Response of Repair-Competent and Repair-Deficient *Escherichia coli* to Three O^6-Substituted Guanines and Involvement of Methyl-Directed Mismatch Repair in the Processing of O^6-Methylguanine Residues†

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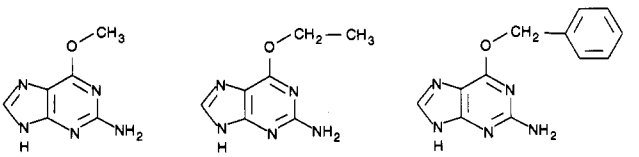
ABSTRACT: Plasmids containing a site-specifically incorporated O^6-methyl- (m^6G), O^6-ethyl- (e^6G), or O^6-benzylguanine (b^6G) within the ATG initiation codon of the *lacZ'* gene were used to transform *Escherichia coli* that were repair proficient or deficient in one or both of the *E. coli* O^6-alkylguanine-DNA alkyltransferases, the *uvr(ABC)* excision repair system, the *recA*-mediated recombination system, or the methylation-directed mismatch repair system. Colonies were scored phenotypically for adduct-induced mutations. With plasmids containing either e^6G or b^6G, the frequency of adduct-induced mutation was low and independent of the repair proficiency of the strain transformed. Plasmids containing an m^6G residue elicited similar responses in all but the mismatch repair-deficient strain. The generally low mutagenicity of all the O^6-substituted guanines was interpreted as reflecting an adduct-induced arrest of replication of the modified strand while the unmodified complementary strand was replicated normally. Studies of the involvement of mismatch repair in m^6G mutagenesis showed that m^6G:T base pairs were more readily processed than m^6G:C base pairs, indicating that mismatch repair involving m^6G residues occurs after replication. These data support a model in which the *E. coli* methylation-directed mismatch repair system diverts plasmids containing promutagenic m^6G:T base pairs into replication-arrested complexes providing another line of defense against O^6-methylguanine mutagenicity in addition to O^6-alkylguanine-DNA alkyltransferase repair and excision repair mechanisms.

Site-specific incorporation of carcinogen-modified bases in plasmid or viral vectors and introduction of these vectors into bacteria or mammalian cells can provide valuable information on how biological systems respond to particular carcinogen–DNA adducts (Basu & Essigmann, 1988; Strauss & Loeb, 1989). The mutagenic potencies of several carcinogen-modified bases have been examined using this approach (Singer & Essigmann, 1991). Of these modified bases, O^6-substituted guanines, particularly O^6-methylguanine, have been most extensively studied (Basu & Essigmann, 1990; Loechler et al., 1984; Hill-Perkins et al., 1986; Rossi et al., 1989; Rossi & Topal, 1991; Chambers et al., 1985, 1988; Bhanot & Ray, 1986; Ellison et al., 1989; Mitra et al., 1989; Pauly et al., 1991; Dosanjh et al., 1991; Chambers, 1991, 1993; Baumgart et al., 1993; Bishop et al., 1993), since these are the highly mutagenic bases resulting from exposure to the carcinogenic alkylating agents (e.g. *N*-alkyl-*N*-nitroso compounds) (Lawley, 1984). A recurring observation in these site-specific mutagenicity experiments has been that, in normal cells, the mutagenicity of these modified guanines is usually quite low presumably due to their efficient repair.

For studies in *Escherichia coli*, enhanced mutagenicity of plasmid-based O^6-substituted guanine residues has been observed in cells pretreated with the methylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) (Loechler et al., 1984; Hill-Perkins et al., 1986; Rossi et al., 1989; Dosanjh et al., 1991; Pauly et al., 1991). It is thought that such treatment, by methylating the host cell's genomic DNA, diverts the cell's repair systems away from the plasmid-based adduct thereby permitting a greater number of adduct-induced mutations to be produced during plasmid replication. Since MNNG pretreatment can alter the efficiency of several repair pathways simultaneously, this system does not permit accurate evaluation of the role played by individual repair pathways in altering the mutagenicity of modified guanines in cells that have not been pretreated. A more effective approach to investigations of repair pathways would involve mutagenicity studies in *E. coli* strains with known repair deficiency (Chambers et al., 1985, 1988; Rossi et al., 1989; Rossi & Topal, 1991; Dosanjh et al., 1991; Chambers, 1993). We have used our previously described sectored colony assay system (Pauly et al., 1991) to compare the mutagenicity of O^6-methyl-, O^6-ethyl-, and O^6-benzylguanine residues in several isogenic repair-deficient *E. coli* strains in order to determine the repair systems most active against guanines bearing increasingly bulky damage at the O^6 position.



O^6-methylguanine (m^6G) O^6-ethylguanine (e^6G) O^6-benzylguanine (b^6G)

The sectored colony assay system utilizes the cassette plasmid pGP10 illustrated in Figure 1A. The plasmid contains a nonfunctional *lacZ'* α-complementation gene in which the *lacZ* promoter sequence (P in Figure 1A) is separated from the coding sequence for the LacZ α-complementation peptide (Lac) by a 0.95-kb insert (insert) which is flanked by two outward-facing sites for the restriction enzyme *Bsp*MI (Figure 1B). Oligodeoxyribonucleotides can be incorporated into this plasmid by first digesting it with *Bsp*MI to produce a 4.3-kb

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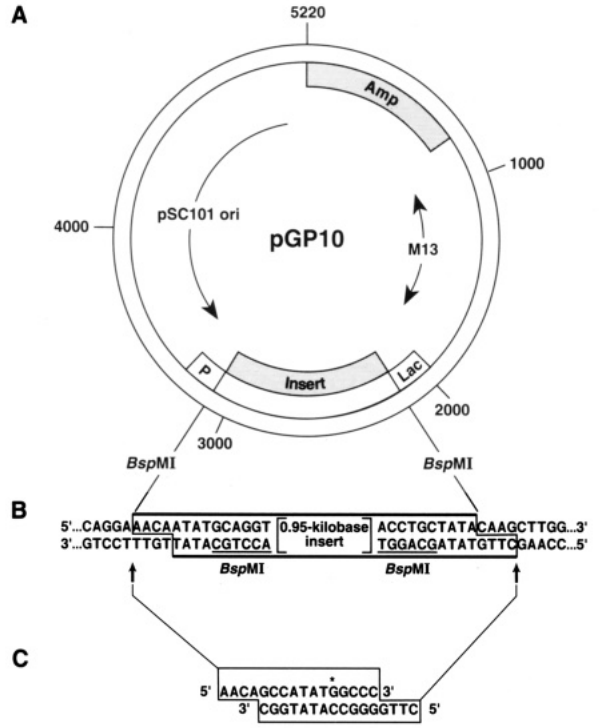

