A Sectored Colony Assay for Monitoring Mutagenesis by Specific Carcinogen–DNA Adducts in *Escherichia coli*†

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ABSTRACT: To study the mutagenicity of various carcinogen–DNA adducts in *Escherichia coli*, a cassette plasmid was developed that permits positioning of specific carcinogen-modified bases within the ATG initiation codon of the *lacZ'* α-complementation gene. Adduct-induced mutations inactivate the gene and lead to formation of blue and white sectored colonies when transformants from an α-complementing version of *E. coli* strain AB1157 are grown on media containing 5-bromo-4-chloro-3-indolyl β-D-galactoside. In the absence of mutation, blue colonies are produced. This system has been used to measure the mutagenicity of O^6-methyl-, O^6-ethyl-, and O^6-benzyl-2'-deoxyguanosine residues incorporated in place of the normal 2'-deoxyguanosine of the ATG initiation codon. Although a low percentage of sectored colonies was produced in this repair-proficient strain, pretreatment of the bacteria with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine to disable DNA repair led to a dose-dependent increase in the percentage of sectored colonies. This percentage increased as a function of modified guanine in the order O^6-benzyl- < O^6-methyl- < O^6-ethyl-2'-deoxyguanosine. The only mutations detected at the site of incorporation of these O^6-substituted guanines were G-to-A transitions. This sectored colony assay system permits convenient screening of large numbers of colonies and simplifies quantification of modified-base-induced mutations whether they be single-base changes, frameshifts, insertions, or deletions.

Carcinogen reaction with cellular DNA is regarded as a critical step in the initiation of chemical carcinogenesis. Such reactions usually produce a number of DNA adducts in different proportions and in different sequence locations, however, and this makes it difficult to determine the influence of a particular adduct on the structure and coding properties of the modified DNA or the possible role played by DNA sequence in modulating these effects. Advances in DNA synthetic chemistry and genetic engineering methods have made possible the incorporation of individual carcinogen-modified bases into precisely defined DNA segments, and this has greatly simplified studies of the effects of modified bases on both the physical chemical properties of DNA and the mutagenicity of modified bases in bacteria and mammalian cells [reviewed in Basu and Essigmann (1988); Basu & Essigmann, 1990; Singer & Essigmann, 1991].

We previously described our utilization of these methods to evaluate the impact on DNA stability of O^6-substituted guanine residues in codon 12 of abbreviated H-*ras* DNA sequences (Pauly et al., 1988; Bishop & Moschel, 1991) as well as the ability of these modified guanines to induce transformation of Rat4 cells when incorporated in codon 12 of the rat H-*ras* gene (Mitra et al., 1989). In this report, we summarize our development of a cassette plasmid for use in *Escherichia coli* that allows the mutagenicity of carcinogen-modified bases to be monitored in a simple sectored colony assay. The plasmid vector we developed for these studies permits the site-selective incorporation of carcinogen-modified bases within the ATG initiation codon of the *lacZ'* α-complementation gene. When plasmids containing a modified base are introduced into an α-complementing version of *E. coli* strain AB1157 and the transformants are grown on media containing 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal), sectored colonies are produced whose numbers reflect the mutagenic potency of the carcinogen-modified base incorporated. This system provides a convenient measure of the frequency of mutations induced by modified bases and has the potential to permit simple enumeration of mutations resulting from single-base-pair changes, frameshifts, insertions, or deletions induced by carcinogen damage.

We have used this system to examine the relative mutagenicity of three O^6-substituted-2'-deoxyguanosines, i.e., O^6-methyl-, O^6-ethyl-, and O^6-benzyl-2'-deoxyguanosine. The salient features of the structure and characterization of the plasmids constructed for these studies will be described below together with the results of our mutagenicity comparisons of the three O^6-substituted guanines.

Materials and Methods

Materials. The enzymes *Bsp*MI, *Nde*I, *Xba*I, and T4 polynucleotide kinase were purchased from New England Biolabs, Beverly, MA. *E. coli* O^6-alkylguanine–DNA alkyltransferase (the *ada* protein) was obtained from Applied Genetics, Inc., Freeport, NY. T4 DNA ligase, 5-bromo-4-chloro-3-indolyl β-D-galactoside, and isopropyl β-D-thiogalactoside (IPTG) were from United States Biochemicals, Cleveland, OH. *N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was from Sigma Chemical Co., St. Louis, MO. Other reagents and solvents were from Aldrich Chemical Co., Inc., Milwaukee, WI, American Burdick and Jackson, Muskegon, MI, Applied Biosystems, Inc., Foster City, CA, or Pharmacia LKB Biotechnology, Inc., Piscataway, NJ.

Oligonucleotide Synthesis and Purification. The 16-base sequences used in these studies (Figure 1) were synthesized on an Applied Biosystems, Inc., Model 380B DNA synthesizer.

