Mutagenesis in *Escherichia coli* by Three O^6-Substituted Guanines in Double-Stranded or Gapped Plasmids†

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ABSTRACT: Plasmids were constructed with guanine (G) or O^6-methyl- (m^6G), O^6-ethyl-(e^6G), or O^6-benzyl- (b^6G) guanine in the initiation codon (ATG) of the *lacZ′* gene. Four deoxyuridine residues were incorporated near the modified guanine in the complementary strand. The deoxyuridine-containing plasmids exhibited similarly high transformation efficiencies in *ung⁻ Escherichia coli*, although the frequency of mutations induced by m^6G, e^6G, and b^6G residues was relatively low. Treatment of the plasmids with uracil-DNA glycosylase (UDG), to remove the uracil residues, or UDG and exonuclease III, to create a gap in the deoxyuridine-containing strand, reduced transformation efficiency for adduct-containing plasmids but did not affect transformation efficiency for control plasmids. However, the same treatments dramatically enhanced mutagenesis by m^6G, e^6G, and b^6G. These results were consistent with blockage of replication by the modified guanines in double-stranded plasmids resulting in preferential replication of the complementary strand. Replication past the modified guanines was forced in the gapped plasmids. The frequency of modified guanine-induced mutations in gapped vectors was similar in strains of *E. coli* that were proficient in DNA polymerase III but deficient in either DNA polymerase I or II or both polymerase I and II suggesting either that polymerase III was primarily responsible for adduct bypass in all strains or that the probability of base misinsertion during bypass by either polymerase I or II was similar to that for polymerase III. Repair studies with gapped plasmids indicated that m^6G was subject to repair by Ada methyltransferase and to postreplication processing by methylation-directed mismatch repair. Neither e^6G nor b^6G were similarly repaired. Both m^6G and e^6G efficiently coded for thymine incorporation although b^6G appeared to be less miscoding.

We recently reported the results of studies of mutagenesis in *Escherichia coli* by three O^6-substituted guanines [i.e., O^6-methylguanine (m^6G), O^6-ethylguanine (e^6G), and O^6-benzylguanine (b^6G)] incorporated site specifically within the ATG initiation codon of the *lacZ′* gene in double-stranded plasmids (Pauly et al., 1991, 1994). Low levels of mutation were induced by these adducts in both wild type and repair deficient strains. The low levels of mutation were interpreted as resulting from partial blockage of replication of the modified guanine-containing strand while the unmodified strand was replicated normally (i.e., strand bias) (Pauly et al., 1994). Blockage of replication leading to strand bias has been previously demonstrated for bulky adducts (Koffel-Schwartz et al., 1987; Naser et al., 1988; Chambers et al., 1988) but was not altogether expected for less obtrusive adducts like O^6-methylguanine. Low levels of O^6-substituted guanine-induced mutations (Loechler et al., 1984; Chambers et al., 1985, 1988; Hill-Perkins et al., 1986; Bhanot & Ray, 1986; Rossi et al., 1989; Rossi & Topal, 1991; Pauly et al., 1991, 1994; Dosanjh et al., 1991; Chambers, 1991, 1993; Baumgart et al., 1993) have often been attributed to rapid repair of the modified base rather than to strand bias effects. However, m^6G has been shown to inhibit or arrest the progression of several DNA polymerases in *in vitro* primer extension experiments (Snow et al., 1984; Singer et al., 1989; Singer & Dosanjh, 1990; Voigt & Topal, 1992, 1993; Dosanjh et al., 1993; Ceccotti et al., 1993), and Dosanjh et al. (1991) noted a decrease in the number of progeny produced by a single-stranded M13 vector carrying an m^6G residue compared to an unmodified vector in *E. coli*.

To further assess the role of modified base-induced strand bias in our system, we constructed plasmids that position an O^6-substituted guanine across from a gap in the complementary strand. Other laboratories have used this approach to enhance the mutagenicity of modified bases (Moriya et al., 1991; Palejwala et al., 1991, 1993, 1994). Single-stranded vector systems have also been used for this purpose (Banerjee et al., 1988; Lawrence et al., 1990; Dosanjh et al., 1991; Moriya, 1993; Moriya et al., 1994; Latham et al., 1993; Basu et al., 1993). Both systems force replication past a modified base. With such systems, a reduced efficiency of transformation with an adduct-containing vector reflects the genotoxicity of the modified base resulting from replication blockage, while the frequency of mutations reflects the inherent miscoding potential of the modified base. In this report we compare these effects produced by the increasingly bulky O^6-substituted guanines, m^6G, e^6G and b^6G, in both double-stranded and gapped plasmids. The data confirm that all three modified bases produce a significant impediment to DNA replication and also show that miscoding potency and repair mechanisms are quite different for the different adducts.

