Site-Specific Frame-Shift Mutagenesis by the 1-Nitropyrene−DNA Adduct *N*-(Deoxyguanosin-8-yl)-1-aminopyrene Located in the $(CG)_3$ Sequence: Effects of SOS, Proofreading, and Mismatch Repair†

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ABSTRACT: 1-Nitropyrene (1-NP), the predominant nitropolycyclic hydrocarbon found in diesel exhaust, is a mutagen and tumorigen. Nitroreduction is a major pathway by which 1-NP is metabolized. Reductively activated 1-NP forms a major DNA adduct, *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG^{AP}), both in vitro and in vivo. In *Salmonella typhimurium* 1-NP induces a CpG deletion in a CGCGCGCG sequence. In *Escherichia coli*, however, mostly −1 and +1 frame-shifts are observed, which occur predominantly in 5′-CG, 5′-GC, and 5′-GG sequences. In order to determine the mechanism of mutagenesis by dG^{AP} in a CpG repetitive sequence, we constructed a single-stranded M13 genome containing the adduct at the underscored deoxyguanosine of an inserted CGCGCG sequence. In *E. coli* strains with normal repair capability the adduct induced approximately 2% CpG deletions, which was 20-fold that of the control. With SOS, the frequency of frame-shift mutations increased to 2.6%, even though the frequency of CpG deletion accompanied 50% reduction. The enhancement in mutagenesis was due to a +1 frame-shift that occurred at a high frequency. In strains with a defect in methyl-directed mismatch repair, 50−70% increase in mutation frequency was observed. When these strains were SOS induced, frame-shift mutagenesis increased by approximately 100%. When transfections were carried out in *dnaQ* strains that are impaired in 3′→5′ exonuclease activity of DNA polymerase III, frame-shift mutagenesis increased 5−7-fold. dG^{AP}-induced frame-shifts in the $(CG)_3$ sequence, therefore, varied from 2% to 17% depending on the state of repair of the host cells. We conclude that dG^{AP} induces both −2 and +1 frame-shifts in a CpG repetitive sequence and that these two mutagenic events are competing pathways. The CpG deletion does not require SOS functions, whereas the +1 frame-shifts are SOS-dependent. On the basis of the data in repair-deficient strains, it appears that both types of frame-shifts occurred as a result of misalignment, which are corrected primarily by the proofreading exonuclease of the DNA polymerase. Misaligned structures that escape the exonuclease are repaired by the methyl-directed mismatch repair, albeit with limited efficiency.

1-Nitropyrene (1-NP),[1] a common environmental contaminant, is tumorigenic in rats and mice (Pitts et al., 1978; Rosenkranz et al., 1980; Hirose et al., 1984; King, 1988; El-Bayoumy et al., 1984, 1988). Nitroreduction is a major pathway of bioactivation by which 1-NP is converted to its activated metabolite, *N*-hydroxy-1-aminopyrene (NHOP) (Heflich et al., 1985). This activated derivative undergoes acid-catalyzed binding to DNA. In both mammalian cells and bacteria, the major DNA adduct formed by 1-NP is *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG^{AP}) (Howard et al., 1983; Stanton et al., 1985). Recent studies indicate that two additional deoxyguanosine adducts are formed, in which the covalent attachment occurs at the 6 or 8 position of the pyrene ring system with the N^2 position of deoxyguanosine (Scheme 1) (Herreno-Saenz et al., 1995).

1-NP is a well-known frame-shift mutagen. It reverts *Salmonella typhimurium* frame-shift tester strains TA98 and TA1538 more efficiently than strains TA100 and TA1535 that detect base pair substitutions (Rosenkranz & Mermelstein, 1983). The most frequent mutation among the revertants in TA98 is a −2 deletion of a GpC or CpG pair within a CGCGCGCG hot-spot sequence upstream of the *hisD3052* mutation (Bell et al., 1991). Nitroreduction is the metabolic pathway that leads to mutagenesis in *Salmonella* (Howard et al., 1983). In a forward mutation assay, DNA sequence analysis of 1-nitrosopyrene-induced mutants in the lambda *c*I gene of *Escherichia coli uvr*⁻ lysogen showed that ∼70% mutants were one-base deletions or additions (Stanton et al., 1988). In a subsequent study in pBR322 −1 deletions and all the targeted base substitutions were observed, but +1 additions were not detected (Melchior et al., 1994). In our laboratory reductively activated 1-NP mutagenesis in *E. coli* was studied in single stranded (ss) DNA (Malia & Basu, 1995). A major fraction of mutagenesis, and specifically one-base deletions and insertions, occurred in 5′-CG, 5′-GC, and 5′-GG sequences. In contrast to the studies in bacteria, sequence analyses of NHOP-induced mutants in mammalian cells indicated that base pair substitution, specifically G·C→T·A transversions, occurred preferentially (Yang et al., 1988; Newton et al., 1992). However, in human T-cells G·C→A·T transitions are the most common mutations (McGregor et al., 1994).

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[1] Abbreviations: 1-NP, 1-nitropyrene; NHOP, *N*-hydroxy-1-aminopyrene; dG^{AP}, *N*-(deoxyguanosin-8-yl)-1-aminopyrene; Gua^{AP}, the corresponding base, *N*-(guanin-8-yl)-1-aminopyrene; AF, 2-aminofluorene; AAF, *N*-acetyl-2-aminofluorene; IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; ss, single stranded; ds, double stranded; Gua, guanine; Ade, adenine; Cyt, cytosine; Thy, thymine; pfu, plaque forming units; MF, mutation frequency; DTT, dithiothreitol; UV, ultraviolet.

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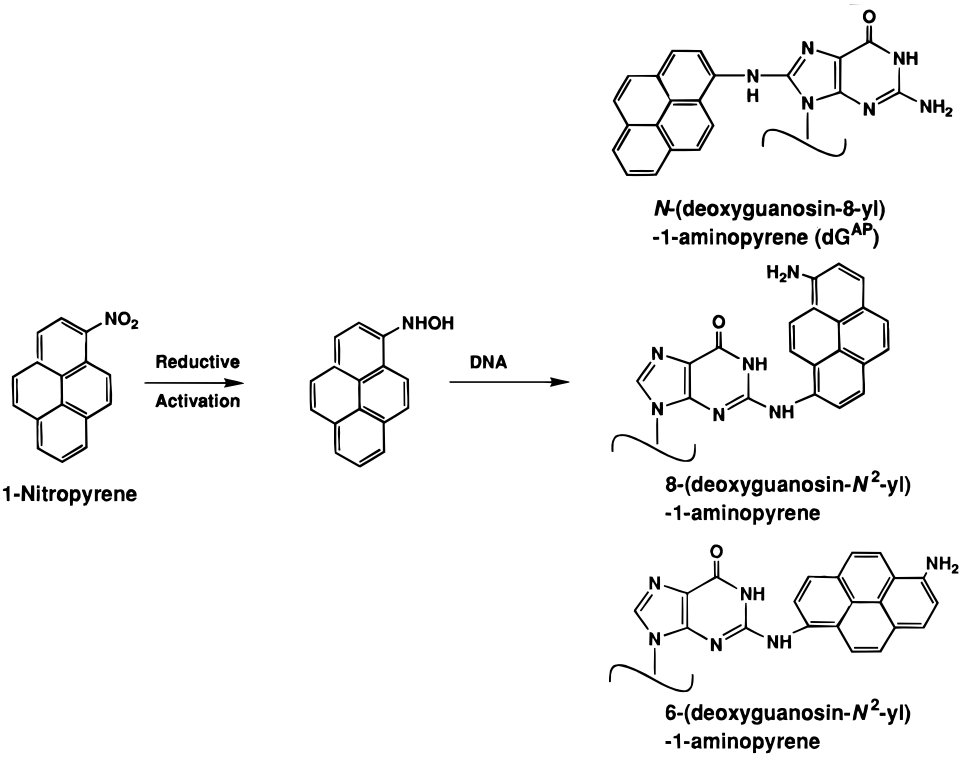
Scheme 1: Structures of 2′-Deoxyguanosine Adducts Formed by Reductively Activated 1-Nitropyrene



