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A Probe for the Mutagenic Activity of the Carcinogen 4-Aminobiphenyl: Synthesis and Characterization of an M13mp10 Genome Containing the Major Carcinogen–DNA Adduct at a Unique Site†

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ABSTRACT: The duplex genome of *Escherichia coli* virus M13mp10 was modified at a unique site to contain *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl ($dG^{8\text{-}ABP}$), the major carcinogen–DNA adduct of the human bladder carcinogen 4-aminobiphenyl. A tetradeoxynucleotide containing a single $dG^{8\text{-}ABP}$ residue was synthesized by reacting 5′-d(TpGpCpA)-3′ with *N*-acetoxy-*N*-(trifluoroacetyl)-4-aminobiphenyl, followed by high-performance liquid chromatography purification of the principal reaction product 5′-d(TpG$^{8\text{-}ABP}$pCpA)-3′ (yield 15–30%). Characterization by fast atom bombardment mass spectrometry confirmed the structure as an intact 4-aminobiphenyl-modified tetranucleotide, while ^1H nuclear magnetic resonance spectroscopy established the site of substitution and the existence of ring stacking between the carcinogen residue and DNA bases. Both 5′-d(TpG$^{8\text{-}ABP}$pCpA)-3′ and 5′-d(TpGpCpA)-3′ were 5′-phosphorylated by use of bacteriophage T4 polynucleotide kinase and were incorporated into a four-base gap uniquely positioned in the center of the recognition site for the restriction endonuclease *Pst*I, in an otherwise duplex genome of M13mp10. In the case of the adducted tetranucleotide, $dG^{8\text{-}ABP}$ was located in the minus strand at genome position 6270. Experiments in which the tetranucleotides were 5′ end labeled with [^{32}P]phosphate revealed the following: (i) the adducted oligomer, when incubated in a 1000-fold molar excess in the presence of T4 DNA ligase and ATP, was found to be incorporated into the gapped DNA molecules with an efficiency of approximately 30%, as compared to the unadducted d(pTpGpCpA), which was incorporated with 60% ligation efficiency; (ii) radioactivity from the 5′ end of each tetranucleotide was physically mapped to a restriction fragment that contained the *Pst*I site and represented 0.2% of the genome; (iii) the presence of the lesion within the *Pst*I recognition site inhibited the ability of *Pst*I to cleave the genome at this site; (iv) in genomes in which ligation occurred, T4 DNA ligase was capable of covalently joining both modified and unmodified tetranucleotides to the gapped structures on both the 5′ and the 3′ ends with at least 90% efficiency. Evidence also is presented showing that the $dG^{8\text{-}ABP}$-modified tetranucleotide was stable to the conditions of the recombinant DNA techniques used to insert it into the viral genome. On the basis of these and other data, the $dG^{8\text{-}ABP}$-modified genome was judged to be a useful probe for investigation of site-specific mutagenesis in *E. coli*.

Chemical carcinogens, when activated by metabolism to electrophilic forms, react with nucleophilic sites in DNA to form an array of carcinogen–DNA adducts (Miller, 1978a). These products may be key intermediates in carcinogenesis, possibly by representing the distinct chemical lesions responsible for the mutagenic activation of oncogenes (Tabin et al., 1982; Sukumar et al., 1983). Indeed, the known mutational specificity of one carcinogen, *N*-methyl-*N*-nitrosourea, has been correlated with the specific DNA sequence change found following induction of rat mammary tumors after a single dose (Zarbl et al., 1985). In most cases, it has been exceedingly difficult to assign specific DNA adducts with responsibility for the mutational specificity of a chemical carcinogen, because so many different products form when carcinogens damage genomes. One simplifying approach to this problem is to situate a unique adduct in a phage or plasmid genome and then to replicate this defined substrate in cells to study site-directed mutagenesis in vivo. We have used this method in the past to show directly that O^6-methylguanine (O^6MeGua),[1] a minor lesion formed by methylating agents, causes G to A transition mutations in vivo (Loechler et al., 1984). This transition also is the genetic change observed in bacteria treated with alkylating agents that react with DNA by an S_N1 mechanism (Miller, 1978b) and, in the experiment cited above, in malignant tumors induced by *N*-methyl-*N*-nitrosourea.

The subject of this work is 4-aminobiphenyl (ABP), a human carcinogen found to produce bladder cancer in 17% of

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the workers exposed during the 2 decades of its manufacture for use as a rubber antioxidant (Melick et al., 1971). Despite cessation of industrial use in 1955, exposure of people to ABP continues today due to cigarette smoking and to other as yet unknown sources (Patrianakos & Hoffmann, 1974; Bryant et al., 1986). Similarly to other aromatic amine carcinogens, ABP is thought to be metabolically activated by N-hydroxylation [for reviews, see Garner et al. (1984) and Beland and Kadlubar (1985)]. There are three known DNA reaction products of metabolically activated ABP, and these are formed in approximately the same proportions whether the carcinogen is activated in vivo in the urinary bladder of the beagle dog or in vitro in the presence of purified calf thymus DNA. The structures of these adducts have been determined to be *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl ($dG^{8\text{-ABP}}$), which accounts for about 70% of the radioactivity bound from ring-labeled ABP, *N*-(deoxyguanosin-N^2-yl)-4-aminobiphenyl, and *N*-(deoxyadenosin-8-yl)-4-aminobiphenyl (Kadlubar et al., 1982; Beland et al., 1983). NMR experiments with the dimer $d(CpG^{8\text{-ABP}})$ and theoretical energy calculations (Broyde et al., 1985; Shapiro et al., 1986) have suggested that the $dG^{8\text{-ABP}}$ adduct resides in the major groove and does not seriously perturb duplex DNA structure, thus possibly allowing the lesion to escape repair. These studies also have suggested that other low-energy conformations may exist that could prove mutagenic at the replication fork.

Salmonella typhimurium his$^-$ tester strains containing G-C base pairs as targets for base pair substitution or frameshift mutagenesis are reverted by ABP in the presence of a microsomal activating system or by *N*-hydroxy-4-aminobiphenyl in the absence of an activating system (McCann et al., 1975; Kadlubar et al., 1982; Beland et al., 1983; Scribner et al., 1979; El-Bayoumy et al., 1981; Pai et al., 1985; Rosenkranz et al., 1985). Only base pair substitution strains containing the plasmid pKM101 are reverted well by activated aminobiphenyl, suggesting a role for the SOS system in processing the DNA lesion(s) of ABP into mutations; the plasmid is not required for mutagenesis in frameshift tester strains. This indication of the ability of chemically or metabolically activated ABP to cause mutation at G-C targets, in combination with its partial mutational spectrum in *Escherichia coli* in the *lacZ* gene fragment of bacteriophage M13mp10 DNA (D. D. Lasko et al., unpublished results), prompted us to design a substrate to investigate mutagenesis by the major guanine reaction product $dG^{8\text{-ABP}}$. The facility with which $dG^{8\text{-ABP}}$ could be incorporated into a short oligonucleotide with high yield and purity and the fact that mutations were observed frequently at G-C base pairs in genomes containing ABP lesions were important factors in our decision to focus initially on the role of this guanine adduct in mutagenesis by ABP.

In this paper we describe a strategy for incorporating a single ABP lesion into M13mp10 DNA. This involved, initially, synthesis and characterization of a tetradeoxynucleotide containing $dG^{8\text{-ABP}}$ at a known site, followed by covalent incorporation of the modified DNA fragment into a four-base gap in a duplex M13mp10 genome.