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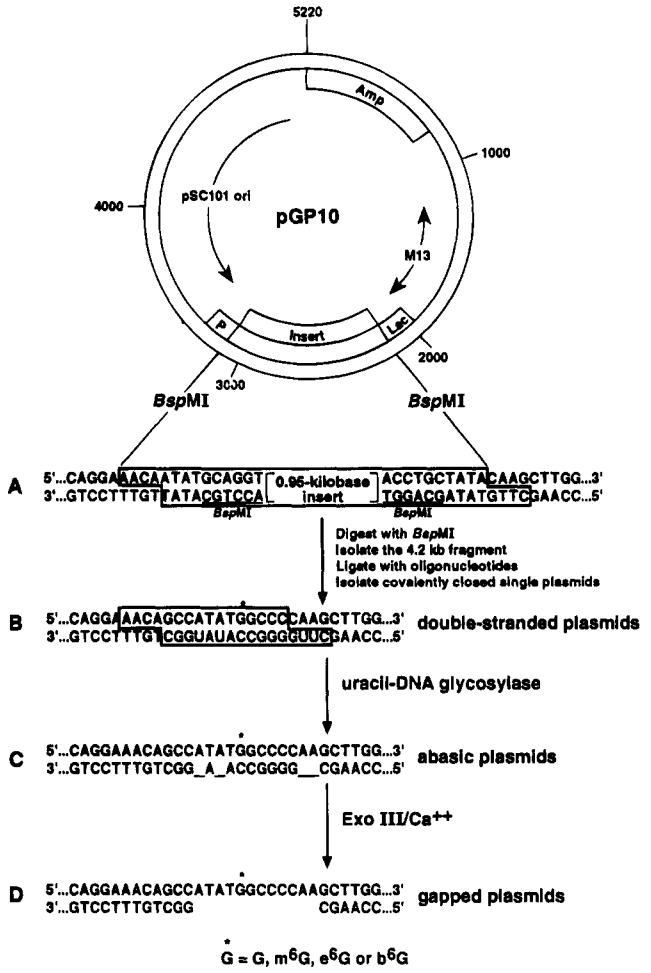

FIGURE 1: Characteristics of the cassette plasmid pGP10 and a schematic for the incorporation of guanine or an O^6-substituted guanine into double-stranded, abasic, or gapped plasmids.

MATERIALS AND METHODS

Materials. All of the enzymes and reagents used in this study were as described in Pauly et al. (1991, 1994) unless specified below.

Plasmid Construction. The synthesis of the O^6-substituted guanine-containing oligonucleotides used in these studies and the preparation of plasmid pGP10 was described in detail previously (Pauly et al., 1991). For the present experiments, pGP10 was digested with *Bsp*MI to remove the DNA sequences enclosed by the box illustrated in Figure 1A. The major fragment produced by *Bsp*MI digestion was ligated to a synthetic oligonucleotide duplex composed of a guanine-containing or an O^6-substituted guanine-containing strand plus a complementary strand containing four deoxyuridine residues, i.e., 5′-d(CUUGGGGCCAUAUGGC)-3′. This produced a covalently closed, circular, double-stranded plasmid containing the sequences enclosed by the box in Figure 1B.

To create abasic sites at positions occupied by deoxyuridine residues, the double-stranded plasmids (66 ng) were treated with 10 U of *E. coli* uracil-DNA-glycosylase (UDG) (Life Technologies, Gaithersburg, MD) in 100 μL of 50 mM Tris-HCl, pH 8.0/10 mM EDTA/10 mM β-mercaptoethanol/15 mM $CaCl_2$ and 100 μg/mL bovine serum albumin at 37 °C for 90 min. This produced abasic plasmids with sequences in the vicinity of the ATG initiation codon illustrated in Figure 1C. To create gapped plasmids, abasic plasmids were subsequently treated with 500 U of exonuclease III (Exo III) (New England Biolabs, Beverly, MA) in the above buffer for 30 min at 37 °C. Calcium in the buffer allows Exo III to function as an AP endonuclease while inhibiting its exonuclease activity (Rogers and Weiss, 1980). This results in the formation of a gap in the complementary strand across from the ATG initiation codon as illustrated in Figure 1D. Completion of this reaction was confirmed by observing the conversion of covalently closed abasic plasmids (form I′ DNA) to nicked circular plasmids (form II DNA) resulting from the creation of the gap (see below). The G* in these various plasmids represents either a guanine residue or an m^6G, e^6G, or b^6G residue.