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Methods for the preparation and incorporation of the suitably protected O^6-substituted 2′-deoxyguanosines used in these studies were described previously (Pauly et al., 1988). Oligonucleotides were purified by reverse-phase high-pressure liquid chromatography (HPLC) followed by polyacrylamide gel electrophoresis using gels that were 20% acrylamide/*N*,*N*′-methylenebisacrylamide (19:1) and 7 M urea, run in 0.089 M Tris–borate/0.089 M boric acid/0.001 M EDTA. Details of these purification methods together with methods for the enzymatic digestion of the purified oligonucleotides and their nucleoside composition analyses were also presented previously (Pauly et al., 1988).

Oligonucleotide Phosphorylation. Oligonucleotides of type 1 or 2 (Figure 1) (1.65 nmol) were phosphorylated with T4 polynucleotide kinase (10 units) in 100 μL of 66 mM Tris-HCl, pH 7.6/6.6 mm $MgCl_2$/10 mM dithiothreitol/5 mM ATP at 37 °C for 1 h. The polynucleotide kinase was inactivated by heating at 65 °C for 15 min. For subsequent ligations into the plasmid (see below), 360 pmol of oligonucleotide of type 1 was annealed with an equimolar amount of oligonucleotide 2 (Figure 1) by heating the mixture to 65 °C and allowing this to cool slowly to room temperature over a 2-h period. The annealed oligonucleotide pairs were then diluted to 3.6 pmol/μL with 66 mM Tris-HCl, pH 7.6/6.6 mM $MgCl_2$/10 mM dithiothreitol.

Preparation of Plasmids. Plasmid pGP10 (Figure 2) was grown in *E. coli* strain DH5α and was isolated by sodium dodecyl sulfate (SDS) lysis (Maniatis et al., 1982). The plasmid (500 μg) was digested to completion with 200 units of *Bsp*MI restriction enzyme for 6 h in 1 mL of 10 mM Tris-HCl, pH 7.9/10 mM $MgCl_2$/50 mM NaCl/1 mM dithiothreitol. Preparative gel electrophoresis of the digested DNA on an 0.8% low-melting agarose gel produced two bands of approximately 4.3 and 0.95 kilobases. DNA in the 4.3-kilobase band was recovered from the gel. Previously annealed oligonucleotide pairs prepared above were used to recircularize the plasmid through a two-step procedure. First, 50 μg (18 pmol) of the 4.3-kilobase fragment was ligated with 2.5 μL (9 pmol) of a phosphorylated oligonucleotide pair (e.g., 1 + 2, see above) in 200 μL of ligation buffer containing 66 mM Tris-HCl, pH 7.6/6.6 mM $MgCl_2$/10 mM dithiothreitol/1 mM ATP with 100 units of T4 DNA ligase at 8 °C. After 2 h, an additional 2.5 μL of oligonucleotide duplex was added and the reaction was allowed to proceed at 8 °C overnight. DNA was then precipitated with 2 volumes of cold ethanol. The DNA was then treated with 200 units of *Xba*I restriction enzyme for 5 h in 200 μL of 10 mM Tris-HCl, pH 7.9/10 mM $MgCl_2$/50 mM NaCl/1 mM dithiothreitol/100 μg/mL bovine serum albumin. Gel electrophoresis of the *Xba*I-digested DNA on a 0.8% low-melting agarose gel gave three bands of approximately 4.3, 2.3, and 2.0 kilobases. DNA in the 4.3-kilobase band was recovered from the gel. In the second step of the ligation procedure, the 4.3-kilobase fragment was dissolved in 3.2 mL of ligation buffer (see above) and was treated with 50 units of T4 DNA ligase for 18 h at 8 °C. At the end of this period, 800 μL of 10 M ammonium acetate solution was added (to reduce coprecipitation of ATP) and the DNA was precipitated by addition of 2 volumes of cold ethanol. The resulting DNA was briefly air-dried and then resuspended in 100 μL of TE buffer (10 mM Tris-HCl, pH 8.0/1 mM EDTA) and applied to a 0.8% low-melting agarose gel containing 1 μg/mL ethidium bromide. Electrophoresis gave bands corresponding to covalently closed circular plasmid with an apparent size of 2.3 kilobases, as well as linear, nicked, and multimeric plasmids. The DNA in the covalently closed circular form was recovered from the gel. This two-step ligation procedure was used to ligate the four oligonucleotide duplexes 1 + 2, 1(12-m^6G) + 2, 1(12-e^6G) + 2, and 1(12-b^6G) + 2 (Figure 1) into covalently closed circular plasmids designated pATG, pATm^6G, pATe^6G, and pATb^6G, respectively. Approximately 1 μg of each purified plasmid was prepared in this way.

Restriction Enzyme Digestion. Each of the above plasmids (50 ng) was treated with 0.43 unit (25-fold excess) of *E. coli* O^6-alkylguanine–DNA alkyltransferase (the *ada* protein) in 100 μL of 5% glycerol/70 mM HEPES, pH 7.1/1 mM EDTA/10 mM dithiothreitol/1 mg/mL bovine serum albumin for 1 h at 37 °C. Plasmids were then precipitated by the addition of 2 volumes of cold ethanol and were air-dried. These alkyltransferase-treated plasmids, or 50 ng of each of the untreated plasmids, were exposed to 2 units of *Nde*I enzyme in 10 μL of Tris–acetate, pH 7.9/10 mM magnesium acetate/50 mM potassium acetate/1 mM dithiothreitol at 37 °C for 1 h. Plasmid samples were subjected to electrophoresis on 0.8% agarose gels containing 1 μg/mL ethidium bromide.

