Mutagenesis at a Site-Specifically Modified *Nar*I Sequence by Acetylated and Deacetylated Aminofluorene Adducts†

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ABSTRACT: A hotspot for mutagenesis by *N*-acetyl-2-aminofluorene (AAF) was site-specifically modified with 2-aminofluorene (AF) and AAF adducts, and the mutation frequencies and specificities were determined and compared. Previous work has shown that the presence of an AAF adduct in a *Nar*I sequence (GGCGCC) results a high mutation frequency for a CG double base pair deletion. In the present study, an M13 derivative was constructed that contained a *Nar*I recognition sequence in the β-galactosidase gene of bacteriophage M13mp9. This derivative was site-specifically modified with either an AF or an AAF adduct, the products were characterized, and these templates were then transformed into *Escherichia coli* wild-type strain JM103 or *uvrA* strain SMH12. The levels and mutation spectra were determined either with or without SOS induction. It was found that, with SOS functions induced, the measured mutation frequencies were substantially higher in all cases. More importantly, the types of mutations induced by the AAF and AF adducts were very different: AAF adducts induced almost exclusively CG double base deletion mutations, whereas AF adducts gave rise specifically to base-substitution mutations. The AF-derived mutation spectrum included both G to T and G to A mutations. The results are discussed in light of the current views on the relationship between the DNA structure and mutagenesis.

N-(Deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF)[1] and *N*-(deoxyguanosin-8-yl)-*N*-acetyl-2-aminofluorene (dG-C8-AAF) are the two major adducts found in the DNA of experimental animals shortly after the administration of the potent carcinogen *N*-acetyl-2-aminofluorene (King, 1985). A third minor adduct is also formed and was shown to be 3-(deoxyguanosin-N^2-yl)-2-(acetylamino)fluorene (Westra et al., 1976). When the mutation spectra in bacteria for the two adducts were determined, the results were qualitatively very different: greater than 85% of the mutations induced by AF adducts that were detected were base-substitution mutations (Bichara & Fuchs, 1985), whereas 90% of the mutations induced by AAF adducts were found to be frameshift mutations (Koffel-Schwartz et al., 1984). Although each adduct was found to be approximately equally distributed among all of the guanine bases, mutation hotspots were detected in the AAF-induced mutation spectrum (Koffel-Schwartz et al., 1984) but not in the AF-induced spectrum (Bichara & Fuchs, 1985). These AAF-induced mutation hotspots belonged to two general classes: repetitive sequence hotspots and nonrepetitive sequence hotspots (Koffel-Schwartz et al., 1984). Mutations recovered from the repetitive mutation hotspots were largely deletions of a repeat unit and could be accounted for by a slippage model (Streisinger & Owen, 1985; Lambert et al., 1992). The nonrepetitive mutation hotspot represented a new class of previously undetected mutation type and was found solely within the *Nar*I restriction enzyme recognition sequence (GGCGCC). This *Nar*I mutation was characterized not only by an increased mutation frequency but also by the induction of only CG double base deletion mutations (GGCGCC → GGCC). Furthermore, the *Nar*I-induced mutation was found to be *umuC*-independent. Using site-specific adducts, it was shown that only AAF adducts positioned in the third guanine of this sequence induced this deletion (Burnouf et al., 1989).

A careful analysis of the AF-induced mutation spectrum detected by Fuchs and co-workers (Bichara & Fuchs, 1985) indicated that even though AF has been found to be characteristically a base-substitution mutagen, a small number of frameshift mutations were detected. Interestingly, three out of the six AF-induced frameshift mutations were located within a *Nar*I sequence. Thus the *Nar*I sequence was far more prone to AF-induced frameshift mutations than any other sequence within the region analyzed.

To directly compare the mutation induction of AF and AAF adducts located in the *Nar*I sequence, we have constructed site-specifically modified DNA molecules containing either an AF or an AAF adduct at the third guanine of the *Nar*I sequence. In this paper we study the mutation spectra induced by these singly modified vectors. We find that only the AAF adduct induced the characteristic CG deletion mutation, while the AF adduct induced base-substitution mutations similar to the types of mutations induced at any other sequences.

EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Media. *Nar*I restriction endonuclease was purchased from Bethesda Research Laboratories (Gaithersburg, MD). *Hin*dIII restriction endonuclease and T4 DNA ligase were purchased from New England Biolabs (Beverly, MA). Sequenase version 1 (modified T7 DNA polymerase), *Escherichia coli* polymerase I large fragment, bacteriophage T4 polynucleotide kinase, bacteriophage T7

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[1] Abbreviations: dG-C8-AAF, *N*-(2′-Deoxyguanosin-8-yl)-*N*-acetyl-2-aminofluorene; AAF, *N*-acetyl-2-aminofluorene; dG-C8-AF, *N*-(2′-deoxyguanosin-8-yl)-2-aminofluorene; HPLC, high-performance liquid chromatography; EDTA, ethylenediaminetetraacetic acid; TE buffer, 10 mM Tris-HCl, pH 8, and 1 mM EDTA; PEG, poly(ethylene glycol); ss, single stranded; ds, double stranded; GHD, gapped heteroduplex; X-gal, 5-bromo-4-chloro-3-indolyl β-galactoside; IPTG, isopropyl β-D-thio-galactopyranoside.

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gene 6 exonuclease, *E. coli* DNA gyrase, and DNA sequencing kits were purchased from United States Biochemicals (Cleveland, OH). All reactions were performed under the conditions suggested by the supplier unless otherwise stated.

[α-^{32}P]dATP (3000 Ci/mmol) and [γ-^{32}P]ATP (4500 Ci/mmol) were purchased from ICN Radiochemicals (Irvine, CA). Nucleotides were purchased from Pharmacia LKB Biotechnology Inc. (Piscataway, NJ), and all other reagents purchased were of the finest quality available.

Oligonucleotides. Synthetic oligonucleotides were obtained from the Center for Molecular Biology at Wayne State University. The crude oligomers were detritylated on a NENSORB PREP column by treatment with 0.5% trifluoroacetic acid as recommended by the manufacturer (NEN/DuPont, Hoffman Estates, IL) and purified by HPLC on a Varian Model 5000 liquid chromatograph connected to a Polychrom 9060 diode array detector utilizing a Zorbax Bio Series Oligo column (8 cm × 6.2 mm) (DuPont), followed by repeated lyophilizations to remove the volatile salt.

Bacteriophage, Bacterial Strains, and DNA Isolation. *E. coli* strains JM103 (endA, hsdR, supE, sbcBC, thi-1, strA, Δ[lac-proAB]/F′ traD36, proAB$^+$, lacI^q, ZΔM15) and SMH12 (F′ lacZΔM15, pro$^+$, supE44, thr-1, leu-6, proA2, his-4, argE3, thi-1, lacY1, galK2, ara-14, xyl-5, mtl-1, txs-33, rpoL21) were obtained from Dr. J. E. LeClerc (University of Rochester). M13mp9xG (M13mp9 containing an extra G in the *Bam*HI site) was prepared as described (Gupta et al., 1989).