FIGURE 1: Characteristics of the cassette plasmid pGP10 and schematic for incorporating carcinogen-modified bases in the ATG initiation codon of the *lacZ'* gene. The asterisk marks the position of incorporation of the modified guanine derivatives.

major fragment and a 0.95-kb insert sequence (i.e., the sequence within the solid lines in Figure 1B). The major fragment with noncomplementary 4-base overhanging ends is then purified and ligated to a synthetic oligodeoxyribonucleotide duplex (e.g., a duplex with strand sequences illustrated in Figure 1C). This results in constructions containing a continuous sequence for the *lacZ'* gene with an initiation codon ATG* (Figure 1C) containing either a normal guanine residue (i.e. G* = G) or an O^6-substituted guanine residue (i.e. G* = m^6G, e^6G, or b^6G). After introduction of these plasmids into a *lacZ*-complementing version of *E. coli* strain AB1157 grown on medium containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), colonies are produced whose color reflects the outcome of replication of the carcinogen-modified plasmid. Blue colonies, i.e. a Lac^+ phenotype, result if the modified base is repaired prior to replication, if the modified base does not cause a mutation during replication, or if the modified base arrests replication of the modified strand while allowing replication of the normal complementary strand to proceed unabated. On the other hand, if replication of the modified strand leads to a mutation while the unmodified strand replicates normally, both *lac*$^+$ and *lac*$^-$ plasmids are harbored by individual bacteria and, during colony growth, plasmid segregation will lead to individual cells being purely Lac^+ or Lac^-; i.e., a blue-and-white sectored colony results. A white colony is produced if both strands carry a mutation after the first round of replication such that a Lac^- phenotype results. In the studies described here, we investigated the distribution of these colony phenotypes in a variety of repair-deficient *E. coli* strains transformed by plasmids containing a normal guanine or an O^6-substituted guanine paired with either cytosine or thymine in the complementary strand.

Table 1: Bacterial Strains

donor strains	genotype	source
AB1157	F$^-$, *thr-1*, *ara-14*, *leuB6*, Δ(*gpt-proA*)*62*, *lacY1*, *tsx-33*, *supE44*, *galK2*, λ$^-$, rac$^-$, *hisG4*(0c), *rfbD1*, *rpsL31*, *kdgK51*, *xyl-5*, *mtl-1*, *argE3*(0c), *thi-1*	B. J. Bachmann[a]
A171	*lacZ*Δ*M15*, *proC*::Tn*10*	T. Patterson[b]
KT 211	As AB1157 plus Δ*ogt*::*cat*	M. Sekiguchi[c]
KT 222	As AB1157 plus (Δ(*ada-alkB*)::*kan*)	M. Sekiguchi
AB1886	As AB1157 plus *uvrA6*	B. J. Bachmann
AB1884	As AB1157 plus *uvrC34*	B. J. Bachmann
RF1020	As AB1157 plus *mutS201*::Tn*5*	R. Fishel[b]
ES1481	*lacZ53*(Am), λ$^-$, *mutS215*::Tn*10*, *thyA36*, *rha-5*, *metB1*, *deoC2*, IN(*rrnD-rrnE*)*1*?	B. J. Bachmann
JC10240	*thr-300*, λ$^-$, *recA56*, *srl-300*::Tn*10*, *relA1*, *rpsE2300* (Spc^R), *spoT1*, *ivl-318*, *thi-1*	B. J. Bachmann
S1540	F$^-$, Δ*lacX74*, *araD139*, Δ(*ara-leu*)*7697*, *galU*, *galK*, *hsdR*, *rpsL*, *dam*::*kan16*	S. Brown[b]

product strains	derivation	repair genotype
GP100	As AB1157 plus *lacZ*Δ*M15*, *proC*::Tn*10*	wild type (wt)
GP101	A spontaneous Tet^s mutant of GP100	wild type
GP102	As GP100 plus *proC*$^+$, Tet^s	wild type
GP112	As GP101 plus Δ*ogt*::*cat*	*ogt*$^-$
GP122	As GP101 plus (Δ(*ada-alkB*)::*kan*)	*ada*$^-$
GP132	As GP122 plus Δ*ogt*::*cat*	*ogt*$^-$-*ada*$^-$
GP140	As AB1886 plus *lacZ*Δ*M15*, *proC*::Tn*10*	*uvrA*$^-$
GP150	As AB1884 plus *lacZ*Δ*M15*, *proC*::Tn*10*	*uvrC*$^-$
GP160	As GP100 plus *mutS201*::Tn*5*	*mutS*$^-$
GP171	As GP101 plus *recA56*, *srl-300*::Tn*10*	*recA*$^-$
GP180	As GP102 plus *dam*::*kan16*	*dam*$^-$
GP181	As GP180 plus *mutS215*::Tn*10*	*dam*$^-$-*mutS*$^-$

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MATERIALS AND METHODS

Materials. All of the enzymes and reagents used in this study were as described in Pauly et al. (1991).

Plasmid Construction. The synthesis of the O^6-substituted guanine-containing oligonucleotides used in these studies and their incorporation into the plasmid pGP10 were described in detail previously (Pauly et al., 1991). In the experiments described here, G* (Figure 1C) is either a normal guanine or an m^6G, e^6G, or b^6G residue. Plasmids containing these nucleotides are designated pG:C, pm^6G:C, pe^6G:C, and pb^6G:C, respectively. Related plasmids containing a thymine in the complementary strand across from the normal guanine or O^6-substituted guanine in the initiation codon were designated pG:T, pm^6G:T, pe^6G:T, and pb^6G:T, respectively. For these constructions the sequence of the strand complementary to the ATG-containing strand was 5'-d(CTTGGGGCTATATG-GC)-3'. For experiments on mismatch correction, pGP10 was grown in the *dam*$^-$ strain GP180 (see below) to produce plasmids which lacked N^6-methyladenine residues (Parker & Marinus, 1988). Oligonucleotides containing either a normal guanine or an m^6G were ligated into the N^6-methyladenine-free pGP10 paired with either a cytosine or thymine residue in the complementary strand. The resulting plasmids were designated UpG:C, Upm^6G:C, UpG:T, and Upm^6G:T, respectively. The U designates that the plasmids were unmethylated (i.e. N^6-methyladenine-free).