Bacteria and Transformations. E. coli strain GP100 was prepared by cotransduction of *lac*ZΔM15 and proC::Tn10 mutations from the strain A171 into AB1157 by P1 transduction (Silhavy et al., 1984). Cells were made competent by a modification of the procedure of Chung et al. (1989). Specifically, a 5-mL overnight culture of GP100 cells in LB broth was used to inoculate fresh LB broth at a dilution of 100:1. The fresh culture was grown to an A_{600} = 0.35. Cultures were cooled on ice for 10 min. Samples (5 mL) were removed to tubes containing either 25 μL of dimethyl sulfoxide or 25 μL of dimethyl sulfoxide containing varying amounts of MNNG to produce final MNNG concentrations of 0, 1, 2.5, 5, 10, and 25 μg/mL. Cultures were kept on ice for 10 min under these conditions and then centrifuged at 16000*g* for 10 min at 4 °C to pellet the bacteria. The media was decanted, and the bacteria were resuspended in 250 μL of ice-cold LB broth. After 5 min, 250 μL of ice-cold 2× transformation and storage solution (Chung et al., 1989) was added. After an additional 10 min on ice, 100-μL samples of competent cells were removed and added to tubes containing 1–2 ng of plasmid. Cells were exposed to plasmid for 30 min. The resulting transformation solutions were diluted with 800 μL of LB broth and allowed to recover at 37 °C for 30 min. Following recovery, 150 μL of transformation reaction was streaked onto each of six plates containing media composed of "E" salts (Vogel & Bonner, 1956) supplemented with 0.1% glucose/1 μg/mL thiamine hydrochloride/100 μg/mL ampicillin/1.5% casamino acids/400 μM IPTB/300 μM X-gal/1.5% agar. Plates were incubated at 37 °C for 36 h and were then stored at room temperature for an additional 2–3 days. Bacterial colonies were then tallied as either blue, white, or sectored (both blue and white).

Selection and Probing for Mutations in Progeny Plasmids. At least 48 sectored colonies derived from cells transformed with each plasmid were removed from plates and were suspended in 10 mM NaCl/10 mM Tris-HCl, pH 8.0, and streaked onto plates containing IPTG and X-gal. After 24 h at 37 °C and an additional 2–3 days growth at room temperature, a single white colony was picked from each of the 48 restreaked sectored colonies. These were resuspended, restreaked, and grown as above. Individual white colonies were again selected and suspended in liquid media and spotted onto nitrocellulose filters (Millipore HATF filters, Millipore Corp., Bedford, MA) on plates containing IPTG and X-gal. Each filter was also spotted with bacteria harboring plasmids which

Table I: Nucleoside Composition of Synthetic Oligodeoxyribonucleotides

oligonucleotide	nucleoside composition found[a] (expected)							ϵ_{260} ($\times 10^{-4}$)[c]
	dCyd	dGuo	dThd	dAdo	m^6G	e^6G	b^6G	
1	6.00 (6)	2.98 (3)	2.03 (2)	4.98 (5)	NP[b]	NP	NP	12.9
1(12-m^6G)	5.99 (6)	1.83 (2)	2.01 (2)	5.17 (5)	1.02 (1)	NP	NP	12.2
1(12-e^6G)	6.00 (6)	1.84 (2)	2.01 (2)	5.09 (5)	NP	1.08 (1)	NP	12.2
1(12-b^6G)	6.00 (6)	1.90 (2)	1.97 (2)	5.11 (5)	NP	NP	1.03 (1)	12.3
2	3.94 (4)	6.14 (6)	3.95 (4)	1.96 (2)	NP	NP	NP	12.8

[a] Results are the average of four determinations. [b] NP, not present. [c] Calculated from the total number of nanomoles of nucleosides liberated in a digest of a known OD_{260} value.

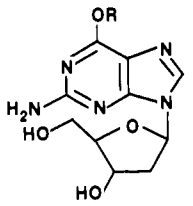
5′-d(AACAGCCATATGGCCC)-3′
1

5′-d(AACAGCCATATm^{6}GGCCC)-3′
1(12-m^{6}G)

5′-d(AACAGCCATATe^{6}GGCCC)-3′
1(12-e^{6}G)

5′-d(AACAGCCATATb^{6}GGCCC)-3′
1(12-b^{6}G)

5′-d(CTTGGGGCCATATGGC)-3′
2



m^{6}G, R = CH_3
e^{6}G, R = CH_2CH_3
b^{6}G, R = $CH_2C_6H_5$

FIGURE 1: Synthetic DNA sequences and structures of the O^6-substituted guanine nucleosides incorporated.