Plasmids treated with UDG or UDG and Exo III were extracted with phenol−chloroform−isoamyl alcohol (25:24:1) followed by chloroform, precipitated with ethanol, and briefly air-dried. Following resuspension in TE buffer, the amount of DNA in each sample was determined by fluorescence spectroscopy using H33258 fluorochrome (Gallagher, 1988).

Bacterial Strains. All of the *E. coli* strains were derived from the parent strain AB1157 (De Witt & Adelberg, 1961). Strains GP102 (wild type for DNA repair), GP112 (*ogt*$^-$), GP122 (*ada*$^-$), GP132 (*ada*$^-$-*ogt*$^-$), GP160 (*mutS*$^-$), and GP171 (*recA*$^-$) have been described previously (Pauly et al., 1994). Additional strains were derived by P1 transduction (Silhavey et al., 1984). GP190 (*ung*$^-$) was derived by transduction of *ung152*::Tn10 from BD2314 (Duncan, 1985) into GP102. GP113 (*ogt*$^-$) was derived by transduction of *ogt1*::Kan^r from GWR107 (Rebeck & Samson, 1991) into GP102. GP123 (*ada*$^-$) was derived by transduction of Δ*ada25*::Cm^r from GW7101 (Shevel et al., 1988) into GP102. GP133 (*ada*$^-$-*ogt*$^-$) was derived by transduction of Δ*ada25*::Cm^r into GP113. GP210 (*uvrB*$^-$) was derived by cotransduction of *uvrB5* and a closely linked Tn10 from LS10 [referred to as *ada*$^+$ *uvrB*$^-$ in Samson et al. (1988)] into GP102. GP220 (*ada*$^-$-*ogt*$^-$-*mutS*$^-$) was derived by transduction of *mutS* from ES1481 (Siegel et al., 1982) into GP133.

DNA polymerase I (Pol I) of *E. coli* is encoded by the *polA* gene. A *polA*$^-$ strain, GP200, was constructed by transduction of Δ*polA*::Kan^r from CJ225 [Δ*polA*::Kan^r, F′(*polA*$^+$,Cm^r)] (Joyce & Grindley, 1984) into GP102. This strain grew well on minimal media but died upon transfer to rich media as is characteristic of *polA*$^-$ strains. Attempts to make electroporation competent cells from any of our strains grown in minimal media were not successful. We therefore transferred an F′ carrying the 5′−3′ exonuclease activity of *polA* from CJ231 [Δ*polA*::Kan^r, F′(5′exo, Cm^r)] (Joyce & Grindley, 1984) into GP200 in order to create GP201. Although GP201 does not express the polymerase domain of Pol I (the Klenow fragment), expression of the 5′−3′ exonuclease domain of Pol I is sufficient to allow growth in rich broth so that competent cells could be prepared. The *E. coli polB* gene encodes DNA polymerase II (Pol II). A *polB*$^-$ strain, GP230, was constructed by transduction of Δ*araD-polB*::Str^r/Spec^r from SH2101 [referred to in Kow et al. (1993)] into GP102. A *polA*$^-$-*polB*$^-$ double-mutant strain, GP240, was constructed by transduction of the above *polB* deletion into GP200. The F′(5′exo, Cm^r) was transferred into GP240 to give GP241 to permit growth in rich broth.

Bacterial Transformations. Electroporation competent cells were prepared by adding 1 mL of a saturated overnight culture in LB broth (Maniatis et al., 1982) into 100 mL of fresh LB broth with incubation and shaking for 3 h at 37 °C. The culture was cooled on ice for 10 min and then centrifuged at 1600*g* for 15 min at 4 °C. The media were decanted, and the cells were resuspended in 100 mL of deionized water at 4 °C. The suspended cells were again centrifuged for 15 min, the water was decanted, and the cell pellet was resuspended in 50 mL of cold deionized water. The cells were pelleted again, resuspended in 5 mL of ice cold 10% glycerol in deionized water (2 mL for the two *polA* strains), and quick frozen on dry ice in 500 μL batches.

Electroporation competent cells were thawed on ice, 100 μL samples were transferred to prechilled tubes, and 1 μL of plasmid DNA (100 pg) was added. The cells and plasmids were transferred to a 0.1 cm gap electroporation cuvette and pulsed at 1.8 kV in an *E. coli* pulser apparatus (Bio-Rad Laboratories, Melville, NY). Following shocking, 900 μL of room temperature LB broth was added, and the cells were transferred to culture tubes and incubated for 30 min at 37 °C. The 1 mL reaction mixture was split onto 10 media plates containing X-gal and IPTG and incubated as previously described (Pauly et al., 1994). The plates were then scored for blue, white, and sectored (both blue and white) colonies.

