 7.5). Gel electrophoresis of reaction products from similar reactions using even longer templates showed that >90% of the primed DNA was chased and ligated into closed circular double-strand DNA under our conditions (5, 5a); intercalating dyes were used in the gels to shift the mobility of covalently closed circles.

Calcium permeabilization and transfection. Bacteria were calcium permeabilized before transfection as described elsewhere (15, 29). Double-stranded M13-pras (0.1 to 0.3 pmol) was added to calcium-permeabilized cells (400 μl) and incubated on ice for 20 min. The cells were heat shocked for 1 min at 45°C. The transfected cells were immediately diluted into 1 ml of TYE medium (10 g of tryptone [Difco], 1 g of yeast extract, 8 g of NaCl, 1 g of glucose, 0.2 g of $CaCl_2$ per liter). The transfection mixture was plated with JM105 onto

20 TYE agar plates in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside 0.04% and 0.008% isopropyl-β-D-thiogalactopyranoside within 10 min of the heat shock. The plates were incubated overnight at 37°C. Ninety to one hundred blue plaques per transfection mixture were each picked into 1 ml of TYE medium. The resulting phage stocks were briefly vortexed, heated at 60°C for 90 min, and centrifuged (10,000 × *g*, 2 min); this left viable bacteriophage but eliminated bacteria.

Plaque lift and probing for G-to-A mutations. A 3-μl volume of each phage stock (100 phage stocks per transfection mixture) was spotted onto a JM105 lawn (18 to 25 spots per plate). Control phage stocks were spotted along with samples. The controls were as follows: (i) wild-type M13mp18 (control for deletion of pras insert); (ii) M13-pras phage with a G at the O^6-meGua position (control for O^6-meGua repaired and replicated as a G · C base pair); (iii) in addition, a test for positive detection of M13-pras phage with an A at the O^6-meGua position was included as an indication of our ability to detect a G-to-A mutation. The plates were incubated at 37°C for 12 to 15 h and cooled at 4°C for 30 min before plaque lift. Plaques were lifted onto nitrocellulose filters (Schleicher and Schuell, Keene, N.H.) and prepared for hybridization as previously described (23). The deprotected and detritylated 12-mer probe (1 nmol) was end labeled with ^{32}P by published procedures (16). The ^{32}P-labeled probe was then diluted into 50 ml of hybridization solution containing 4X SSC (final concentrations, 0.6 M NaCl and 0.06 M sodium citrate [pH 7.0]) and 10× Denhardt solution (final concentrations, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin pentax fraction V). The prepared nitrocellulose filters were hybridized (four filters per 15 ml of hybridization solution) for 2 to 3 h at 25 or 37°C. The filters were washed twice sequentially with 1× SSC. The filters were autoradiographed with an intensifying screen for 1 to 12 h, depending on the specific activity of the labeled probe. The accuracy of the hybridization assay for quantitation of mutations was confirmed by sequencing 75 of the mutants.

RESULTS

Oligonucleotide self-selection. The mutagenicity of O^6-meGua at any of eight different sequence sites, CCAGCTGG TGGTGGTG^{1}G^{2}G^{3}CG^{4}CTG^{5}G^{6}AG^{7}G^{8}CGTGGG (Fig. 1), was determined in *E. coli* cells with various repair backgrounds. Oligonucleotide self-selection (23) was used to select phage replicated from the O^6-meGua-containing minus strand (Fig. 2). Summarized here (see reference 23 for details), oligonucleotide self-selection used the G · T mismatch $\frac{CTG}{GAT}$ to place CTG (which gives CAG coding for Glu in the coding strand) on the O^6-meGua-containing strand 5′ to the *lac Z* (α complementation) gene. If the G · T mismatch was not repaired, phage replicated from this minus strand were capable of α complementation and gave blue plaques that were evaluated for O^6-meGua mutagenesis. Phage replicated from the plus strand and from DNA in which the mismatch was corrected (the T of the mismatch was not corrected, since it was in the plus strand, which was methylated at 5′-GATC-3′ sequences) contained a TAG stop codon and thus were incapable of α complementation, which resulted in white plaques that were not evaluated.