had either an ATG, ATA, ATT, or ATC sequence for the *lacZ′* initiation codon. After overnight incubation at 37 °C the colonies were lysed with alkali, neutralized, and baked as described (Weis, 1987). Filters were washed at 55 °C overnight in several changes of 3× sodium chloride/sodium citrate (SSC) buffer/0.1% SDS (Duby et al., 1988) in order to remove bacterial debris. The filters were then rinsed in 6× SSC and prehybridized for 2 h at 37 °C in a prehybridization solution composed of 6× SSC, 5× Denhardt's solution (Duby et al., 1988), 50 μg/mL sonicated salmon sperm DNA, and 10 mM EDTA. Filters were then hybridized overnight at 37 °C in the above prehybridization solution containing 0.5 pmol/mL of ^{32}P-end-labeled 16-mer probe (Duby et al., 1988). The probe used for hybridization to the plasmid ATG sequence was 5′-^{32}P-d(CTTGGGGCCATATGGC)-3′; for the ATA sequence, 5′-^{32}P-d(GCCATATAGCCCCAAG)-3′; for the ATT sequence, 5′-^{32}P-d(CTTGGGGCAATATGGC)-3′; and for the ATC sequence, 5′-^{32}P-d(CTTGGGGCGATATGGC)-3′. After hybridization the filters were washed six times with 6× SSC at room temperature followed by a 30-min wash in 6× SSC at 47 °C in the case of the ATT probe or at 52 °C for the ATG, ATA, or ATC probes. The filters were then autoradiographed at −70 °C overnight with an intensifier screen. When colonies failed to exhibit positive hybridization with any of these probes, plasmid from these colonies was isolated (Maniatis et al., 1982) and the DNA was sequenced by the dideoxy sequencing method (Sanger et al., 1977).

Results

To compare the relative mutagenicity of O^6-methyl- (m^6G), O^6-ethyl- (e^6G), and O^6-benzyl-2′-deoxyguanosine (b^6G) (Figure 1) in this sectored colony assay system, synthetic 16-base DNA sequences were required that contained either a normal guanine at position 12, i.e., oligonucleotide 1 (Figure 1), or the appropriately substituted guanine in place of the normal guanine at position 12, i.e., 1(12-m^6G), 1(12-e^6G), or 1(12-b^6G), together with complement 2 (Figure 1). These were synthesized using phosphite triester chemistry on an automated DNA synthesizer using methods we developed previously (Pauly et al., 1988). They were purified by HPLC

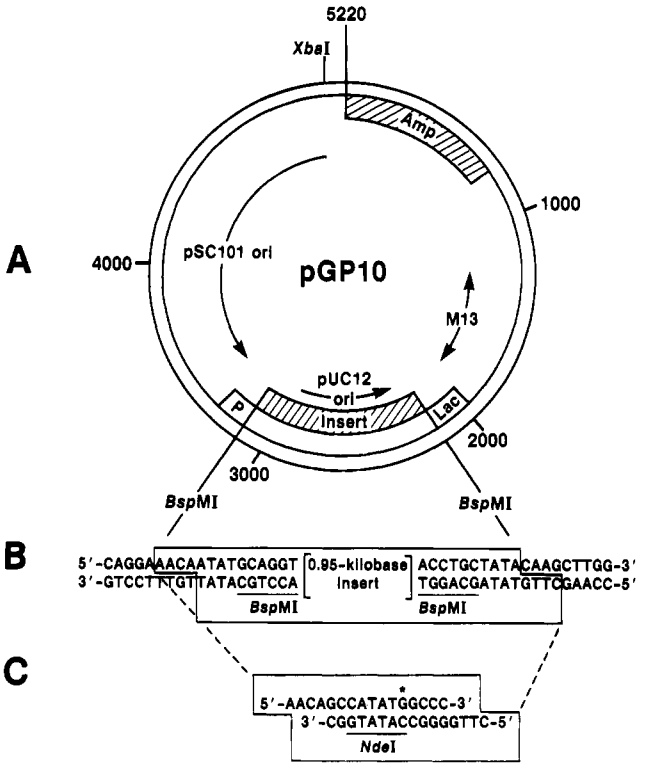


FIGURE 2: Diagram of the plasmid pGP10 and a schematic for incorporating carcinogen-modified bases into the ATG initiation codon of the *lacZ′* gene. The G* indicates the position of incorporation of the carcinogen-modified guanine.

and gel electrophoresis and were characterized by digestion to their component 2′-deoxyribonucleosides by methods also described previously (Pauly et al., 1988). A tabular summary of the nucleoside composition data for these oligonucleotides is presented in Table I together with their molar absorptivities. These data show agreement between the expected and experimentally determined nucleoside compositions and confirm that the oligonucleotides are free of significant contamination by incompletely deprotected nucleosides or possible nucleoside contaminants produced during synthesis and deprotection steps (Borowy-Borowski & Chambers, 1987; Jones, 1984).