M13RT-A$_7$ is a derivative of M13mp9xG which contains a *Nar*I sequence in the cloning region of this vector. The first step in the construction of M13RT-A$_7$ involved the disruption of the original *Nar*I sequence by cleaving 1.2 μg with 36 units of *Nar*I overnight at 37 °C, giving rise to linear molecules with ends containing two-base 5′ overhangs. The 3′ OH recessed ends were extended to generate blunt ends by the addition of 5 units of *E. coli* DNA polymerase I large fragment to 0.32 μg of the *Nar*I-digested DNA for 15 min at 37 °C in a buffer containing 0.8 mM dCTP, 0.8 mM dGTP, 10 mM Tris-HCl (pH 8.0), 6.6 mM $MgCl_2$, and 1 mM β-mercaptoethanol (total volume of 20 μL). The blunt-ended molecules (0.15 μg) were ligated at 4 °C for 4 h with T4 DNA ligase at a DNA concentration of 1 μg/mL to form double-stranded circular DNA. The product was then transformed into JM103 host cells, and a progeny phage (M13mp9xG *Nar*I$^-$) was isolated that was found to be resistent to *Nar*I digestion. Sequencing the resistant phage confirmed that the *Nar*I site was disrupted.

The second step in creating M13RT-A7 was the introduction of a new *Nar*I sequence in the cloning region at position 6240 by annealing a 25-base oligonucleotide primer, 5′-CGACCTGCAGTGGCGCCAAGCTTGG-3′, in a 100-fold excess to 0.15 μg of single-stranded M13mp9xG(NarI$^-$) followed by the addition of 1 unit of DNA polymerase I (large fragment) and a mixture of dNTPs (0.25 mM each) and allowing DNA synthesis to proceed at 25 °C for 10 min. T4 DNA ligase was then added, and the product was transformed into JM103. The 25-base primer contains five extra noncomplementary bases (underlined) required for insertion of the new *Nar*I site. The addition of five bases by this step in combination with the extra G from the parent molecule restored the reading frame of the LacZ′ marker enzyme, which therefore resulted in progeny that gave rise to blue plaques. A blue plaque was isolated and sequenced and was found to contain a *Nar*I sequence inserted as designed. This hybrid phage was labeled M13RT-A$_7$.

Replicative form (RF) DNA was isolated by alkaline lysis (Birnboim & Doly, 1979) and purified on cesium chloride–ethidium bromide density gradients. Ethidium bromide was removed from DNA on a DOWEX 50X8-200 cation-exchange column. Viral DNA was purified by a modified phenol extraction procedure as described (Dugaiczyki et al., 1975). Following purification, both preparations were dialyzed extensively against 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (TE buffer). Single-stranded DNA was isolated on a small scale to be used for sequencing experiments. Five milliliters of YT media was inoculated with 50 μL of JM103 starter culture and infected with a single viral plaque. The solution was incubated at 37 °C for 7 h and centrifuged at 10 000 rpm for 10 min to remove the cellular pellet. The supernatant containing the phage was precipitated by adding 200 μL of a PEG mixture (20% PEG and 2.5 M NaCl) followed by centrifugation at 10 000 rpm for 10 min. The pellet was resuspended in 400 μL of TE and phenol extracted, and the single-stranded DNA was ethanol precipitated.

Preparation of AF- and AAF-Modified Oligonucleotides. *N*-Acetoxy-*N*-acetyl-2-aminofluorene was a gift from Dr. C. M. King (Michigan Cancer Foundation). *N*-Acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene was synthesized as described (Reid et al., 1990). In a typical modification reaction 14 nmol of the pentamer 5′-CGCCA-3′ in 2 mM citrate buffer (pH 7.0) was mixed with 140 nmol of *N*-acetoxy-*N*-acetyl-2-aminofluorene (to produce the AAF adduct) or *N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene (to produce the AF adduct) in 95% ethanol such that the final solution became 10% in ethanol. The reaction mixture was incubated at 37 °C for 1–3 h in the dark under an argon atmosphere. All subsequent steps requiring the handling of the adducted oligonucleotide were performed under reduced light. Unreacted fluorene derivatives were removed from the product by repeated extraction with diethyl ether. The AF- and AAF-modified oligonucleotides were HPLC purified using a Zorbax Bio Series oligo column (DuPont). The HPLC was run isocratically with 20% acetonitrile and with a gradient of 1 M ammonium acetate: 5 min at 0% 1 M ammonium acetate followed by a 0–75% gradient of 1 M ammonium acetate over 30 min, at a flow rate of 0.75 mL/min. Under these conditions the 5-mer eluted at 16.5 min, the dG-C8-AAF-containing 5-mer eluted at 21.0 min, and the dG-C8-AF-containing 5-mer eluted at 23.8 min (Figure 1). Samples were collected from the HPLC and lyophilized at least four times. The isolated materials were ^{32}P-labeled with polynucleotide kinase and run on a 23% polyacrylamide gel (Figure 2). Both the AF-modified and the AAF-modified materials ran slower than the unmodified material as expected (Johnson et al., 1986), and no other species were observed following HPLC purification.

Covalent modification at the lone guanine residue by the AF and AAF adducts was confirmed by piperidine digestion (Johnson et al., 1986, 1987). Each of these ^{32}P-labeled samples was treated with piperidine for 3 h at 90 °C to induce strand cleavage at the adduct site which in both cases should generate ^{32}pCp. Under these conditions approximately 50% of the strands should be cleaved (Johnson et al., 1987). Longer treatment resulted in higher levels of cleavage (not shown). The products of piperidine cleavage were run on a polyacrylamide gel along with a dimethyl sulfate G-specific cleavage (Maxam & Gilbert, 1980) of the unmodified 5-mer serving as a control (Figure 3). It is evident from the analysis shown (Figure 3A, lane 7, and Figure 3B, lane 10) that piperidine treatment generated a material that comigrated

