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Preparation and Characterization of a Viral DNA Molecule Containing a Site-Specific 2-Aminofluorene Adduct: A New Probe for Mutagenesis by Carcinogens†

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Received July 10, 1985

ABSTRACT: The synthetic oligonucleotide heptamer 5′-ATCCGTC-3′ was reacted in vitro with *N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene and the resulting product isolated by reverse-phase high-performance liquid chromatography (HPLC). This purified oligonucleotide, which was shown by chemical and enzymatic analysis to be a heptamer containing a single *N*-(deoxyguanin-8-yl)-2-aminofluorene adduct, was then used to situate the putatively mutagenic aminofluorene lesion within the genome of M13 mp9 by ligating it into a complementary single-stranded region located at a specific site in the negative strand of the duplex M13 mp9 DNA molecule. The presence of the adduct at the anticipated location was confirmed by taking advantage of the facts that AF adducts inhibit many restriction enzymes when located in or near their restriction sites and that the AF moiety should be contained within the *Hin*cII recognition sequence on M13 mp9 DNA. Upon attempted cleavage of the M13 DNA containing the site-specific AF adduct with *Hin*cII, we find that the large majority of the DNA remained circular, demonstrating the incorporation of the AF adduct in high yield into the DNA molecule at this location. This system should prove useful in vivo for the study of mutagenesis by chemical carcinogens and in vitro to study the interaction of purified DNA metabolizing proteins with a template containing a site-specific lesion.

The formation of covalent adducts between reactive electrophilic metabolites of chemical carcinogens and the nucleophilic sites of DNA is considered to be a critical step in chemical carcinogenesis (Miller, 1978). Chemical modification of cellular DNA presents a serious challenge to cells since mutation or cell death may result upon exposure to an agent with such potential. There is substantial evidence suggesting that modification of cellular DNA is the premier event of the multistep carcinogenic process (Weinstein, 1981; King, 1985), and it is generally accepted that the majority of ultimate carcinogens are mutagens (Ames & McCann, 1979), which mediate their effect through covalent binding to DNA (Singer & Grunberger, 1984).

Aromatic amines have been shown conclusively to induce urinary bladder cancer in humans and tumors in a wide variety of organs in experimental animals [for reviews, see King (1982, 1985) and Garner et al. (1984)]. The carcinogenic activity of this class of compounds, as with agents having other structures, appears to depend on their conversion to reactive metabolites that alter the macromolecules of the target tissues (King, 1985). 2-(Acetylamino)fluorene (AAF),[1] one of the most studied aromatic amines, is believed to form DNA adducts following N-oxidation and a subsequent metabolic activation involving conjugation of the hydroxamic acid with sulfate in rat liver or O-acetylation of the hydroxylamine in

† This study was supported by grants from the National Cancer Institute (CA35451 and CA23386). L.J.R. is the recipient of a National Institutes of Health Research Career Development Award (CA00995).
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[1] Abbreviations: AAF, 2-(acetylamino)fluorene; AF, 2-aminofluorene; RF, replicative form; TE buffer, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA; GHD, gapped heteroduplex DNA (M13 mp9 DNA containing a seven-nucleotide gap in the negative strand from position 6251 to position 6257); Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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the target tissues of several species (King, 1985). Activation by sulfate conjugation can result in the introduction of the AAF moiety at C8 and N2 of guanine in DNA; O-acetylation leads to the introduction of 2-aminofluorene (AF) substituents bound through the arylamine nitrogen to the C8 of guanine. This latter AF structure, which represents the major adduct in all tissue studied thus far, causes much less distortion to the DNA helix than that produced by an AAF adduct. Apparently, a 2-(acetylamino)fluorene bound to the C8 position of guanine forces the base to adopt a syn conformation with respect to the sugar (Fuchs & Daune, 1972; Fuchs et al., 1976; Grunberger & Weinstein, 1976; Singer & Grunberger, 1984). This structure predicts that the AAF moiety displaces the guanine in the DNA helix. However, in the case of the analogous aminofluorene adduct, it has been demonstrated that the guanine retains its normal anti conformation (Evans et al., 1980; Daune et al., 1981). The extent of helix distortion correlates well with the half-lives of the two lesions in DNA and with their mutagenic potentials: it has been demonstrated that AF lesions are removed much more slowly than the AAF adduct in rat liver DNA (Beland et al., 1982), that AF substituents are better tolerated that AAF structures on transfection of modified ϕX174 RF DNA into *Escherichia coli* (Tang et al., 1982), and that AF adducts produce mainly point mutations (Bichara & Fuchs, 1985), while the AAF adducts give rise to frame-shift mutations in pBR322 forward mutation assays (Koffel-Swartz et al., 1984).

In order to investigate the specific mechanism of mutagenesis by AF adducts, we have utilized the techniques of recombinant DNA technology to situate a single AF adduct in the negative strand of replicative form (RF) M13 mp9 DNA at base position 6253. This base lies within the *Hin*cII recognition site of this vector. The experimental approach taken to construct this DNA molecule has been to synthesize a heptamer having the sequence 5′-ATCCGTC-3′ and to introduce an AF adduct at the C8 position of the single guanine in this sequence. This modified heptamer was then ligated into a gapped heteroduplex DNA molecule that lacked only the heptamer. The construction of this probe provides us with a tool for the future evaluation of both the in vivo and in vitro effects of aminofluorene–DNA adducts and establishes techniques for the positioning of other carcinogenic and mutagenic lesions at specific sites in DNA molecules.