It is intriguing that in *Salmonella* 1-NP predominantly induces −2 frame-shifts, whereas in *E. coli* +1 and −1 frame-shifts are common, but −2 deletions have never been detected. One can rationalize this apparent discrepancy if the adduct-induced −2 frame-shifts occur only in repetitive CpG sequences. Indeed, none of the relevant DNA sequences in *E. coli* contain CpG repetitive sequences. The +1 and −1 frame-shifts in *E. coli* are almost entirely dependent on the SOS functions of the host cells (Malia & Basu, 1995). Additional genetic requirements for these frame-shifts have not been investigated. Several other questions related to 1-NP mutagenesis have not been resolved. For example, three different deoxyguanosine adducts have been identified, and there is no evidence to indicate which one induces the −2 frame-shifts. It is also uncertain which adduct is responsible for the +1 or −1 mutations. The differences in genetic requirements, if any, for the −2, −1, and +1 frame-shifts are also not yet known.

Frame-shift mutations account for a major proportion of spontaneous mutagenesis. It has been recognized for a long time that certain sequences are particularly prone to undergo frame-shift mutations [reviewed by Kunkel (1990)]. Frame-shifts resulting from slippage were first proposed by Streisinger to account for the observation that such mutations are more common in vivo in repeated sequences than in nonrepetitive sequences (Streisinger et al., 1966). Examination of frame-shift hot spots in *E. coli* and yeast suggested that misalignments in repeated DNA sequences can cause both deletions and insertions (Farabaugh et al., 1978; Stewart & Sherman, 1974). Ripley as well as others have studied various mechanisms of frame-shifts, which include direct and inverted repeats, palindromic and quasipalindromic sequences, and Z-DNA-forming sequences (Ripley, 1982; Ripley et al., 1983, 1986; De Boer & Ripley, 1984; Lloyd & Augustine, 1987; Balbinder et al., 1989; Freund et al., 1989). Instability in certain repetitive sequences has recently been shown to be associated with several genetic disorders, such as triplet repeat diseases and hereditary colorectal cancer (Thibodeau et al., 1993). Some studies suggest that exonucleolytic proofreading enhances frame-shift fidelity (Strauss & Sagher, 1995). Furthermore, *mutHLS*-dependent methyl-directed repair of heteroduplexes with one-, two-, and three-base deletions is as highly efficient as the repair of G·T mismatches (Parker & Marinus, 1992).

As in the case of spontaneous frame-shift hot spots, carcinogen−DNA adduct-induced frame-shift mutations are also sequence-dependent. For example, a propanodeoxyguanosine adduct induced base substitutions in a nonrepetitive sequence, whereas predominantly −2 frame-shifts were detected when it was located in a $(CpG)_4$ sequence (Moriya et al., 1994; Benamira et al., 1992). Fuchs and co-workers noted that AAF-induced CpG deletions occur in alternating CpG sequences, whereas −1 deletions occur in reiterated base sequences (Fuchs et al., 1981; Koffel-Schwartz et al., 1984). The location of the adduct is crucial, because −2 frame-shifts were observed only when the C8−dG adduct of AAF was located at G^3 of the *Nar*I sequence $G^1G^2CG^3$-CC (Burnouf et al., 1989). For the −1 frame-shifts, an increased number of contiguous Gua residues 5′ to the adduct site result in increased mutagenesis (Lambert et al., 1992). The inducible nature of these two types of deletions is different. In the contiguous Gua sequences the RecA protein participates with the *umuDC* gene products (Burnouf et al., 1989). In contrast, in alternating CpG sequences RecA is not essential, except to cleave the LexA repressor (Maenhaut-Michel et al., 1992).

To elucidate the mechanism of frame-shift mutations induced by 1-NP, in the current work we have employed site-specific experiments to determine the biological effects of dG^{AP} in a repetitive CpG sequence. We have constructed a ss M13 bacteriophage genome in which dG^{AP} was located at the underscored dG of an inserted CGCGCG sequence in the *lacZ* α fragment. Following transformation of this genome in several *E. coli* strains, we determined the genetic

requirements for the −2 and +1 mutagenesis. Specifically, we have examined dG^{AP} mutagenesis in strains with a defect in the *mutD* (or *dnaQ*) gene that encodes the ϵ-subunit of DNA polymerase III holoenzyme. *Pol* III from these strains has been shown to be defective in 3′→5′ proofreading exonuclease activity (Echols et al., 1983). We have also determined the effects of this adduct in strains with impaired postreplicative mismatch repair.

MATERIALS AND METHODS

Materials

E. coli strains. GW5100 (JM103, $P1^-$) was reported (Malia & Basu, 1995). NR9295 (*ara*, *thi*, Δ*prolac*, F′128−27) and its derivatives NR9292 (*mutH101*), NR9293 (*mutL101*), NR9294 (*mutS101*), NR9288 (*zaf-13*::Tn*10*, *mutD21*), and NR11446 (*zae*::Tn*10dCam*, *dnaQ49*, *zae502*::Tn*10*, F′cc105) were provided by R. Schaaper (NIEHS, Research Triangle Park, NC).

1-NP, 1-aminopyrene, and *m*-(chloroperoxy)benzoic acid were from Aldrich Chemical Co. (Milwaukee, WI). Ethidium bromide and poly(ethylene glycol) 8000 were obtained from Sigma (St. Louis, MO). M13 DNA sequencing kit, *E. coli* single-strand binding protein, and Sequenase version 2.0 were purchased from Amersham Corp. (Cleveland, OH). Isopropyl β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) were from Gold Biotechnologies (St. Louis, MO). T4 polynucleotide kinase and DNA ligase were obtained from Bethesda Research Laboratory (Gaithersberg, MD). *Eco*RI was from Boehringer Mannheim (Indianapolis, IN). *Bss*HII and exonuclease III were purchased from New England Biolabs (Beverly, MA). [α-^{35}S]dATPαS was from Du Pont New England Nuclear (Boston, MA).