Mismatch repair involves replacement of a significant stretch of DNA (reviewed in reference 18); thus, selection for the mismatch-containing minus strand should rescue most other lesions within that DNA stretch from the effects of such repair. Under our conditions of selection, 1% of the phage from each *E. coli* strain were blue; the remaining plaques were white.

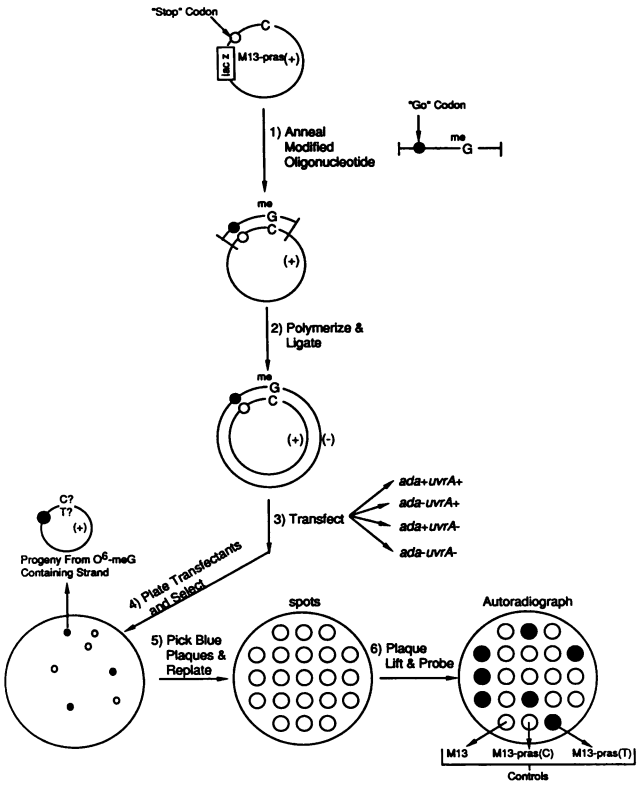

FIG. 2. Methodology for selection and analysis of progeny from an O^6-meGua-containing viral strand. In step 1, an O^6-meGua-containing primer with a go codon (i.e., any codon allowing synthesis of the active peptide) was annealed to M13-pras containing a stop codon upstream of the α-complementing partial *lacZ* sequence. In step 2, the annealed complex was polymerized and ligated to yield an M13-pras duplex. In step 3, double-stranded. O^6-meGua-containing M13-pras was transfected into repair-proficient strain AB1157 (*ada*$^+$ *uvrA*$^+$) and repair-deficient mutant strains BS24 (*ada-5 uvrA*$^+$), UNC1158 (*ada*$^+$ *uvrA*::Tn*10*), and UNC25 (*ada-5 uvrA*::Tn*10*). In step 4, transfectants were plated with a nonsuppressing *E. coli* strain in the presence of inducer and indicator (see Materials and Methods). In step 5, 90 to 200 blue plaques were randomly chosen for mutation analysis, and concentrated phage stocks grown from each plaque were replated as individual spots along with controls. In step 6, the resulting 90 to 200 large spots were probed with labeled oligonucleotides (see Materials and Methods) and subsequently autoradiographed. O^6-meGua-induced mutations appeared as dark spots on the film.

Oligonucleotide self-selection allows the progeny from the strand of interest that have escaped mismatch repair to be selected in the presence of progeny from the unmodified complementary strand. The progeny isolated from the O^6-meGua-containing strand (i.e., the 1% of the progeny that were blue) were probed as described in Materials and Methods. Upon autoradiography, O^6-meGua-induced mutations were visible as dark spots on the film whereas nonmutated guanines were apparent as barely detectable spots on the film (Fig. 3).