Materials and Methods

T4 DNA ligase, *E. coli* DNA polymerase large fragment, and restriction endonucleases *Bam*HI and *Hin*cII were purchased from New England Biolabs. T4 polynucleotide kinase was obtained from P-L Biochemicals. [γ^{32}P]ATP was obtained from ICN Pharmaceuticals. All reactions were performed under conditions suggested by the supplier except where noted. 2-Mercaptoethanol (16 mM) was substituted for 5 mM dithiothreitol in polynucleotide kinase reactions that were to be analyzed by HPLC.

Electrophoresis of DNA was carried out as described (Maniatis et al., 1982) on 1% horizontal agarose slab gels in 77 mM Tris, 75 mM borate, and 2 mM EDTA and visualized with 0.5 μg/mL ethidium bromide after completion of electrophoresis. After being destained in distilled water, the gels were photographed and, if required, were dried under heated vacuum prior to autoradiography. Polyacrylamide gel electrophoresis was carried out as described (Maniatis et al., 1982) in 22% denaturing gels of 0.8 mm thickness. Autoradiography of both agarose and polyacrylamide gels was performed at −70 °C with Du Pont Cronex Lightning-Plus CA screens and Kodak X-Omat XAR-5 film.

Bacteriophage, Bacterial Strains, and DNA Isolation. *E. coli* strains JM103 and M13 mp9 (Messing & Vieira, 1982) were obtained from Dr. J. E. LeClerc (Rochester University). Replicative form (RF) DNA was isolated by alkaline lysis (Birnboim & Doly, 1979) and purified on cesium chloride density gradients (Hayes & LeClerc, 1983). Viral DNA was purified by a modified phenol extraction procedure as described (Dugarczyk et al., 1975). Following purification, both preparations were dialyzed extensively against 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (TE buffer).

Results

An approach to the incorporation of a site-specific lesion in viral and plasmid genomes has been previously described for the smaller premutagenic lesion, O^6-methylguanine (Loechler et al., 1984). This covalent modification used a tetranucleotide prepared by the phosphotriester method starting with an O^6-methylguanine-containing nucleotide precursor. A similar approach is less appropriate for incorporating an AF lesion by using currently available oligonucleotide synthesis technology due to the instability of such lesions to the chemistry involved (Itakura et al., 1984; Kreik & Westra, 1980). Stöhrer et al. (1983) have been able to prepare a protected AAF-containing oligonucleotide using a solid-phase DNA synthesizer. This 14-mer was unstable to the deprotection step, resulting in the formation of an uncharacterized "oxidation" product. If the deprotection was carried out in the presence of 2-mercaptoethanol, a 14-mer was produced having a UV spectrum consistent with an AF-linked guanine.

In the present study, we have taken advantage of the high specificity for the reaction of aromatic amine derivatives with the C8 position of guanine to allow the specific incorporation of an aminofluorene adduct into a heptamer having the sequence 5′-ATCCGTC-3′. This oligonucleotide was subsequently ligated into a duplex DNA molecule into which a specific single-stranded region complementary to the heptamer was created by the techniques of recombinant DNA technology (Figure 1). The formation of this gapped heteroduplex DNA molecule was accomplished by double digesting RF M13 mp9 with *Bam*HI and *Hin*cII followed by gel filtration to separate the large DNA molecule from the low molecular weight fragment produced by the digestion. Following confirmation of digestion by both enzymes, a single guanine was replaced by filling the recessed *Bam*HI 3′-terminus by reaction with dGTP and *E. coli* DNA polymerase I large fragment. This *Bam*HI–*Hin*cII-digested fragment was then denatured and allowed to reanneal to an excess of single-stranded circular (viral) DNA. After ligation of the modified heptamer into the single-stranded gap, the resultant DNA molecule has exactly the same sequence as the parent molecule with the single exception that the guanine of the native genome at position 6253 in the negative stand of M13 mp9 was replaced with *N*-(deoxyguanin-8-yl)-2-aminofluorene.

Construction of Duplex M13 mp9 Molecules Containing a Specific Single-Stranded Region. M13 mp9 RF DNA (400 μg) was treated with 240 units of *Bam*HI in a reaction mix (final volume 0.3 mL) containing 100 mM NaCl, 10 mM Tris-HCl, pH 7.6, 1.25 mM dithiothreitol, 6 mM $MgCl_2$, and 50 mg/mL bovine serum albumin until complete digestion was confirmed by agarose gel electrophoresis (8 h at 37 °C). Digestion with *Hin*cII was accomplished during an additional 16-h incubation by adding 100 units of the enzyme to the above reaction mixture in two 50-unit aliquots separated by 8 h. The reaction mixture was then adjusted to a final concentration of 10 mM EDTA and the mixture extracted with phenol. The