Experimental Procedures

Materials. Protected monomers and support for solid-phase oligonucleotide synthesis by the phosphotriester method were purchased from Cruachem. *N*-Hydroxy-4-aminobiphenyl and [2,2′-^{3}H]-*N*-hydroxy-4-aminobiphenyl (12 mCi/mmol) were obtained from Midwest Research Institute. Acetic anhydride and trifluoroacetic anhydride, gold label, were from Aldrich. Deoxyribonucleoside triphosphates were purchased from Boehringer Mannheim. [α-^{32}P]dCTP (3000 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were purchased from Amersham. Bio-Gel P-6DG and acrylamide were from Bio-Rad. The dT_{4-22} molecular weight standard was from Bethesda Research Laboratories. Hydroxylapatite and PEI–cellulose TLC plates were purchased from Boehringer Mannheim and Brinkmann, respectively. Bacteriophage T4 DNA polymerase was purchased from Bethesda Research Laboratories or New England Biolabs. Nuclease P_1 was the product of Bethesda Research Laboratories, and bacterial alkaline phosphatase and snake venom phosphodiesterase were obtained from Sigma Chemical Co. Bacteriophage T4 polynucleotide kinase and restriction endonucleases were obtained from New England Biolabs.

Synthesis and Purification of the ABP-Modified Tetranucleotide 5′-d(TpG$^{8\text{-ABP}}$*pCpA)-3′.* The completely protected derivative of the tetradeoxynucleotide 5′-d(TpGpCpA)-3′ was synthesized by solid-phase phosphotriester chemistry with a poly(dimethylacrylamide)–kieselguhr support on a Cruachem manual synthesizer (Sproat & Gait, 1984). After release of the oligonucleotide from the resin by the 2-nitrobenzaldoximate of 1,1,3,3-tetramethylguanidine, which also deblocked the phosphate-protecting 2-chlorophenyl functionality, the mixture was shaken overnight with aqueous ammonia at 55 °C to hydrolyze the exocyclic amino protecting groups. Finally, the 5′-(dimethoxytrityl) group was released by 80% aqueous acetic acid treatment for 20 min to generate the tetradeoxynucleotide d(TpGpCpA). The crude oligodeoxynucleotide was purified by HPLC on a Partisil 10 SAX column. The product was further purified by reversed-phase HPLC on a Waters μBondapak C_{18} column followed by desalting on a Waters Sep-Pak C_{18} cartridge. The nucleoside composition was confirmed by digesting the tetramer with snake venom phosphodiesterase and bacterial alkaline phosphatase followed by reversed-phase HPLC analysis (Fowler et al., 1982).

The singly adducted tetranucleotide $d(TpG^{8\text{-ABP}}pCpA)$ was formed by reacting d(TpGpCpA) with the electrophilic reactant *N*-acetoxy-*N*-(trifluoroacetyl)-4-aminobiphenyl (*N*-OAc-TFAABP), which was prepared from either unlabeled or ^{3}H-labeled *N*-hydroxy-4-aminobiphenyl by a modification of the method of Lee and King (1981). All steps of the synthesis were performed under an argon atmosphere. The second step in the preparation of *N*-OAc-TFAABP, which involves incubation of *N*-hydroxy-*N*-(trifluoroacetyl)-4-aminobiphenyl with acetic anhydride, was always performed immediately prior to its reaction with the tetramer. The d(TpGpCpA) (0.25–1 mg) was dissolved at a concentration of 1 mg/mL in 1 mM potassium citrate buffer, pH 7, and was then mixed with an equal volume of 95% ethanol. At 30-min intervals, five additions of a 10-fold molar excess of *N*-OAc-TFAABP, dissolved at 30 mM in ethanol, were made to the reaction mixture, which was stirred continuously at 37 °C. At

[1] Abbreviations: bp, base pair; b, base; ss, single stranded; ds, double stranded; FAB, fast atom bombardment; RF, replicative form; ABP, 4-aminobiphenyl; *N*-OAc-TFAABP, *N*-acetoxy-*N*-(trifluoroacetyl)-4-aminobiphenyl; 5′-d(TpG$^{8\text{-ABP}}$pCpA)-3′, tetranucleotide in which the sole guanine residue has been converted to *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl; $dG^{8\text{-ABP}}$, *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl; M13mp10-ABP_1, duplex genome of M13mp10 in which the guanine at position 6270 in the minus strand has been replaced with *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl; M13mp10-G, same as M13mp10-ABP_1 but where the genome contains guanine at this site (this genome has experienced the same recombinant DNA manipulations as M13mp10-ABP_1); O^6MeGua, O^6-methylguanine; 5′-d(TpG$^{O^6Me}$pCpA)-3′, tetranucleotide containing O^6-methylguanine; PEI, poly(ethylenimine); TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

30 min after the last addition, the mixture was flushed with argon to remove the ethanol cosolvent. The aqueous mixture was extracted 5 times with diethyl ether to remove *N*-OAc-TFAABP and its decomposition products and was subsequently freed of dissolved ether under a stream of argon.

d(TpG$^{8\text{-ABP}}$pCpA) was then purified by preparative reversed-phase HPLC on a Waters μBondapak C_{18} Semiprep column. The HPLC solvent program was 10% CH_3CN in 0.1 M ammonium acetate, pH 5.7, for 0–7 min, followed by a linear gradient to 100% CH_3CN from 7 to 17 min, and continued at 100% CH_3CN for 8 min before initial conditions were returned to over a 10-min period (HPLC method A). The flow rate was 3 mL/min. UV absorption spectra obtained on a Hewlett-Packard 1040A photodiode array detector were used to monitor the desired reaction product, which eluted at 14–15 min. The chromatographically purified oligonucleotide was reinjected onto the same column (flow rate 3 mL/min) and desalted by elution with water for 10 min followed by a 10-min linear gradient of 0–100% aqueous CH_3CN; the adducted tetranucleotide was eluted after 17–18 min. The solvent was removed under a flow of argon, and the sample was stored dry at −70 °C under an argon atmosphere.

Synthesis and Purification of the ABP-Modified Mononucleotide 5′-d(pG$^{8\text{-ABP}}$p)-3′. *N*-(Deoxyguanosin-8-yl)-4-aminobiphenyl 3′,5′-bisphosphate [5′-d(pG$^{8\text{-ABP}}$p)-3′] was synthesized from *N*-OAc-TFAABP and deoxyguanosine 3′,5′-bisphosphate essentially as described above except that the initial reaction mixture did not contain ethanol and the *N*-OAc-TFAABP was added twice in a 50-fold molar excess. The aqueous fraction obtained prior to HPLC was also purified further by adjusting the pH to 2 with 1 N HCl, extracting 5 times with equal volumes of H_2O-saturated ethyl acetate, recovering the product(s) by five, equal-volume, extractions with 1-butanol, evaporating the extract to dryness under reduced pressure, and reconstituting the sample in 10% CH_3CN. Purification by HPLC yielded the desired product, which eluted at 16 min, as well as the corresponding ABP-modified 5′- and 3′-monophosphate derivatives, which eluted at 16.8 and 17.5 min, respectively. The identity of these products was confirmed by UV and ^{1}H NMR spectroscopy.

Characterization of 5′-d(TpG$^{8\text{-ABP}}$pCpA)-3′ and 5′-d(pG$^{8\text{-ABP}}$p)-3′. Proton magnetic resonance spectra were obtained at 500 MHz on a Bruker WM500 NMR spectrometer. d(TpG$^{8\text{-ABP}}$pCpA) and unmodified d(TpGpCpA) were dissolved in D_2O (600 μg/mL). d(pG$^{8\text{-ABP}}$p) was dissolved in D_2O (30 μg/mL), and for comparison to the tetranucleotide, the pD was adjusted to 5.2 with DCl to obtain the monoanionic species. Dioxane was added as an internal reference (3.76 ppm), and spectra were recorded at 21 °C. Resonance assignments were made by homonuclear decoupling experiments and by comparisons with spectra obtained for *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl (Kadlubar et al., 1982), for the ABP-modified guan-8-yl derivative of d(CpG) (Shapiro et al., 1986), and for unmodified dinucleotides (Cheng & Sarma, 1977).

Fast atom bombardment (FAB) mass spectra were obtained with a Kratos MS-50 triple analyzer mass spectrometer, which has been described elsewhere (Gross et al., 1982). The FAB ion source is a standard Kratos design equipped with an Ion Tech fast atom gun. FAB ionization employed 7–8-kV xenon atoms. Samples were dissolved in methanol (~1 mg/mL), and 1 μL of the solution was placed on the copper FAB probe tip (target) after application of dithioerythreitol as the FAB liquid matrix.