The cassette plasmid into which these oligonucleotides were inserted is the 5220-base pGP10 illustrated diagrammatically in area A of Figure 2. The plasmid carries an ampicillin-resistance gene (AMP) for bacterial selection on media containing ampicillin, and it harbors an M13 single-strand origin of replication for use, if necessary, in generating single-stranded DNA in vivo (Viera & Messing, 1987). The plasmid contains a nonfunctional *lacZ′* α-complementation gene, where the *lac* promoter sequence (i.e., P in Figure 2) is separated from the coding sequence for the α-complementation peptide by a 0.95-kilobase insert sequence (insert, Figure 2). The insert sequence contains the origin of replication from the high copy number plasmid pUC12 and is bordered by two outward-

facing recognition sequences for the restriction enzyme *Bsp*MI. The plasmid is replicated from the pUC12 origin and has the higher copy number associated with this relaxed plasmid replicon (Cabello et al., 1976) facilitating isolation of fairly large quantities of pGP10 from *E. coli*. The plasmid also contains the lower copy number origin of replication from pSC101 (Cabello et al., 1976) which governs the copy number of the plasmid during mutagenicity experiments since the insert containing the pUC12 origin is replaced by synthetic oligonucleotides in subsequent plasmid constructions (see below). Finally, pGP10 contains a unique *Xba*I restriction site for use in subsequent plasmid constructions that is positioned opposite the *Bsp*MI-flanked insert sequence.

To incorporate oligonucleotides into this plasmid, it is first digested with *Bsp*MI to produce a 4.3-kilobase fragment and release the shorter 0.95-kilobase insert sequence flanked by *Bsp*MI recognition sequences (i.e., the sequences within the solid lines in area B of Figure 2). The 4.3-kilobase fragment which has nonsymmetric, noncomplementary 4-base overhanging ends is purified by agarose gel electrophoresis and recovered. A phosphorylated oligonucleotide duplex composed of strands of type 1 and 2 (area C, Figure 2) is then ligated to the ends of the 4.3-kilobase fragment in place of the previously excised 0.95-kilobase insert. We have used a two-step ligation procedure to maximize recovery of covalently closed circular plasmid product containing only a 1:1 ratio of 4.3-kilobase fragment and 16-mer oligonucleotide duplex (see Materials and Methods). After 5′ phosphorylation, an oligonucleotide duplex composed of strands 1 and 2 is ligated to the 4.3-kilobase fragment at a high concentration (i.e., >250 μg/mL) which favors formation of concatemers of the 4.3-kilobase fragment joined by 16-base DNA duplexes. Treatment of these concatemers with *Xba*I produces linear fragments of 4.3 kilobases which represent properly ligated products together with shorter fragments of 2.0 and 2.3 kilobases which result from *Xba*I cleavage of 4.3-kilobase fragments that did not undergo ligation with the introduced 16-mers. After electrophoresis and recovery of the 4.3-kilobase ligation product, a second ligation under dilute conditions (1–3 μg of DNA/mL) leads to unimolecular circularization of this fragment. Covalently closed circular plasmid is then purified by agarose gel electrophoresis for use in characterization and mutagenicity experiments.

Using these procedures, we incorporated sequences 1, 1-(12-m^{6}G), 1(12-e^{6}G), and 1(12-b^{6}G) into plasmids when paired with complement 2. These resulting plasmids referred to as pATG, pATm^{6}G, pATe^{6}G, and pATb^{6}G, respectively, contain continuous sequences for the *lacZ′* gene with either a wild-type ATG initiation codon or an initiation codon containing an O^6-substituted guanine in place of the normal guanine. Because the initiation codon in these plasmids is part of an *Nde*I recognition sequence (area C, Figure 2), the successful incorporation of the O^6-substituted guanines can be confirmed by observing their ability to interfere with normal *Nde*I cleavage of the unmodified plasmid. The resulting constructions were characterized in this way.

This is illustrated in Figure 3, where the electrophoretic mobilities of the plasmids pATG, pATm^{6}G, pATe^{6}G, and pATb^{6}G are shown in lanes 2, 3, 4, and 5, respectively, along with molecular weight markers from a *Hin*dIII digest of λ DNA in lane 1. The plasmid preparations primarily contain covalently closed circular (form IV) species and small amounts of nicked circular species (form II) (Figure 3) which are produced during isolation. Lanes 6–9 of Figure 3 illustrate the response of these four plasmids to a 1-h digestion with an

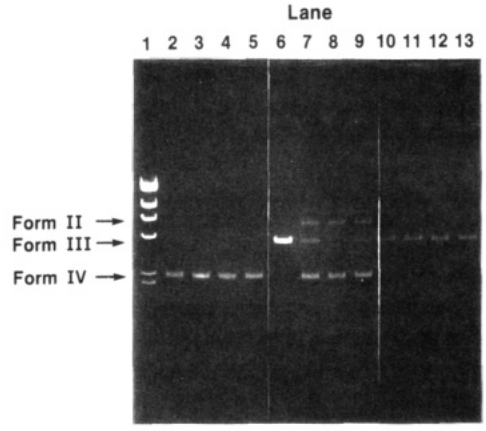


FIGURE 3: Agarose gel electrophoretic behavior of plasmids containing a normal or carcinogen-modified initiation codon in the *lacZ′* gene. Lane 1: molecular weight markers derived from a *Hin*dIII digest of λ phage DNA. Lane 2: plasmid pATG with a normal ATG initiation codon. Lanes 3–5: plasmids pATm^{6}G, pATe^{6}G, and pATb^{6}G, respectively. Lanes 6–9, pATG, pATm^{6}G, pATe^{6}G, and pATb^{6}G, respectively, after treatment with an 80-fold excess of *Nde*I restriction enzyme. Lanes 10–13: the same series of plasmids treated first with a 25-fold molar excess of *E. coli* O^6-alkylguanine–DNA alkyltransferase followed by an 80-fold excess of *Nde*I.