Methods

Unlabeled 1-nitrosopyrene was synthesized according to the published procedure (Howard et al., 1983). Oligodeoxynucleotides were synthesized on an Applied Biosystems, Inc., Model 380B, DNA synthesizer, using the phosphoramidite method. HPLC separations were performed using reversed-phase columns (Phenomenex Ultracarb C-18, 4.6 × 250 mm). Bacteriophage M13mp7L2 DNA was prepared as described (Sambrook et al., 1989).

Synthesis of d(CGCG^{AP}CG). d(CGCGCG) (50 nmol) was stirred at an ambient temperature with 15 μmol of 1-nitrosopyrene and 3 μmol of ascorbic acid in 100 μL of DMF−sodium acetate−acetic acid buffer (0.1 M), pH 5.0 (1:9), under nitrogen and protected from light. An additional aliquot of 3 μmol of ascorbic acid was added every 15 min during the first hour, and the reaction was allowed to be continued for 16−20 h. Noncovalently bound material was removed by extensive extraction with chloroform as reported elsewhere (Vyas et al., 1993). The adducted and unadducted oligonucleotides were separated by reversed-phase HPLC. Further purification of the oligonucleotides was carried out by denaturing polyacrylamide gel electrophoresis. The oligonulceotides were desalted on a Sephadex G-10 column, dried, and stored at −20 °C until further use.

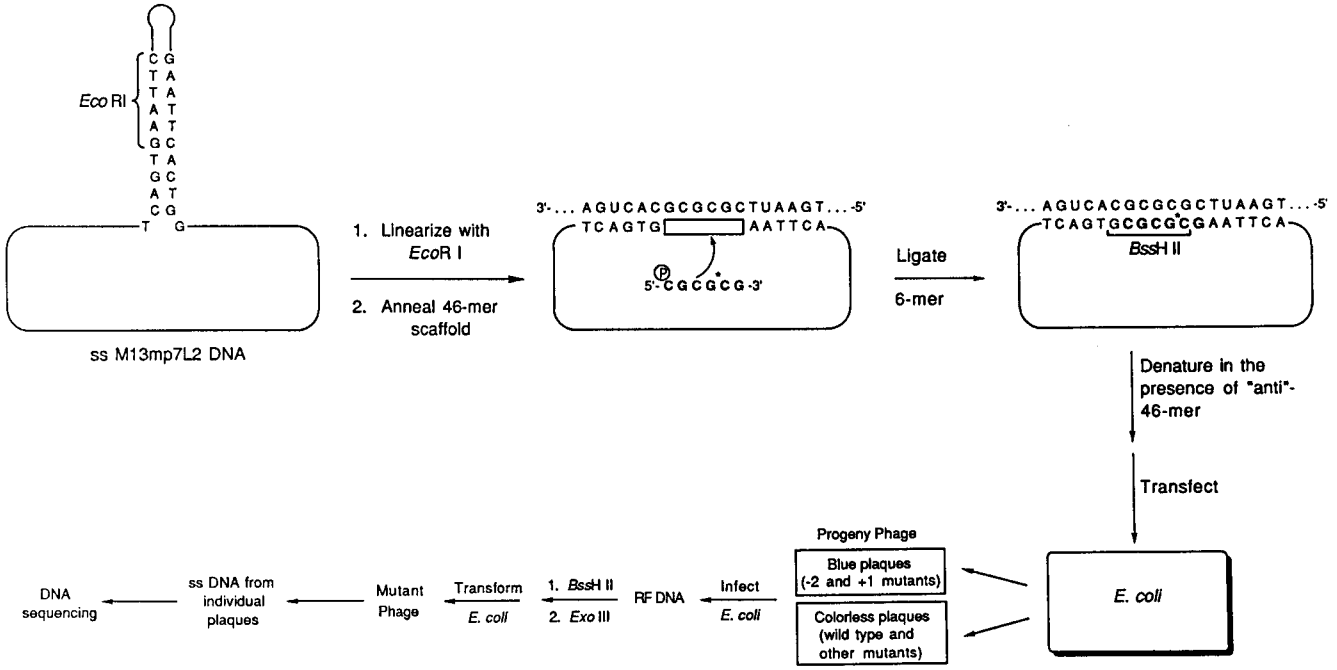
Construction of Site-Specifically Modified M13 Genomes. For construction of site-specifically modified genomes, we followed the procedure developed by Lawrence and coworkers (Banerjee et al., 1988, 1990). Bacteriophage M13mp7L2[2] (200 μg) was digested with a large excess of *Eco*RI (2400 units) for 2 h at 25 °C in 1 mL of 100 mM Tris·HCl, pH 7.5, 5 mM $MgCl_2$, and 50 mM NaCl. Agarose gel electrophoresis indicated no visible band for the remaining circular DNA. A 2-fold molar excess of a scaffold 46-mer was annealed to the linear ss DNA at a concentration of 100 ng/mL by heating at 67 °C for 4 min followed by slow cooling to room temperature over a period of 3−4 h. The proportion of circularized vector as determined by agarose gel electrophoresis was 35−45%. A 50-fold molar excess of the modified or unmodified hexanucleotide was ligated into the gap of this annealed DNA in the presence of 20 units of T4 DNA ligase (BRL) in 40 mM Tris·HCl buffer (pH 7.8), 8 mM $MgCl_2$, 16 mM dithiothreitol, and 1 mM ATP at 16 °C overnight. A "mock ligation" was also carried out in which the 5′-CGCGCG hexamer was replaced with the hexanucleotide 5′-ATGCAT that cannot anneal to the gap.

SOS Induction and Transformation in E. coli. *E. coli* cells were grown in 100 mL cultures to 1×10^8 cells/mL and then harvested by centrifugation at 5000*g* for 15 min at 0 °C. All *E. coli* cells were grown in Luria broth, except the *dnaQ49* and *mutD* strains (NR9288 and NR11446, respectively) which were grown in minimal medium. The cells were resuspended in an equal volume of ice-cold deionized water and recentrifuged at 5000*g* for 30 min. This procedure was repeated except the cells were resuspended in 50 mL of water. The bacterial pellet was resuspended in 1 mL of glycerol/water (10% v/v) and kept on ice until further use. To induce SOS, the following additional steps were introduced after the first centrifugation. The cells were resuspended in 50 mL of 10 mM $MgSO_4$ and treated with UV light (254 nm) (50 J/m^2) in 25 mL aliquots in 150 × 50 mm plastic petri dishes. The cultures were incubated in Luria broth at 37 °C for 40 min in order to express SOS functions maximally. Following SOS induction, these cells were centrifuged, deionized, and resuspended in glycerol/water in a manner similar to that described earlier except all manipulations were carried out in subdued light.