Preparation and Characterization of [5′-^{32}P]-5′-d(TpG$^{8\text{-ABP}}$pCpA)-3′. Both modified and unmodified tetranucleotides were 5′-phosphorylated by incubation with T4 polynucleotide kinase and ATP for 45 min at 37 °C; [γ-^{32}P]ATP (3000 Ci/mmol) was diluted with unlabeled ATP to give a final specific activity of 50–250 Ci/mmol. Reaction conditions were 16 μM ATP, 8 μM tetranucleotide, 10 mM Tris-HCl buffer, pH 7.4, 16 mM dithiothreitol, and 20 units of polynucleotide kinase, in a total volume of 0.05 mL. Each phosphorylated oligonucleotide was separated from the unphosphorylated starting material by chromatography on a reversed-phase HPLC column (essentially the HPLC conditions of Figure 2, frame B; the phosphorylated product generally eluted 2 mL earlier than the precursor). The large amount of radioactivity in the sample made it impossible to preparatively purify the phosphorylated tetranucleotide and, in the same chromatographic run, to determine the efficiency of phosphorylation. Accordingly, in order to quantitate phosphorylation, an initial analytical HPLC injection of ~10^5 cpm was made, and the radioactivity in the ATP was compared to that in the ^{32}P-phosphorylated tetranucleotide. To collect the bulk of the phosphorylated product, the remaining portion of the reaction mixture was injected onto the column, and the ^{32}P-labeled tetranucleotide was collected. The volume of the solution was reduced with a stream of argon to lower the concentration of CH_3CN, and finally the sample was desalted by rechromatography on an analytical reversed-phase HPLC column. The phosphorylation reaction and all subsequent manipulations required for ligation of the adducted tetranucleotide into the M13 genome were carried out under amber lights.

Certain aspects of the structure and purity of the adducted tetranucleotide were further investigated by one-dimensional homochromatography following partial digestion with nucleases. Kinetic experiments with nuclease P_1 (a 3′ → 5′ exonuclease and endonuclease) were conducted on [^{32}P]d(TpG$^{8\text{-ABP}}$pCpA) in parallel with a tetranucleotide containing O^6MeGua, [^{32}P]d(TpG$^{O^6\text{Me}}$pCpA) (Fowler et al., 1982), and [^{32}P]d(TpGpCpA). At time points of 0, 2, 10, 20, and 60 min, aliquots of the reaction mixture were placed on ice, and an equal volume of 50 mM EDTA buffer, pH 8, was added to stop digestion. Samples at each time point were then applied to PEI–cellulose TLC plates and eluted at 65 °C with Homomix C-15 as the mobile phase (Jay et al., 1974). Following homochromatography, which essentially separated partial digestion products by size, the TLC plate was covered with Kodak XAR film and autoradiographed for 24 h at −70 °C. The distribution of radioactivity into the respective fragments was estimated by visual inspection of the autoradiogram.

Preparation of the Gapped M13mp10 Genome. The protocol for preparation of the gapped M13mp10 genome is outlined in Figure 1. Double-stranded (ds) M13mp10 replicative form I (RF) DNA was prepared from cells infected with phage by using a modification of a Triton X-100 procedure (Maniatis et al., 1982). This DNA was twice purified from CsCl/ethidium bromide density gradients. Single-stranded (ss) DNA was isolated by phenol extraction of M13mp10 phage from the supernatant of the same infection used for preparation of RF DNA (Messing, 1983). The DNA was further purified by chromatography on hydroxylapatite in 0.14 M potassium phosphate buffer, pH 7. DNA was stored at 4 °C in 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA. Blunt-ended DNA missing the internal four nucleotides of the *Pst*I recognition sequence, 5′-CTGCAG-3′, was produced by a method similar to that of Green et al. (1984) except that the concentration of dCTP was raised from 14.4 to 60 μM, the 3′ → 5′ excision reaction on *Pst*I-cleaved sub-

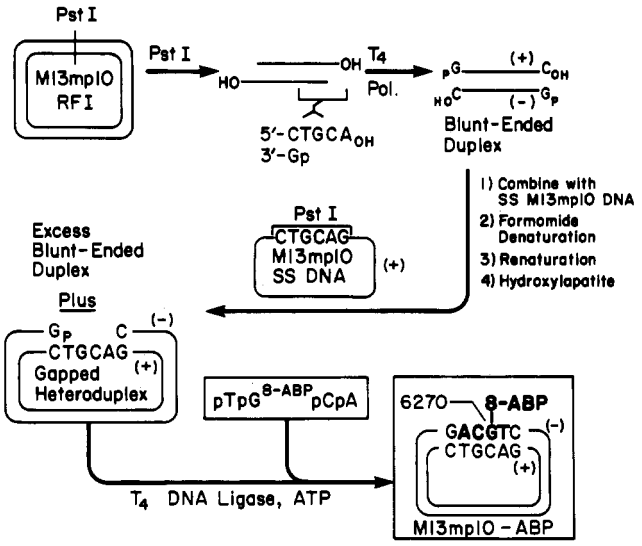

FIGURE 1: Site-specific modification of the M13mp10 genome to contain a single 4-aminobiphenyl adduct. As described in the text, M13mp10 RF DNA was cleaved with *Pst*I, which produced four-base overhangs at the 3′ ends. The overhanging bases were removed by using the 3′ → 5′ exonuclease of bacteriophage T4 DNA polymerase. This blunt-ended, ds DNA was then hybridized to an excess of ss M13mp10 (plus strand) DNA. The products of the hybridization treatment were gapped-heteroduplex DNA, a small amount of reannealed blunt-ended duplex DNA, and the excess ss DNA. Circular gapped-duplex DNA containing a small amount (<10%) of linear blunt-ended DNA was isolated. The $dG^{8\text{-ABP}}$ adduct was situated at genome position 6270.

strates proceeded for 30 min, and the subsequent DNA resynthesis was for 45 min.

As a quality control step to evaluate the success of the resynthesis step of the blunt-ending reaction, a small portion of the excision reaction (0.05 mL or 2.5 μg of DNA) was resynthesized in the presence of [α-^{32}P]dCTP at a final specific activity of 33 Ci/mmol. This labeled DNA was purified by chromatography on a Bio-Gel P-6DG column, and the radioactivity eluting in the void volume was quantitated by Cerenkov counting to determine the extent of resynthesis, typically 30 bases per end under these conditions. Portions (~3000 cpm) were cleaved with either *Eco*RI, *Bam*HI, or *Hin*dIII and electrophoresed through a 20% polyacrylamide [19:1 acrylamide:bis(acrylamide)]–7 M urea gel; resultant fragment lengths upon electrophoresis were determined by comparison to a dT_{4-22} ladder 5′-^{32}P end labeled as recommended by the manufacturer. Preparations of blunt-ended DNA showing >10% of the radioactive products to be smaller than the expected length (36, 18, and 9 bases with the enzymes listed above, respectively) were discarded.

The remainder of the blunt-ended DNA, prepared with nonradioactive DNA synthesis precursors, was extracted twice with phenol and precipitated with ethanol. This material (~50 μg) was then hybridized to a 20-fold excess of ss M13mp10 DNA (plus strand) with the formamide denaturation/renaturation technique of Lundquist and Olivera (1982). DNA concentrations for this hybridization were 8 μg/mL for blunt-ended duplex DNA and 80 μg/mL for ss DNA. After dialysis against 0.14 M potassium phosphate buffer, pH 7, excess ss DNA was removed by passage over a column of 2 g of hydroxylapatite previously equilibrated with the same buffer; ss DNA was eluted with the 0.14 M phosphate buffer, and the desired ds gapped-duplex DNA was eluted with 0.4 M potassium phosphate, pH 7. Fractions containing DNA were detected by ethidium bromide fluorescence of aliquots spotted onto a 1% agarose slab containing 1 μg/mL ethidium bromide. The gapped-duplex DNA was dialyzed against 10 mM Tris-HCl buffer, pH 8, containing 1 mM EDTA. DNA was quantitated by ethidium bromide fluorescence following agarose gel electrophoresis of an aliquot and comparison with standards of known concentration.