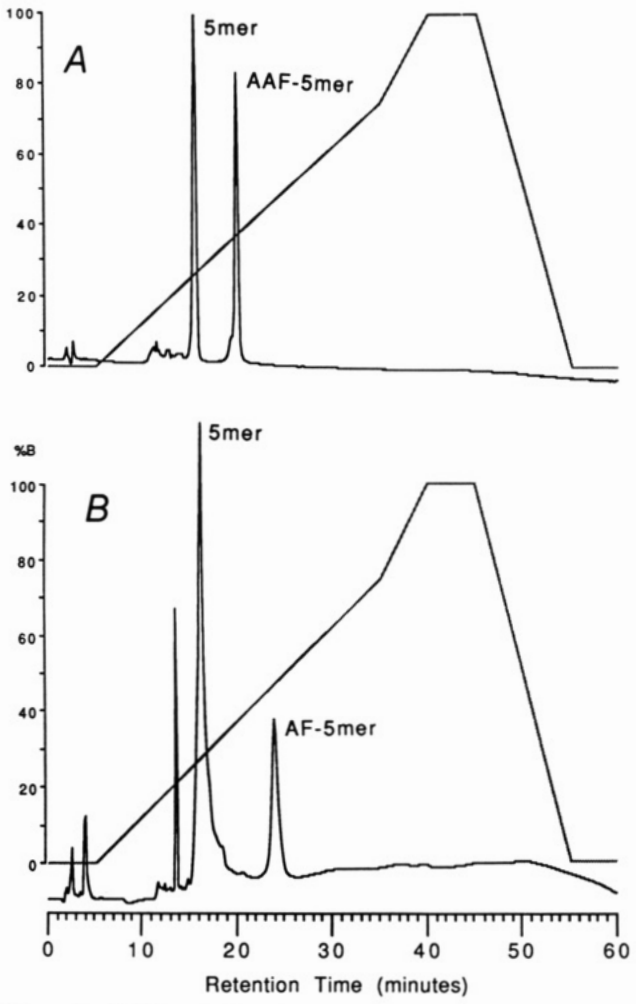

FIGURE 1: HPLC analysis and purification of AF- and AAF-modified 5-mers. The AF- and AAF-modified oligonucleotides were purified on a Zorbax Bio Series oligo column (DuPont) in 20% acetonitrile using a gradient of 1 M ammonium acetate.

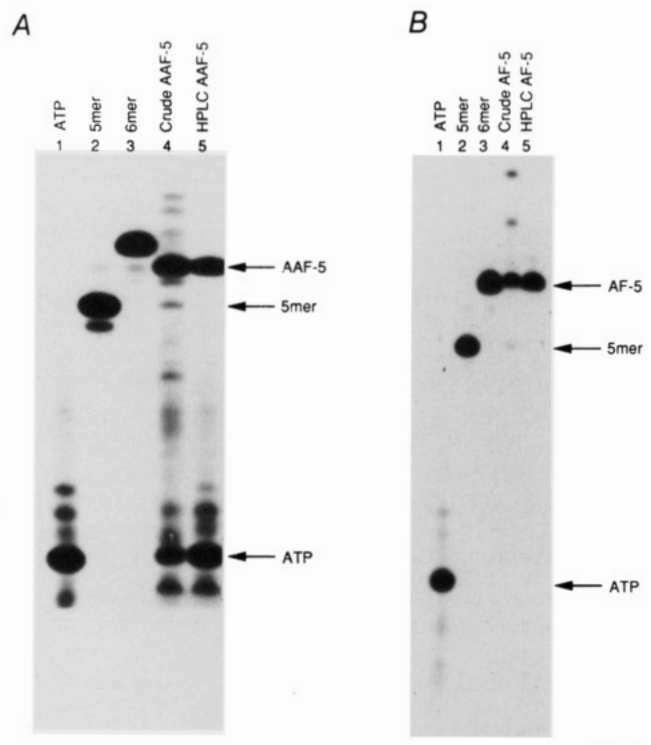

FIGURE 2: Polyacrylamide gel analysis of AF- and AAF-modified 5-mers. The crude reactions and the HPLC-purified materials from the AF and AAF modifications of the 5-mer (5′-CGCCA-3′) were labeled with ^{32}P using polynucleotide kinase and run on a 23% polyacrylamide gel along with labeled 5-mer and 6-mer controls, and the positions of 5-mers were determined by autoradiography. (A) Analysis of AAF-modified 5-mer: lane 1, ATP; lane 2, unmodified 5-mer, 5′-CGCCA-3′; lane 3, unmodified 6-mer; lane 4, crude reaction mixture containing AAF-modified 5-mer; lane 5, HPLC-purified 5′-CG$^{(AAF)}$CCA-3′. (B) Analysis of AF-modified 5-mer: lane 1, ATP; lane 2, unmodified 5-mer, 5′-CGCCA-3′; lane 3, unmodified 6-mer; lane 4, crude reaction mixture containing AF-modified 5-mer; lane 5, HPLC-purified 5′-CG$^{(AF)}$CCA-3′. Presumably no [γ-^{32}P]ATP is present in lanes 2–5 in panel B because the label was entirely transferred during the kinase reaction.

with authentic pCp in the gel. Finally, UV absorption spectrophotometry performed on the AF- and AAF-modified 5-mers gave rise to characteristic absorbances at 330 and 310 nm, respectively (Johnson et al., 1987) (data not shown).

Construction of the Site-Specifically Modified Vector. The procedure used to construct the site-specifically modified vector is shown schematically in Figure 4. A gapped-heteroduplex molecule was prepared having five bases removed from the negative strand at position 6239–6243, creating a five-base single-stranded region. RF M13RT-A_7 (206 μg) was cut with 250 units of *Nar*I restriction endonuclease in a buffer containing 10 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 1 mM dithiothreitol at 37 °C. *Nar*I was added in 50-unit aliquots at 90-min intervals, and the reaction was stopped after a total incubation time of 10 h by the addition of EDTA to 10 mM. Under these conditions approximately 85% of the M13RT-A_7 DNA was cut to yield linear DNA, with the remaining DNA appearing as nicked on an agarose gel; no supercoiled DNA remained. (*Nar*I has been shown to exhibit marked site preference (Hu, 1984), and unfortunately this particular recognition site was a poor substrate for *Nar*I, therefore requiring rigorous digestion procedures.) The *Nar*I-digested product was extracted twice with phenol/chloroform/isoamyl alcohol (25/24/1) and three times with ether. The sample was cut a second time with 175 units of *Hin*dIII in a buffer containing 10 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 1 mM dithiothreitol, and 50 mM NaCl at 37 °C for 5 h. The reaction was stopped by the addition of EDTA to 10 mM and extracted as before. The large DNA fragment was separated from the smaller excised fragment on a Sephacryl S-300 column (0.5 × 60 cm) using a TE buffer containing 50 mM NaCl. The large fragment, eluting in the void volume, was concentrated in a Centricon-30 microconcentrator (Amicon, Danvers, MA) and found to contain 110 μg of product. The extent of cleavage by each enzyme was determined using run-off synthesis with DNA polymerase I, large fragment, in the presence of dNTPs and utilizing either a ^{32}P-end-labeled reverse sequencing primer to visualize cleavage at the *Hin*dIII site or a ^{32}P-end-labeled normal sequencing primer to assess cleavage at the *Nar*I site. These experiments indicated that cleavage by *Hin*dIII was greater than 95%, while 85% of the *Nar*I sites were cleaved. The gapped-heteroduplex molecule (GHD) was constructed by mixing 110 μg of the large *Nar*I–*Hin*dIII double-digested DNA fragment with a 3-fold excess (330 μg) of single-stranded (+) M13RT-A_7 DNA in a solution containing 100 mM NaCl and 90% formamide at a final DNA concentration of 25 μg/mL. The sample (17.6 mL) was placed in dialysis tubing (25 mm, 12 000–14 000 MW cutoff), heated to 45 °C inside a flask containing the same buffer to ensure denaturation of the double-stranded fragment, and then allowed to cool to 25 °C over 30 min. The sample was subsequently dialyzed against a decreasing formamide concentration at a rate decreasing in formamide of 5% every 20 min until the final buffer was TE containing 100 mM NaCl.