Before transformation, the constructed genome was subjected to another round of *Eco*RI treatment to digest any uncut or religated M13mp7L2 DNA. A 10-fold molar excess of a 46-mer that contained the DNA sequence complementary to the scaffold oligomer was added to each DNA solution, and the mixture was heated at 100 °C for 2 min to remove the scaffold and rapidly cooled to 0 °C. To monitor the extent of removal of the scaffold, a gapped genome was taken through the same protocol. An aliquot of each of these DNA solutions was subjected to agarose gel electrophoresis to ensure that the scaffold was quantitatively denatured. For each transformation, 40 μL of the cell suspension was mixed with 4 μL (500 ng) of DNA solution and transferred to the bottom of an ice-cold Bio-Rad Gene-Pulser cuvette (0.1 cm electrode gap). Electroporation of cells was carried out in a Bio-Rad Gene-Pulser apparatus at 25 μF and 1.8 kV with the pulse controller set at 200 Ω. Immediately after electroporation, 1 mL of SOC medium was added, and the mixture was transferred to a 1.5 mL microcentrifuge tube.

[2] In order to stabilize the duplex hairpin region of the *Eco*RI site of ss M13mp7, M13mp7L2 was constructed by replacing the dinucleotide 5′-AC located 5′ to the first *Eco*RI sequence at the multiple cloning region of the former with the tetranucleotide 5′-CAGT (Banerjee et al., 1990). M13mp7L2, therefore, is a +2 mutant of M13mp7.

Scheme 2: Strategy for Construction of Site-Specifically Modified Genome and Selection of Mutant Progeny



Part of the cells was plated following a 15 min recovery at 37 °C in the presence of the plating bacteria *E. coli* GW5100, IPTG, and X-Gal to determine the number of independent transformants. The remainder of cells were centrifuged at 15000*g* for 5 min to isolate the phage-containing supernatant. The −2 and +1 mutant phage were detected directly from the progeny as blue plaques after 18 h of incubation at 37 °C. All other colorless mutants were isolated as described in Scheme 2 following a sequence of DNA isolation, restriction endonuclease digestion, and subsequent transformation. A small batch of ss DNA from each mutant plaque was prepared, and the DNA sequence was determined.

RESULTS

Synthesis and Characterization of CGCG^{AP}CG. The adducted hexanucleotide was synthesized by allowing the unmodified duplex hexamer to react with NHOP as reported in a prior publication from our laboratory (Vyas et al., 1993). The reversed-phase HPLC profile of the reaction mixture, following chloroform extraction of unreacted materials, is shown in Figure 1. Three major peaks (identified as A, B, and C) eluted after the unmodified hexamer peak at 15.3 min. Peak A (at 19.3 min), which was isolated in ∼5% yield, was identified as d(CGCG^{AP}CG) (vide infra), whereas peaks B and C contained the adduct at the other two Gua sites (data not shown).

The absorption spectrum of peak A clearly showed the 241 nm band of dG^{AP}, but the 342 nm maximum was red shifted to ∼365 nm as noted with several other dG^{AP}-containing oligonucleotides (inset, Figure 1) (Vyas et al., 1993; Nolan et al., 1995). Enzymatic digestion of peak A to the component nucleosides showed dC and dG peaks and a late-eluting peak that cochromatographed with an authentic sample of dG^{AP} (data not shown). Polyacrylamide gel electrophoresis showed slower mobility of peak A compared to the unmodified hexamer, as expected (Figure 2A). It is noteworthy that the adducted hexamer did not contain any visible contamination of the unmodified hexamer. In order to determine the site of adduction, we treated peak A with

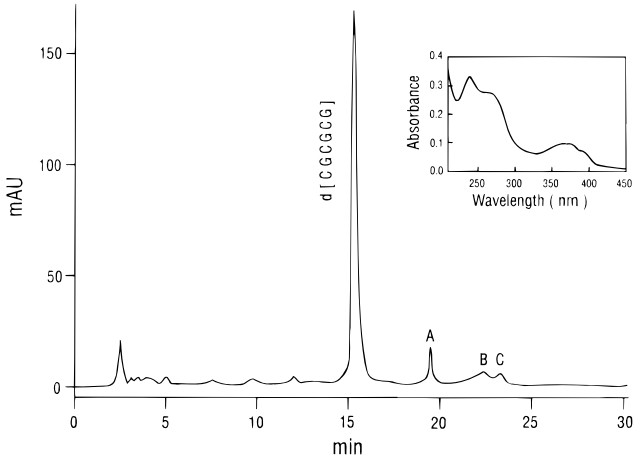


FIGURE 1: Reversed-phase HPLC profile of the reaction mixture of NHOP with the hexamer d(CGCGCG). Elution conditions: C18 Ultracarb column (7 μm particle size; 4.6 × 250 mm) (Phenomenex), 1 mL/min flow rate, 0−35% acetonitrile in 0.1 M aqueous triethylammonium acetate (pH 7.0) over 45 min. The unmodified oligonucleotide elutes at 15.4 min. Peaks A, B, and C were identified as monoadducted hexamers. The inset displays the absorption spectrum of peak A which was characterized as d(CGCG^{AP}CG).

piperidine at 90 °C for 3 h. We have shown that piperidine treatment induces a DNA strand break at the dG^{AP} sites, presumably via a mechanism similar to the cleavage of AF adducts (Vyas et al., 1993). To indicate the electrophoresis migration characteristics of the cleavage products at different Gua in the hexamer, the Maxam−Gilbert G-specific sequencing reaction was carried out with the unmodified CGCGCG. As shown in Figure 2B, cleavage of peak A occurred specifically at the second Gua, suggesting that peak A is d(CGCG^{AP}CG).

Construction and Characterization of an M13 Genome Containing a Single dG^{AP}. Following the protocol of Lawrence and co-workers, the hairpin region of a +2 frameshift mutant clone of M13mp7 was digested with *Eco*RI (Banerjee et al., 1988, 1990). This was recircularized noncovalently by annealing a scaffold 46-mer, the two ends

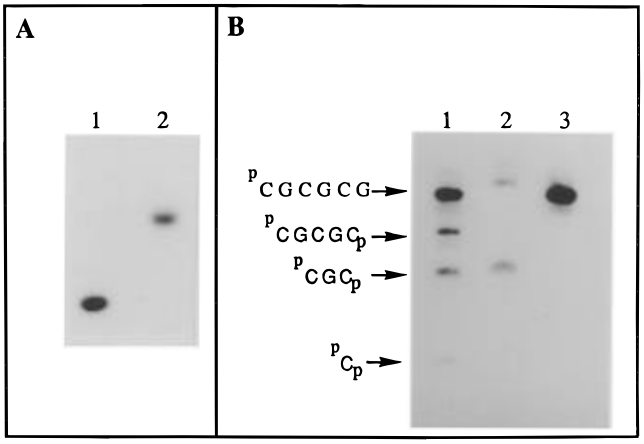


FIGURE 2: (A) Denaturing polyacrylamide gel electrophoresis of ^{32}P-end-labeled d(CGCGCG) (lane 1) and d(CGCG^{AP}CG) (peak A from Figure 1) (lane 2). Electrophoresis was carried out at room temperature at 1800 V for 3.5 h on a 16% polyacrylamide gel (30 cm × 40 cm × 0.4 mm) containing 8 M urea. (B) Denaturing polyacrylamide gel electrophoresis of ^{32}P-end labeled d(CGCGCG) that was subjected to the Maxam−Gilbert G-specific sequencing reaction (lane 1); peak A from Figure 1 treated with piperidine at 90 °C for 3 h (lane 2); unmodified hexamer, d(CGCGCG), treated with piperidine under identical conditions as in lane 2 (lane 3). Electrophoresis was carried out at room temperature at 1800 V for 1 h and 20 min on a polyacrylamide gel as described above.