Bacterial Strains. The bacterial strains used in this study and their sources are described in Table 1. The wild-type (wt) repair-competent strain used in these experiments was strain GP100 (Table 1) (Pauly et al., 1991). The repair-deficient strains included strains GP112, GP122, and GP132

(Table 1), which were deficient in the *ogt*, *ada*, or both the *ogt* and *ada* genes and were designated as having the *ogt*⁻, *ada*⁻, or *ogt*⁻-*ada*⁻ genotypes, respectively. Ogt is the constituitively expressed O^6-alkylguanine-DNA alkyltransferase of *E. coli* (Potter et al., 1987) while the Ada protein is an alkyltransferase induced in response to DNA alkylation (Lindahl et al., 1988). Both proteins reverse alkylation damage to the O^6 position of DNA guanines by transferring the alkyl group to an active-site cysteine residue. Strains GP140 (*uvrA*⁻, Table 1) and GP150 (genotype *uvrC*⁻) were each deficient in one of the three genes of the *uvr*(*ABC*) excision repair system that primarily excises UV-light-induced DNA photoproducts and bulky carcinogen–DNA adducts (Myles & Sancar, 1989). Strain GP171 (*recA*⁻) was deficient in the RecA protein, which is involved in DNA recombination and the regulation of the SOS response to DNA damage (Myles & Sancar, 1989). Strain GP160 (*mutS*⁻) (Table 1) was deficient in the MutS protein, which is involved in recognition and repair of mismatched bases in *E. coli* DNA (Modrich, 1991).

All of the product strains were derived from the parent AB1157 (De Witt & Adelberg, 1962). With the exception of GP140 and GP150 (designated genotype *uvrA*⁻ and *uvrC*⁻, respectively), the repair-deficient strains were derived by transfer of the indicated repair deficiency into the repair-competent strains GP100, GP101, or GP102 by P1 transduction (Silhavey et al., 1984). GP140 and GP150 were produced by cotransduction of *lacZΔM15* and *proC*::Tn*10* from GP100 into the excision repair-defective strains AB1884 (*uvrC34*) and AB1886 (*uvrA6*), which were both originally derived by mutagenesis of the parent strain AB1157 (Howard-Flanders et al., 1966). In agreement with their repair genotypes, GP140 (*uvrA*⁻), GP150 (*uvrC*⁻), and GP171 (*recA*⁻) were hypersensitive to ultraviolet (UV) light when compared to GP100 (Howard-Flanders et al., 1966; Howard-Flanders & Theriot, 1966). Sensitivity to UV light was determined by streaking samples of overnight cultures across an LB agar (Maniatis et al., 1982) culture dish in parallel lines. The cultures were then masked with aluminum foil except for the last centimeter of the culture streak. The plate was exposed to 254-nm light from a hand-held lamp (Mineralight Model UVGL-55) held directly over the plate for 2 s. The foil was then moved to expose another centimeter of culture streak, and the culture was exposed as above. This process was repeated across the plate. UV-light-sensitive strains were killed by even the shortest exposure to UV light while repair-competent strains grew completely across the plate.

Plasmids isolated from strain GP180 (*dam*⁻) were sensitive to digestion by *Mbo*I, which can only cut DNA lacking Dam methylation, but were completely resistant to *Dpn*I, which cuts only Dam-methylated DNA (Parker & Marinus, 1988).

GP160 (*mutS*⁻) and GP181 (*dam*⁻-*mutS*⁻) exhibited a high rate of spontaneous mutation to rifampicin resistance, a characteristic of *mutS* strains (Siegel et al., 1982). Increased spontaneous mutation was demonstrated by picking a single colony of a strain to be tested into LB broth and growing it overnight at 37 °C with aeration. One hundred microliters of the overnight culture was then spread onto an LB agar plate containing 150 μg/mL rifampicin and incubated overnight at 37 °C. Strains GP160 and GP181 generally produced from one hundred to several hundred colonies on rifampicin plates while GP100 and GP102 produced only a few colonies.

Strains GP122 (*ada*⁻) and GP132 (*ada*⁻-*ogt*⁻) showed increased sensitivity to the alkylating mutagen MNNG as judged by failure to grow across an MNNG gradient plate as described by Rebeck and Samson (1991). The *ogt* deficiency of GP112 (*ogt*⁻) and GP132 (*ogt*⁻-*ada*⁻) was assumed from the acquisition of chloramphenicol resistance following transduction, indicating that the *ogt* gene carrying the insertion had been transferred.

Bacterial Transformation. The *E. coli* strains described above were made competent for transformation by a modification of the procedure previously described in Pauly et al. (1991). Specifically, a 5-mL overnight culture of bacteria grown in LB broth was used to inoculate 5 mL of fresh NZYM broth (Maniatis et al., 1982) at a dilution of 100:1. The fresh culture was grown in a shaker at 37 °C for 2 h to an A_{600} of approximately 0.6. The culture was centrifuged at 1600*g* for 10 min at 4 °C in order to pellet the bacteria. The bacterial pellet was resuspended in 250 μL of ice-cold LB broth, and 250 μL of ice-cold 2× transformation and storage solution (Chung et al., 1989) was added. One hundred-microliter samples of cells were removed and added to tubes containing 1–2 ng of plasmid on ice. For experiments involving the N^6-methyladenine-free plasmids, 10 ng of DNA was used per transformation. The transformation reactions were kept on ice for 30 min, and then 900 μL of room-temperature LB broth was added to each tube. The cells were allowed to recover at 37 °C for 30 min, and then 100-μL samples of the transformation reaction were plated onto each of 10 plates containing media composed of "E" salts (Vogel & Bonner, 1956) supplemented with 0.1% glucose, 1 μg/mL thiamine hydrochloride, 100 μg/mL ampicillin, 50 μg/mL each L-threonine, L-leucine, L-proline, L-histidine, and L-arginine, 400 μM IPTG, and 300 μM X-gal in 1.5% agar. The plates were incubated for 2 or 3 days at 37 °C and an additional 2–3 days at room temperature. The plates were then scored for blue, white, and sectored (both blue-and-white) colonies. The slow growth of colonies on the media described above improves the color development in sectored colonies.

Identification of Mutations in Plasmids. Ten white and ten sectored colonies were picked from plates resulting from transformation of each of the strains GP100, GP112, GP122, GP132, GP140, GP150, GP171, and GP160 with each of the modified plasmids pm⁶G:C, pe⁶G:C, and pb⁶G:C. Pure white colonies were derived from the sectored colonies by restreaking for single colonies. Single white colonies were dispersed into LB broth and spotted onto nitrocellulose, allowed to grow overnight on LB agar, and then lysed and probed with ^{32}P-end-labeled 16-base oligonucleotides directed against the sequences ATG, ATA, ATT, and ATC in the *lacZ'* initiation codon of the plasmids as previously described (Pauly et al., 1991). Plasmid mutations which could not be identified by hybridization with the probes were analyzed by sequencing using a Δ*Taq* Cycle Sequencing Kit (US Biochemical Co., Cleveland, OH). Sixteen white and 11 sectored colonies were isolated from transformations with the plasmid pG:C. Plasmid DNA was isolated from the cells and sequenced using a Δ*Taq* Cycle Sequencing Kit.