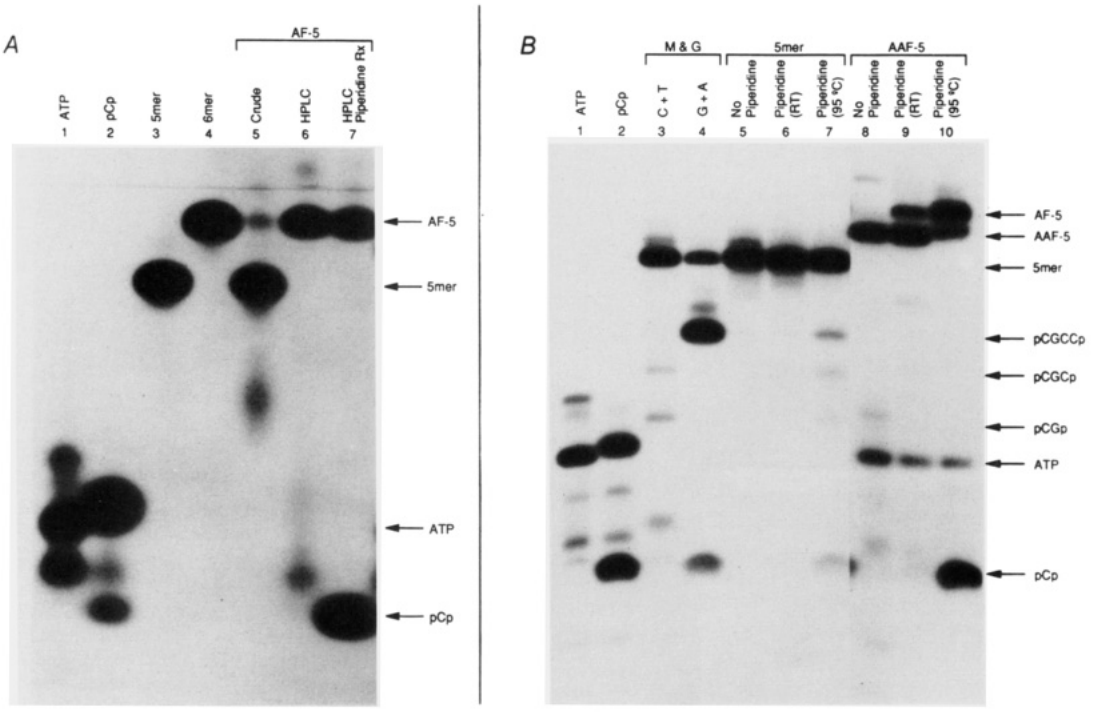

FIGURE 3: Piperidine cleavage of AF- and AAF-modified 5-mer. Each of the ^{32}P-labeled samples was run on a 23% polyacrylamide gel, and their positions were determined by autoradiography. (A) Analysis of AF-modified 5-mer: lane 1, ATP; lanes 2–6, indicated samples treated with polynucleotide kinase; lane 7, HPLC-purified, ^{32}P-labeled AF-5-mer treated with piperidine. (B) Analysis of AAF-modified 5-mer: lane 1, ATP; lane 2, Cp treated with polynucleotide kinase; lanes 3 and 4, C + T and G + A Maxam and Gilbert sequencing reactions (Maxam & Gilbert, 1980), respectively; lanes 5–10, 5-mer and AAF-5-mer treated as indicated.

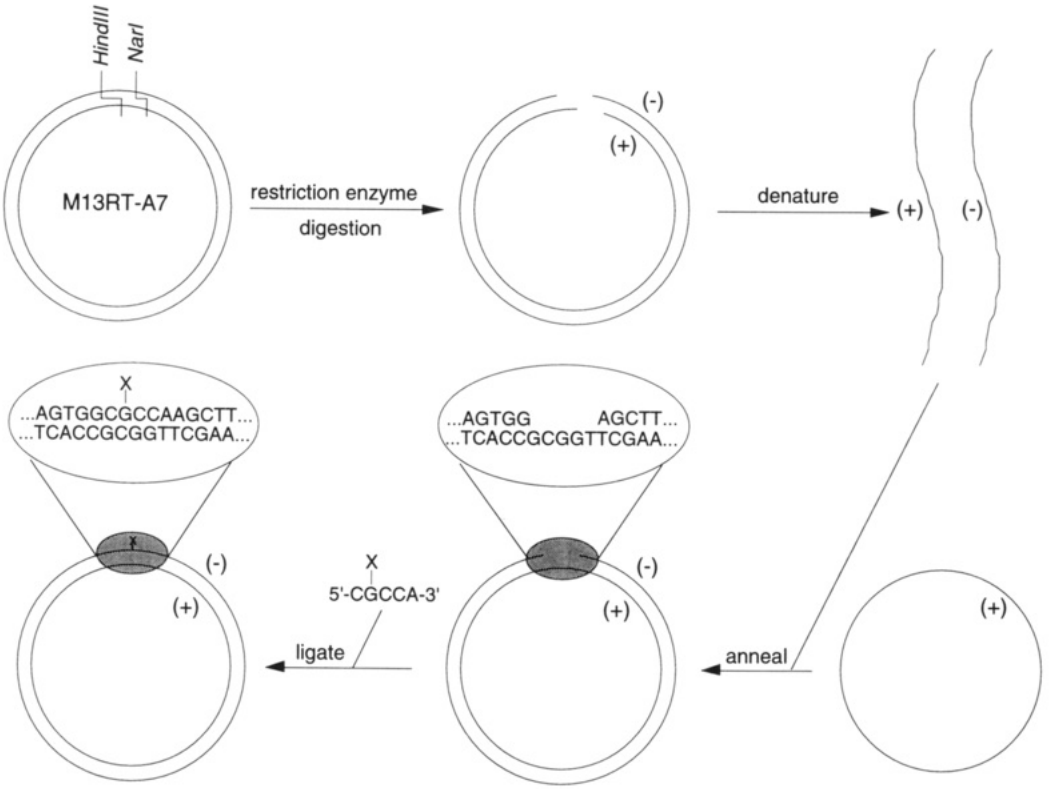

FIGURE 4: Construction of M13RT-A$_7$ vector containing a site-specific adduct. Five bases are removed from the negative strand by digesting RF DNA with *Nar*I and *Hin*dIII. Annealing the doubly digested molecule with excess single-stranded DNA yields a gapped-heteroduplex molecule into which a modified pentamer can be inserted to yield a heteroduplex product. X represents AF, AAF, or no modification (mock).