DNA sequencing of a significant number of mutants and probing for the wild type, as well as for G-to-A mutations, established that the only mutations detected by our analysis were G-to-A mutations at the site of O^6-meGua. Direct

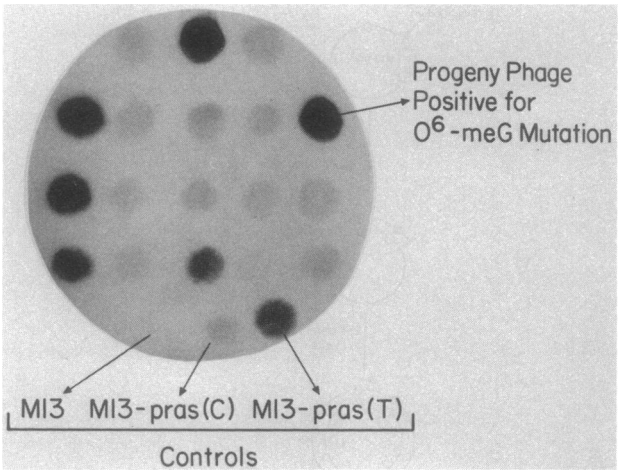

FIG. 3. Sample autoradiograph from analysis of O^6-meGua mutagenesis. Progeny phage from transfection of M13-pras (O^6-meGua at site 3) into excision repair-deficient mutant UNC1158 (*ada*$^+$ *uvrA*::Tn*10*) were probed with a ^{32}P-radiolabeled 12-mer oligonucleotide (Fig. 1). Progeny phage positive for an O^6-meGua-induced mutation specifically bound the probe and appeared as dark spots, as did the M13-pras(T) control. M13 and M13-pras(C) controls did not appreciably bind the probe and appeared as light spots on the film.

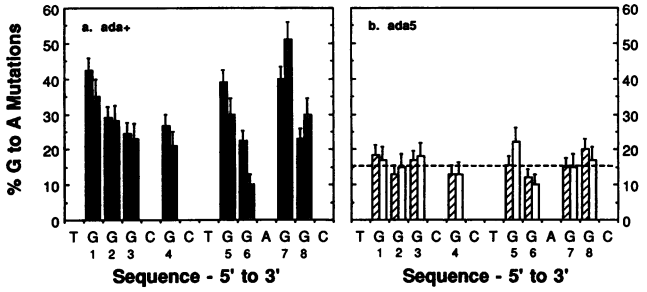

FIG. 4. Effect of DNA sequence context and *E. coli* repair background on mutation by O^6-meGua. Duplex M13-pras DNAs containing a single O^6-meGua at sites 1 through 8 (x axis) were transfected into *ada*$^+$ cells (a; symbols: ■, strain AB1157 [*ada*$^+$ *uvrA*$^+$]; ▩, strain UNC1158 [*ada*$^+$ *uvrA*::Tn*10*]) and *ada-5* cells (b; symbols: ▨, strain BS24 [*ada-5 uvrA*$^+$]; □, strain UNC25 [*ada-5 uvrA*::Tn*10*]). The average G-to-A mutation frequency in the *ada-5* mutants is shown by the broken line. Standard error was determined from $[\%(1 - \%)/n]^{1/2}$, where % is the G-to-A mutation frequency and n is the number of plaques tested (19). For studies with excision repair-competent (*uvrA*$^+$) cells, n was 200; for studies with *uvrA* mutants, n was 90 to 100. Reproducibility of ±2.5% was measured from two independent determinations of the mutagenicity at each of the O^6-meGua positions in the wild type and in BS24, starting from the annealed oligonucleotide. The total mutation frequency at each site is 1% of the value indicated (see text).

sequencing of 75 of the clones and reprobing of several hundred of the clones that did not test positively with the probe that detects the G-to-A mutation, this time with a probe for the wild-type sequence, showed that the level of spontaneous mutation at a given site was well below our level of detection (<5% of the blue plaques, estimated from an overall reproducibility of ±2.5% for detection of O^6-meGua mutations). There were eight oligonucleotides, with a different O^6-meGua site in each. Therefore, there were seven different internal controls for background mutation at any of the O^6-meGua positions. Mutations at other than the particular O^6-meGua site being studied would have been detected by either DNA sequencing or inability of the wild-type probe to hybridize. Mutations other than G to A at the O^6-meGua site being studied would have been detected by either DNA sequencing or inability of either of the probes used for that site, the probe that recognizes wild-type sequence or the probe that recognizes the G-to-A base change, to hybridize.