RESULTS

The average percentage of sectored and white colonies produced by plasmids containing an O^6-methylguanine residue paired with cytosine (pm⁶G:C), an O^6-ethylguanine paired with cytosine (pe⁶G:C), or an O^6-benzylguanine paired with cytosine (pb⁶G:C) in various repair-deficient strains of *E. coli* is presented in Figure 2. The unmodified plasmid pG:C produced an average of only 0.1% sectored or white colonies in these strains. As indicated (Figure 2), when the repair-

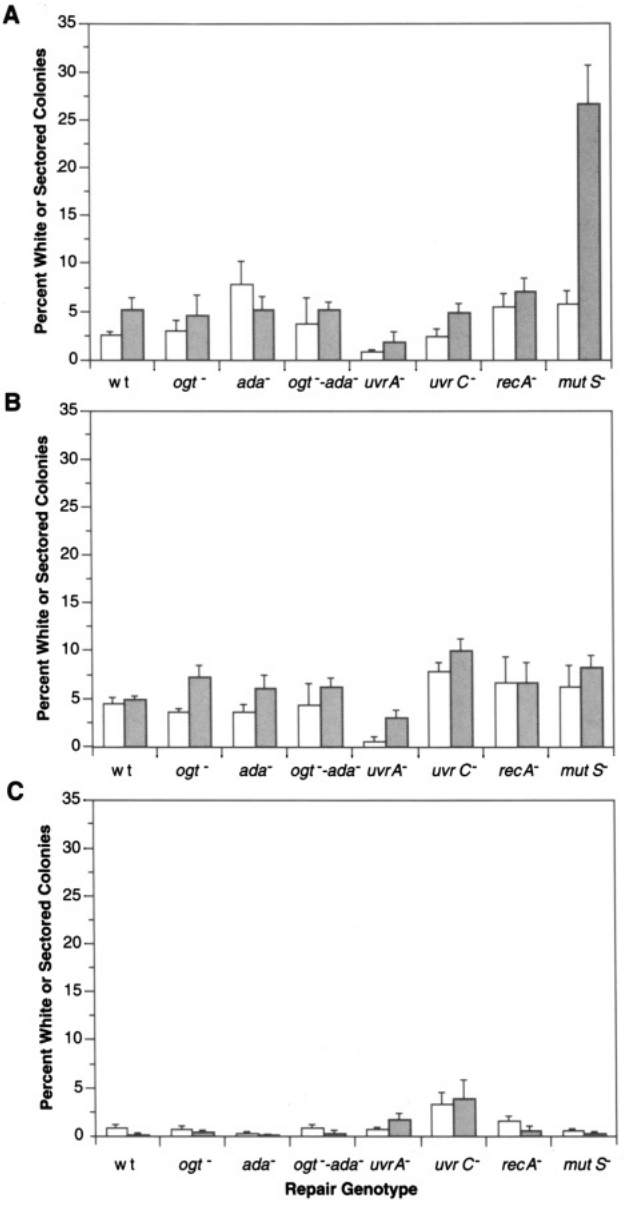

FIGURE 2: Percentage of white or sectored colonies produced in various strains of *E. coli* having the indicated DNA repair genotype following transformation with plasmids containing an O^6-methylguanine residue paired with cytosine (pm^{6}G:C) in the ATG initiation codon of the *lacZ'* gene (A), an O^6-ethylguanine residue paired with cytosine (pe^{6}G:C) in the initiation codon (B), or an O^6-benzylguanine residue paired with cytosine (pb^{6}G:C) in the initiation codon (C). The majority of colonies were blue in every bacterial strain. The data presented are the average of three or more experiments. Error bars represent the standard deviation. White colonies are indicated by the open bars, and sectored colonies are indicated by gray bars.

competent strain GP100 (wild type, wt) was transformed with the plasmid pm^{6}G:C, mutation resulted in the formation of about 5% sectored and 2.5% white colonies (Figure 2, panel A), which is similar to the results reported previously by Pauly et al., 1991. Significantly, when cells deficient in either one or both of the *E. coli* O^6-alkylguanine-DNA alkyltransferases (i.e., strains *ogt*$^-$, *ada*$^-$, or *ogt*$^-$-*ada*$^-$) were transformed with pm^{6}G:C, there was no significant change in mutation frequency from that observed in the wt strain. In the *uvrA*$^-$ strain we observed a small decrease in the frequency of m^{6}G-induced mutations, although this decrease was not seen in the *uvrC*$^-$ strain (Figure 2, panel A). A mutation in *recA* also had no significant effect on mutation induced by m^{6}G. There was, however, a significant increase in the frequency of sectored

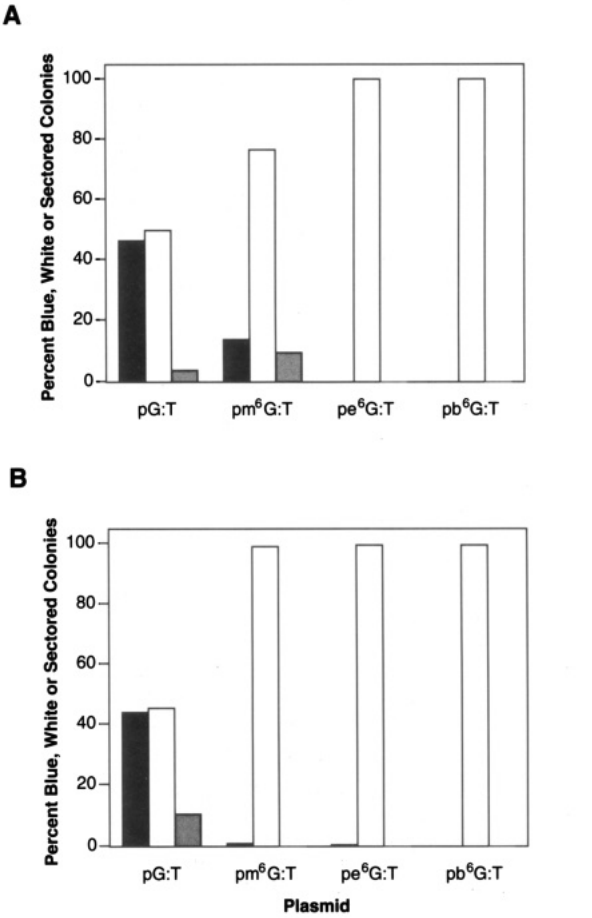

FIGURE 3: Percentage of blue, white, and sectored colonies produced in the wild-type strain GP100 (A) and the *ogt*$^-$-*ada*$^-$ strain GP132 (B) when transformed with plasmids pG:T, pm^{6}G:T, pe^{6}G:T, and pb^{6}G:T, respectively. The data are the results of one experiment. Blue colonies are indicated by the black bars, white colonies by the open bars, and sectored colonies by the gray bars.

colony formation in the strain deficient in the MutS protein, indicating that the *mutS* mismatch correction system recognized an O^6-methylguanine-containing base pair and acted to repair it. However, the frequency of white colony formation was not significantly increased in the *mutS*$^-$ strain.