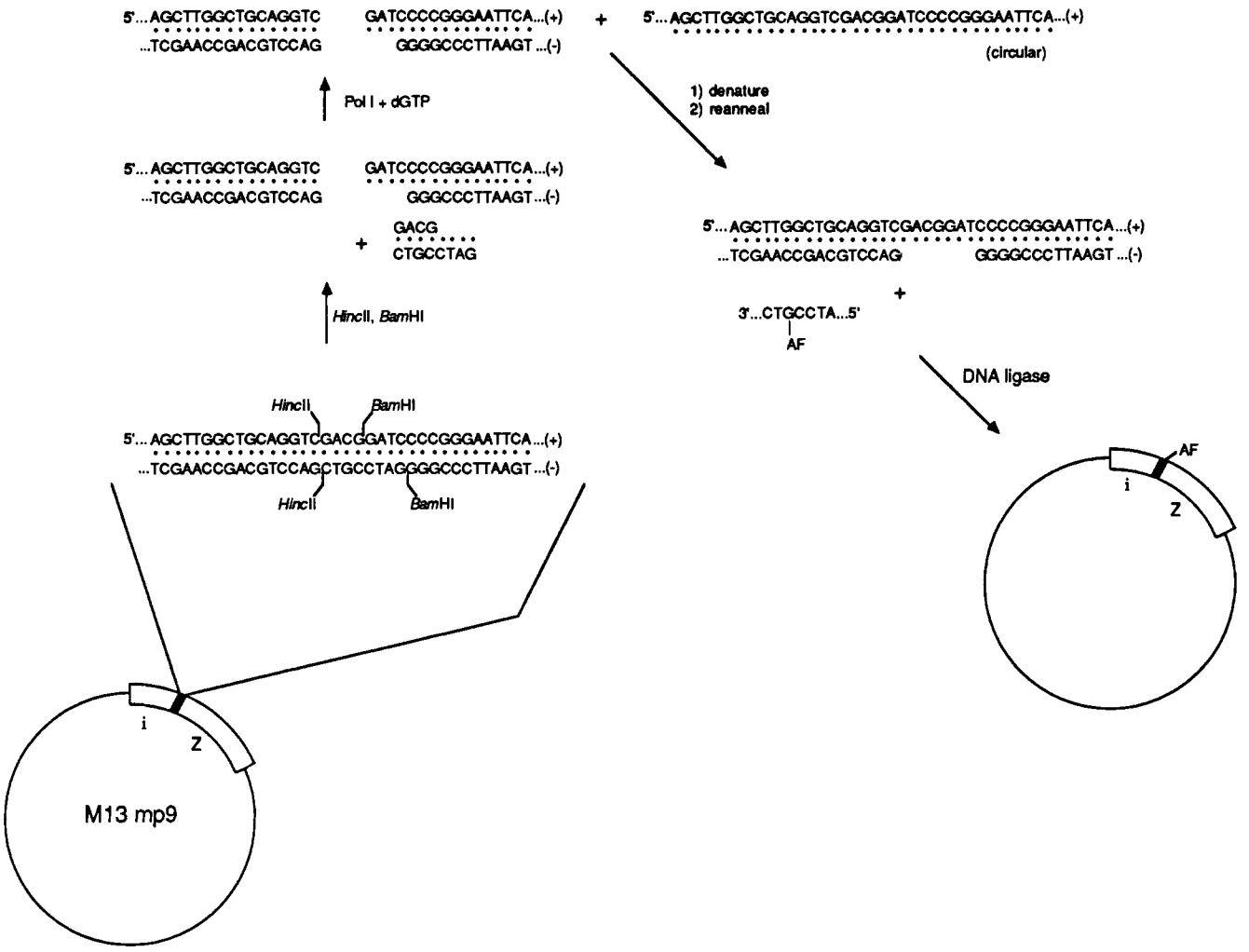

FIGURE 1: Schematic representation of the method used to prepare a DNA molecule containing a site-specific 2-aminofluorene adduct.

aqueous phase, containing the double-digested linear DNA, was loaded directly onto a 0.5 cm × 20 cm column of Sephadex G-100 (P-L Biochemicals), which was preequilibrated with TE buffer containing 0.1 M NaCl. The large fragment of DNA eluted in the void volume as determined by absorbance at 260 nm. We have confirmed that this column quantitatively separated the DNA from the small excised fragment by including within the loaded sample a ^{32}P-labeled pentadecamer. No ^{32}P eluted in the column void volume. The void volume fractions were concentrated by successive butanol extraction and precipitated with ethanol. The recovered DNA was resuspended in TE buffer.

The double-stranded DNA preparation was further analyzed to confirm complete *Hin*cII cleavage and to assure that the eight-nucleotide excised fragment had been efficiently removed by the Sephadex G-100 gel filtration column. Treatment of this linear product with DNA ligase either under reaction conditions reported to favor circularization of genome-length fragments or under conditions that favor dimerization (Sugino et al., 1977) resulted in the quantitative formation of only dimer products as determined by agarose gel electrophoresis (data not shown). These dimers were subsequently shown to be fully digested by *Bam*HI but resistant to restriction by *Hin*cII (data not shown). This evidence indicates that the original preparation of *Bam*HI- and *Hin*cII-treated DNA had been completely digested by both enzymes, because had *Hin*cII not cleaved, circular structures would have been formed via hybridization and ligation of the *Bam*HI sticky ends. Furthermore, the fact that no detectable DNA remained as the monomer after ligation indicates the absence of the eight-nucleotide excised fragment. Ligation of this short fragment to the genome-length DNA molecule via the *Bam*HI sticky ends would have produced a percentage of molecules with blunt ends at both of their termini. These molecules ligate several hundred fold less efficiently than DNA containing cohesive ends (Sugino et al., 1977) and would have resulted in a percentage of the reacting DNA remaining linear.

Digestion of M13 mp9 with *Bam*HI and *Hin*cII removes a four-nucleotide fragment from the positive strand and an eight-nucleotide segment from the negative strand with the sequence 5′-GATCCGTC-3′ (Figure 1) (Messing & Viera, 1982). In order to produce the specific single-stranded region complementary to an oligonucleotide containing a single guanine, the guanine at the 3′-end of the *Bam*HI site must be reinserted into the DNA molecule. This was accomplished with *E. coli* DNA polymerase I large fragment and dGTP. The *Bam*HI–*Hin*cII-generated fragment (100 μg) was treated with *E. coli* DNA polymerase I large fragment (20 units) in a reaction mixture containing 50 mM Tris-HCl, pH 7.2, 10 mM $MgSO_4$, 0.1 mM dithiothreitol, 50 mg/mL bovine serum albumin, and 3 mM dGTP. After incubation at 37 °C for 30 min, the reaction mixture was made 12 mM in EDTA, extracted with phenol, and precipitated with ethanol, and the DNA was resuspended in TE buffer.