80-fold excess of *Nde*I. Plasmid pATG (lane 6, Figure 3) with a normal guanine in the *lacZ′* initiation codon was completely converted to linear DNA (form III) by this treatment. A much lower proportion of linearization occurred with the modified plasmids although significant amounts of nicked material (form II) were produced by *Nde*I treatment (lanes 7, 8, and 9, respectively). The reduced amount of linearization is consistent with the expectation that the modified guanine would inhibit linearization by *Nde*I. However, the observation that *Nde*I can produce single-strand nicks complements the findings of Voigt and Topal (1990), who observed that several other restriction enzymes are able to nick DNA duplexes containing O^6-methylguanine residues in the recognition sequence for the restriction enzyme. Lanes 10–13 (Figure 3) summarize the results of treatment of these plasmids for 1 h with a 25-fold molar excess of *E. coli* O^6-alkylguanine–DNA alkyltransferase (the *ada* protein) prior to *Nde*I treatment. Pretreatment with the alkyltransferase would be expected to produce a normal guanine residue at the site of the O^6-substituted guanine (Pegg et al., 1985) and thereby restore susceptibility to *Nde*I digestion. Lanes 10–13 (Figure 3) indicate that following exposure to O^6-alkylguanine–DNA alkyltransferase all the modified plasmids behaved like the unmodified plasmid and were completely linearized by *Nde*I treatment. These data confirm that the modified plasmids contained the O^6-substituted guanine at the expected site and also indicate that O^6-benzylguanine in DNA is a substrate for the *E. coli ada* alkyltransferase. Since alkyltransferase pretreatment permits complete linearization by *Nde*I, it is unlikely that a significant proportion of plasmids contain modified bases other than an O^6-substituted guanine in the ATG codon. Contaminating bases produced during synthesis and deprotection of the synthetic oligonucleotides (Pauly et al., 1988; Borowy-Borowski & Chambers, 1987; Jones, 1984) would not be expected to permit *Nde*I linearization.

When *E. coli* with the *lacZ*ΔM15 genotype were transformed with these modified plasmids and were plated onto media containing X-gal, mutations induced by the modified base in the *lacZ′* initiation codon could be quantified through sectored colony formation. This derives from the fact that bacteria harboring a plasmid with a functional *lacZ′* gene metabolize X-gal to produce blue colonies whereas bacteria

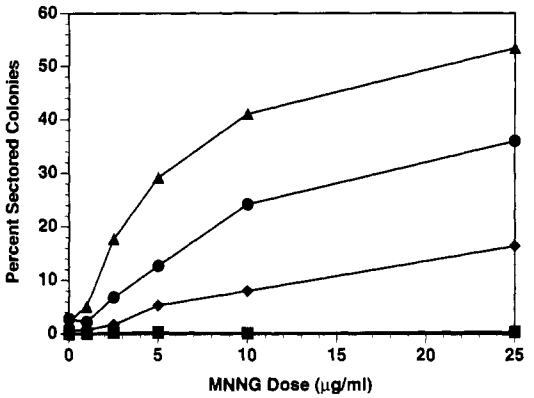


FIGURE 4: The percentage of sectored colonies produced by transformation of bacteria with plasmids pATG (■), pATm^{6}G (●), pATe^{6}G (▲), or pATb^{6}G (◆) as a function of MNNG concentration. Each point is an average of two determinations.

harboring only mutant plasmids, resulting from mutations in the ATG sequence, would carry a nonfunctional *lacZ'* gene and would give rise to white colonies. Since our plasmid constructions contain a carcinogen-modified base in only one strand of the plasmid, plasmid replication in the absence of repair would cause bacteria to harbor a mixture of progeny coding for both a functional and a nonfunctional *lacZ'* gene. After several rounds of cell division, segregation of these plasmids produces sectored (i.e., blue and white) colonies. The proportion of sectored colonies reflects the mutagenic potency of the carcinogen-modified base incorporated in the ATG initiation codon.