Panel B of Figure 2 shows the results of transformation of the plasmid pe^{6}G:C, which contains an O^6-ethylguanine residue, into the same *E. coli* strains. The percentage of white and sectored colonies was similar in all strains. Significantly, the enhanced sectored colony formation exhibited by pm^{6}G:C in the *mutS*$^-$ strain was not observed with pe^{6}G:C. When the O^6-benzylguanine-containing plasmid pb^{6}G:C was transformed into the majority of these strains, only about 1% of the resulting colonies were sectored or white (Figure 2, panel C) although these percentages were slightly increased in the *uvrC*$^-$ strain.

O^6-Substituted-Guanine-Induced Strand Bias during DNA Replication. To test the possibility that the O^6-substituted guanines impeded replication of the modified strand while replication of the unmodified strand proceeded preferentially, we prepared plasmids containing guanine or an O^6-substituted guanine paired with a thymine residue in the complementary strand. These plasmids were designated pG:T, pm^{6}G:T, pe^{6}G:T, and pb^{6}G:T. The results of transformation of the wild-type strain GP100 with these plasmids are summarized in Figure 3A. Transformations with plasmid pG:T produced nearly equal amounts of blue and white colonies and relatively few sectored colonies. The data obtained following trans-

formation of GP100 with pm⁶G:T, pe⁶G:T, and pb⁶G:T (Figure 3A) indicate that the majority of colonies produced were white. This contrasts dramatically with the preponderance of blue colonies produced with plasmids pm⁶G:C, pe⁶G:C, and pb⁶G:C in this strain (Figure 2). This finding is consistent with a preferential replication of the unmodified strand in both cases but also suggests that these modified guanines are highly miscoding, since if even limited replication over these bases led to incorporation of a cytosine in the complementary strand, sectored or blue colonies would be produced in addition to white colonies. The blue and sectored colonies observed in the repair-competent GP100 following transformation by pm⁶G:T were likely a result of alkyltransferase repair of the O^6-methylguanine residue and subsequent replication over a normal guanine residue, since, when this plasmid was used to transform the *ogt*⁻-*ada*⁻ strain GP132 (Figure 3B), only white colonies were produced. Since plasmids pe⁶G:T and pb⁶G:T produced only white colonies in either strain, this indicates that alkyltransferase plays a far lesser role in repair of O^6-ethyl- and O^6-benzylguanine residues than O^6-methylguanine residues.

Recognition of O^6-Methylguanine-Containing Base Pairs by the E. coli Methylation-Directed Mismatch Repair System. Experiments were undertaken to further characterize the role of the *E. coli* mismatch repair system in processing O^6-methylguanine damage. In *E. coli*, it is known that mismatch repair correction in λ bacteriophage and associated phage viability are influenced by strand methylation. Methylation is carried out by the *dam* gene product at the N^6 position of adenine residues within the sequence GATC. Bacteriophage λ DNA isolated from *dam*⁻ *E. coli* is N^6-methyladenine-free. Such unmethylated DNA containing a mismatch transforms repair-competent *E. coli* less efficiently than the same DNA that is either hemi- or fully-methylated at Dam methylation sites (Doutriaux et al., 1986). The reduced transfection efficiency reflects lethal processing of the phage DNA by the mismatch repair system, which makes double-strand breaks in unmethylated DNA when a mismatch is detected (Doutriaux et al., 1986; Au et al., 1992).

We took advantage of this lethal processing to observe how viable an N^6-methyladenine-free version of our vector would be when it contained a mismatched base pair or m⁶G-containing base pairs. For these experiments, plasmid pGP10 was isolated from the *dam*⁻ strain GP180 (Table 1). This N^6-methyladenine-free pGP10 was then used to construct plasmids which carried a normal G:C base pair, a mismatched G:T base pair, or an m⁶G:C or m⁶G:T base pair. Parallel transformation reactions were carried out with approximately equal amounts of the four plasmids to transform either *dam*⁻ GP180 cells or GP181 cells which were both *dam*⁻ and *mutS*⁻. The number of colonies produced by transformation of either strain with the G:C-containing plasmid (UpG:C) was set to 100%, and the percentages of colonies produced with the other three plasmids, UpG:T, Upm⁶G:C, and Upm⁶G:T, were calculated.

The results of these experiments are shown in Figure 4. In strain GP180, which has a functional mismatch repair system, the mismatch-containing plasmid UpG:T produced only 9% as many colonies as plasmid UpG:C, which did not contain a mispair (Figure 4A). This is consistent with the expected lethal processing of our mismatch-containing plasmid by the undirected mismatch repair system (Doutriaux et al., 1986). Transformation of strain GP180 with Upm⁶G:C produced 91% of the colonies produced by UpG:C, indicating that an m⁶G:C base pair was not processed like the G:T mismatch.

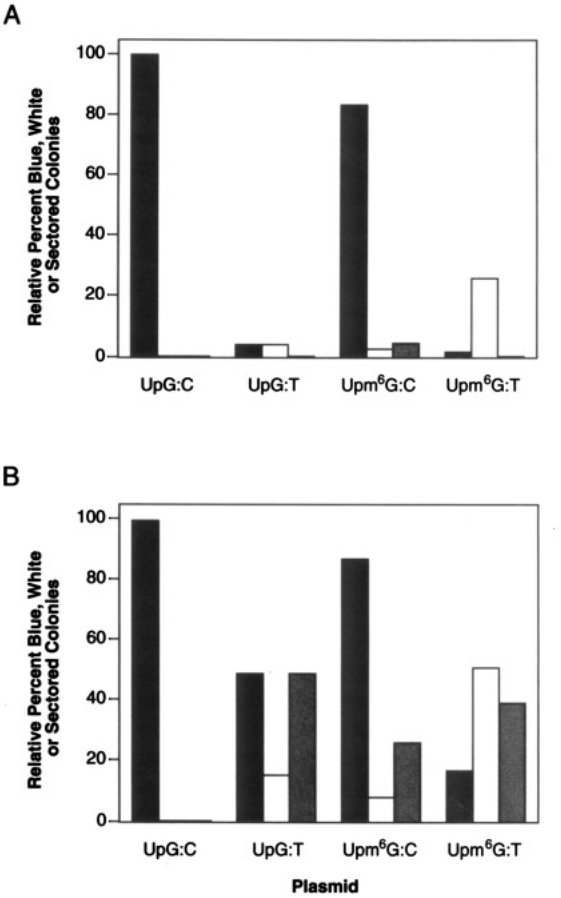

FIGURE 4: Percentage of blue, white, and sectored colonies produced in the *dam*⁻ strain GP180 (A) and the *dam*⁻-*mutS*⁻ strain GP181 (B) when transformed with N^6-methyladenine-free plasmids UpG:C, UpG:T, Upm⁶G:C, and Upm⁶G:T, respectively. The percentages reported represent the fraction of colonies produced relative to UpG:C, which was set to 100%. The data in panel A are the average of five experiments while the data in panel B are the average of three experiments. Blue colonies are indicated by the black bars, white colonies by the open bars, and sectored colonies by the gray bars. The U designates that the plasmids are unmethylated (i.e. N^6-methyladenine-free).