of which were complementary to the terminal 20 nucleotides of the linearized vector (Scheme 2). The central segment of the oligomer was complementary to 5′-CGCGCG, which allowed the ligation of 5′-CGCG^{AP}CG to the ends of the M13 vector by T4 DNA ligase. A control unmodified genome was constructed in a similar manner. A mock ligation was also carried out, in which the hexamer 5′-CGCGCG was replaced with 5′-ATGCAT. The purpose of the latter was to determine the extent of ligation at the two ends of the gap, since the hexamer 5′-ATGCAT cannot anneal to the gapped region.

In order to visualize the unmodified and modified DNA constructs, a portion of each of these genomes was run on a 1% agarose gel in the presence of ethidium bromide (Figure 3, lanes 3 and 4). Densitometry analysis indicated that the efficiency of recircularization of both the unmodified and modified vectors was 34−35%. When these DNA constructs were treated with an excess of *Bss*HII, >95% of the unmodified genome was digested to a linear material (lane 5) whereas the presence of dG^{AP} at the restriction site of the modified genome prevented it from being digested with this enzyme (lane 6). This is not surprising because inhibition of restriction enzyme cleavage by the presence of DNA adducts has been shown in many earlier studies [for example, see Basu et al. (1993) and Benamira et al. (1992)]. It is noteworthy that only a small amount (<2%) of circular DNA was detected in the mock ligation, suggesting that end ligation of the gap by forming a loop in the GCGCGC segment of the scaffold is a rare event (data not shown).

To remove the 46-mer scaffold from the M13 DNA, each DNA solution was heated at 100 °C for 2 min and rapidly cooled to 0 °C. Prior to heating, a 10-fold molar excess of a 46-mer that contained the DNA sequence complementary to the scaffold oligomer was added to the DNA solution to ensure that the scaffold, once denatured, did not reanneal on the M13 DNA. To monitor whether removal of the scaffold was quantitative, an aliquot of gapped genome was subjected to the same steps of denaturation and analyzed by

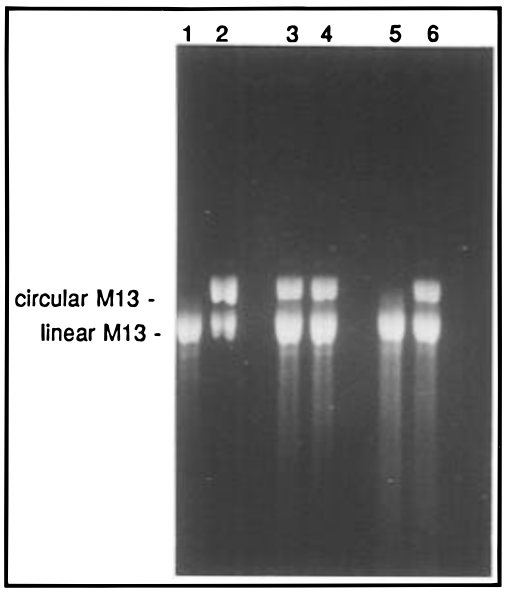


FIGURE 3: Characterization of the M13 constructs. The DNA samples were electrophoresed on 1% agarose gel in the presence of ethidium bromide (1 μg/mL). Lanes 1 and 2 show *Eco*RI-digested M13mp7L2 before and after annealing the 46-mer scaffold, respectively. The M13 genomes after ligation of unmodified and modified hexamers are shown in lanes 3 and 4, respectively. Lanes 5 and 6 show the unmodified and modified M13 genomes, respectively, after treatment with 15 units of *Bss*HII for 3 h at 37 °C.

Table 1: Survival of Site-Specifically Modified M13 in *E. coli*[a]

		relative survival (%)			
		control[a]		dG^{AP} adducted	
strain (genotype)	expt	−SOS	+SOS[b]	−SOS	+SOS[b]
NR9295	1	100 (1.6×10^7)[c]	100 (1.0×10^6)	24	38
(wild type)	2	100 (1.0×10^7)	100 (1.8×10^6)	28	45
NR9292 (*mutH*)	1	100 (7.9×10^6)	100 (7.1×10^6)	22	33
	2	100 (8.5×10^6)	100 (2.0×10^6)	21	36
NR9293 (*mutL*)	1	100 (7.1×10^6)	100 (8.4×10^6)	25	35
	2	100 (8.0×10^6)	100 (7.8×10^6)	23	30
NR9294 (*mutS*)	1	100 (8.4×10^6)	100 (9.0×10^5)	20	30
	2	100 (7.5×10^6)	100 (7.0×10^6)	24	32
NR11446	1	100 (9.0×10^5)	100 (1.4×10^5)	19	22
(*dnaQ49*)	2	100 (1.7×10^6)	100 (5.4×10^5)	20	25
NR9288 (*mutD*)	1	100 (2.8×10^6)	100 (7.7×10^5)	22	30
	2	100 (1.0×10^6)	100 (5.6×10^5)	25	33

[a] Survival (in percent) was determined by comparing the number of infective centers from the adducted DNA with that of the control genome (assumed to be 100% survival). [b] SOS was induced by UV (50 J/m^2) irradiation as described in Methods. [c] Transformation efficiencies (per microgram of DNA) are shown in parentheses for the control genome.

agarose gel electrophoresis. Since migration characteristic of linear and circular DNA in an agarose gel is different, we could demonstrate a complete conversion of any unligated circular genome (or a gapped genome) to linear M13 DNA by the above protocol. In a separate experiment the adducted hexamer was exposed to the conditions of ligation and denaturation of the scaffold and analyzed by HPLC. No detectable (>1%) degradation of the adduct was noted, suggesting that dG^{AP} is stable to the conditions of genome construction.