Identification of Mutations. Abasic or gapped plasmids containing either m^{6}G, e^{6}G, or b^{6}G were used to transform the repair competent strain GP102. Fifty white colonies were picked from each transformation. These colonies were spotted onto nitrocellulose filters and probed with ^{32}P-end labeled 16-base oligonucleotide probes directed against G, A, T, or C at the site of the O^6-modified guanine, as previously described (Pauly et al., 1991). A single mutant plasmid not identified by hybridization was isolated and sequenced.

RESULTS

The principal aim of this study was to compare the mutagenicity of O^6-methyl-, O^6-ethyl-, and O^6-benzylguanine when the modified base was incorporated into either a double-stranded or gapped plasmid vector. To accomplish this, double-stranded plasmids were constructed containing a guanine or a modified guanine across from a complementary sequence containing four deoxyuridine residues as illustrated in Figure 1B. These double-stranded plasmids exhibited electrophoretic mobilities through 0.8% agarose indicative of covalently closed plasmids (form I′ DNA) together with a small amount of nicked material (form II DNA) (Figure 2, lanes 2−5). When these plasmids were treated with UDG to create abasic plasmids (Figure 1C), the covalently closed circular form (form I′) still predominated (Figure 2, lanes 6−9). However, treatment of double-stranded plasmids with UDG to create abasic sites followed by Exo III to produce nicks at these sites resulted in the quantitative conversion of the double-stranded plasmids to the nicked form (Figure 2, lanes 10−13) consistent with formation of gapped plasmids (Figure 1D).

Equal amounts of deoxyuridine-containing double-stranded plasmids, UDG-treated abasic plasmids, or gapped plasmids containing either guanine or an m^{6}G, e^{6}G, or b^{6}G were used to transform the *ung*$^-$ strain GP190 (Materials and Methods) in parallel electroporations. Table 1 shows the percentage of colonies produced by these plasmids. As indicated, all of the double-stranded plasmids produced similar numbers of colonies, indicating that as long as one strand of the plasmid was adduct free, plasmid viability was high. There was also no effect on transformation efficiency when the double-stranded guanine-containing plasmid was treated with UDG or UDG and Exo III indicating that, in the absence of an O^6-modified guanine, abasic or gapped plasmids were efficiently repaired and replication of the unmodified guanine-containing strand was not inhibited. However, for the modified guanine-containing abasic or gapped plasmids, there were notably fewer colonies produced compared to the number produced by the control plasmid. This reduction in transformation efficiency indicated that all three O^6-substituted guanines produced a significant impediment to DNA replication.

Because the O^6-modified guanines in these plasmids were positioned within the ATG initiation codon of the *lacZ′* gene, mutations induced by the modified guanines result in the deactivation of the *lacZ′* gene producing white or sectored bacterial colonies when grown on media containing X-gal. In the absence of mutation, blue colonies result. Figure 3 shows the distribution of colony phenotypes (blue, white, or sectored) following the above parallel transformations. As expected, almost all of the colonies produced by transformation with the double-stranded guanine-containing plasmid were blue (i.e., *lac*$^+$) (Figure 3A), although a few

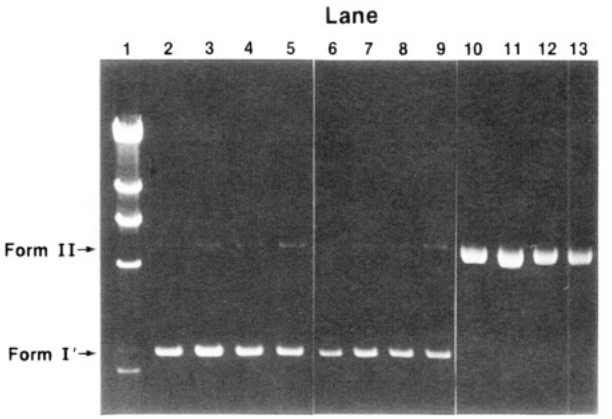

FIGURE 2: Agarose gel electrophoresis of deoxyuridine-containing untreated (double-stranded) plasmids, plasmids treated with UDG alone (abasic plasmids), or plasmids treated with both UDG and Exo III (gapped plasmids). Lane 1: molecular weight markers derived from *Hin*dIII digestion of λ phage DNA. Lanes 2−5: double-stranded plasmids containing a G (lane 2), m^{6}G (lane 3), e^{6}G (lane 4), or b^{6}G (lane 5) in the ATG initiation codon of *lacZ′*. Lanes 6−9: abasic plasmids containing a G (lane 6), m^{6}G (lane 7), e^{6}G (lane 8), or b^{6}G (lane 9) in the ATG initiation codon. Lanes 10−13: gapped plasmids containing a G (lane 10), m^{6}G (lane 11), e^{6}G (lane 12), or b^{6}G (lane 13) in the initiation codon.