Ligation of 5′-d(pTpG$^{8\text{-ABP}}$pCpA)-3′ and 5′-d(pTpGpCpA)-3′ into the Gapped Genome. Ligation conditions were somewhat altered from those previously described (Green et al., 1984). To minimize exposure of the adducted tetranucleotide to alkaline conditions that could catalyze hydrolytic opening of the ABP-substituted guanine ring, the pH of the ligation buffer (measured at 22 °C) was lowered from 7.8 to 7.4. Ligation conditions were 70 mM Tris-HCl buffer, pH 7.4, 16 mM dithiothreitol, 8 mM $MgCl_2$, 5–15 nM gapped-duplex DNA (1 μg = 0.2 pmol), and 4–7 μM adducted or unadducted ^{32}P-labeled tetranucleotide. The buffer did not contain bovine serum albumin. T4 DNA ligase (400 units) was added, and the mixture was incubated at 16 °C for 12 h. After 12 h, another 400 units of T4 DNA ligase was added and incubation continued for a further 10–12 h. The reaction mixture was chromatographed on a 26 × 1.5 cm column of Bio-Rad P-6DG, which had been equilibrated with sterile H_2O. Fractions eluting at and around the exclusion volume, which contained radioactive, high molecular weight DNA, were combined, and their volume was reduced under a flow of argon. Efficiency of ligation was defined as the percentage of available gapped-duplex DNA molecules that had incorporated a tetranucleotide and was calculated by dividing the picomoles of incorporated tetramer (as calculated from its specific activity) by the picomoles of gapped-duplex DNA (quantitated as described above).

Phosphorylated tetranucleotide recovered from ligation reactions was analyzed by reversed-phase HPLC (conditions of Figure 2) in order to assess whether its structural integrity had been compromised during the in vitro manipulations. In another experiment, which addressed the question of whether the tetranucleotide had ligated into the correct area of the genome, 100–300 cpm of ligated material (~5 fmol) was used for restriction enzyme digestion. Before restriction, 0.4 μg of RF DNA was added to each reaction tube as an internal standard in order to allow visualization of complete digestion by staining of the DNA with ethidium bromide. The "three-buffer" system (Maniatis et al., 1982) was used for DNA restriction, and digestions were performed for 1 h at 37 °C with 10 units of *Pst*I, *Ava*II, *Bgl*I, *Acc*I, *Bam*HI, *Hin*cII, or *Hin*dIII. The cleavage products were electrophoresed on 14 × 20 cm 0.8% agarose gels at 25 V for 12 h in Tris–acetate buffer (Maniatis et al., 1982) supplemented to 1 μg/mL with ethidium bromide. Gels were dried under vacuum; autoradiography was performed for 12–24 h by using Kodak XAR film with intensifying screens at −70 °C. Electrophoresis on denaturing 20% polyacrylamide gels [19:1 acrylamide:bis(acrylamide)] was carried out at 40 mA.

RESULTS

Synthesis and Characterization of a Tetranucleotide Containing the C8-Substituted Guanine Adduct of the Carcinogen ABP. The reaction of 5′-d(TpGpCpA)-3′ with *N*-OAc-TFAABP afforded the isolation of 5′-d(TpG$^{8\text{-ABP}}$pCpA)-3′ as a major synthetic product, with a yield corresponding to 15–30% of the tetranucleotide modified in different preparations (n = 6). Figure 2A shows the preparative HPLC elution profile of the crude reaction mixture (cf. Experimental Procedures) in which both the unmodified tetranucleotide and the synthetic product were observed as primary components.

Analytical HPLC of d(TpG$^{8\text{-ABP}}$pCpA) after desalting (Figure 2B) did not indicate the presence of any UV-absorbing contaminants in the purified product. The UV spectra of the unmodified and ABP-modified tetranucleotides, obtained with a photodiode array detector, are shown in Figure 2C and are compared with the spectrum of a reference compound, d(pG$^{8\text{-ABP}}$p). The spectrum of the ABP–tetranucleotide adduct displayed an absorption maximum at 260 nm that is consistent with its nucleotide structure as well as a shoulder at 310 nm that is characteristic of maxima exhibited by the reference compound and of other C8-substituted nucleotide adducts of carcinogenic arylamines.

The structural characterization of this product as an *N*-(deoxyguanosin-8-yl) derivative of ABP was established by ^{1}H NMR spectroscopy at 500 MHz. In Figure 3, the downfield regions of the NMR spectra of d(pG$^{8\text{-ABP}}$p) (frame A), d(TpGpCpA) (frame B), and d(TpG$^{8\text{-ABP}}$pCpA) (frame C) are compared. For the latter, all nine ABP aromatic ring protons could be assigned as well as the proton resonances of each of the purine and pyrimidine bases, with the notable exception of the C8 proton of the guanine ring. The resonance line widths of the two tetramers were narrow in all cases. Thus, any possible interstrand effects, which would be expected to induce line broadening, should be minor under the conditions utilized here. In addition, all four signals characteristic of the H-1′ protons of the 2′-deoxyribose moiety were observed. Compared to those of d(pG$^{8\text{-ABP}}$p), the ABP protons of the adducted tetranucleotide were shifted upfield by 0.06–0.21 ppm. Small upfield shifts (0.04–0.07 ppm) of the adenine and thymine protons relative to the unmodified tetramer were also detected. Interestingly, the two cytosine protons were shifted downfield by about 0.1 ppm as a result of adduct formation. These results provide evidence for alterations in the normal stacking interactions throughout the oligomer, especially the stacking of the ABP ring system.

FAB mass spectrometry was used to confirm the structural integrity and purity of the ABP-modified tetranucleotide for use in subsequent ligation experiments. The positive ion spectrum showed a prominent peak at m/z 1385, which corresponds to the $(M + H)^+$ ion of the disodium salt of d(TpG$^{8\text{-ABP}}$pCpA). Remarkably, this was the only quasi-molecular ion observed, and no other ions were detected in the high-mass region of the spectrum. No significant fragment ions were observed.

Polynucleotide kinase-mediated phosphorylation of the 5′-hydroxyl of the modified and unmodified tetranucleotides was monitored by HPLC analysis of the reaction mixtures. Conversion of either oligonucleotide to the corresponding 5′-phosphate was between 95 and 98%. When [^{32}P]ATP was included in the reaction, the phosphorylated tetranucleotides achieved a specific activity of 50 or 250 Ci/mmol (40×10^6 or 200×10^6 Cerenkov cpm/μg). The adduct-containing tetranucleotide was stable under storage conditions (for at least 1 year) and could be highly purified as judged by HPLC analysis (Figure 2) and by one-dimensional homochromatography as described below. This purity was essential for subsequent biological experiments.

Partial digestion of the adduct-containing tetranucleotide with nuclease P_1 followed by analysis by homochromatography revealed a pattern of nuclease inhibition similar to that for d(pTpG$^{O^6Me}$pCpA) (Fowler et al., 1982), further establishing a modification at the guanine residue. These time course experiments compared the relative sensitivities to nuclease P_1 of the three phosphodiester bonds of [^{32}P]d(TpG$^{8\text{-ABP}}$pCpA) to those of the control oligonucleotides, [^{32}P]d(TpG$^{O^6Me}$pCpA) and [^{32}P]d(TpGpCpA). At the 0-min time point, the ^{32}P-la-