This DNA was then mixed with a 3-fold molar excess of single-stranded circular (+) M13 mp9 DNA in 0.1 M NaCl, 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA, at a DNA concentration of 25 μg/mL. The DNA mixture was denatured

by heating at 100 °C for 4 min and allowed to reanneal as described (Green et al., 1984). The volume was reduced by successive butanol extraction; the DNA was precipitated with ethanol and resuspended in TE buffer. The resultant gapped heteroduplex DNA molecule (GHD) migrates in a 1% agarose gel to the same position as nicked circular double-stranded DNA and is well resolved from either single-stranded circular or linear forms of M13 mp9 DNA that are present in the sample after the reannealing reaction. Finally, we observe a species on the agarose gel following reannealing that remains at the top of the gel during electrophoresis. Although we have not characterized this species, we presume it to be multimeric species formed by the interstrand association of partially complementary sequences during reannealing. We also find that this species is unstable and dissociates if the DNA is stored at 4 °C for several weeks.

Modification of the Synthetic Oligonucleotide Heptamer. The synthetic oligonucleotide heptamer 5′-ATCCGTC-3′ was obtained from P-L Biochemicals as a custom synthesis. [*ring*-^{3}H]-*N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene (60 mCi/mmol) was synthesized and characterized[2] as described previously for the analogous biphenyl derivative (Lee & King, 1981). In citrate buffer, these compounds undergo solvolysis of the trifluoro moiety to yield reactive *N*-(acetoxyaryl)amines (Lee & King, 1981). These activated derivatives, which can also be produced enzymatically by O-acylation, can react readily with both protein and nucleic acids (King & Glowinski, 1983). Only the deacylated aminofluorene adduct at the C8 position of guanine [i.e., *N*-(guanin-8-yl)-2-aminofluorene] is produced upon reaction with guanine-containing nucleotides.[2] In a typical modification of the heptamer, 50 μg (23 mmol) of the oligonucleotide was reacted with 50 μg (149 nmol) of [^{3}H]-*N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene (sp act. 60 μCi/μmol) in a solution containing 8 mM citrate (pH 7.0) in 20% ethanol. After incubation under argon atmosphere at 37 °C for 60 min, the reaction mixture was extracted 10 times with 1-mL aliquots of diethyl ether. At this point, no radioactivity was detectable in the organic phase. The remaining solution was made 50 mM with respect to triethylammonium acetate, pH 6.5, and analyzed by reverse-phase HPLC on a Perkin-Elmer 3B liquid chromatograph with a LC-85 UV detector plus a FLD-I Radiomatic Instrument radioactive flow detector utilizing a Waters Nova-Pak C_{18} column (3.9 mm × 15 cm) at a flow rate of 0.8 mL/min. The elution conditions were 100% H_2O for 10 min followed by 0–30% methanol in 30 min. Two major peaks of absorbance were noted at 260 nm, the first eluting at 26 min and a second at 34 min (Figure 2). The first peak coeluted with authentic unmodified heptamer analyzed under the same conditions. Under stopped-flow analysis, this peak produced a UV spectra identical with the authentic heptamer (Figure 2). The second absorbance peak at 34 min contained all the radioactivity and under stopped-flow conditions produced a UV spectra having an absorbance shoulder at 320–330 nm (Figure 2) that is characteristic of an *N*-(deoxyguanosin-8-yl)-2-aminofluorene-containing nucleic acid (Kriek, 1967). The yield for a typical reaction was 10–15% modified heptamer on the basis of both absorbance and radioactivity measurements.

The peak eluting at 34 min from a preparative-scale reverse-phase HPLC analysis was collected, lyophilized, and redissolved in double-distilled water. A portion (2 μg) of this material was labeled with ^{32}P by treatment with T4 polynucleotide kinase and [γ-^{32}P]ATP (sp act. 4500 Ci/mmol) and electrophoresed on a 22% polyacrylamide gel. The peak eluting at 26 min was also treated in this manner. The radioactive products were visualized by autoradiography (Figure 3). Not only did the AF-modified material eluting at 34 min migrate more slowly through the gel matrix, as would be expected due to the increase in molecular weight upon binding of AF, but a single band was noted in the autoradiograph, indicating the high purity of the preparation and the lack of contaminating unmodified heptamer. The material eluting at 26 min treated in the same manner appeared identical with the authentic unmodified ^{32}P-labeled heptamer (Figure 3).