Table 1: Percentage of Colonies Resulting from Transformation of *ung*$^-$ Cells with Equal Amounts of the Indicated Plasmids[a]

base in initiation codon	plasmids		
	double-stranded	abasic	gapped
G	100[b]	109.6 ± 8.8	89.8 ± 32.6
m^{6}G	93.8 ± 20.2	22.6 ± 6.6	20.5 ± 7.1
e^{6}G	85.1 ± 22.0	30.5 ± 2.6	29.3 ± 20.0
b^{6}G	107.4 ± 18.0	22.8 ± 6.9	23.3 ± 6.3

[a] Values are the average of five experiments plus or minus the standard deviation. [b] This value is defined as 100 for each experiment. The actual number of colonies ranged between 600 and 1400.

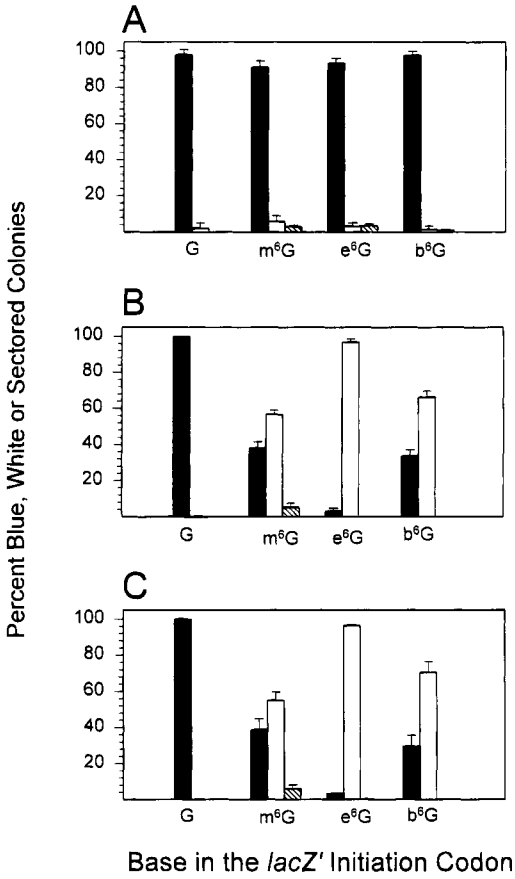

FIGURE 3: Percentage of blue, white, or sectored colonies produced by transformation of *ung*$^-$ *E. coli* with plasmids containing a normal guanine or an O^6-substituted guanine in the initiation codon of *lacZ'*. Panel A: double-stranded plasmids. Panel B: abasic plasmids. Panel C: gapped plasmids. Between 100 and 1400 colonies were produced per transformation. The data represent the average of five experiments. Error bars represent the standard deviation. Blue colonies are indicated by black bars, white colonies are indicated by open bars, and sectored colonies are indicated by hatched bars.

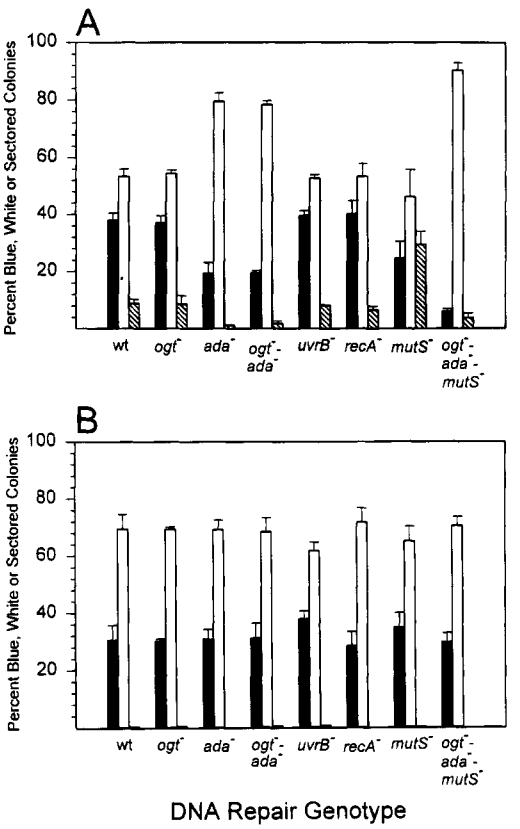

FIGURE 4: Percentage of blue, white, or sectored colonies produced in strains of *E. coli* having the indicated DNA repair genotype following transformation with gapped plasmids containing an m^{6}G residue (A) or a b^{6}G residue (B) in the initiation codon for *lacZ'*. Between 225 and 2500 colonies were produced per transformation. The data represent the average of three or more experiments. Error bars represent the standard deviation. Blue colonies are indicated by black bars, white colonies are indicated by open bars, and sectored colonies are indicated by hatched bars.