Mutation frequency of Ada$^+$ cells due to site-specific O^6-meGua adducts. Analysis of mutation frequencies due to transfection of O^6-meGua-containing DNA into Ada$^+$ strains, including wild-type AB1157 (*ada*$^+$ *uvrA*$^+$) and UNC1158 (*ada*$^+$ *uvrA*::Tn*10*), showed levels of mutagenesis that depended on the sequence position of O^6-meGua (Fig. 4). In wild-type cells, O^6-meGua lesions that were the first guanine in a series of two (^{me}GG) or three (^{me}GGG) gave an average mutation frequency (sites 1, 5, and 7) of 41 ± 2% (standard deviation) among these three sites. When the 1% selection rate is considered, that is equivalent to an average total mutation frequency of 0.41% among these three sites.

$$\text{Total population} \xrightarrow{\text{Select}} \text{Mismatch mutants (1\%)} \xrightarrow[41\% \text{ positive}]{\text{Probe}} O^6\text{-meGua + mismatch mutants (0.4\%)}$$

O^6-meGua lesions that were located 3′ to an unmodified guanine (G^{me}GG, GG^{me}G, and G^{me}G), as was found at sites 2, 3, 6, and 8, gave a lower total average mutation frequency of 0.25 ± 0.03% among the four sites. A singlet guanine (C^{me}GC) at site 4 gave a total mutation frequency of 0.27%. Therefore, in wild-type cells, O^6-meGua in place of the 5′ leading guanine in a guanine doublet or triplet is about 1.6-fold more mutagenic than O^6-meGua at other positions.

Analysis of mutation frequencies due to O^6-meGua after transfection into *uvrA* mutant UNC1158 (*ada*$^+$ *uvrA*::Tn*10*) showed mutation frequencies (Fig. 4) that generally paralleled the mutation frequencies in the wild type. Quantitatively, only site 6 showed obvious differences in mutation levels between these two genotypes.

Mutation frequency of *ada-5* mutant due to site-specific O^6-meGua adducts. Transfection of O^6-meGua-modified M13-pras DNAs into *ada-5* mutant BS24 (*ada-5 uvrA*$^+$) and double mutant UNC25 (*ada-5 uvrA*::Tn*10*) resulted in lower mutation frequencies than in wild-type strain AB1157. The mutation frequencies of the *ada-5* mutants were lower at all sites than in the wild type by 31 to 62%, depending on the sequence position of the adduct. The O^6-meGua total mutation frequencies averaged 0.16 ± 0.02% in *ada-5* mutants and 0.16 ± 0.04% in *ada-5 uvrA*::Tn*10* double mutants among the eight sites studied.

Effect of mismatch repair on site-specific O^6-meGua adducts. Mismatch repair has been reported to affect O^6-meGua lesions (8, 11). Therefore, to determine the effect of mismatch repair on the mismatch and O^6-meGua lesions under our conditions, DNA containing both O^6-meGua at site 2 and the mismatch reference was transfected into *E. coli mutS* strain KMBL3775 (9), which lacks the ability to recognize the presence of the mismatch and thus repair it (18), and its parent, KMBL3752.

O^6-meGua was synthesized into the undermethylated strand and so was subject to mismatch repair. Transfection of the O^6-meGua-containing DNA into *mutS* cells resulted in a mismatch mutation frequency of 6.8%; 49% of these mismatch mutants also contained the O^6-meGua mutation (Table 1). This level of O^6-meGua mutations indicates that

TABLE 1. Effect of mismatch repair on mutation frequency

Strain	% of population with mismatch mutation	% of mismatch mutants with O^6-meGua mutation	Total O^6-meGua mutation frequency (%)
KMBL3752 (wild type)	0.9	20	0.2
KMBL3775 (*mutS*)	6.8	49	3.4

about half (51%) of the synthesized O^6-meGua was repaired, most likely by constitutive alkyltransferase activity (Ogt).

Transfection of this same DNA into wild-type *E. coli* KMBL3752 resulted in a mismatch mutation frequency of 0.9%, similar to that found with AB1157; 20% of these mismatch mutants also contained an O^6-meGua mutation (Table 1). Thus, the presence of mismatch repair had the expected large effect on mismatch mutations but also had some effect on the O^6-meGua lesion. The effect of mismatch repair on O^6-meGua was significantly less than its effect on the mismatch.