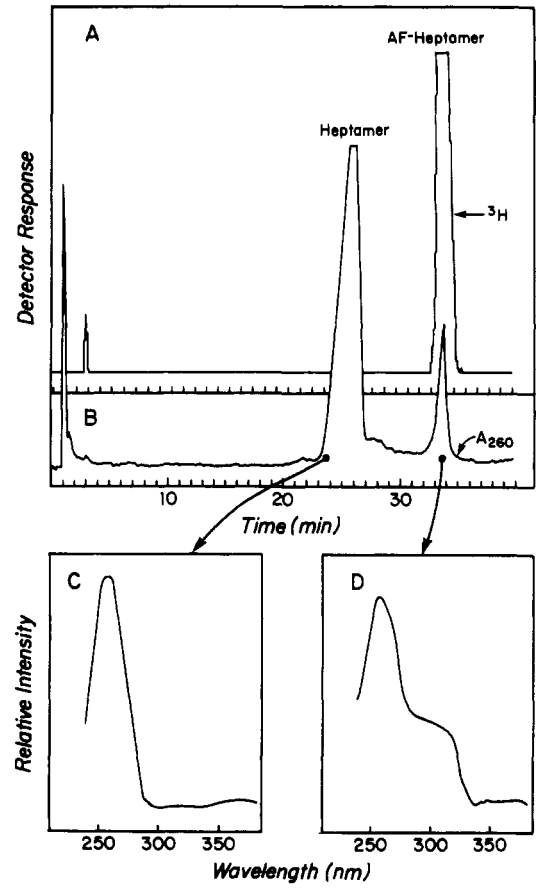

FIGURE 2: HPLC analysis of the products of the reaction of [^{3}H]-*N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene with the synthetic oligonucleotide heptamer 5′-ATCCGTC-3′: (A) radioactivity detector response, ^{3}H; (B) absorbance (260 nm) profile; (C and D) spectra of absorbance peaks eluting at 26 and 34 min, respectively. Note absorbance shoulder at 320–330 nm in (D) (see text for detailed description).

Piperidine-Induced Cleavage of the AF-Modified Heptamer. Treatment of modified DNA with piperidine has previously been shown to induce DNA strand scission at numerous types of DNA lesions, including aflatoxin B1, benzo[*a*]pyrene diolepoxide, 6–4 pyrimidine photoproducts, and aminofluorene adducts (D'Andrea & Haseltine, 1978; Muench et al., 1983; Lippke et al., 1981; Sage & Haseltine, 1984; Bichara & Fuchs, 1985). This sensitivity has allowed the determination of the in vitro binding spectrum of various compounds with DNA including that of *N*-hydroxy-2-aminofluorene (Bichara & Fuchs, 1985). To confirm the positioning of the AF adduct in the heptamer, we have treated this modified oligonucleotide with piperidine and analyzed the products by polyacrylamide gel electrophoresis. Either native or AF-modified ^{32}P-labeled heptamer (0.5 pmol) was treated with 0.1 mL of 1 M piperidine at 90 °C in a sealed polypropylene tube. The piperidine

[2] M.-S. Lee, P. Gupta, T. M. Reid, and C. M. King, unpublished experiments.

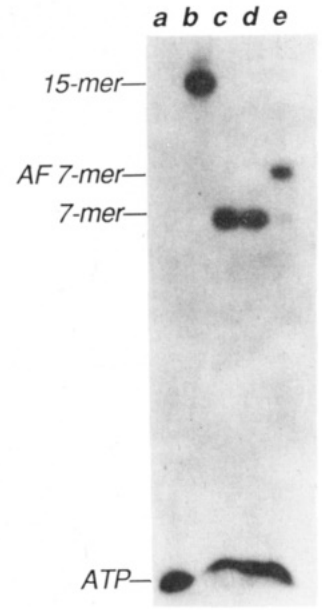

FIGURE 3: ^{32}P end labeling of the synthetic oligonucleotide heptamer containing an *N*-(deoxyguanin-8-yl)-2-aminofluorene adduct. Labeled samples were electrophoresed through a 22% polyacrylamide gel and subjected to autoradiography: (lane a) [$\gamma^{32}P$]ATP; (lane b) commercial M13 pentadecamer sequencing primer; (lane c) synthetic heptamer; (lane d) synthetic heptamer following HPLC analysis (absorbance peak eluting at 26 min); (lane e) synthetic heptamer containing an aminofluorene adduct (absorbance peak eluting at 34 min).

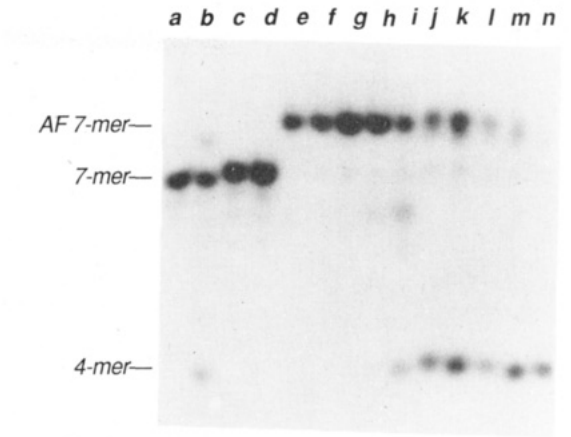

FIGURE 4: Analysis of AF-modified heptamer by piperidine cleavage. ^{32}P end labeled unmodified (lanes c and d) or AF-containing (lanes f–n) heptamer were treated with 1 M piperidine at 90 °C for the indicated times as described under Materials and Methods. The resulting products were electrophoresed on a 23% polyacrylamide gel and visualized by autoradiography: (lane a) unmodified heptamer; (lane b) standard Maxam and Gilbert G-specific DNA strand cleavage reaction of the unmodified heptamer; (lanes c and d) treatment of unmodified heptamer with piperidine for 8 and 16 h, respectively; (lane e) AF-modified heptamer; (lanes f–n) treatment of AF-modified heptamer with piperidine for 10 min, 20 min, 30 min, 1 h, 2 h, 3 h, 4 h, 8 h, and 16 h, respectively.

was removed by repeated lyophilization, and the products of these reactions were analyzed on a 23% polyacrylamide gel (Figure 4). Treatment of the AF-containing heptamer with piperidine for 16 h resulted in its quantitative conversion to a ^{32}P-labeled oligonucleotide (Figure 4, lane n), which comigrated in the gel with the product obtained upon treatment of the unmodified heptamer with the Maxam and Gilbert G-specific sequencing reaction (Maxam & Gilbert, 1977) (Figure 4, lane b). This is the anticipated reaction product on the basis of prior studies on piperidine treatment of AF-modified DNA (Bichara & Fuchs, 1985). No other ^{32}P-containing products were observed from the piperidine reactions, and further, the unmodified heptamer was stable to a 16-h incubation with piperidine (Figure 4, lane d). These results indicate that the piperidine-labile lesion was positioned at the guanine in the heptamer sequence 5′-ATCCGTC and that no other positions had been modified.