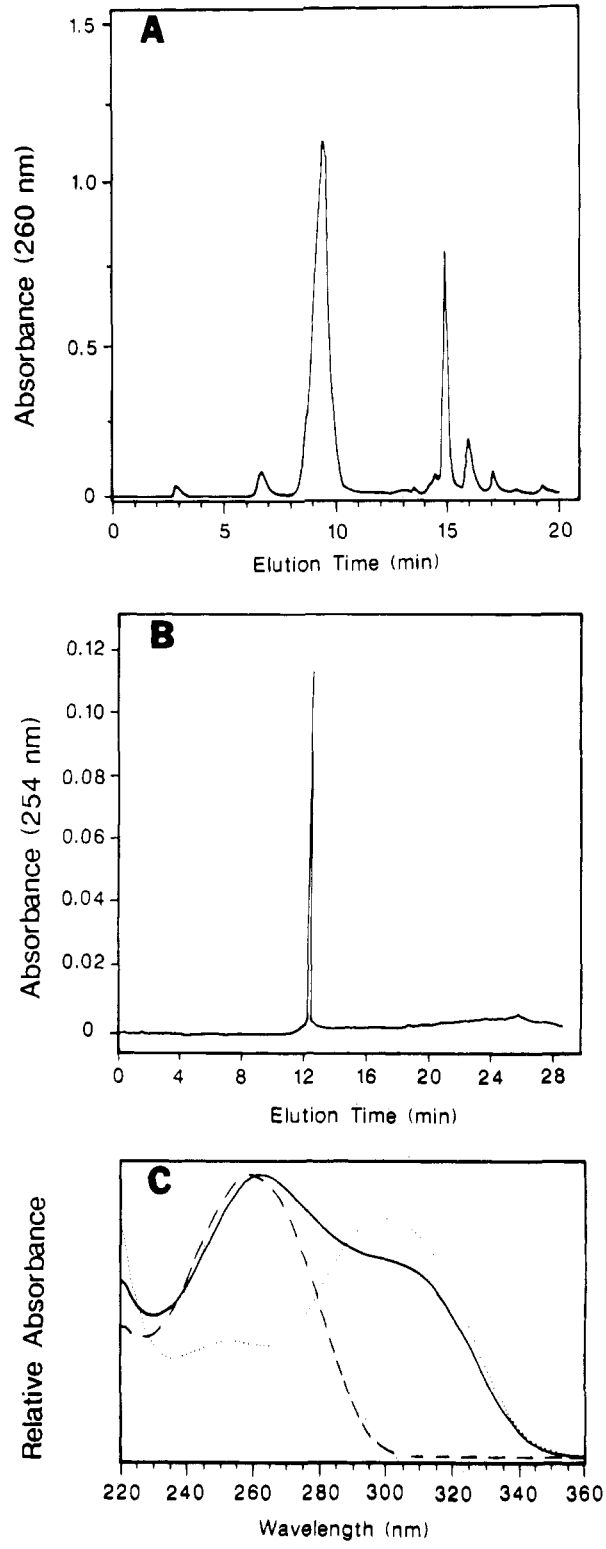

FIGURE 2: HPLC profiles and ultraviolet absorption spectrum of 5′-d(TpG$^{8\text{-ABP}}$pCpA)-3′. In frame A, a preparative HPLC profile is presented of the crude reaction mixture of 5′-d(TpGpCpA)-3′ with *N*-OAc-TFAABP. Chromatographic conditions: method A under Experimental Procedures. The peak eluting near 15 min was collected, desalted, and reanalyzed by analytical HPLC as shown in frame B. Chromatographic conditions: 0–5 min, 0.1 M ammonium acetate, pH 5.7, 10% CH_3CN; 5–12 min, linear gradient to 100% CH_3CN; 12–17 min, 100% CH_3CN; 17–22 min, reverse gradient to initial conditions; 22–28 min, initial conditions. The column was a Macherey-Nagel Nucleosil 10 C_{18}, and the flow rate was 2 mL/min. Frame C shows UV spectra of the d(TpG$^{8\text{-ABP}}$pCpA) that eluted near 15 min in frame A (solid line), the unmodified d(TpGpCpA) that eluted between 9 and 10 min in frame A (broken line), and the reference compound d(pG$^{8\text{-ABP}}$p) (dotted line).

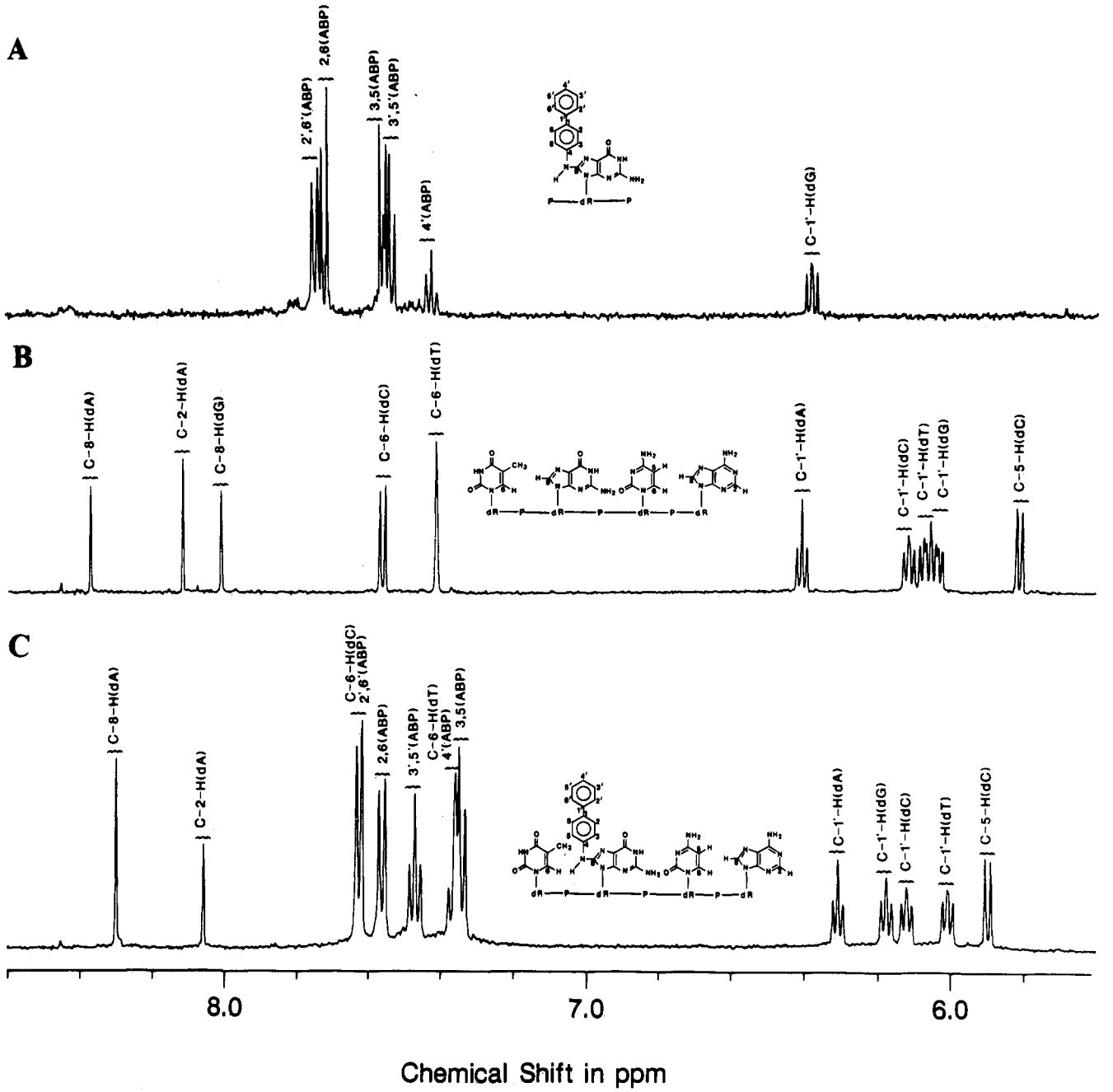

FIGURE 3: The 500-MHz ^1H NMR spectra of the downfield region of 5'-d(pG$^{8\text{-ABP}}$p)-3' (frame A), 5'-d(TpGpCpA)-3' (frame B), and 5'-d(TpG$^{8\text{-ABP}}$pCpA)-3' (frame C). The spectra were recorded in D_2O at 21 °C.