DISCUSSION

This report describes the use of site-directed mutagenesis technology and an oligonucleotide self-selection method to quantitate the mutagenicity of O^6-meGua synthesized at each of eight different DNA sequence positions, CCAGCT GGTGGTGGTG^{1}G^{2}G^{3}CG^{4}CTG^{5}G^{6}AG^{7}G^{8}CGTGGG, in an M13mp18 DNA construct. These viral DNA constructs were introduced into isogeneic *E. coli* strains derived from AB1157 either proficient (*ada*$^+$ *uvrA*$^+$) or deficient (*ada*$^+$ *uvrA*::Tn*10*; *ada-5 uvrA*$^+$; *ada-5 uvrA*::Tn*10*) in DNA repair. The mutagenicity of O^6-meGua at position 2 was also determined in wild-type and *mutS* cells.

Exposure of growing *E. coli* bacteria to low doses of alkylating agents adapts the cells by inducing resistance to the toxic and mutagenic effects of higher doses of alkylating agents (24). The basis for this effect is induction of expression of *E. coli* Ada-methyltransferase, an inducible protein that scavenges methyl groups from the O^6 position of O^6-meGua and the O^4 position of O^4-methylthymine and transfers these methyl groups irreversibly to one of the cysteine residues of the Ada protein (reviewed in reference 12). Transfer of a methyl group from methyl phosphotriesters in DNA to another separate alkyltransferase site on the Ada protein acts as the signal that triggers Ada protein to act as a transcriptional activator (12).

In our study, O^6-meGua was introduced as an example of a specific lesion without methylation of the DNA phosphodiester backbone. Thus, no signal was produced to activate Ada protein to turn on the *ada* gene. Under these conditions, we determined the mutagenicity of O^6-meGua in a wild-type *E. coli* strain and a strain containing the *ada-5* mutation. The Ada-5 protein repairs O^6-meGua lesions 3,000- to 4,000-fold slower than the wild-type enzyme (6). In addition, the Ada-5 protein is defective in transcriptional activation of the *ada* and *alkA* genes (6). Thus, O^6-meGua transfected into these *E. coli* strains faced constitutive levels of Ogt protein and either a very low level of uninduced Ada protein or a very low level of a sluggish *ada-5* mutant protein.

A surprising dichotomy was found when we looked at the effects of the *ada*$^+$ and *ada-5* genotypes on O^6-meGua mutation levels. Ada$^+$ cells had a higher mutation frequency than Ada-5 cells at all O^6-meGua sites studied; the largest difference was 2.6-fold. In addition, the mutation frequencies varied in Ada$^+$ cells with the position of the O^6-meGua adduct. It was highest (1.6-fold) when O^6-meGua replaced the 5′-most guanine in each of the G doublets and the G triplet. Mutation levels were almost uniform, however, among the sites studied (Fig. 4) in *ada-5* mutants.

Our results for O^6-meGua mutagenesis in Ada-5 and wild-type strains can be compared with O^6-meGua mutation frequencies determined in other studies. Loechler et al. (13) reported a mutation frequency of at least 0.08% in *E. coli* MM294A (wild type for repair) for O^6-meGua synthesized into the *Pst*I restriction site in M13mp8 replicative form DNA. Hill-Perkins et al. (10) reported a mutation frequency of 0.46% in *E. coli* JM101 (wild type for repair) for O^6-meGua synthesized into the α-complementing region of M13mp9 replicative form DNA. Chambers et al. (4) reported a mutation frequency of 15.8% in *E. coli* AB1157 (wild type for repair) for O^6-meGua synthesized to rescue a lethal mutation in the third codon of gene G of bacteriophage φX174. Bhanot and Ray (2), who used a selection system almost identical to that of Chambers et al. (4), reported a mutation frequency of 8% in *ada-5* cells (BS24) for O^6-meGua synthesized into gene G of bacteriophage φX174. In the wild type, they detected no mutants in 100 infective centers tested, which implies a mutation frequency of <1%; the strain used was C6100, not the true parent of BS24, which is AB1157. Mismatch repair was not a factor in the studies by Chambers et al. and Bhanot and Ray, since φX174 DNA lacks *dam* methylase sites (GATC). Finally, we incorporated O^6-medGTP into a pBR322-f1 chimera (8). Transfection of that DNA into *ada-5* mutant and wild-type cells gave mutation frequencies of 0.45 and 0.27%, respectively.