When an α-complementing version of *E. coli* strain AB1157 (i.e., GP100 cells, see Materials and Methods) was transformed with the "wild-type" plasmid containing a normal ATG initiation codon (i.e., pATG) or the three modified plasmids containing either an O^6-methyl-, O^6-ethyl-, or O^6-benzylguanine residue (pATm^{6}G, pATe^{6}G, or pATb^{6}G), the average percentage of sectored colonies observed for between 200 and 800 total colonies produced was 0, 2.8, 2.3, and 0.6%, respectively (Figure 4). The low percentages produced with the modified plasmids are consistent with data from other laboratories where the AB1157 strain was used to monitor the mutagenic frequencies of O^6-substituted guanine residues in bacteriophage DNA (Rossi et al., 1989; Rossi & Topal, 1991; Chambers, 1991). These percentages reflect, at least in part, efficient repair of the O^6-substituted guanines in this repair-competent strain. To diminish the efficiency of this repair, cells were pretreated with increasing concentrations of MNNG for 10 min prior to transformation with plasmid. This approach has been used previously by others to enhance the mutagenicity of O^6-methylguanine residues in bacteriophage DNA (Loechler et al., 1984; Hill-Perkins et al., 1986; Rossi et al., 1989). The results of these experiments in our system are also illustrated in Figure 4, which shows that the percentage of sectored colonies produced with the modified plasmids increased with increasing MNNG dose in the order pATb^{6}G < pATm^{6}G < pATe^{6}G. The lack of effect of MNNG treatment in cells transformed with pATG indicates that MNNG is not significantly modifying the transforming plasmids to produce mutations but is most probably modifying the host cell's genomic DNA and thereby diverting DNA repair from the transforming plasmids. Only 1% or fewer sectored colonies were produced by bacteria transformed with pATG when treated with MNNG over the dose range indicated in Figure 4. In addition to sectored colonies, 1% or fewer of the total colonies produced with the modified plasmids were white. The mechanism of formation of white colonies is not absolutely certain at present, but it may involve improper segregation of wild-type and mutant plasmids where the wild-type is somehow lost, or it may involve mismatch repair (Modrich, 1989). White colonies could be produced by a mismatch repair mechanism if the repair system were directed to replace the unmodified strand using the carcinogen-modified strand as template. Since the relative proportion of white colonies is low and variable and independent of MNNG treatment (not shown), they are not included in tallies for the number of mutations induced by the modified guanines

To identify the mutations produced by each of the O^6-substituted guanines, sectored colonies that arose from bacteria treated with 5 μg/mL MNNG were picked and restreaked. From these resultant streaks, pure stocks of white colonies were grown on nitrocellulose filters for ^{32}P-labeled probe hybridization experiments to determine the base changes induced by the modified guanines. At least 48 independent clones were examined from each experiment with a modified-guanine-containing plasmid. For the case of the O^6-ethylguanine-containing plasmid, pATe^{6}G, 47 of 48 colonies probed positively for an ATA sequence at the initiation codon. The O^6-methylguanine-containing plasmid, pATm^{6}G, produced similar results, with 45 of 48 colonies exhibiting the ATA sequence. With the O^6-benzylguanine-containing plasmid, 35 of 48 colonies contained the ATA sequence. Regardless of the modified guanine incorporated, no colonies exhibited positive hybridization with probes for an ATT or ATC initiation codon.

For the O^6-benzylguanine-containing plasmid, 2 of 48 colonies failed to hybridize with any of the labeled oligonucleotide probes. Sequencing of plasmids from these clones indicated that both had undergone a deletion of one of the four cytosine residues 3' to the guanine of the ATG initiation codon (see area C of Figure 2). At present, it is uncertain which of the four cytosines was lost. Since similar mutations were not observed in the case of the O^6-methyl- or O^6-ethylguanine-containing plasmids, it may be that these deletions were induced by the bulky O^6-benzylguanine residue. Mutations beyond the site of incorporation of bulky carcinogen-modified bases are known to occur (Reid et al., 1990). However, the individual cytosine residues either are very near to or are part of a ligation site for incorporation of the modified oligonucleotides, and it is therefore also possible that these deletions are artifacts of ligation anomalies. Additional experiments will be required to distinguish these possibilities.

Of the remaining colonies examined, eleven of the 48 colonies produced by pATb^{6}G-transformed cells probed positively for the "wild-type" ATG sequence. Three of the pATm^{6}G-transformed colonies contained plasmid with the normal ATG sequence while only 1 of 48 colonies derived from O^6-ethylguanine-containing plasmids tested positively for this sequence. As indicated above, up to 1% of the sectored colonies observed in these experiments can arise as "background" associated with transformation and/or MNNG treatment alone. This necessarily represents a higher fraction of total sectored colonies when the number produced by a modified plasmid is low. Thus, as much as one-fifth of the total sectored colonies produced by pATb^{6}G-transformed cells can result from transformation and MNNG treatment of the host cells alone, compared to 1/13 of the pATm^{6}G-treated cells or 1/30 of the pATe^{6}G-treated cells (Figure 4). These fractions are in close agreement with the number of ATG-positive colonies produced in each of these groups of 48 colonies tested, which suggests that these ATG-positive "mutants" are a result of host cell mutations rather than mutations produced in the transfected

plasmids. To test this, plasmids harbored by these ATG-positive white colonies were isolated and used to transform fresh host bacteria. In every case, only blue colonies were produced, confirming that the introduced plasmids carried a functional *lacZ'* coding sequence. With these considerations in mind, it is clear that the only mutations detected in progeny plasmids at the site of incorporation of any of these O^6-substituted guanines were G-to-A transitions.