TFA Digestion of AF-Modified Heptamer. A portion of this modified heptamer was subjected to anhydrous trifluoroacetic acid digestion (Tang & Lieberman, 1983), and the products were analyzed by reverse-phase HPLC utilizing a Waters μBondapak C_{18} column (3.9 mm × 30 cm) and a 0–100% methanol gradient in 30 min at a flow rate of 1 mL/min. The eluant was monitored at 300 nm. The modified heptamer was compared with a similar digestion and HPLC analysis of *N*-(deoxyguanosin-8-yl)-2-aminofluorene 5′-monophosphate that had been prepared by the reaction of *N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene with dGMP. The major peak seen from the TFA digestion of the modified dGMP had a retention time of 28.0 min and a UV spectrum characteristic of *N*-(guanin-8-yl)-2-aminofluorene (i.e., absorption maxima at 280 and 326 nm) (Kriek et at., 1967). A minor product of the hydrolysis of the AF-modified dGMP had a UV spectrum and retention time (23.2 min) consistent with a ring-open degradation product of the modified nucleoside (Kreik & Westra, 1980). The major radioactive peak observed from the digestion of the modified heptamer had a retention time of 27.9 min that corresponded to *N*-(guanin-8-yl)-2-aminofluorene. The other radioactive peaks in this digestion were consistent with breakdown products seen following trifluoroacetic acid digestion of aminofluorene-containing nucleic acids that had been observed in these and previous studies (King & Philips, 1969, 1970; Kreik & Westra, 1980; Vaught et al., 1981). These data, taken together, indicate that this preparation of modified heptamer contains a single *N*-(deoxyguanin-8-yl)-2-aminofluorene adduct located on the guanine in the sequence 5′-ATCCGTC.

Ligation of an Unmodified Heptamer into the Gapped Heteroduplex DNA Molecule. Utilizing a 250-fold molar excess of unmodified heptamer, we were able to ligate the synthetic oligonucleotide into the complementary single-stranded region, which had been specifically created in the M13 mp9 DNA. In a typical reaction, 15 μg of gapped heteroduplex containing 3.1 pmol of the single-stranded region was mixed with 1.7 μg (775 pmol) of heptamer, and this mixture incubated with 40 units of T4 DNA ligase at 16 °C for 18 h (Figure 5, lane e). Aliquots of this reaction mixture containing 1 μg of DNA were digested with either *Bam*HI or *Hin*cII and analyzed on a 1% agarose gel (Figure 5, lanes f and g, respectively). This analysis demonstrates that complete ligation of the unmodified heptamer into the gap within the GHD had occurred under the reaction conditions. If the T4 DNA ligase was not included in the reaction mixtures or if the DNA had not undergone prior treatment with *E. coli* DNA polymerase I and dGTP, sensitivity to neither restriction enzyme was restored (data not shown). In this manner it was possible to monitor the efficiency of both the ligation and the *Bam*HI terminus-filling reactions. Using the reaction conditions described above, we obtained essentially complete digestion by either *Bam*HI or *Hin*cII (Figure 5), suggesting that ligation of the heptamer had occurred in high yield.

Ligation of the AF-Modified Heptamer into the Gapped Heteroduplex DNA. The ligation of the aminofluorene-containing heptamer into the gapped heteroduplex M13 mp9 DNA molecule was carried out by a procedure analogous to that used for ligating the unmodified oligonucleotide described

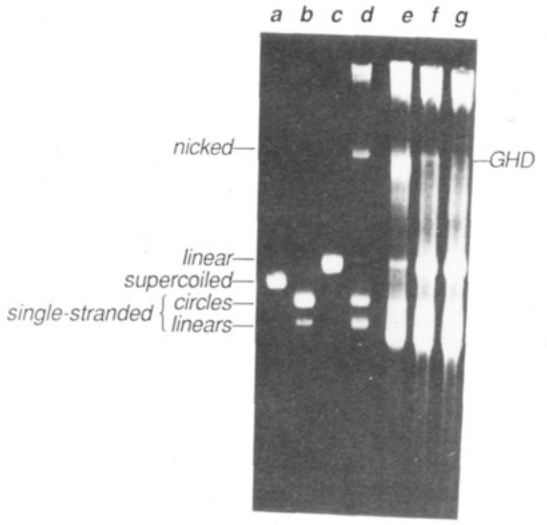

FIGURE 5: Restriction analysis of the product from ligation of GHD and unmodified heptamer. GHD M13 mp9 DNA was incubated with T4 DNA ligase and a 250 molar excess of synthetic heptamer (lane e). The DNA product in lane e was treated with *Bam*HI and *Hin*cII (lanes f and g, respectively) and sensitivity to the restriction endonucleases demonstrated. (Lane a) Supercoiled M13 mp9 DNA; (lane b) single-stranded circular (viral) M13 mp9 DNA; (lane c) *Bam*HI–*Hin*cII linearized M13 mp9 DNA; (lane d) GHD DNA. Nicked, linear, and supercoiled refer to the positions to which the double-stranded forms of M13 mp9 control DNA migrated. GHD is the gapped heteroduplex DNA position.