Chambers et al. (4) attributed the high mutation frequency (15.8%) of O^6-meGua in strain AB1157 found in their study to interference by *uvrABC* in repair of the lesion. We also found evidence for the ability of *uvrABC* to affect O^6-meGua mutagenesis at some DNA positions (this study; 23), but to a much lower extent. Perhaps the high O^6-meGua mutation frequency reported by Chambers et al. in AB1157 is somehow related to the lack of mismatch repair in their studies.

The total mutation frequencies determined here for O^6-meGua in wild-type cells are in general agreement with the above-mentioned studies, except for that measured by Chambers et al. (4). We selected for mismatch mutants, which represented that percentage of the population of bacteriophage that was replicated from the modified DNA strand and that, to some extent, escaped mismatch repair. That select population was analyzed for the presence of O^6-meGua mutations. Therefore, the total O^6-meGua mutation frequency is the selection frequency multiplied by the O^6-meGua mutation frequency measured at a given site. This product, for example, was about 0.3% at site 2 in wild-type *E. coli*. This mutation frequency was confirmed in the absence of selection. One hundred plaques were randomly selected (in the absence of color change), and no mutations were detected; this implies that the overall mutation frequency of O^6-meGua at site 2 was <1%, confirming our calculations. Since the selection frequencies for the mismatch were all about 1% in strains wild type for mismatch repair, the overall mutation frequencies at all of the sites studied are 1% of the mutation frequencies shown in Fig. 4.

The O^6-meGua mutation frequencies we measured in Ada-5 cells appear lower than expected on the basis of the above-mentioned studies and our understanding of alkyltransferase repair of O^6-meGua in DNA (12). For example, Loechler et al. (13) and Hill-Perkins et al. (10) found that exhaustion of constitutive alkyltransferase levels by treat-

ment of cells with low levels of MNNG significantly increased the mutagenicity of O^6-meGua. Treatment with MNNG, of course, is not the same as use of an *ada* mutant but does suggest that mutation frequencies would be higher in such a mutant. We also found that treatment of wild-type cells with small amounts of MNNG increased the mutagenicity of O^6-meGua (23). The same treatment of *ada-5* mutants resulted in levels of O^6-meGua mutagenesis higher than those of similarly treated wild-type cells (23). Thus, whereas under our conditions O^6-meGua was more mutagenic in wild-type cells than in *ada-5* mutant cells in the absence of MNNG, it is more mutagenic in *ada-5* mutant cells than in wild-type cells in the presence of low levels of MNNG (see Fig. 3 in reference 23). The quantitative difference in mutagenicity of O^6-meGua between strains wild type for Ada repair and the *ada-5* mutants in the absence of MNNG varied with the sequence position of the lesion and the capacity of the cells for UvrABC repair as well as Ada repair (Fig. 4).

The mutagenicity of O^6-medGTP incorporation was higher in *ada-5* mutant cells than in wild-type cells, which appears to agree with our expectations. However, O^6-MedGTP in incorporated in place of dATP opposite T (1, 27, 28). The resulting O^6-meG · T base pairs depend on methyltransferase repair to give mutagenic G · T intermediates. Therefore, one possible explanation for the lower mutation frequency in wild-type cells is less methyltransferase repair of O^6-meGua in wild-type cells than in *ada-5* mutant cells. If this is true, then the lower repair of O^6-meGua opposite Thy in *ada-5* mutant cells would be consistent with the results found here for O^6-meGua opposite Cyt.

The higher mutation frequencies for O^6-meGua opposite C found in wild-type cells (and the lower mutation frequencies for O^6-meGua opposite T) than in *ada-5* mutants appear surprising compared with the ability of the adaptive response to protect wild-type *E. coli* strains from the mutagenic consequences of methylating agents. One might expect that the sluggish Ada-methyltransferase repair in *ada-5* mutants would either increase the mutagenicity of O^6-meGua opposite C above that in the wild type or have no effect, since Ogt-methyltransferase levels should be the same in both cell types. Such extrapolation, however, is not necessarily warranted, since introduction of a single O^6-meGua lesion into an *E. coli* cell is not analogous to treatment of cells with low doses of a methylating agent. Perhaps the most important difference is that the *ada* gene is not induced by O^6-meGua, whereas it is induced by treatment of cells with methylating agents.