Finally, the sample was dialyzed repeatedly in TE buffer and concentrated in a Centricon-30 microconcentrator. The formation of gapped heteroduplex from this reaction can be seen on an agarose gel as shown in Figure 5A. A new product corresponding to double-stranded relaxed circular DNA is formed by mixing ss circular DNA and ds linear DNA as outlined above (Figure 5A, lane 4). The GHD was purified from ss circular DNA by centrifugation through a 5–25% sucrose gradient (Figure 5B). Sixty-nine micrograms of GHD was recovered.

Ligation of Oligonucleotides into GHD. Complementary pentamers having no adduct, an AF adduct, or an AAF adduct

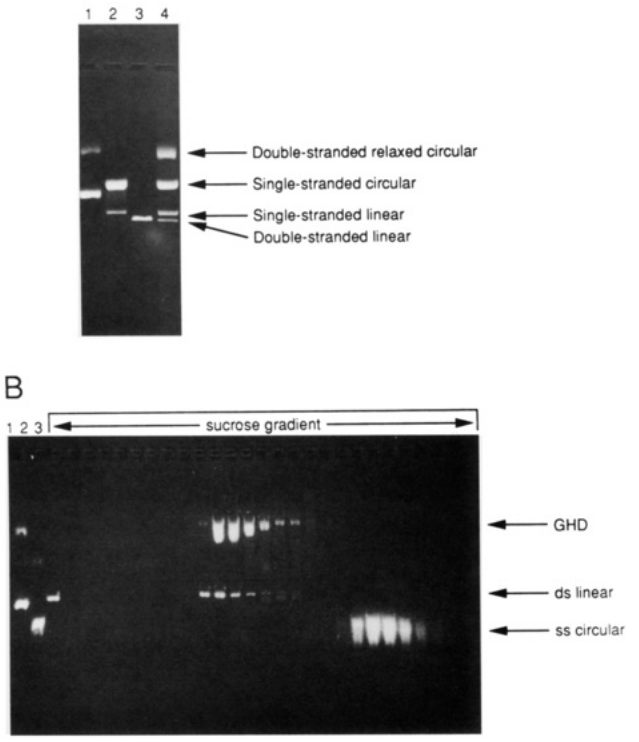

FIGURE 5: Agarose gel electrophoresis demonstrating the formation and purification of gapped heteroduplex. (A) 1% agarose gel, 100 V, 2 h. Each lane contains approximately 0.1 μg of sample DNA. Lane 1, RF M13RT-A_7; lane 2, ss M13RT-A_7; lane 3, ds linear M13RT-A_7; lane 4, GHD prepared from ss M13RT-A_7 and ds linear *Nar*I–*Hin*dIII-cut M13RT-A_7 denatured and reannealed as shown in Figure 3. (B) 1% agarose gel, 60 V, 14 h. Samples collected from a 5–25% sucrose gradient are shown at the left. The bottom of the gradient is to the right. Lane 1, RF M13RT-A_7; lane 2, ss M13RT-A_7; lane 3, ds linear M13RT-A_7.

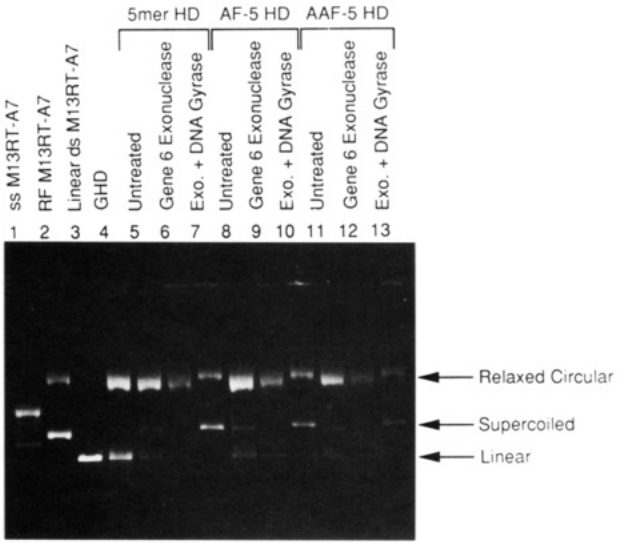

FIGURE 6: Supercoiling of the GHD ligation products with DNA gyrase. Samples were treated with T7 gene 6 exonuclease, phenol extracted, and ethanol precipitated, after which 0.23 μg was incubated with 4 units of DNA gyrase at 37 °C for 90 min. The reactions were stopped by the addition of EDTA to 20 mM, and reaction mixtures were loaded directly onto a 1% agarose gel for electrophoresis. Samples were visualized by ethidium bromide staining. Lane 1, ss M13RT-A7; lane 2, RF M13RT-A7; lane 3, ds linear M13RT-A7; lane 4, GHD; lanes 5, 6, and 7, 5-mer ligated into GHD; lanes 8, 9, and 10, AF-5 ligated into GHD; lanes 11, 12, and 13, AAF-5 ligated into GHD. Lanes 6, 9, and 12 contain samples that were treated with T7 gene 6 exonuclease. Lanes 7, 10, and 13 contain samples that were treated with T7 gene 6 exonuclease followed by DNA gyrase.

were ligated into this gap, creating a heteroduplex molecule containing a site-specific AF or AAF adduct or, in the case where unmodified 5-mer was inserted, a mock GHD control molecule (Figure 6, lanes 5, 8, and 11). In a typical ligation 2.5 μg of GHD was mixed with a 250-fold molar excess (132 pmol) of pentamer, the pentamer being either 5-mer (mock), AF-5-mer, or AAF-5-mer. Ligation was carried out overnight at 16 °C with 25 units of T4 DNA ligase followed by two successive boosts with 25 units of ligase and 4-h incubations at 37 °C. The sample was then phenol extracted and ethanol precipitated.

Each sample was treated first with the T7 gene 6 exonuclease followed by *E. coli* DNA gyrase under conditions suggested by the manufacture. T7 gene 6 exonuclease is a 5′ to 3′ exonuclease specific for duplex DNA, while gyrase will supercoil closed covalent circular duplex DNA molecules. The exonuclease treatment was able to digest the gapped strand of the GHD under these conditions, and gyrase was unable to supercoil the GHD (not shown). When each ligation product was treated with these enzymes, a portion of each sample was resistant to exonuclease digestion (Figure 6, lanes 6, 9, and 12), and when these exonuclease-treated samples were incubated with gyrase, a portion of each was supercoiled, proving covalent attachment of the oligonucleotides into the five-nucleotide gap. These samples were phenol extracted, and ethanol precipitated, and used for the transfection experiments.