In contrast, plasmid Upm⁶G:T produced only 28% of the colonies produced by the control plasmid, indicating that the m⁶G:T base pair is recognized and processed as a mismatched base pair more readily than an m⁶G:C base pair, although not as efficiently as an authentic G:T mismatch. Thus, the *mutS* system clearly recognized (and lethally processed) both G:T and m⁶G:T base pairs. Transformation of GP181 (*dam*⁻-*mutS*⁻) with the four plasmids yielded similar numbers of colonies, regardless of the plasmid used (Figure 4B).

The colonies produced following transformation of strain GP180 with UpG:C were all blue, as expected (Figure 4A). Transformation with the mismatch-containing UpG:T plasmid produced equal numbers of blue and white colonies although the total number of colonies in this case was very low. This indicates that, for those plasmids that escape lethal processing by the methylation-directed mismatch repair system, correction utilized either strand as a template with equal frequency prior to plasmid replication. Transformation of GP180 cells with Upm⁶G:C produced mainly blue colonies together with the same percentage of white and sectored colonies that were produced in the wild-type strain GP100 with the fully methylated plasmid pm⁶G:C (Figure 2A). This is in spite of the fact that the *dam*⁻ GP180 strain should exhibit increased expression of SOS genes (Peterson & Mount, 1993), suggesting that induced SOS has little or no effect on the mutagenicity

Table 2: Mutations Identified in White and Sectored Colonies Resulting from Transformation with Unmodified and Carcinogen-Modified Plasmids

ligation site — initiation codon — ligation site

5′-G A A$_{-11}$ A$_{-10}$ C$_{-9}$ A G$_{-7}$ C C A T A T$_{-1}$ G$_0$ G C C C$_4$ C A A G C T-3′[a]

		mutations							
colony	plasmid	$G_0 \rightarrow A$	$-G_{0,1}$	$-C_{2\rightarrow5}$	$T_{-1} \rightarrow C$	$-G_{-7}$	$-(A_{-11}A_{-10}C_{-9})$	host mutation	wt ±30
white	pG:C	0	4	5			1	5	1
	pm^{6}G:C	76		2					2
	pe^{6}G:C	78	1						1
	pb^{6}G:C	67	3	7		1		1	1
sectored	pG:C	0	2	5				1	3
	pm^{6}G:C	76	2	2					
	pe^{6}G:C	78		2					
	pb^{6}G:C	64	5	8	1			2	

[a] Base sequence surrounding the ATG initiation codon.

of m^{6}G residues. The majority of colonies produced by Upm^{6}G:T plasmids that escaped lethal processing were white (Figure 4A), suggesting either that the mismatch repair system preferentially resolved the m^{6}G:T mismatch by using the unmodified thymine-containing strand as template prior to replication (which contrasts with data for UpG:T) or that the thymine-containing strand was replicated preferentially.

Figure 4B shows the phenotypes of colonies resulting from transformation of the *dam*$^-$-*mutS*$^-$ strain GP181. Again, transformation with the plasmid UpG:C produced only blue colonies while plasmid UpG:T produced equal amounts of blue and sectored colonies in preference to white colonies. These results are different from those obtained with the GP180 strain (Figure 4A) and probably reflect the differences in mismatch correction brought about by the MutS protein in strain GP180 compared to the cases of the MutS-independent mismatch repair mechanisms that operate in strain GP181 (Fishel & Kolodner, 1983). Colony distribution observed after transformation with Upm^{6}G:C in strain GP181 is very similar to that observed for transformation of the fully methylated plasmid pm^{6}G:C in the *dam*$^+$-*mutS*$^-$ strain GP160 (Figure 2A). Strain GP181, like strain GP180, should also exhibit increased SOS expression. These data provide further evidence for the lack of SOS involvement in enhancing m^{6}G mutagenicity. Additionally, strains GP181 and GP160 carry different alleles of *mutS* although both process m^{6}G:C base pairs similarly. The colony distribution after transformation with Upm^{6}G:T in strain GP181 reflects the complex processing of an m^{6}G:T mismatch by the repair systems not involving *mutS*.

Identification of Mutations. White and sectored colonies were isolated after transformation of all of the test strains of Figure 2 with each of the modified plasmids. Sectored colonies were restreaked in order to isolate pure white clones. The plasmids carried by these cells were then characterized by a combination of oligonucleotide hybridization and direct sequencing. The results of these experiments are shown in Table 2. As indicated, the predominant mutation found in both white and sectored colonies resulting from transformation with any of the three O^6-substituted guanine-containing plasmids was a G-to-A transition mutation at the site of the O^6-substituted guanine ($G_0 \rightarrow A$) (Table 2). This mutation was not seen in cells transformed with the control plasmid pG:C. Rare mutations resulting from transformation with pb^{6}G:C included a deletion of the guanine at position −7 ($-G_{-7}$) (Table 2) and a T-to-C transition at T_{-1} ($T_{-1} \rightarrow C$) (Table 2). Rare mutations seen in colonies derived from both modified guanine-containing plasmids and the control pG:C plasmid included a deletion of the G in the initiation codon or the adjacent downstream G ($-G_{0,+1}$) (Table 2), a deletion of a C from the set of four between positions +2 and +5 downstream from the G of the ATG codon ($-C_{2\rightarrow5}$) (Table 2), or the loss of the AAC triplet at positions −9 through −11 [$-(A_{-11}A_{-10}C_{-9})$]. Reasons for loss of G_0 or G_{+1} are unclear although the $-C_{2\rightarrow5}$, $-(A_{-11}A_{-10}C_{-9})$, and $-G_{-7}$ mutations could be the result of errors produced during ligation of synthetic oligonucleotides into plasmid pGP10.