The decrease in mutagenesis observed concomitant with the loss of normal *ada* gene function suggests that a DNA-binding protein whose presence depends on at least a low level of functional Ada^+ protein is able to interfere with O^6-meGua repair in a sequence-dependent manner. The most likely candidates are the *alkA*, *alkB*, and *aidB* gene products (12) or another, uncharacterized, gene product induced by the *ada* gene product. A less likely possibility is competition between Ada- and Ogt-methyltransferases for repair of O^6-meGua. Such competition, if detrimental within the small time available before replication fixes the mutation, might result in decreased repair.

Interference between repair systems resulting in increased mutation levels has been seen before. O^6-meGua mutagenesis with a site-specifically located adduct is 40-fold higher in wild-type cells than *uvrA* mutants (4). Our work also indicates that $UvrA^+$ protein can hinder mutation by O^6-meGua; the largest effect was 2.5-fold at position 6 (this study; 23). In the wild type, UvrA protein (the damage recognition subunit of ABC excinuclease) may bind to the DNA region containing the methylated base and interfere with its repair by alkyltransferase (4, 23, 32).

Subsequent studies of the mutagenic frequencies of O^6-meGua in wild-type *E. coli* and a *uvrA* mutant (23) and studies using antibodies to monitor O^6-meGua induced by *N*-methyl-*N*-nitrosourea (25) in wild-type *E. coli* and a *uvrB* mutant indicated that ABC excinuclease is involved in repair of O^6-meGua in vivo. In vitro studies of synthetic substrates that contain O^6-meGua at defined positions found that the *E. coli* ABC excinuclease repair enzyme binds specifically to the region of the DNA containing the adduct and produces incisions at the eighth phosphodiester bond 5′ and the fifth or sixth phosphodiester bond 3′ to the modified guanine (32). Extrapolation from these studies to the increase in O^6-meGua mutation frequencies found here in *E. coli* strains containing the wild-type *ada* gene compared with that found in *E. coli* strains containing the *ada-5* mutation suggests that additional activities may be found to bind O^6-meGua in a sequence-dependent manner.

Another possible explanation for the unexpected mutation differences in this study is our placement of a mismatch close to O^6-meGua. All of our studies placed O^6-meGua in an undermethylated strand; thus, mismatch repair could contribute to our results. For example, binding of *mutS* protein (the mismatch recognition protein in mismatch repair) to the mismatch could interfere with repair of O^6-meGua. However, the mutation frequency of O^6-meGua in *mutS* cells was twofold higher than in the wild type. Thus, mismatch repair contributed to repair of O^6-meGua and MutS protein did not interfere with alkyltransferase repair, since such interference would have given a higher mutation frequency in wild-type cells than in *mutS* cells. This does not rule out the possibility that the closeness of the mismatch affected O^6-meGua mutation levels in ways not understood. If this is true, such interference might have important implications for repair of chemical damage occurring near mismatches.

In summary, the presence of a functional uninduced ada^+ gene resulted in higher *E. coli* mutation frequencies in response to O^6-meGua adducts than those found in *E. coli* strains containing the *ada-5* mutation. In addition, the mutagenicity of O^6-meGua was dependent on the surrounding sequences in *E. coli* containing a wild-type *ada* gene. O^6-meGuas at the 5′ ends of runs of consecutive guanines were the most mutagenic, whereas O^6-meGuas at the 3′ ends of runs of consecutive guanines and an O^6-meGua without guanine neighbors were the least mutagenic. Either a gene product dependent on the presence of Ada-methyltransferase or Ada-methyltransferase itself is implicated in the sequence-specific mutagenicity of O^6-meGua, since mutagenesis was sequence specific in the wild type but not in *ada-5* mutants. The nearby mismatch may also have had an effect on O^6-meGua mutation levels. Nonuniform mutagenesis is well documented (3, 17, 29). However, this is the first time that a factor in such nonuniformity has been traced to the repair background of the cell.

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