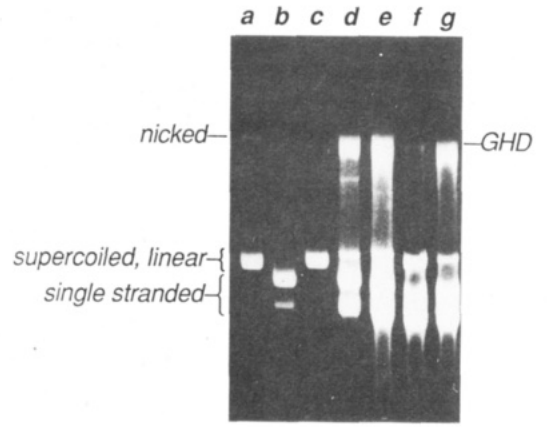

FIGURE 6: Restriction analysis of the product from ligation of GHD and AF-containing heptamer. GHD M13 mp9 DNA was incubated with T4 DNA ligase and a 250 molar excess of AF-modified heptamer (lane e). The DNA product in lane e was treated with *Bam*HI and *Hin*cII (lanes f and g, respectively). Sensitivity to *Bam*HI cleavage was restored while resistance to *Hin*cII cleavage was maintained. (Lane a) Supercoiled M13 mp9 DNA; (lane b) single-stranded circular (viral) M13 mp9 DNA; (lane c) *Bam*HI–*Hin*cII linearized M13 mp9 DNA; (lane d) GHD DNA. Nicked, linear, and supercoiled refer to the positions to which the double-stranded forms of M13 mp9 control DNA migrated. GHD is the gapped heteroduplex DNA position.

above. Agarose gel electrophoresis of the resultant product is shown in Figure 6, lane e. As was observed with the unmodified DNA, the covalently closed reaction product migrates in a 1% gel to the same position as the nicked material in the supercoiled sample (Figure 6, lane a).

The presence of the AF adduct in the DNA molecule at the anticipated location was confirmed by restriction enzyme analysis. First, the ability of *Bam*HI to digest virtually all of this DNA product (Figure 6, lane f) indicates that the heptamer was efficiently ligated into the gap in the GHD molecule. Second, this same DNA sample was almost totally resistant to cleavage by *Hin*cII whose restriction recognition

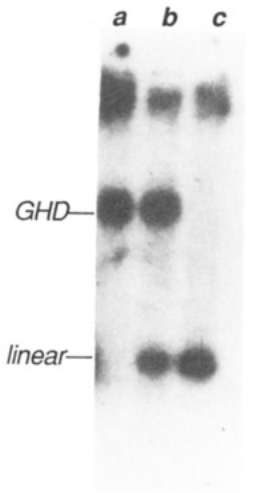

FIGURE 7: Association of ^{32}P from end-labeled synthetic heptamer with M13 mp9 gapped heteroduplex DNA. ^{32}P-labeled heptamer containing an aminofluorene adduct was ligated to the GHD (lane a) and digested with either *Hin*cII (lane b) or *Bam*HI (lane c) and electrophoresed through a 1% agarose gel. Bands were visualized by autoradiography. GHD, gapped heteroduplex position; linear, position to which *Bam*HI–*Hin*cII linearlized control DNA migrated.

site, 3′-CAGCTG-5′, should contain the AF adduct on the 5′-terminal G. The inability of *Hin*cII to cleave this DNA was anticipated on the basis of prior studies that indicated that AF adducts inhibit digestion by numerous restriction endonucleases when the adduct occurs within the recognition sequence of a given enzyme.[3] This result, and the fact that no unmodified heptamer could be detected in our preparation of the AF-modified oligomer product, supports the contention that essentially all of the closed covalent DNA molecules bear a single aminofluorene lesion in the negative strand at base position 6253.

We have further characterized the DNA product by labeling the unmodified and AF-containing heptamer with ^{32}P at their 5′-termini. The presence of this label allows the visualization of the heptamer-containing DNA after ligation. Autoradiography of an agarose gel of the ligation product from reaction with the ^{32}P-containing oligonucleotide is shown in Figure 7. Reactions using either unmodified (not shown) or AF-containing (lane a) heptamer produced a band at the position in the gel corresponding to the nicked position. Digestion of the ligation product from the AF-modified heptamer with *Bam*HI resulted in complete cleavage of the product DNA (Figure 7, lane c). However, the large majority of this DNA was resistant to cleavage by *Hin*cII (Figure 7, lane b). This result not only confirms the restriction analysis presented above but also demonstrates the covalent association of heptamer with the DNA ligation product since the ^{32}P remained associated with the DNA throughout the electrophoresis.

Discussion

It is well accepted that most mutagens and carcinogens exert their biological effect by covalently binding to and altering the genomic DNA. However, there are a multitide of difficulties associated with specifying the chemical nature of the lesion affecting DNA metabolism. These problems result primarily from the variety of DNA reaction products induced by most of these DNA-damaging agents and from the rarity of the cellular response to this damage.

Studies using a DNA molecule containing chemically

[3] T. Surendranath and L. J. Romano, unpublished experiments.

well-defined damage located at a unique position could circumvent most of these difficulties. In this situation there is no ambiguity as to the structure of the damage that has caused the observed biological effect. Furthermore, since each DNA molecule would be identically modified at a unique position, we would anticipate a low probability that the damage would be lethal upon transfection but a high probability that the lesion would have an observable effect in vivo or in vitro.

On the basis of several lines of evidence, the present work has yielded such a DNA molecule—an intact viral genome containing a single aminofluorene lesion located at a unique position. The approach taken was to ligate into a gapped duplex M13 mp9 DNA molecule a complementary synthetic oligonucleotide heptamer modified at a single position with an *N*-(deoxyguanin-8-yl)-2-aminofluorene adduct. This modified oligonucleotide had been prepared by the reaction of *N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene with the chemically synthesized heptamer, followed by purification by preparative HPLC. The identity of the reaction product was determined both spectrophotometrically by using the characteristic 320–330-nm absorption band of an AF-linked guanine and chemically by degrading the heptamer with either piperidine or TFA and analyzing the resulting products. Piperidine treatment produced cleavage of the heptamer only at the guanine base, confirming the specificity of the placement of the AF moiety. TFA digestion yielded an AF-containing species having identical HPLC retention times as authentic *N*-(guanin-8-yl)-2-aminofluorene.