beled ABP–tetranucleotide was free of radiochemical impurities (>95% purity) detectable by this chromatographic technique; these data are in accord with those from HPLC indicating a similar level of purity. Migration of [^{32}P]d(TpG$^{8\text{-ABP}}$pCpA) was retarded on the TLC plate by 50% compared to the methylated and unmodified tetranucleotides. The trimer digestion product was similarly retarded, but the 5'-[^{32}P]dTMP monomer digestion product comigrated with authentic 5'-dTMP. This result localized the position of the modification to either of the internal two nucleotides of the tetranucleotide. After 10 min of hydrolysis, [^{32}P]d(TpGpCpA) was completely digested to [^{32}P]dTMP monomer. In contrast, [^{32}P]d(TpG$^{O^6Me}$pCpA) was digested to trimer and [^{32}P]dTMP monomer by the exonuclease/endonuclease activities of the enzyme, whereas most of the [^{32}P]d(TpG$^{8\text{-ABP}}$pCpA) remained in tetramer form (ca. 60% tetramer, 10% trimer, and 30% monomer). At 60 min, [^{32}P]d(TpG$^{O^6Me}$pCpA) was almost completely digested to monomer (ca. 0% tetramer, 10% trimer, 5% dimer, and 85% monomer), and [^{32}P]d(TpG$^{8\text{-ABP}}$pCpA) was reduced to trimer (10%) and monomer (90%) forms. Comparison of the nuclease P_1 digestion of [^{32}P]d(TpG$^{O^6Me}$pCpA) and [^{32}P]d(TpG$^{8\text{-ABP}}$pCpA) showed that inhibition of cleavage for the G–C bond was much greater for the oligonucleotide containing the dG$^{8\text{-ABP}}$ lesion than for that containing dG$^{O^6Me}$. It was in fact not possible to observed a dimer digestion product for d(pTpG$^{8\text{-ABP}}$pCpA) under these experimental conditions.

Incorporation of the Tetranucleotides into Gapped-Duplex DNA. The results of several ligation experiments are listed in Table I. The mean ligation efficiency for d(pTpG$^{8\text{-ABP}}$pCpA) was 30% compared to 60% for d(pTpGpCpA). The presence of the ABP lesion inhibited ligation to a greater extent than did the presence of an O^{6}MeGua lesion, since the ligation efficiency for d(pTpG$^{O^6Me}$pCpA) was found to be at least 50% by similar techniques (Green et al., 1984). M13mp10-ABP$_1$ is defined

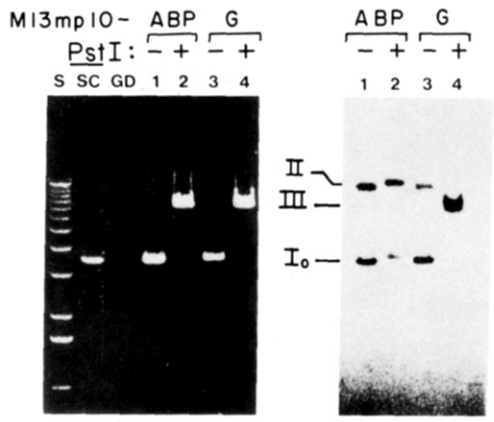

FIGURE 4: 4-Aminobiphenyl adduct inhibits *Pst*I cleavage of the singly adducted M13mp10 genome. Shown are an ethidium bromide stained agarose electrophoresis gel (left panel) and an autoradiogram (right panel) of ~5 fmol each of purified d(pTpG$^{8\text{-ABP}}$pCpA) and d-(pTpGpCpA) ligation products (M13mp10-ABP and M13mp10-G, respectively), cleaved with *Pst*I (lanes 2 and 4) or buffer controls (lanes 1 and 3). Forms II and III are relaxed circular and linear DNA molecules, respectively; S, SC, and GD refer to DNA molecular weight standard, supercoiled DNA, and gapped-duplex DNA, respectively.

Table I: Ligation of Tetranucleotides Containing either the dG$^{8\text{-ABP}}$ Adduct or Unmodified Guanine into Gapped M13mp10 Genomes

expt	gapped-duplex DNA (pmol)	TpG$^{8\text{-ABP}}$-pCpA (pmol)	TpGpCpA (pmol)	ligation efficiency (%)[a] TpG$^{8\text{-ABP}}$-pCpA	ligation efficiency (%)[a] TpGp-CpA
1	0.1	130	94	30	70
2	0.1	140	ND	40	ND
3	0.3	130	83	30	50

[a] Ligation efficiency was defined as 100 times the ratio of picomoles of tetranucleotide eluting in the void volume of a P-6DG gel filtration column to the picomoles of gapped-duplex DNA in the reaction.

as the genome containing the dG$^{8\text{-ABP}}$ located specifically in the minus strand at the *Pst*I site; M13mp10-G refers to control material that had undergone the same in vitro manipulations but contained guanine at position 6270 [i.e., the material derived from insertion of d(pTGCA) into the genome].

A series of experiments addressed the issues of whether the insertion of the ABP-modified tetranucleotide was covalent and of whether it occurred specifically at the *Pst*I site. When the products of ligation were examined by using agarose gel electrophoresis followed by autoradiography, it was clear that a major portion of the labeled tetranucleotide had been covalently incorporated, because approximately 50% of the radioactivity migrated at the position of covalently closed circular DNA (form I_0), both for the unadducted and for the adducted tetramers (Figure 4, lanes 1 and 3). Cleavage with *Pst*I for 1 h (conditions that resulted in complete scission of unlabeled M13mp10 RF added as an internal standard, as indicated in the left panel, lanes 2 and 4) resulted in complete linearization of the originally circular M13mp10-G. However, M13mp10-ABP$_1$ was almost totally refractory to cleavage, in that only trace amounts of linear DNA formed (Figure 4, lanes 2 and 4). It is conceivable that the increase in the proportion of RFII to RFI seen after *Pst*I cleavage of M13mp10-ABP (right panel, lanes 1 and 2) may have been due to specific nicking opposite the *Pst*I site; it may also have arisen from random nicking by another endonuclease activity in the *Pst*I preparation.

In a related experiment (data not shown), a survey of restriction endonucleases was undertaken to ascertain whether the presence of the lesion at the *Pst*I site inhibited the cleavage

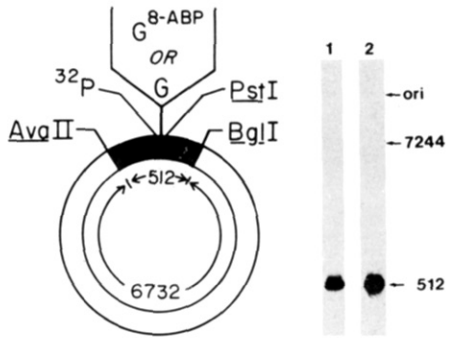

FIGURE 5: Postligation localization of radioactivity from [5′-^{32}P]d-(pTpG$^{8\text{-ABP}}$pCpA)-3′ and 5′-d(pTpGpCpA)-3′ to the region of the M13mp10 genome containing the *Pst*I site. (Right panel) Autoradiogram of ~5 fmol of adducted (lane 1) and control (lane 2) ligation products doubly digested with *Ava*II and *Bgl*I restriction endonucleases. (Left panel) Cartoon illustrating that double digestion produces two products, a 512-bp fragment containing the *Pst*I site and a 6732-bp fragment. Also indicated is the position of the ^{32}P radiolabel from the 5′ end of the tetranucleotide.

of any enzyme other than *Pst*I. *Acc*I, which cleaves between positions 6264 and 6265 on the minus strand and *Hin*dIII, which cleaves between positions 6280 and 6281 on the minus strand, both cut M13mp10-ABP$_1$ DNA to completion. Thus, although *Pst*I cleavage was inhibited, this effect did not extend to two enzymes that cut within 6 bases 3′ to the lesion (*Acc*I) and 10 bases 5′ to the lesion (*Hin*dIII).

Although the radioactivity eluting in the void volume of the P-6DG column was resistant to *Pst*I cleavage and comigrated with M13mp10 DNA, it was also necessary to show that the label was specific to the *Pst*I site and not the result of covalent or noncovalent interactions with any other region of the genome. This question was approached through a series of double-digestion experiments. The first experiment, illustrated in Figure 5, localized all radioactivity in M13mp10-ABP$_1$ and M13mp10-G to the 512-bp *Ava*II/*Bgl*I digestion product (7% of the genome) including the *Pst*I site. The second experiment (Figure 6, discussed in detail below) more specifically localized the radiolabel to a 17-base fragment in the minus strand.