SOS Induction and Transfection. Overnight cultures of *E. coli* JM103 (or SMH12) (500 uL) were added to 50 mL of YT media and incubated at 37 °C until they reached a cell density of approximately 5×10^8 cells/mL. The cells were pelleted and resuspended in 10 mL of 67 mM KCl, 17 mM NaCl, 7.6 mM Tris/HCl, 0.8 mM $MgSO_4$, and 1 mM $CaCl_2$. Five milliliters of this cellular suspension was UV irradiated at 50 J/m^2 (6 J/m^2 for SMH12 cells) in a Petri dish with gentle shaking, after which 5 mL of 2× YT media was mixed with the cell suspension, and the mixture was incubated on ice for 10 min. The remaining 5-mL suspension of cells was treated similarly except for UV irradiation and classified as uninduced. Both irradiated and unirradiated cells were made competent by calcium chloride treatment (Sambrook et al., 1989). M13RT-A_7 double-stranded DNA containing either AF or AAF adducts or mock-treated DNA was transfected into competent cells after heat shock at 42 °C for 2 min. An aliquot of the cell suspension was added to 3 mL of soft agar containing the indicator dye 5-bromo-4-chloro-3-indolyl β-galactoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) and then poured onto an agar plate. The top agar was allowed to harden at room temperature, and the plates were incubated overnight at 37 °C.

Detection of Mutants. M13RT-A_7, having been derived from M13mp9, contains the *lacZ′* gene which encodes the α-peptide of the β-galactosidase protein. The *Nar*I sequence in M13RT-A_7 has been positioned inside the *lacZ′* gene. This allows for the detection of many mutations by phenotypic selection based on the α-complementation function between the virus and the host. Phenotypic mutants are clear or light blue plaques. Mutants selected phenotypically were plaque purified before DNA isolation was performed.

Several mutations are phenotypically silent and were detected by probe hybridization (Gupta et al., 1989). One hundred milliliters of YT media was inoculated with 1 mL of host JM103 bacteria and allowed to incubate for 3 h at 37 °C with vigorous shaking ($OD_{590} \sim 1.0$). This bacterial suspension was added in 125-μL aliquots to each well of a 96-well

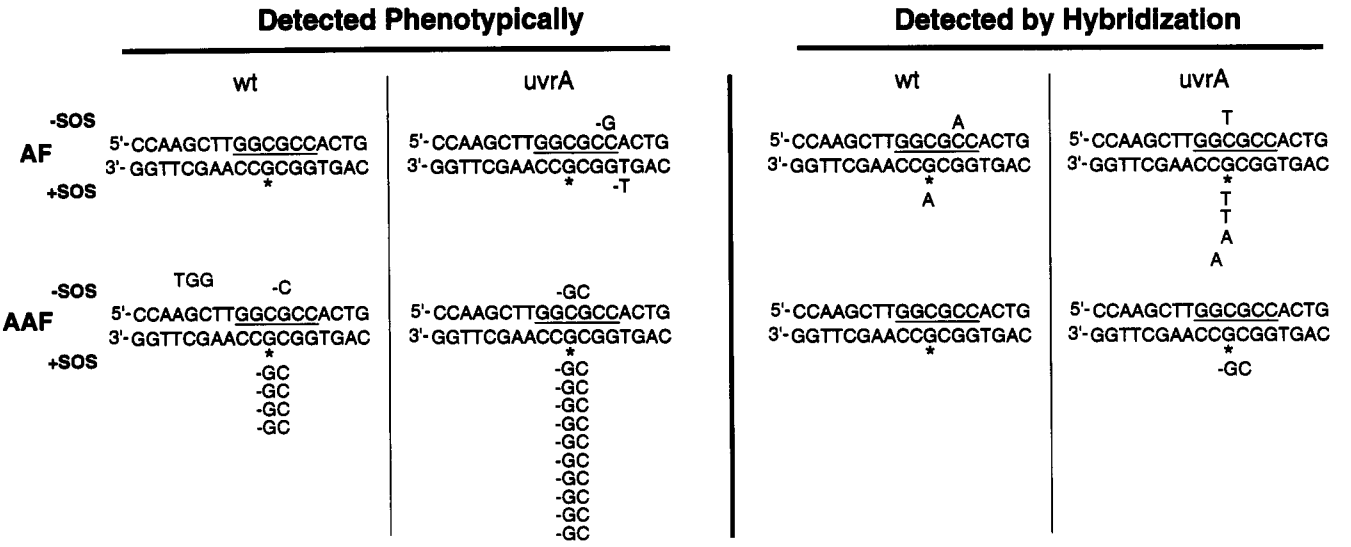

FIGURE 7: Mutation spectra obtained from transfection of site-specifically modified M13 DNA. The underlined regions correspond to the *Nar*I site. The nucleotides above and below each sequence correspond to the independently isolated mutants and the adduct-containing strand.

culture plate. Plaques were individually and randomly picked with an inoculating needle and used to infect the wells containing the cell suspension. Each plate contained three controls, a wild-type transfectant, a two-base frameshift mutation, and a single-base substitution. After transfection of each well with a different plaque, the 96-well plate was incubated in a 37 °C oven for 3 h. Using a 48-prong REPLACLONE (L.A.O. Enterprises), the cells were transferred onto a YT agar plate freshly topped with 3 mL of soft agar containing 52 μL of 2% X-gal, 20 μL of 100 mM IPTG, and 200 μL of overnight JM103, forming a grid of plaques after overnight incubation at 37 °C. The plaques were transferred to Amersham Hybond-N filters, which were placed on a stack of filter paper soaked with denaturation buffer (1.5 M NaCl and 0.5 M NaOH) for 1 min followed by a stack of filter paper soaked with neutralization buffer (1.5 M NaCl and 0.5 M Tris-HCl, pH 8.0) for 5 min. The filters were allowed to air dry, and the DNA was fixed to the filters by irradiation with short-wavelength 300-nm light using a Fotodyne Foto UV 300 DNA transilluminator for 2 min.

The 15-base oligonucleotide probe 5′-CAGTGGCGC-CAAGCT-3′ was ^{32}P-end-labeled and hybridized to the filter in hybridization buffer containing 3× SSC and 10× Denhardt's solution. Hybridizations were done at 25 °C overnight in a Petri dish with very gentle swirling. The filters were then rinsed three times by gently placing them in Petri dishes containing 30 mL of 3× SSC for 5 min each rinse, following which they were air dried and exposed to X-ray film overnight to reveal the autoradiographic pattern under relaxed conditions. The filters were then exposed to a stringent wash in which they were incubated at 49 °C for 5 min, air dried, and autoradiographed to allow the detection of single-base mismatches. The two-base frameshift mutation control and the single-base mismatch control showed no hybridization under stringent conditions.