Survival of dG^{AP}-Adducted M13 Genome. As shown in Table 1, survival of the M13 genome containing a single adduct was 26% of that of the control in *E. coli* cells with

Table 2: Mutagenesis Detected Phenotypically at the $(CG)_3$ Sequence

		control[a]		dG^{AP} adducted[a]	
strain (genotype)	expt	−SOS	+SOS	−SOS	+SOS
NR9295 (wild type)	1	0.09 (9/10093)[b]	0.6 (11/1834)	1.6 (21/1337)	2.0 (32/1607)
	2	0.08 (7/8277)	0.7 (14/2013)	2.0 (56/2794)	3.2 (71/2243)
NR9292 (*mutH*)	1	0.08 (5/6021)	0.5 (7/1517)	3.1 (39/1262)	3.6 (59/1654)
	2	0.10 (4/3746)	0.8 (7/899)	2.9 (33/1122)	4.2 (63/1501)
NR9293 (*mutL*)	1	0.09 (4/4265)	0.6 (7/1122)	2.6 (45/1727)	6.0 (61/1016)
	2	0.10 (5/4958)	0.8 (8/1014)	2.6 (36/1378)	5.0 (53/1056)
NR9294 (*mutS*)	1	0.08 (6/7306)	0.7 (8/1132)	2.8 (30/1065)	5.7 (90/1583)
	2	0.09 (7/8003)	0.9 (10/1094)	2.5 (26/1042)	6.3 (55/871)
NR11446 (*dnaQ49*)	1	0.3 (13/3949)	0.5 (8/1658)	8.5 (122/1436)	15.4 (279/1810)
	2	0.3 (9/2949)	0.5 (10/1888)	7.6 (76/1004)	16.5 (234/1417)
NR9288 (*mutD*)	1	0.5 (7/1493)	0.6 (7/1120)	9.3 (83/894)	16.5 (190/1154)
	2	0.5 (6/1320)	0.6 (8/1327)	10.4 (105/1014)	17.0 (189/1113)

[a] Mutation frequency (%). [b] Number of blue plaques detected per total number of plaques.

Table 3: Sequence Analysis of Mutants[a]

				+1 addition[d]		
strain (genotype)	genome[b]	SOS	CpG deletion (MF)[c]	+C (MF)	+G (MF)	other[e] (MF)
NR9295 (wild type)	G	−	9/9 (0.09)[c]			
	G	+	16/16 (0.65)			
	G*	−	15/16 (1.7)		1/16 (0.1)	
	G*	+	6/14 (1.1)	2/14 (0.37)	6/14 (1.1)	
NR9294 (*mutS*)	G	−	15/15 (0.09)			
	G	+	16/16 (0.8)			
	G*	−	14/16 (2.3)		2/16 (0.3)	
	G*	+	5/16 (1.9)	6/16 (2.25)	4/16 (1.5)	1/16 (0.04)
NR11446 (*dnaQ49*)	G	−	13/15 (0.28)			2/15 (0.04)
	G	+	12/12 (0.51)			
	G*	−	14/16 (7.1)			2/16 (1.0)
	G*	+	6/18 (5.3)	12/18 (10.7)		

[a] An approximately equal number of phenotypically detectable mutants from two separate experiments were subjected to DNA sequencing. [b] G and G* denote control and adducted genome, respectively. [c] Mutation frequency of each type, as determined by DNA sequencing, is shown in parentheses. [d] The sequence alteration of +1 mutants was 5′-CGCCGCG (plus C) or 5′-CGCGGCG (plus G) from 5′-CGCG^{AP}CG. [e] The only other type of phenotypically detectable mutation was a −8 frame-shift.

normal repair functions. With SOS[3] (50 J/m^2), survival increased to 42%. This suggests that dG^{AP} constitutes a strong block of DNA replication and that SOS partially alleviates the replication block. In several other strains with defects in either mismatch repair or proofreading, viability of the adducted vector followed a similar pattern. In mismatch repair-deficient and *dnaQ* strains, particularly with SOS, however, survival of the adducted genome was 20−40% lower. A reduced survival in *mutH*, *mutL*, and *mutS* can be rationalized if mismatch repair plays a role in adduct repair. In the *dnaQ* strains, on the other hand, transformation efficiency was approximately an order of magnitude lower for the control genome. Because of such a drastic reduction in transformation efficiency, it is uncertain if the further decrease in survival of the adducted genome was due to the presence of dG^{AP}. The *dnaQ* strains are potent mutators and are defective in the *dnaQ* gene, which encodes the proofreading 3′→5′ exonuclease (ϵ-subunit) of DNA polymerase III holoenzyme (Degnen & Cox, 1974; Echols et al., 1983).

$(CG)_3$ Is a Hot Spot for Spontaneous Mutagenesis. Our strategy for screening and selection of mutants is shown in Scheme 2. The M13 vector we have constructed is a +2 derivative, which generates colorless plaques in the presence of IPTG and X-Gal. Either a −2 or a +1 frame-shift can restore the reading frame to a Lac$^+$ phenotype generating blue plaques. Furthermore, the adduct was situated within a unique 5′-GCGCGC site, the recognition sequence for the restriction endonuclease *Bss*HII. Therefore, all other types of mutant phage population within the hexanucleotide segment can be enriched by the *Bss*HII digestion.

Spontaneous mutagenesis of the unmodified vector as determined phenotypically by the reversion to blue plaques was 1×10^{-3} (Table 2). With SOS, the MF increased to 6×10^{-3}. The spontaneous MF remained almost unchanged in strains with a defect in the methyl-directed mismatch repair system. However, the MF increased 3−5-fold in *dnaQ* strains (i.e., *dnaQ49* and *mutD* strains). Assuming that a misaligned intermediate was responsible for the mutagenesis, these data imply that a part of the misaligned structures was corrected by the exonucleolytic proofreading and not by the methyl-directed mismatch repair system. When SOS functions were induced, however, the variation in spontaneous MF was almost undetectable in all these strains.

DNA sequencing showed that CpG deletion occurred in these mutants, except in the population collected from the *dnaQ* strains in which a −8 deletion was also detected (Table 3). The mutagenesis in the $(CG)_3$ sequence was not unexpected, because repetitive CpG sequences have been shown to be spontaneous −2 deletion hot spots (Isono & Yourno, 1974; Fuscoe et al., 1988; Cebula & Koch, 1990). However, the lack of enhancement of mutagenesis in mismatch repair-deficient strains was surprising particularly because M13mp2 containing the $(CA)_{14}$ insert undergoes reversion at a frequency of ∼1% in control strains, which is

[3] The UV dose of 50 J/m^2 was chosen for SOS induction because this irradiation dosage provided maximum enhancement of dG^{AP} mutagenesis in our prior study (Malia & Basu, 1995).

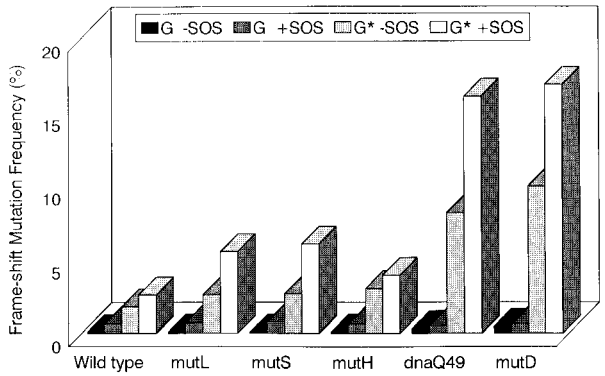


FIGURE 4: Comparison of phenotypically detectable mutation frequency of control and dG^{AP}-adducted M13 genome in wild type and repair-deficient strains. G and G* denote unmodified and dG^{AP}-adducted M13 genome, respectively. The results are the mean of two separate experiments.

increased 4−5-fold in *mutS*, *mutL*, and *mutH* strains (Strauss & Sagher, 1995). In addition, in an earlier investigation, frame-shifts within the poly(CA/TG) sequence increased more than an order of magnitude in *mutL* and *mutS* cells (Levinson & Gutman, 1987). However, both these studies employed long stretches of repetitive sequences, and one explanation of our result is that involvement of methyl-directed mismatch repair may be dependent on the length of the repeat unit. Indeed, in the study carried out by Levinson and Gutman (1987) reversion frequency increased more than linearly with the length of the repeat unit. Furthermore, our study involved CpG repeat sequences, whereas the other two studies employed CpA repetitive sequences.