It was possible to establish that ligation had taken place on *both* the 5′ and the 3′ sides of the adduct-containing tetranucleotide. Ligation on both sides had been suggested by the fact that much of the ligation product was present in the form of covalently closed circular DNA (the form I_0 DNA in Figure 4). To further investigate this issue, an experiment was devised in which the purified product of the ligation reaction was digested with restriction enzymes that cleave within 20 base pairs of the anticipated adduct site. It was expected that if ligation had in fact occurred on both ends of the tetranucleotide, cleavage with two enzymes recognizing sites that straddled the adduct would be required to release a radiolabeled small fragment of a particular size, which could be analyzed on a denaturing polyacrylamide gel. Should ligation have occurred solely on the 5′ or on the 3′ end in some or all of the molecules, the double digest would reveal molecules smaller than the anticipated size; similarly, a single enzyme cleavage also would yield a radiolabeled small fragment following denaturation. The results of such an experiment are shown in Figure 6, in which M13mp10-ABP$_1$ (left panel) and M13mp10-G (right panel) were singly digested with *Hin*dIII (lane 1) and *Bam*HI (lane 2) and doubly digested with both *Hin*dIII and *Bam*HI (lane 3). No radioactivity was released by the single digestions, and the double digestion released an oligonucleotide fragment of 27 nucleotides, except in the case of M13mp10-G. (In this case the principal product was the

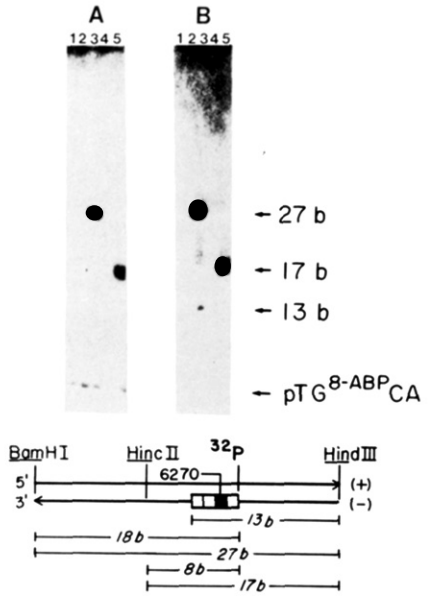

FIGURE 6: Assessment of the ability of T4 DNA ligase to form phosphodiester bonds between synthetic tetranucleotides and M13mp10 duplexes containing complementary or partially complementary four-base gaps. Ligation of [5′-^{32}P]d(TpG$^{8\text{-ABP}}$pCpA)-3′ (or its analogue lacking ABP) on both the 5′ and the 3′ sides was assessed by digesting the ligation products with restriction enzymes that cleave within 20 bp of the expected adduct site. Fragment sizes were calibrated by using a dT_{4-22} ladder (not shown). The tetranucleotide introduction site is depicted in the lower portion of the figure by the rectangle in the minus strand; the solid box is the relative position of the adduct in M13mp10-ABP$_1$ and of guanine in the control genome, M13mp10-G. Anticipated results from this experiment were as follows: a single digest with *Hin*dIII should give a radioactive band of 13 bases if ligation had occurred on the 5′ end of the oligomer but not the 3′ end [horizontal lines interrupted by italicized fragment lengths (in bases, b) are drawn to scale]; *Bam*HI digestion should yield an 18-b radioactive band if ligation occurred at the 3′ end but not the 5′ end; double digestion with both of these enzymes should yield a 27-b fragment *only* in those genomes in which ligation occurred on both ends of the tetranucleotide. Digestion with *Hin*cII alone should result in an 8-b fragment if ligation had not occurred on the 5′ end, and double digestion with *Hin*cII and *Hin*dIII should yield a 17-b fragment if ligation had occurred on both sides of the oligomer. Panel A shows the results of such an experiment performed on M13mp10; panel B shows the results for M13mp10-G (unadducted, ligated material). *Hin*dIII (lane 1); *Bam*HI (lane 2); *Hin*dIII and *Bam*HI (lane 3); *Hin*cII (lane 4); *Hin*cII and *Hin*dIII (lane 5).

27-mer, although a small proportion of the radioactivity was also present in a band of 13 nucleotides, indicating that in some molecules ligation had occurred on the 5′ but not the 3′ end.) This experiment permitted the conclusion that the adduct-containing tetranucleotide was ligated into the gaps on both ends in a minimum of 90% of the gapped structures receiving the tetramers. Moreover, the *Hin*cII/*Hin*dIII double digest (lane 5) localized the ABP–tetranucleotide to a 17-base fragment, representing 0.2% of the genome. The P-6DG column was not absolutely efficient in removing 5′-d-(pTpG$^{8\text{-ABP}}$pCpA)-3′ from the ligated material as indicated by the radioactivity at the bottom of the autoradiogram in Figure 6; this residual oligonucleotide altered the original estimate of ligation efficiency by <10%.

Due to the small amount of singly adducted genome available (microgram quantities), it was not possible by conventional analytical procedures to directly investigate the question of whether the adduct had remained structually intact during the in vitro manipulations involved in construction of M13mp10-ABP$_1$. Nonetheless, it was possible to address this point indirectly by recovering the excess adducted tetranucleotide from the ligation reaction [i.e., the unligated [^{32}P]d-(TpG$^{8\text{-ABP}}$pCpA)] and subjecting this material to analysis. HPLC radioactivity profiles revealed that 96% of the ^{32}P radiolabel coeluted with authentic [^{32}P]d(TpG$^{8\text{-ABP}}$pCpA), while the remaining 4% eluted in the column void volume (data not shown). Our experience with HPLC separations of modified and unmodified oligonucleotides has shown this technique, under the elution conditions employed, to be capable of resolving species differing by very subtle structural features. After one ligation experiment, the optical purity of the excess (unligated) ABP-modified tetranucleotide was monitored by absorbance detection of the HPLC eluant at 254 nm followed by integration of detector output. Peaks due to the ligation buffer were subtracted, and a final optical purity of at least 90% was determined. Thus, by these indirect measurements we conclude that adduct structure was unlikely to have been altered by the recombinant DNA techniques used to incorporate it into an M13mp10 genome.

Discussion

The ability of chemical and physical agents to produce an array of DNA damages often makes it difficult to assign the mutagenic risk to individual lesions. Further complicating the situation are questions of DNA sequence context and its effect in determining which lesions will be mutagenic (Miller, 1983). Because of these obstacles, it is convenient to have a simple model system in which to evaluate the effect of adduct structure on the process of mutagenesis. The model system described here, which involves incorporation of an adducted tetramer into a duplex genome containing a four-base gap at the *Pst*I site, is particularly suitable for the study of guanine lesions. 4-Aminobiphenyl, an aromatic amine known as an occupational bladder carcinogen and presently encountered daily by humans due to cigarette smoking and other sources (Patrianakos & Hoffmann, 1974; Bryant et al., 1986), was shown in previous work to produce two guanine adducts in vivo and was apparently mutagenic at G-C base pairs, as detected in the *Salmonella his* reversion assay (McCann et al., 1975; Scribner et al., 1979; El-Bayoumy et al., 1981; Beland et al., 1983; Pai et al., 1985); Rosenkranz et al., 1985). Furthermore, in work reported in the next paper in this series, we have shown that G-C base pairs are the principal sites at which base pair substitution mutations arise in *E. coli* (Lasko et al., unpublished results). Accordingly, the dG$^{8\text{-ABP}}$ lesion provided a genetically relevant, although admittedly challenging, model for extending the study of site-specific mutagenesis beyond the case of the structurally simpler lesion O^{6}MeGua, as studied previously by us (Loechler et al., 1984) and others (Chambers et al., 1985; Hill-Perkins & Karran, 1986; Banhot & Ray, 1986).