Mutations described as host mutations (Table 2) result from a loss of *lacZ*-complementation in the transformed host cells and not from a mutation in the ATG initiation codon of the harbored plasmids. Mutations indicated as wt ±30 are mutations in the harbored plasmids which produce a Lac$^-$ phenotype but which lie outside a ±30 base-pair sequence flanking the ATG initiation codon. The source of these host cell or wt ±30 mutations is not known. In any event, these relatively infrequent background mutations represent a small fraction of the total mutations identified in experiments with pm^{6}G:C and pe^{6}G:C. Since the frequency of adduct-induced mutations produced by pb^{6}G:C was significantly lower, these background mutations represented a higher proportion of the total mutations observed with this plasmid. We did not observe any significant difference in the types of mutations identified in white versus sectored colonies. Similarly, there was no particular type of background mutation associated with any individual repair-deficient strain.

DISCUSSION

Repair of O^6-substituted guanine residues in *E. coli* DNA is known to involve several repair systems. The most studied of these is the reaction with the two O^6-alkylguanine-DNA alkyltransferase proteins Ogt and Ada (Potter et al., 1987; Lindahl et al., 1988; Myles & Sancar, 1989; Rebeck & Samson, 1991; Pegg & Beyers, 1992). The *uvr(ABC)* excision repair system also plays a significant role, particularly with bulky O^6-alkylguanine adducts (Todd & Schendel, 1983; Chambers et al., 1985, 1988; Van Houten & Sancar, 1987; Samson et al., 1988; Myles & Sancar, 1989; Chambers, 1993), and evidence has been presented that the *E. coli* nucleotide mismatch repair system contributes to the avoidance of mutations induced by m^{6}G (Eadie et al., 1984; Rossi & Topal, 1991). Unfortunately, site-specific mutagenesis studies in repair-deficient *E. coli* strains have often failed to provide consistent confirmation for the involvement of these systems.

With respect to alkyltransferase repair, Rossi et al. (1989) and Rossi and Topal (1991) observed an unexpected lower frequency of O^6-methylguanine-induced mutation in *ada*$^-$ cells than in an isogenic *ada*$^+$ strain. In contrast, Bhanot and Ray

(1986) and Dosnajh et al. (1991) observed an increase in O^6-methylguanine-induced mutations in *ada*⁻ strains relative to *ada*⁺ strains. We noted no significant effect of *ada* competence on the frequency of mutations induced by any of the three O^6-substituted guanines we studied. Our experiments in *ogt*⁻ strains are the first to be presented. The results failed to reveal any significant influence of Ogt repair on mutation by any of the O^6-substituted guanines we studied using either an *ogt*⁻ strain or an *ogt*⁻-*ada*⁻ strain.

With respect to studies in *uvr(ABC)*-deficient *E. coli*, Chambers et al. (1985) and Chambers (1993) observed a decrease in mutation frequency induced by an O^6-methylguanine in a *uvrA*⁻ strain compared to a repair-competent strain. Rossi and Topal (1991), however, using an *E. coli* strain carrying a different *uvrA* allele did not observe a consistent difference in the mutagenicity of O^6-methylguanine residues in *uvrA*-competent or -deficient cells although they noted a variation in the modified guanine's mutagenicity as a function of sequence context. Like Chambers et al. (1985) and Chambers (1993), we also noted a reduction in mutation induced by O^6-methylguanine residues in a *uvrA*⁻ strain relative to a *uvrA*-competent strain. We noted a similar effect with O^6-ethylguanine residues. Chambers et al. (1985) and Chambers (1993) saw a dramatic increase in mutation by a bulky O^6-*n*-butyl-substituted guanine in a *uvrA*⁻ strain compared to a normal strain. However, with a bulky O^6-benzylguanine residue, we noted only a very slight increase in mutagenicity in our *uvrA*⁻ strain relative to the wt strain. Since our results in the *uvrC*⁻ strain differ from those in the *uvrA*⁻ strain, it is difficult to conclude with assurance that defects in the *uvr(ABC)* excision repair system significantly affect the mutagenicity of any of the modified guanines we studied.

Finally, Chambers et al. (1985) noted that the mutation frequency for O^6-methylguanine was decreased in a *recA*⁻ strain relative to a wild-type strain but that bulky O^6-*n*-butylguanine-induced mutations increased. We failed to detect any significant effect of *recA* deficiency on mutation regardless of the size of the O^6 substituent.

It has generally been assumed in site-specific mutagenesis studies that the commonly observed low frequencies of adduct-induced mutations result from efficient repair of the adduct. This seems an unlikely explanation for the majority of our results since, with the exception of increased mutations by m^6G residues in a *mutS*⁻ strain, we observed no significant enhancement in the mutagenicity of the three O^6-substituted guanines in bacterial strains deficient in repair systems known to repair these bases. It is also unlikely that the low levels of mutation we observed were a result of an intrinsically low miscoding potential for these bases, since several *in vitro* studies have demonstrated that O^6-substituted guanine residues code for thymine incorporation as well or better than cytosine incorporation using a variety of procaryotic and eucaryotic DNA polymerases (Singer et al., 1989; Singer & Dosanjh, 1990). On the other hand, an adduct-induced blockage to replication of the adduct-bearing strand such that replication of the unmodified strand occurs preferentially seems the most likely explanation for our results and probably makes a major contribution to the low levels of mutation observed by others. This would be consistent with the adduct-induced blockage to modified strand replication noted for bulky DNA adducts such as those derived from acetylaminofluorene (Koffel-Schwartz et al., 1987) or O^6-*n*-butylguanine (Chambers, 1988) as well as for O^6-methylguanine in a phage system (Dosanjh et al., 1991) and in several *in vitro* replication systems (Voigt & Topal, 1992, 1993; Dosanjh et al., 1993; Ceccotti et al., 1993; Snow et al., 1984).

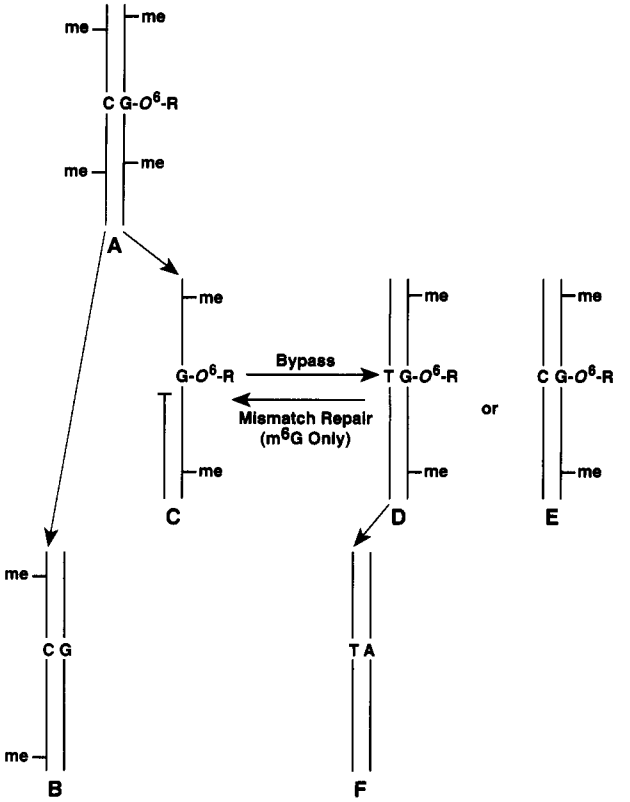

FIGURE 5: Schematic for the replication of carcinogen-modified plasmids.