RESULTS

Mutant phage were detected either by phenotypic selection caused by a mutation that inactivates the β-galactosidase marker or by probe hybridization. The location of the adduct in the α-complementation region of the β-galactosidase gene in the M13mp9 derivative allowed the selection of frameshift mutations by the production of clear plaques when the infected cells were plated on media containing IPTG and X-gal (Gupta et al., 1989). However, this method did not detect base substitutions at this site, and therefore a hybridization procedure (Gupta et al., 1989) was used to simultaneously select these mutations as well as frameshift events.

Table 1: Mutagenesis Induced in the *Nar*I Sequence

		mutagenesis detected phenotypically[a]		mutagenesis detected by hybridization[a]	
DNA	SOS	wt	uvrA	wt	uvrA
mock	no	(0/44 369)[b]	(0/11 095)	(0/504)	(0/504)
	yes	(0/29 870)	(0/6666)	(0/504)	(0/503)
AF	no	(0/34 219)	(0/13 031)	(0/1045)	9.6 (1/1045)
	yes	(0/26 098)	(0/9792)	9.5 (1/1048)	38 (4/1048)
AAF	no	0.22 (1/46 213)	1.1 (1/8893)	(0/603)	(0/603)
	yes	1.3 (4/30 916)	16 (10/6404)	(0/603)	17 (1/603)

[a] Mutation frequency (×10^4). [b] Number of mutations detected per total number of plaques.

Phenotypic Mutations. Site-specifically adducted M13RT-A_7 viral DNA containing either an AF adduct or an AAF adduct at position 6240 and control (mock) M13RT-A_7 viral DNA containing no adduct were used to transform competent JM103 or SMH12 (*uvrA*$^-$) host bacteria. Mutant phage progeny were selected on the basis of the inactivation of β-galactosidase which resulted in a clear plaque. Host cells used for transformation were either SOS induced or untreated (uninduced).

The DNA from the putative phenotypic mutants that were scored was sequenced, and the mutations targeted to the adduct location are shown in Figure 7 and tabulated in Table 1. Mutations shown are with respect to the adduct-containing strand. All mutations that occurred in the region shown are displayed, but mutants were counted in Table 1 only if they were either targeted to the adduct location or located one nucleotide on either side of the adduct site (semitargeted mutations). We were unable to detect any targeted mutations using this screening procedure for either the control ("mock modified") DNA transfections or the AF-modified material. However, for the AAF adduct we obtained significant mutation frequencies in both the wild-type and the *uvrA* cells. In both cases, the mutation frequencies were SOS dependent and induction resulted in a 6-fold (wild type) or a 14-fold (*uvrA*) enhancement of mutation frequencies.

As shown in Figure 7, of the 16 mutations detected, 15 corresponded to deletion of GC dinucleotide (i.e., 5′-GGCG*-CC → GGCC). Note that it cannot be determined whether the C before or after the modified G is the base that is deleted. The other mutation detected was a deletion of a C at a site adjacent to the adduct position.

Mutation Detection by Probe Hybridization. The position of the modified guanine apparently did not allow the detection of simple base substitutions since this position lies in the cloning region, which is not required for the α-complementation of β-galactosidase. To detect these mutations, a hybridization procedure was used which was able to detect any substitution or frameshift at the adduct site (Gupta et al., 1989). However, because of the difficulty of the procedure, a much smaller pool was used than was used in the prior analysis. Putative mutants showing lower levels of hybridization were isolated, and the DNA from each was sequenced. Figure 7 shows all mutations that occurred in the region shown, but only mutations targeted to the adduct site or one nucleotide on either side were scored as mutations in Table 1. As was found with the phenotypically selected mutations, SOS induction increased the mutation frequency, as did transfection into a *uvrA* host. Surprisingly, the AF-induced base substitution mutation frequency was higher than the frequency of the frameshift mutagenesis generated by the AAF adduct.

The types of mutations that were detected by hybridization are displayed in Figure 7. As with the mutations detected phenotypically, the base changes shown are with respect to the adduct-containing strand. Of the seven mutations scored, six were targeted to the adduct site, and each of these was either a G → T transversion (3 total), a G → A transition (2), or a –CG deletion (1). The regionally targeted mutation was on the 3′ side of the adduct, and this was a C → A transversion.

DISCUSSION

The *Nar*I mutation hotspot was first observed in a study where a 276 base pair fragment of the tetracycline-resistance gene from pBR322 was randomly modified with AAF (Koffel-Schwartz et al., 1984). This sequence (GGCGCC) was found to be 100 times more mutagenic than the least mutation prone sequences and produced a unique –CG dinucleotide deletion. More recently, templates were prepared in which each of the guanines in this sequence was modified (Koehl et al., 1989a), and it was shown that only modification of the third G in this sequence resulted in the –CG deletion (Burnouf et al., 1989). Conversely, studies using randomly modified AF-containing templates did not indicate any mutational hotspots (Bichara & Fuchs, 1985) and, moreover, indicated that most of the mutations produced were base substitutions. However, of the six frameshift mutations observed, three occurred at *Nar*I sequences, and two of these were the –CG deletions. It was this latter result which prompted this present study to directly compare the mutagenesis induced by the presence of either an AF or an AAF adduct located on the third G of the *Nar*I sequence by preparing site-specifically modified templates containing these adducts in the *Nar*I site.

We employed two screening procedures to detect the mutations formed. First, a phenotypic analysis based on the inactivation of the α-complementing portion of the β-galactosidase gene present on an M13mp9 derivative was used to screen large numbers of progeny for frameshift mutations. Second, a procedure using the inhibition of hybridization of an oligonucleotide to the region which had contained the adduct was used to detect base substitutions. This latter procedure was technically more difficult, and therefore fewer progeny could be screened using this method. The results shown in Table 1 and Figure 7 clearly indicate that the AAF adduct was more prone to induce the –CG dinucleotide deletion than was the AF adduct. At the levels of mutagenesis we were able to detect, we found that the AF adduct was at least 10-fold less efficient at producing this mutation. On the other hand, the AAF adduct was much less able to induce base substitutions. Both of these observations are in good agreement with prior studies (Koffel-Schwartz et al., 1984; Bichara & Fuchs, 1985).

The predominant base substitutions that we observed for the AF adduct were G → T transversions and G → A transitions. This is in good agreement with prior studies which have reported that either or both of these mutations can be induced by this lesion (Bichara & Fuchs, 1985; Gupta et al., 1988, 1989; Ross et al., 1988; Mah et al., 1989). It is interesting to note that 2D NMR studies were able to obtain a unique conformation for a duplex oligonucleotide in which an A was opposite an AF-modified G, while the duplex having C opposite this position was not interpretable (Norman et al., 1989).