Frequency and Specificity of Frame-Shift Mutagenesis of the dG^{AP}-Adducted M13 Genome. The presence of dG^{AP} increased the MF as determined by an increased proportion of blue plaques (Table 2). MF of the adducted genome without SOS (2×10^{-2}) was approximately 20-fold that of control genome in a repair-proficient strain. Without SOS, in *mutS*, *mutH*, or *mutM* strains, MF increased 50−70% compared to the same in the wild-type strain, whereas in the *dnaQ49* and *mutD* strains MF was 3−5-fold that of the repair-proficient strain (Figure 4). It is interesting that the MF of dG^{AP} in the mismatch repair-deficient strains without SOS is approximately the same as for the SOS-induced wild-type strain.

With SOS induction, MF increased in all the repair-deficient strains as well. For the mismatch repair-deficient strains with SOS, an approximately 2-fold increase in MF relative to the same in the wild-type strain was noted, whereas for the *dnaQ* strains there was an approximately 6−7-fold enhancement of mutagenesis (Figure 4). In comparison to spontaneous frame-shift events, the presence of dG^{AP} resulted in a 30-fold enhancement of mutagenesis in the SOS-induced *dnaQ* strains. The adduct-induced frame-shift mutagenesis varied from 2% in uninduced wild-type *E. coli* to 17% in the SOS-induced *mutD* strain.

To determine the types of mutations occurring in wild-type, mismatch repair defective, and *dnaQ* strains, a representative population of blue plaques from control and adducted genome was subjected to DNA sequencing. The sequencing results indicated that, for the adducted vector, CpG deletion was the major type of mutation detected in the absence of SOS (Table 3). However, in the presence of SOS, the predominant mutation was a one-base insertion event. It is interesting to note that the −2 frame-shifts were reduced when SOS was induced, although the total frequency of frame-shifts exhibited a 50% enhancement (Table 3). Single-base additions included both +G and +C. The sequence alteration was 5′-CGCGGCG or 5′-CGCCGCG from 5′-CGCG^{AP}CG, and therefore the exact site where the single nucleotide insertion occurred cannot be determined. Also, on the basis of the small pool of mutants sequenced, it is unclear if one type is favored over the other type or if there is a change in the type of +1 mutagenesis when transformations were carried out in one strain relative to another. In addition to −2 and +1, an infrequent −8 frame-shift was detected, which involved replacement of 5′-TTAAGCGCGCGTGACTTA with 5′-TTAGGCATTA. Since this was also observed in the progeny from the control genome, it is unlikely that this was induced by dG^{AP}.

Does dG^{AP} Induce Any Mutations That Are Phenotypically Undetectable? The strategy of digesting the wild-type DNA for the enrichment of mutant population has been used successfully in many earlier site-specific studies (Basu & Essigmann, 1988; Basu et al., 1989, 1993). Since the progeny isolated from the *dnaQ* strains showed the highest level of mutagenesis, we employed this strategy to determine if there were any mutants in the progeny (obtained from either uninduced or SOS-induced cells) that exhibit colorless plaque phenotype. Extensive *Bss*HII digestion of the DNA isolated from progeny phage followed by exonuclease III treatment and subsequent transformation in *E. coli* resulted in a large enhancement of the proportion of blue plaques. DNA sequencing of a population (∼30) of the remaining clear plaques indicated that approximately 1% of the initial phage population did not contain the CGCGCG hexamer segment, which presumably arose from ligation at the two ends of the gap as noted in several earlier site-specific experiments (Basu et al., 1989, 1993). In addition, we detected a small fraction of −1 mutants that involved a C deletion at the 5′ ligation site of the inserted hexanucleotide. This event occurred at a frequency of <0.1%.[4] However, this deletion also does not appear to represent adduct-induced mutagenesis, since such mutations were believed to arise from nicks remaining from genetic engineering techniques (Benamira et al., 1992). All other colorless plaques exhibited wild-type sequences. This implies that phenotypically undetectable mutations, if any, occur at a very low frequency at this site. We cannot rule out the possibility, however, that one-base deletions may occur at a higher frequency in cells other than the *dnaQ* strains.

DISCUSSION

In most organisms high fidelity of DNA replication is maintained by at least three major steps. First, the DNA polymerase can discriminate against insertion of incorrect nucleotides. Second, the 3′→5′ exonuclease activity associated with the DNA polymerase can remove the incorrectly incorporated nucleotide. Third, a DNA mismatch repair system detects and corrects mismatched nucleotides shortly after replication. It has been estimated that base selection discriminates against errors by 200 000−2 000 000-fold, proofreading by 40−200-fold, and mismatch repair by 20−400−fold, each depending on the type of error (Schaaper,

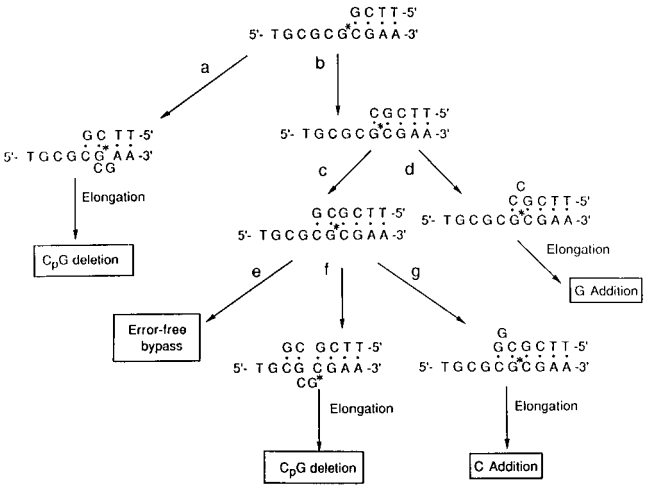
[4] The frequency of these events was calculated essentially as described in Basu et al. (1993).

1993). It is well established that the ability of the DNA polymerase to incorporate the correct nucleotide opposite many carcinogen DNA adducts is impaired. In addition, types and frequencies of misincorporation are often dependent on the DNA sequence context [for example, see Singer et al. (1989)]. For the last two decades mispairing potential of many adducts has been studied extensively (Basu & Essigmann, 1988, 1995). In contrast, relatively little is known on the roles of 3′→5′ exonuclease activity of the DNA polymerase and mismatch repair proteins in adduct-induced mutagenesis.