white colonies were observed. With double-stranded m^{6}G-containing plasmids, the majority of colonies produced were also blue, although a slightly higher fraction of the colonies were white or sectored (5.9% and 3.0%, respectively), indicating a low frequency of m^{6}G-induced mutations. The double-stranded e^{6}G-containing plasmid also produced some white and sectored colonies (3.2% and 3.5%, respectively), while the b^{6}G plasmid produced relatively fewer white or sectored colonies (1.5% and 0.9%, respectively) (Figure 3A). These results are similar to previous results with plasmids containing thymidine residues rather than deoxyuridine residues in the complementary strand (Pauly et al., 1991, 1994).

Treatment of the plasmids with UDG alone (to produce abasic plasmids) (Figure 3B) or UDG followed by Exo III (to produce gapped plasmids) (Figure 3C) had a dramatic effect on the distribution of colony phenotypes produced by the modified guanines. As indicated, abasic and gapped plasmids containing an e^{6}G residue produced almost exclusively mutant (white) colonies (96.8 ± 1.8% and 96.3 ± 0.7%, respectively) in *ung*$^-$ *E. coli* (Figure 3B,C, respectively). The fraction of white colonies produced with analogous m^{6}G- or b^{6}G-containing plasmids was also greatly enhanced, although a higher percentage of blue colonies was produced with these plasmids than with the e^{6}G-containing derivative. Thus, these gapped plasmids, by eliminating the O^6-substituted guanine-induced preferential replication of the complementary strand in double-stranded plasmids (Pauly et al., 1994), enforce replicative bypass of the modified guanines producing significantly higher levels of mutation.

Since the frequency of e^{6}G-induced mutations was nearly 100% (Figure 3), it was obvious that little if any repair of O^6-ethylguanine was occurring in our abasic or gapped vectors. On the other hand, it seemed plausible that the significantly lower mutation frequency observed for m^{6}G and b^{6}G residues might be a result of repair. To test this possibility, gapped plasmids containing m^{6}G or b^{6}G were used to transform several strains of *E. coli* that were deficient in one or more specific DNA repair activities (Materials and Methods). The distribution of colony phenotypes observed for m^{6}G- and b^{6}G-containing plasmids in these strains is presented in Figure 4. Figure 4A indicates that when gapped plasmids containing an m^{6}G were used to transform *E. coli* that were deficient in the Ogt O^6-alkylguanine-DNA alkyltransferase (*ogt*$^-$), deficient in excision repair (*uvrB*$^-$), or deficient in RecA-mediated recombination (*recA*$^-$), the distribution of phenotypes among the resulting colonies was the same as that seen in the DNA repair competent (wt) strain. Excision repair would not be expected to be active on these gapped plasmids. However, a strain with a deletion of the Ada O^6-alkylguanine-DNA alkyltransferase (*ada*$^-$), or a strain defective in both Ogt and Ada alkyltransferases (*ogt*$^-$-*ada*$^-$) produced fewer blue and sectored colonies and

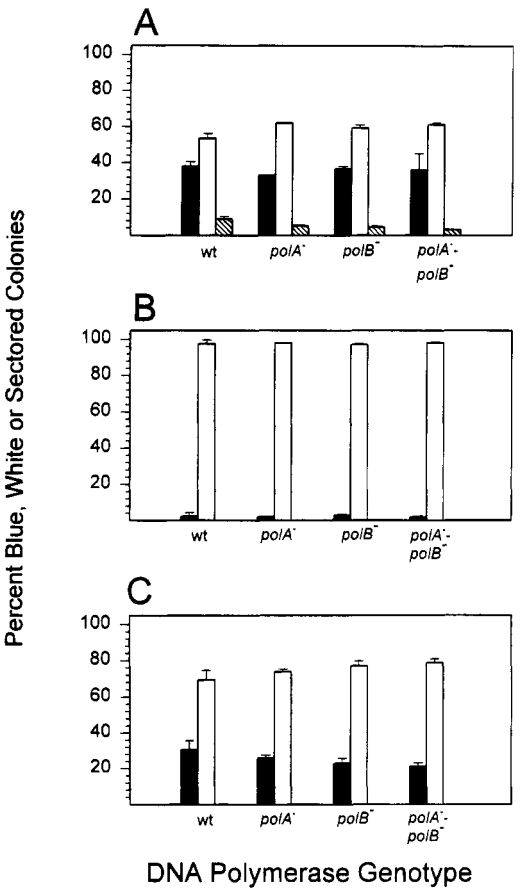

FIGURE 5: Percentage of blue, white, or sectored colonies produced in strains of *E. coli* having the indicated DNA polymerase genotype following transformation with gapped plasmids containing an m^6G residue (A), an e^6G residue (B), or a b^6G residue in the *lacZ'* initiation codon. Between 450 and 2860 colonies were produced per transformation. The data represent the average of three experiments. Error bars represent the standard deviation. Blue colonies are indicated by black bars, white colonies are indicated by open bars, and sectored colonies are indicated by hatched bars.