A comparison between the mutation frequencies determined here and those observed in prior studies shows some differences. In the prior random-modification studies, the average mutational cross-section (MCF), defined as the frequency of conversion of a premutagenic lesion into a measurable mutation times 1000, for AF and AAF adducts was 0.5 and 1.1, respectively, if SOS functions were induced. However, MCF was dependent on the sequence context and increased with the modification levels (Veute & Fuchs, 1993). The MCF value of a cold spot modified with AAF was found to be lower than MCF_{ave}, while a mutation-prone *Nar*I sequence was found to have a much higher MCF (Koffel-Schwartz et al., 1984). Placement of an AAF adduct specifically in a *Nar*I sequence produced an MCF of 100, a level substantially higher than what was determined in the random-modification studies (Burnouf et al., 1989). However, this analysis is complicated by the fact that mutation levels are also dependent upon whether the adduct is located in the leading or the lagging strand during DNA replication (Veaute & Fuchs, 1993). This strand-specific study showed that, in a *uvrA* strain with SOS functions induced, an AAF adduct at the third G of the *Nar*I sequence produced a mutation frequency of 0.5% (MCF = 51) when positioned in the leading strand, a value that is 3-fold higher than that which we report.

We have found that modification at the *Nar*I sequence with AF adducts leads to the induction of mutations that are similar to the types of mutations induced by AF adducts at any other average DNA sequence. We observed both G → T (3 of 5) and G → A (2 of 5) targeted base substitution mutations in the AF-induced mutation spectrum. In prior studies both of these mutations have been found to be induced by AF. One report found the G → A transition mutation to be the major base-substitution mutation (Ross et al., 1988), whereas several other investigators found the G → T transversion mutation to be the dominant base-substitution mutation induced by AF (Bichara & Fuchs, 1985; Sahm et al., 1989; Reid et al., 1990). Finally, one report indicated that these two guanine mutations occurred at equal levels (Gupta et al., 1988).

It seems reasonable that the mutation spectra induced by these adducts are related to the structures induced by them in the DNA, although this line of reasoning must be tempered by the fact that during DNA replication these structures are actually positioned in the single-stranded DNA template. A great deal of information is available which shows that AF

Table 2: Sequence Analysis of Double-Base Deletion Mutations

position	mutations	ref	sequence
*Nar*I 413	2,13	*a,b*	CGGCATCACCGGCGCCACAGGTGCGG
*Nar*I 434	3	*b*	TGCGGTTGCTGGCGCCTATATCGCCG
*Nar*I 547	13	*b*	GGGACTGTTGGGCGCCATCTCCTTGC
–69	2	*c*	CAACGCAATTAATGTGAGTTAGCTCA
–34	1	*c*	CACCCCAGGCTTTACACTTTATGCTT
59	1	*c*	TGATTACGAATTCACTGGCCGTCGTT
117	1	*c*	ACCCAACTTAATCGCCTTGCAGCACA
122	1	*c*	ACTTAATCGCCTTGCAGCACATCCCC
140/188	2,6	*d,c*	ACATCCCCCTTTCGCCAGCTGGCGTA
373	8	*e*	ACAATCTTCTCGCGCAACGCGTCAGT
488	1	*e*	TCTCTGACCAGACACCCATCAACAGT
492	1	*b*	AGCCCGAGTACTCGCGAACAAAGCCG
608	3	*e*	GTTCTGTCTCGGCGCGTCTGCGTCTG
776	1	*e*	ACGATCAGATGGCGCTGGGCGCAATG
783	4	*e*	GATGGCGCTGGGCGCAATGCGCGCCA
791	4	*e*	GGGCGCAATGCGCGCCATTACCGAGT
814	1	*e*	GAGTCCGGGCTGCGCGTTGGTGCGGA

[a] Koffel-Schwartz et al. (1984). [b] Bichara and Fuchs (1985). [c] Sahm et al. (1989). [d] Ross et al. (1988). [e] Schaaper et al. (1990).

and AAF adducts form fundamentally different structures in DNA and that these conformations are modulated by the particular sequence within which they lie. Early models suggested that a major conformational change occurred at the site of an AAF adduct in which the guanine is rotated into a syn conformation so that the fluorene ring is inserted into the DNA helix (Grunberger et al., 1970; Fuchs & Daune, 1971). The AF adduct, on the other hand, seems to induce a much smaller distortion in the DNA so that the adduct resides in the minor groove. These models are supported by a wide variety of experimental results, including NMR and CD analyses of nucleotides (Evans et al., 1980; Leng et al., 1980) and oligonucleotides (Norman et al., 1989; O'Handley et al., 1993) and computational (Hingerty & Broyde, 1986; Shapiro et al., 1989) and molecular dynamics simulations (Fritsch & Westhof, 1991).

The fact that the AF and AAF adducts adopt different structures not only in duplex DNA but also during DNA replication as well is suggested by in vitro DNA synthesis studies which show that these two adducts affect synthesis very differently. Whereas both adducts inhibit DNA synthesis, the AAF adduct is much more effective at blocking DNA synthesis by a variety of polymerases (Michaels et al., 1986; Koehl et al., 1989a,b).

There has been substantial interest in the sequence dependency of the structures induced by AAF adducts, and many studies have focused on the *Nar*I sequence. Fuchs has shown that placement of an AAF adduct on the third guanine of the *Nar*I site results in a major distortion of the DNA helix with the possible induction of a local Z-like DNA structure. Supporting experiments include circular dichroism (Koehl et al., 1989b), chemical cleavage (Belguise-Vallandier & Fuchs, 1991), and DNA footprinting studies (Veaute & Fuchs, 1991). This conclusion is strengthened further by prior studies of randomly modified poly(GC) DNA where it has been shown that AAF modification tends to induce a B to Z conversion (Sage & Leng, 1980). Also, UvrABC endonuclease cleavage was more extensive when the AAF adduct was positioned on the third rather than the second guanine of the *Nar*I sequence (Seeberg & Fuchs, 1990). Whether this adduct does, in fact, induce a B to Z conformational change when positioned in the *Nar*I sequence awaits a three-dimensional structure determination.

Fuchs and co-workers have proposed that the CG double-base deletions seen at the *Nar*I sequence following modification with AAF result from the ability of AAF to induce the Z DNA-like conformation within that sequence. By extension, apparently AF is less capable of inducing this conformational change within the *Nar*I sequence because only base-substitution mutations were detected. An analysis of other sequences that gave rise to a two-base deletion following modification with AAF or AF indicates that in every case the deletion of a pyrimidine purine pair had occurred and that, with one exception, this pair was followed by a cytidine on the 3′ side. These sequences are displayed in Table 2. As expected, there is a strong preference for CG deletions in the *Nar*I, site although CG deletions also occur in other closely related sequences. This analysis indicates a strong preference for a CG deletion over the other possible combinations as well as a preference for CG deletions in general: of the 68 dinucleotide deletions, 31 are in a *Nar*I site, and 62 are CG deletions.

In conclusion, it appears likely that the presence of an AAF adduct within a *Nar*I or *Nar*I-like sequence causes a specific conformational change in the DNA that results in the deletion of a CG dinucleotide during replication. The relationship between these structural changes and the replication enzymes must somehow direct this unique deletion. However, the molecular mechanism involved in this process is unclear at present.

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