Ligation of this AF-containing heptamer into the gapped heteroduplex DNA molecule produced a covalently closed DNA molecule, which was subsequently characterized both enzymatically and chemically to support the contention that the AF lesion had been positioned as planned in the negative strand of M13 mp9 at position 6253. First, we showed that ligation had efficiently occurred between the AF-containing heptamer and the gapped DNA molecule by demonstrating that the *Bam*HI recognition site was restored in the product DNA. The ligation efficiency was further confirmed by demonstrating that a ^{32}P-labeled heptamer became linked to the product DNA and that this label remained associated with the DNA throughout agarose gel electrophoresis and during restriction enzyme analysis. Finally, we confirmed the placement of the AF adduct into the *Hin*cII recognition site by showing that the large majority of the AF-containing product DNA was resistant to cleavage by this enzyme. This evidence, taken together, strongly supports the contention that an aminofluorene adduct was successfully incorporated into the *Hin*cII site on the negative strand of M13 mp9 DNA.

Current efforts in site-directed mutagenesis have positioned AAF and AF adducts into discrete gene products and have provided much needed information concerning the mutational spectra of such adducts (Bichara & Fuchs, 1985; Koffel-Swartz et al., 1984). However, the system described here has the advantage of positioning a single well-defined AF adduct at a known position, allowing a direct correlation between the in vivo and in vitro effects of this specific lesion on DNA synthesis. For example, this modified duplex DNA molecule may be used directly to transfect *E. coli* strains with different repair backgrounds in order to determine the enzymatic mechanism by which an aminofluorene adduct leads to a mutation and should provide further evidence as to the identity of the base change it causes.

The vector also has potential utility for in vitro mechanistic studies on the effects of AF lesions on DNA metabolizing enzymes. For example, it has been reported that AF and AAF adducts prevent bypass of DNA polymerases during elongation (Moore et al., 1982). This system will prove particularly useful in examining this phenomenon by allowing a direct measurement of the effectiveness of an AF lesion in blocking DNA polymerases. Preliminary studies[4] using this vector have shown that both *E. coli* DNA polymerase I and T7 DNA polymerase can readily bypass an AF adduct during DNA synthesis. Others have also demonstrated that polymerase I can synthesize past an AF lesion located on an oligonucleotide (O'-Connor & Stöhrer, 1985).

The system we report here will be used as the prototype for the inclusion of numerous other carcinogenic and mutagenic adducts into this site on the negative strand of the M13 mp9 DNA molecule. In addition, an analogous protocol using M13 mp8 will allow us to position the same adduct-containing heptamer into the positive strand of the viral DNA. In this case we also will be able to remove the complementary strand prior to transfection, thus making the vector less susceptibile to accurate DNA repair pathways that require double-stranded DNA.

Registry No. 5′-ATCCGTC-3′, 99475-94-2; *N*-acetoxy-*N*-(trifluoroacetyl)-2-aminofluorene, 99475-95-3.

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Synthesis, Characterization, and Black Lipid Membrane Studies of [7-L-Alanine]gramicidin A†

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Received August 27, 1985

ABSTRACT: With a view to study the relevance of side-chain orientation in the transport of cations through a gramicidin transmembrane channel and to identify an analogue with favorable characteristics, [L-Ala^7]gramicidin A was synthesized, purified, verified, and characterized by high-performance liquid chromatography, by carbon-13 and proton magnetic resonance spectra, and by circular dichroism spectra in methanol. Complete incorporation as the channel state was achieved when packaged in lysolecithin-containing lipid bilayers. The single-channel conductance data in diphytanoyllecithin/*n*-decane membranes are presented along with those of synthetic gramicidin A (GA). [L-Ala^7]GA exhibits the highest most probable single-channel conductance so far reported for an analogue occurring at 28 pS as compared to 21 pS for GA under similar conditions. Also, a dramatic reduction in the dispersity of conducting states is observed with about 76% of the events falling in a narrow 1.75-pS conductance window as compared to about 31% of the events for GA under identical conditions. Thus, with the above characteristics, [L-Ala^7]GA appears to be a very good candidate for a thorough study of ionic mechanism. The present results indicate that elements intrinsic to the channel proper are rate-limiting for GA and that there is no interfacial polarization or diffusion-controlled association at 1 M KCl and a 100-mV applied potential.

Gramicidin, a pentadecapeptide, isolated from a strain of *Bacillus brevis* (Hotchkiss & Dubos, 1940) was shown to be a mixture of gramicidins A, B, and C (Gregory & Craig, 1948) with alternating L- and D-amino acids differing in position 11, with Trp, Phe, or Tyr (Gross & Witkop, 1965), respectively, in the ratio of 72:9:19 (Glickson et al., 1972). Each in turn had either Val or Ile in position 1. The structure of [Val^1]-gramicidin A was determined to be (Sarges & Witkop, 1965a,b)

H-C(O)-L-Val^1-Gly^2-L-Ala^3-D-Leu^4-L-Ala^5-D-Val^6-L-Val^7-D-Val^8-L-Trp^9-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15}-$NHCH_2CH_2OH$

† This work was supported in part by NIH Grant GM 26898.

0006-2960/86/0425-0456$01.50/0