Discussion

Site-directed mutagenesis by O^6-methylguanine has been studied in several laboratories (Basu & Essigmann, 1988, 1990) using M13 (Loechler et al., 1984; Hill-Perkins et al., 1986; Rossi et al., 1989; Rossi & Topal, 1991) or ϕX174 virus vectors (Chambers et al., 1985, 1988; Bhanot & Ray, 1986; Chambers, 1991). While each of these systems has its proven utility and particular advantages, the determination of the frequency of adduct-induced mutations often involves several steps. Our sectored colony assay system greatly simplifies this process since it allows mutation frequency to be determined directly in one step through visualization of the number of blue and white sectored colonies produced as a fraction of total blue colonies. Consequently, large numbers of colonies can be scored rapidly. Following isolation of pure white colonies from sectored colonies, hybridization (Maniatis et al., 1982; Duby et al., 1988; Rossi & Topal, 1991) and/or DNA sequencing (Sanger et al., 1977) of resultant plasmids permits ready identification of the mutations, whether they are single-base changes, frameshifts, insertions, or deletions.

Our data for the mutagenicity of O^6-methylguanine in *E. coli* AB1157 are similar to those reported by Rossi et al. (1989), Rossi and Topal (1991), and Chambers (1991). Chambers (1991) also compared the mutagenicity of O^6-ethylguanine and the bulky O^6-*n*-propyl- and O^6-*n*-butylguanine and observed descending mutant frequencies in the order O^6-ethylguanine > O^6-methylguanine > O^6-*n*-propylguanine = O^6-*n*-butylguanine. While we found O^6-methylguanine to be slightly more mutagenic than O^6-ethylguanine in non-MNNG-treated cells (see above), we observed the related mutagenicity order O^6-ethylguanine > O^6-methylguanine > O^6-benzylguanine in MNNG-treated cells (Figure 4).

The repair-competent AB1157 cells contain two forms of O^6-alkylguanine–DNA alkyltransferase which can act on these modified guanines: a constitutive protein termed *ogt* (Margison et al., 1990) and an inducible protein, the *ada* protein, which is produced as part of the adaptive response to alkylation damage (Lindahl et al., 1988). The *ogt* protein is known to be responsible for the repair of the majority of simple O^6-substituted guanine damage in uninduced cells (Wilkinson et al., 1989); its activity can be depleted by treating cells with MNNG (Rossi et al., 1989). The *ogt* protein removes O^6-methyl substituents from modified guanines approximately six times faster than O^6-ethyl substituents (Wilkinson et al., 1989). Since the reaction specificity of the *ogt* protein is known to resemble that of the human O^6-alkylguanine–DNA alkyltransferase (Pegg, 1990), and since the human protein can remove O^6-benzylguanine derivatives very readily from DNA (Dolan et al., 1990), it may be that the mutagenicity order we observe reflects the inverse of the ease of repair of these adducts by *ogt*. The inducible *ada* repair protein removes O^6-methyl groups from DNA guanine residues nearly 1000 times faster than O^6-ethyl groups (Wilkinson et al., 1989). If this protein were being induced to contribute significantly to the repair of these adducts in our system, one would expect to observe a marked enhancement in the mutagenicity of the pATe^{6}G plasmid over that exhibited by pATm^{6}G with increasing MNNG dose. Since we observed only a 2-fold difference in mutagenic potency of these two plasmids (Figure 4), our data suggest that the *ada* protein is not playing a dominant role in repair in these experiments. This may be because there is not sufficient time to induce *ada* as part of the adaptive response or because the adaptive response is saturated by the high doses of MNNG used in our experiments.

While it is recognized that differences in rates of repair for these adducts will contribute to their relative mutagenic potency, it is also recognized that the intrinsic miscoding properties of these O^6-substituted guanines may be quite different. While O^6-methyl- and O^6-ethylguanine residues might both code fairly efficiently for thymine incorporation during DNA replication in *E. coli*, O^6-benzylguanine residues might not. It has been argued recently that as the steric bulk of a substituent group attached to the O^6-position of guanine residues increases, the efficiency of misincorporation of thymine residues relative to cytosine residues might decrease (Basu & Essigmann, 1990; Swann, 1990). The net effect would be to reduce the frequency of G-to-A transitions induced by bulky substituents relative to those induced by smaller alkyl substituents. The lower mutagenic potency we observed for O^6-benzylguanine as well as the lower potency observed by Chambers (1991) for O^6-*n*-propyl- and O^6-*n*-butylguanine may reflect just such a steric effect. The extent to which these effects or individual repair mechanisms govern the relative mutagenic potency of these and additional adducts will be tested with our assay system in *E. coli* strains that have deficiencies in various repair pathways.

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Registry No. **1**, 137332-34-4; **1** (12-m^{6}G), 137332-35-5; **1** (12-e^{6}G), 137332-36-6; **1** (12-b^{6}G), 137332-37-7; O^6-methyl-2′-deoxyguanosine, 964-21-6; O^6-ethyl-2′-deoxyguanosine, 50704-46-6; O^6-benzyl-2′-deoxyguanosine, 129732-90-7.

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