a greater percentage of white colonies than the wild type strain. A strain defective in methylation-directed mismatch correction (*mutS*$^-$) gave a decrease in the percentage of wild type blue colonies and a significant increase in the percentage of sectored colonies. Finally, a double-alkyltransferase mutant that was also defective in methylation-directed mismatch correction (*ogt*$^-$-*ada*$^-$-*mutS*$^-$) gave the highest percentage of white colonies with the m^6G-containing plasmid. In contrast to these observations with m^6G-containing plasmids, none of the repair deficient strains significantly influenced the distribution of colony phenotypes produced by the gapped b^6G-containing plasmid (Figure 4B).

To determine if the mutation frequency produced by these O^6-substituted guanines was influenced by different *E. coli* DNA polymerases, we compared the mutagenicity of m^6G, e^6G, and b^6G in *E. coli* strains that were proficient in DNA polymerase III (genotype wt, Figure 5) but otherwise deficient in either DNA polymerase I (genotype *polA*$^-$) (Figure 5) or polymerase II (genotype *polB*$^-$) or both polymerases I and II (genotype *polA*$^-$-*polB*$^-$). As indicated, when either or both of these DNA polymerase activities was absent, there was little effect on the frequency of mutations induced by any of the O^6-substituted guanines.

The repair proficient strain GP102 was transformed with abasic and gapped plasmids containing the three O^6-substituted guanines. Fifty white colonies resulting from each of the transformations were transferred to nitrocellulose filters and analyzed with oligonucleotide probes in order to identify the mutations they carried (Pauly et al., 1991, 1994). All 300 of the colonies analyzed carried a G → A transition mutation at the original site of the O^6-substituted guanine. One colony which had been transformed with an m^6G-containing abasic plasmid also harbored a C → T transition five bases to the 5′ side of the original adduct site.

DISCUSSION

In attempts to increase the mutagenic potency of selected carcinogen–DNA adducts, several investigators synthesized site specifically modified single-stranded vectors (Loechler et al., 1984; Banerjee et al., 1988; Lawrence et al., 1990; Dosanjh et al., 1991; Moriya et al., 1993; Latham et al., 1993; Basu et al., 1993) or vectors that position an adduct across from a gap in the complementary strand (Moriya et al., 1991, 1994; Palejwala et al., 1991, 1993, 1994). Our present studies are the first to compare mutagenesis by O^6-substituted guanines in double-stranded and gapped plasmid vectors. The observation (Table 1) of a 3–5-fold reduction in transformation efficiency for O^6-substituted guanine-containing gapped plasmids relative to the guanine-containing analog confirms that O^6-methyl-, O^6-ethyl-, or O^6-benzylguanine residues provide a significant impediment to DNA replication which, in double-stranded plasmids, leads to preferential replication of an unmodified complementary strand (Pauly et al., 1994). However, gapped plasmids, which require replicative synthesis past the modified guanines to generate viable progeny, produced a dramatic enhancement in mutagenesis by these modified guanines, reflecting some of the highest mutation frequencies exhibited by O^6-substituted guanines in *E. coli*.

Recently, higher levels of mutation were observed for ethenocytosine adducts positioned in gapped plasmid vectors (Palejwala et al., 1991, 1993, 1994) compared to single-stranded vectors (Basu et al., 1993; Moriya et al., 1994). It was suggested that this may be a reflection of the consequences of replication past the adduct in the gapped vector by *E. coli* Pol I (the *polA* gene product) rather than Pol III. The former is known to be involved in DNA repair synthesis of shorter segments such as those required in nucleotide or base excision repair processes (Myles & Sancar, 1989), while Pol III would be expected to predominate during replication of longer single-stranded substrates (Marians, 1992; Moriya et al., 1994). The role of a third *E. coli* polymerase, DNA Pol II (the *polB* gene product), is less well understood, although it is likely to be involved in DNA repair since the *polB* gene is induced as part of the SOS response to DNA damage (Bonner et al., 1990; Iwasaki et al., 1990), and the polymerase has been implicated in translesion bypass at abasic or noninstructional sites (Bonner et al., 1988; Kow et al., 1993; Tessman & Kennedy, 1994). In our experiments with gapped vectors, we failed to observe any significant difference in mutagenesis by the O^6-substituted guanines in strains that were competent in Pol III but deficient in either Pol I, Pol II, or both Pol I and Pol II. This may indicate that only Pol III is involved in bypass of these adducts or that if either Pol I or Pol II were responsible for bypass, then they exhibit the same tendency for inserting a thymine rather than a cytosine residue in the complementary strand as does Pol III. While this seems true for the O^6-substituted guanines studied here, it is reasonable that these polymerases

might respond differently to other adducts in other sequence contexts, as has been suggested for ethenocytosine adducts (Moriya et al., 1994; Basu et al., 1993).