The work described herein is the first unambiguous demonstration that mutations induced by dG^{AP}, a bulky carcinogen−DNA adduct, increase significantly if the 3′→5′ exonuclease activity of the DNA polymerase III is impaired. A modest enhancement of mutagenesis also was observed with a defective methyl-directed mismatch repair system. Exonucleolytic editing is a major contributor to the fidelity of DNA synthesis by DNA polymerase III, which was shown in many earlier studies (Echols & Goodman, 1991). In addition, the current study showed that this editing function plays a major role in correcting misaligned structures induced by dG^{AP}. How does a 3′→5′ exonuclease recognize mispairs or misaligned structures? Two possible mechanisms have been suggested. The exonuclease may recognize a departure from the Watson−Crick geometry (Echols & Goodman, 1991). The exonucleolytic editing may also depend on the melting capacity of the 3′ terminus since a mismatched or misaligned structure exists more often in a single-stranded form (Echols & Goodman, 1991). Both structural and kinetic studies suggest that melting of the 3′ terminus is the primary determinant for editing (Brutlag & Kornberg, 1972; Brenowitz et al., 1991). The results of the current study suggest that dG^{AP} promotes misaligned intermediates. However, on the basis of the melting model, one can postulate that its inability to form a stable pair can be detected by the exonuclease, which efficiently eliminates a major fraction of these promutagenic intermediates. Misaligned intermediates that escape the proofreading function of the polymerase are subject to repair by the *mutSLH* pathway. Increase in mutagenesis in the mismatch repair defective strains is particularly noteworthy in the SOS-induced cells. Parker and Marinus (1992) showed that heteroduplexes containing one-, two-, and three-base deletions are repaired very efficiently. Our results indicate that bulges opposite a bulky adduct can also be repaired, albeit with reduced efficiency.

It has been argued that a required function of the SOS regulon is to inhibit 3′→5′ exonuclease activity associated with the DNA polymerase (Lu et al., 1986). This step is believed to be important for translesion synthesis. The observations that the RecA protein binds to UV photoproducts and inhibits the editing subunit of DNA polymerase III are consistent with this hypothesis (Lu et al., 1986). Furthermore, overproduction of 3′→5′ exonuclease counteracts the SOS response (Jonczyk et al., 1988). Notwithstanding this relationship between editing and SOS, it was clearly shown in the current study that dG^{AP}-induced +1 mutations do not occur by an impaired exonuclease activity alone. Some other factors are therefore required to trigger the SOS mutagenesis. In principle, this finding is similar to the in vitro observation that loss of 3′→5′ exonuclease activity of the DNA polymerase increases the frequency of UV-induced frame-shifts, but lack of such activity is not sufficient for their production (Sagher et al., 1994).

Scheme 3: Model To Account for the −2 and +1 Mutagenesis in the $(CG)_3$ Sequence



Another noteworthy feature of the current study is that dG^{AP} induced at least two distinct frame-shift mutations in the CpG repetitive sequence. Without SOS, dG^{AP} induced −2 deletion ∼20-fold that of control. With SOS, however, the frequency of CpG deletion decreased and one-base additions occurred at a high frequency. This suggests that dG^{AP}-induced −2 and +1 frame-shifts are competing pathways. A model consistent with our data is shown in Scheme 3. In the absence of SOS, dG^{AP} is a strong block of DNA replication, and therefore pathway a would be favored. The misaligned structure allows the DNA polymerase to bypass the adduct even though it may be unable to incorporate a nucleotide opposite the adduct. When the SOS functions are induced, the rate of translesion synthesis increases. In pathway b the correct nucleotide dCMP is incorporated opposite the adduct. This is consistent with an in vitro study, which showed that the Klenow fragment of DNA polymerase I prefers to incorporate dC opposite dG^{AP}, although the adduct was located in a different sequence (Vyas & Basu, 1995). We postulate that upon induction of SOS, pathway b is preferred over pathway a. Subsequently, DNA replication can continue in an error-free manner (pathway e). Alternatively, this structure can also undergo misalignment to generate a −2 (pathway f) or cause a C addition (pathway g). The data in the current work suggest that pathway g is preferred over pathway f. Pathway d shows a slightly altered route to cause a G addition.

The results of this study also point out the similarities and differences between the mutagenic mechanism of dG^{AP} with that of the C8-Gua adduct of AAF. For example, both these adducts cause CpG deletion in alternating CG sequences. In addition, neither UmuDC nor the RecA protein is needed for optimal mutagenesis by these adducts. However, −2 frame-shift by AAF at the *Nar*I site requires a LexA-controlled function other than UmuDC (Koffel-Schwartz et al., 1984). Furthermore, induction of SOS results in two different types of dG^{AP}-induced +1 frame-shifts that were never detected with the AAF adduct in alternating CG sequences (Burnouf et al., 1989; Bintz & Fuchs, 1990; Tebbs & Romano, 1994). Unlike the AAF adduct, dG^{AP}-induced CpG deletion is unique in that the frequency of this deletion is reduced upon induction of SOS response. Therefore, it appears that there are several critical differences between the mechanism of mutagenesis by these two C8−Gua adducts. For more than two decades much attention was

focused on the differences in mutagenesis between the C8−Gua adduct of AAF and its deacetylated derivative, AF. The C8−Gua adducts of AF and AAF adopt different conformations and their mutagenic specificity is significantly different (Fuchs et al., 1981, 1983; Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985; Norman et al., 1989; Reid et al., 1990; O'Handley et al., 1993; Garcia et al., 1993; Cho et al., 1994; Eckel & Krugh, 1994; Tebbs & Romano, 1994). It is conceivable that the conformations of these adducts could be linked to their mutagenic specificity. Unlike the AF and AAF adducts, relatively little is known about the conformation(s) of dG^{AP}. However, a recent 2D-NMR study[5] in a CGC sequence showed that the aminopyrene moiety of dG^{AP} can reside in an intercalated base-displaced orientation opposite dC in a DNA duplex. Further studies must be carried out to understand the relationship between the conformation(s) of dG^{AP} with its mutational specificity.

In conclusion, CpG repetitive sequences are spontaneous −2 frame-shift hot spots, and the MF increases with SOS. A misaligned structure appears to be the promutagenic intermediate. The presence of dG^{AP} increases the occurrence of such intermediates, resulting in a 20-fold enhancement of CpG deletions. With SOS, further increase in adduct-induced mutagenesis was observed, but a +G or +C frame-shift occurred at a high frequency concomitantly with a reduction in the −2 frame-shifts. In mismatch repair deficient cells, a modest increase in mutagenesis was observed, whereas in cells with an impaired 3′→5′ exonuclease activity of *pol* III a large enhancement in frame-shift mutagenesis occurred. Each of the three factors, i.e., SOS, mismatch repair, and proofreading function of the DNA polymerase, therefore, plays a role in dG^{AP}-induced frame-shift mutagenesis.

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