Figure 5 provides a model for the mechanisms contributing to our experimental results. In the absence of repair, replication of the individual strands of a fully methylated O^6-substituted-guanine-containing plasmid (A, Figure 5) in repair-competent *dam*⁺ *E. coli* can produce several outcomes. First, replication of the cytosine-containing complementary strand will produce a *lac*⁺ viable plasmid indicated by B (Figure 5), which differs from a normal methylated plasmid (i.e. pG:C) in that the newly synthesized G-containing strand is temporarily not *dam* methylated. On the other hand, at least three outcomes of replication over the strand containing an O^6-substituted guanine residue are possible. If the O^6-substituted guanine significantly retards or arrests polymerase replication of the modified strand, a complex such as C would be produced initially. This homolog of the plasmid would be lost unless the adduct were repaired by reaction with alkyltransferase, for example, or if eventual base incorporation permitted bypass of the modified guanine. Miscoding for thymine incorporation would produce a plasmid D while incorporation of cytosine would lead to plasmid E. The latter plasmid resembles the initially transformed plasmid except it is temporarily hemi-methylated. Its processing during subsequent rounds of replication would resemble that for the starting plasmid A. Plasmid D, however, is a promutagenic plasmid. Subsequent replication of its thymine-containing complementary strand would produce mutant plasmids F containing a G-to-A transition mutation at the site of the original modified guanine residue. These plasmids would be *lac*⁻. Bacteria harboring both plasmids B and F would eventually produce sectored colonies. Blue colonies result when progeny plasmid B is produced in preference to F, as is the case when the path to plasmid F is blocked by the adduct in complex C. On the other hand, if formation of plasmid B is

somehow aborted while formation of plasmids D and F occurs, then plasmid F will be produced preferentially, leading to a white colony.

The phenotypic outcome of our transformation experiments suggests that the impediment to strand replication imposed by all three O^6-substituted guanines is quite significant, since when plasmids of type A are used to transform bacteria, progeny of type B (Figure 5) predominate and the majority of colonies are blue. Inhibition of modified strand replication in an intermediate such as C is likely responsible for blocking production of plasmids of type D and F. Furthermore, when fully-methylated or -unmethylated plasmids of type D are introduced intentionally, formation of progeny F by preferential replication of the thymine-containing complementary strand produces almost exclusively white colonies. The formation of sectored colonies following introduction of plasmids of type D requires alkyltransferase repair of the modified guanine in D, or following conversion of D to C during modified strand replication, a cytosine would have to be inserted across from the modified guanine as in plasmid E.

Our mismatch repair studies indicated that an m^{6}G:T pair is more readily processed than an m^{6}G:C pair (Figure 4), indicating that mismatch repair provides a line of defense against mutation by O^6-methylguanine damage after it has coded for thymine incorporation. At short times after replication, the resulting m^{6}G:T pair would be present in a hemi-methylated plasmid as illustrated by D in Figure 5. Consequently, the methylation-directed mismatch correction system of *E. coli* (Modrich, 1991; Myles & Sancar, 1989) would be expected to excise a portion of the thymine-containing strand, leading again to a replication-inhibited intermediate C during resynthesis of the excised sequence. Reinsertion of another thymine residue in the newly formed complement would produce a new substrate D for an additional round of mismatch correction. This results in a cycling of plasmids D to complex C (Figure 5) which is lethal to the plasmid. The cycle would be expected to continue until halted by *Dam* methylation of the thymine-containing strand. In our assay system, mismatch repair cycling of D to C in wild-type *E. coli* no doubt contributed to strand bias during replication of m^{6}G-containing plasmids. The greater proportion of sectored colonies produced by these plasmids in the *mutS*$^-$ strains resulted from an elimination of the *mutS*-mediated lethal cycling of plasmids D to C and allowed formation of type F plasmids. This produced an apparent "tolerance" to the genotoxicity of an m^{6}G residue in the modified strand at the expense of increased mutations.

Eadie et al. (1984) proposed that mismatch correction contributed to the avoidance of O^6-methylguanine-induced mutations by resolving G:T mismatches which resulted from alkyltransferase action on m^{6}G:T base pairs following replication of a damaged template. Our results show that the m^{6}G:T base pair is itself a target for mismatch correction independent of alkyltransferase repair. Additionally, our data support the suggestion made by Karran and Marinus (1982) that, in the absence of *dam* methylation, undirected attempts to repair m^{6}G:C or m^{6}G:T pairs could be lethal, although we have observed this to be true only for m^{6}G:T pairs. Rossi and Topal (1991) previously implicated mismatch correction by *MutS* in the processing of O^6-methylguanine-containing plasmids, but a clear interpretation of their results was complicated by the incorporation of an authentic mismatched base pair near the site of the O^6-methylguanine residue in their plasmid vector.

Several groups have implicated mismatch correction in human cells as being responsible for the toxicity exhibited by alkylating mutagens and carcinogens such as MNNG. Goldmacher et al. (1986), Roberts and Basham (1990), and Karran and Bignami (1992) proposed that m^{6}G:T pairs could be acted on by a repair system similar to the methyl-directed mismatch repair system of *E. coli* (Holmes et al., 1990; Thomas et al., 1991) to remove a sequence of bases in the thymine-containing strand near the modified guanine in the complement. Unresolved attempts to remove thymine residues could lead to accumulation of repair tracts in the unadducted strand and interfere with subsequent DNA replication while a mutation in the mismatch repair system would prevent this cycle. This is consistent with the observations that cells which are resistant to the toxic effects of methylating carcinogens such as MNNG lack specific mismatch repair activities and are hypermutable by this compound (Goldmacher et al., 1984; Branch et al., 1993; Kat et al., 1993). We have provided a model which explains these results and demonstrates that mismatch repair makes a significant contribution to the redundancy of repair mechanisms that operate to correct O^6-methylguanine damage in *E. coli*. It is of interest that a mutation in human mismatch repair has recently been linked to a predisposition to colon carcinogenesis (Fishel et al., 1993; Leach et al., 1993; Parsons et al., 1993).

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