O^6-Ethylguanine residues in either abasic or gapped plasmids produced more than 95% white colonies in our experiments and were the most mutagenic of the O^6-substituted guanines studied. This was true in the *ung*$^-$ strain (Figure 3) as well as in an *ogt*$^-$-*ada*$^-$ strain, GP133 (data not shown). These results suggested that O^6-ethylguanine residues were not effectively repaired in our experiments and that O^6-ethylguanine residues efficiently coded for thymine incorporation during bypass.

In contrast to the apparent lack of repair of O^6-ethylguanine residues, our data indicated that O^6-methylguanine residues in gapped vectors were repaired by the Ada alkyltransferase and processed by the methylation-directed mismatch repair system of *E. coli*. Ada alkyltransferase involvement was demonstrated in Figure 4A which showed that significantly higher percentages of white colonies were produced by m^{6}G residues in the *ada*$^-$ and *ada*$^-$-*ogt*$^-$ strains. Colony phenotype distribution in *ogt*$^-$ cells was not different from that observed in wild type cells indicating that Ada played a more important role in m^{6}G repair in this system than did Ogt. This result is somewhat surprising in light of the similar activities of Ada and Ogt proteins toward m^{6}G (Wilkinson et al., 1989; Sassanfar et al., 1991) and the greater abundance of Ogt in cells not induced for the adaptive response (Rebeck et al., 1989). However, we obtained the same results with strains carrying different mutant alleles of *ogt* (strains GP112 and GP113), *ada* (GP122 and GP123), or both *ogt* and *ada* (strains GP132 and GP133) (data not shown) indicating that the preferential repair by Ada was reproducible. This may be a consequence of more efficient repair of m^{6}G residues in single-stranded DNA segments by Ada compared to Ogt. Kinetic studies of m^{6}G repair in single-stranded substrates by Ada and Ogt will be required to confirm this interpretation.

The involvement of the *E. coli* methylation-directed mismatch repair system in processing m^{6}G residues was demonstrated previously (Pauly et al., 1994). This involvement was confirmed in the present studies since a higher percentage of sectored and white colonies relative to blue colonies was produced by m^{6}G-containing plasmids in a *mutS*$^-$ strain compared to the wild type strain (Figure 4). In the strain that was both alkyltransferase and methylation-directed mismatch repair deficient, the mutagenicity of m^{6}G residues was enhanced to near the level observed for e^{6}G residues, indicating that m^{6}G also efficiently codes for thymine incorporation unless repaired prior to bypass.

The mutagenicity of O^6-benzylguanine residues in gapped plasmid vectors was not affected by the repair status of any of the *E. coli* strains (Figure 4) indicating that none of the repair systems examined repaired the b^{6}G residues in these plasmids. These results suggest that the ratio of blue to white colonies (3:7) produced in these strains reflects the ratio of cytosine to thymine incorporation during bypass of the bulky b^{6}G residue. Chambers et al. (1988) calculated a value of 0.5−0.62 for the ratio of cytosine to thymine incorporation during bypass of a similarly bulky O^6-(*n*-butyl)guanine adduct in a ΦX174 vector. These data together with our own strongly suggest that bulkier O^6-substituted guanine adducts are less miscoding than O^6-methylguanine residues. Basu and Essigmann (1990) previously discussed mechanisms to account for this possibility. The weaker miscoding properties of O^6-benzylguanine may help explain the low mutagenicity observed for this modified guanine in other biological systems (Mitra et al., 1989; Bishop et al., 1993; Pauly et al., 1991, 1994).

In summary, our data indicate that O^6-methyl-, O^6-ethyl-, and O^6-benzylguanine residues can produce a significant obstruction to DNA replication. In our gapped vector system, O^6-ethylguanine residues are not effectively repaired and code efficiently for thymine incorporation during bypass. In contrast, O^6-methylguanine residues are repaired by the Ada alkyltransferase and processed by the methylation-directed mismatch repair system of *E. coli*, although m^{6}G residues also code efficiently for thymine incorporation during bypass. Finally, the bulky O^6-benzylguanine residue is not repaired in our gapped vector system but appears to code less efficiently for thymine incorporation than the less bulky O^6-methyl- and O^6-ethylguanines.

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