In contrast to our synthetic strategy for an O^{6}MeGua-containing tetranucleotide, which involved incorporation of a protected adduct during stepwise oligonucleotide syntheis, we chose to react the deprotected, purified oligonucleotide d-(TpGpCpA) with the highly reactive *N*-OAc-TFAABP in order to generate d(TpG$^{8\text{-ABP}}$pCpA). The advantages of the former approach (total synthesis) include a greater degree of flexibility with regard to location of the adduct within the oligomer. The potential disadvantage is that certain adducts might be labile under conventional oligonucleotide deprotection conditions, which may need to be modified according to the stability profile of the adduct in question (Kuzmich et al., 1983). The latter approach has been used successfully by others to produce oligonucleotides containing *N*-acetyl-2-aminofluorene or 2-aminofluorene lesions (Sharma & Box, 1985; Johnson et al., 1986). In one of these studies (Johnson et al., 1986), the C8-substituted deoxyguanosine adduct of

2-aminofluorene, which differs from ABP by the lack of the methylene bridge carbon and by the planarity of the aromatic rings, was formed in a heptadeoxynucleotide, which in turn was inserted into the genome of M13mp9. This study did not include, however, detailed structural information on the synthetic oligonucleotide product. In this work we demonstrate that the electrophilic ABP derivative *N*-OAc-TFAABP provided a high yield of the $dG^{8\text{-}ABP}$ reaction product, making available micromole quantities of site-specifically modified tetranucleotide for full characterization and for forthcoming biological studies.

It is significant that, even in an oligonucleotide as short as a tetramer, the presence of the ABP lesion did not distort DNA conformation to the extent that ligation was precluded; indeed, ligation occurred with half the efficiency of the unmodified d(pTpGpCpA). The observation of efficient ligation is for the most part in accord with the conclusions of Broyde et al. (1985), who used minimized semiempirical potential energy calculations to predict that the ABP residue of this adduct resides in the major groove in a way that does not result in a major disturbance of DNA structure. In this study, however, our ^1H NMR studies of $d(TpG^{8\text{-}ABP}pCpA)$ revealed a significant degree of stacking of the ABP ring system that may be concomitant with a reduction in the fraction of cytosine stacking, as indicated by the appreciable upfield and downfield chemical shifts, repectively, of their ring protons. This can be rationalized by a model in which there is stacking of the ABP ring with the adenine and thymine bases, which is supported by the small upfield shifts of the adenine and thymine resonances in the adducted tetranucleotide. The extent of ABP interaction with the cytosine ring in the modified tetranucleotide is unclear. Shapiro et al. (1986) have concluded that significant stacking does occur with the ABP adduct of d(CpG), although it is noteworthy that this adduct has the opposite local sequence as compared to $d(TpG^{8\text{-}ABP}pCpA)$. More detailed studies on the conformation of this adducted tetranucleotide are in progress in our laboratory. We add, however, that unusually large upfield shifts have been observed for the cytosine protons of the C8-substituted guanine derivative of 2-(acetylamino)fluorene in d(CpG) (Evans and Levine, submitted for publication), which, when compared to the present data and those of Shapiro et al. (1986), clearly indicate substantial differences in the mode and extent of stacking between the ABP and 2-(acetylamino)fluorene adducts.

Additional experiments established that adduct structure was likely to have remained intact through the ligation reaction and that ligation of $d(pTpG^{8\text{-}ABP}pCpA)$ had occurred both on the 5′ and the 3′ ends. Although we did observe some labeled nicked DNA (Figure 4), which could have been due to failure to ligate at either end of the tetranucleotide, our data from analysis of these products on denaturing gels (Figure 6) ruled out this possibility. We suspect that the principal source of the relaxed circular DNA evident in Figure 4 was random nicking of the genome. Such events are common during the storage of supercoiled genomes.

The $dG^{8\text{-}ABP}$ adduct conferred resistance of the genome to *Pst*I cleavage, despite complete cleavage of internal standard labeled DNA. We did note, however, that a small amount of the radioactively labeled genome was sensitive to *Pst*I. This may have been due to the possibility that cleavage was merely slowed rather than absolutely blocked. The kinetics of the cleavage reaction were not extensively investigated with M13mp10-ABP_1, but we have observed that *Pst*I seems to be particularly sensitive to modifications within its recognition sequence since lesions with structures as disparate as O^6MeGua and $dG^{8\text{-}ABP}$ inhibit cleavage. It contrast, recently we have observed that genomes containing, within *other* restriction endonuclease recognition sites, thymine glycol, 1,N^6-ethenoadenine, and even O^6MeGua *can* be cleaved by the appropriate restriction endonuclease, albeit at a lower rate than the corresponding unmodified sequence (A. Basu, E. Dogliotti, K. Ellison, and J. Essigmann, unpublished observations).

In related work, a collection of 20 mutants induced by ABP lesions in the *lacZ* gene fragment of M13mp10 was isolated, and the specific DNA sequence changes were determined. Base pair substitutions occurring at G-C residues constituted the major class of mutations identified. Taken together with previous work in *S. typhimurium his* tester strains, these results provide a rationale for the further investigation of the $dG^{8\text{-}ABP}$ lesion. In parallel, the mutagenic properties of the $dG^{8\text{-}ADP}$ adduct in the singly adducted genome, M13mp10-ABP, were evaluated. The strategy for these mutagenesis experiments exploited the location of the lesion at a unique restriction site as a means for isolation of mutant genomes. Base pair substitution mutagenesis was investigated by transfecting adducted and control DNAs into *E. coli* induced for SOS mutagenic processing and determining the frequency of phage with *Pst*I-resistant RF DNA among the progeny. A specific base pair substitution, the G-C to T-A transversion at position 6270, could be assessed without selection by plating phage on an opal suppressor strain (NR8044); this sequence alteration caused a TGC (Cys) to TGA (opal stop) change in the M13mp10 *lacZ* α fragment. These results will be reported in full in an accompanying paper (D. D. Lasko, S. H. Harvey, F. F. Kadlubar, and J. M. Essigmann, unpublished results).

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Registry No. d(TpGpCpA), 80692-82-6; *N*-OAc-TFAABP, 78281-05-7; 5′-$d(pG^{8\text{-}ABP}p)$-3′, 107556-01-4; 5′-$d(TpG^{8\text{-}ABP}pCpA)$-3′, 107541-11-7; deoxyguanosine 3′,5′-bisphosphate, 16174-59-7; 4-aminobiphenyl, 92-67-1.

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Isolation and Characterization of a cDNA Encoding Rat Cationic Trypsinogen†

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ABSTRACT: A cDNA encoding rat cationic trypsinogen has been isolated by immunoscreening from a rat pancreas cDNA library. The protein encoded by this cDNA is highly basic and contains all of the structural features observed in trypsinogens. The amino acid sequence of rat cationic trypsinogen is 75% and 77% homologous to the two anionic rat trypsinogens. The homology of rat cationic trypsinogen to these anionic trypsinogens is lower than its homology to other mammalian cationic trypsinogens, suggesting that anionic and cationic trypsins probably diverged prior to the divergence of rodents and ungulates. The most unusual feature of this trypsinogen is the presence of an activation peptide containing five aspartic acid residues, in contrast to all other reported trypsinogen activation peptides which contain four acidic amino acid residues. Comparisons of cationic and anionic trypsins reveal that the majority of the charge changes occur in the C-terminal portion of the protein, which forms the substrate binding site. Several regions of conserved charge differences between cationic and anionic trypsins have been identified in this region, which may influence the rate of hydrolysis of protein substrates.

The trypsins (EC 3.4.21.4) are important members of a large family of pancreatic serine proteases that share a common catalytic mechanism and posess similar tertiary structures. These enzymes are endopeptidases which are synthesized as proenzymes by pancreatic acinar cells and secreted into the gut. The trypsins are distinguished from the other pancreatic serine proteases both by their specificity for arginine or lysine residues and by their capability to activate the other pancreatic zymogens.

Although multiple ionic forms of trypsin have been isolated from humans (Travis & Roberts, 1969; Mallory & Travis, 1973), cows (Louvard & Puigserver, 1974), rats (Brodrick et

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