Deoxyhexanucleotide Containing a Vinyl Chloride Induced DNA Lesion, 1,N^6-Ethenoadenine: Synthesis, Physical Characterization, and Incorporation into a Duplex Bacteriophage M13 Genome as Part of an *amber* Codon†

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ABSTRACT: Organic synthesis and recombinant DNA techniques have been used to situate a single 1,N^6-ethenoadenine (ϵAde) DNA adduct at an *amber* codon in the genome of an M13mp19 phage derivative. The deoxyhexanucleotide d[GCT(ϵA)GC] was chemically synthesized by the phosphotriester method. Mild nonaqueous conditions were employed for deprotection because of the unstable nature of the ϵAde adduct in aqueous basic milieu. Physical studies involving fluorescence, circular dichroism, and ^{1}H NMR indicated ϵAde to be very efficiently stacked in the hexamer, especially with the 5′-thymine. Melting profile and circular dichroism studies provided evidence of the loss of base-pairing capabilities attendant with formation of the etheno ring. The modified hexanucleotide was incorporated into a six-base gap formed in the genome of an M13mp19 insertion mutant; the latter was constructed by blunt-end ligation of d(GCTAGC) in the center of the unique *Sma*I site of M13mp19. Phage of the insertion mutant, M13mp19-*Nhe*I, produced light blue plaques on *SupE* strains because of the introduced *amber* codon. Formation of a hybrid between the single-strand DNA (plus strand) of M13mp19-*Nhe*I with *Sma*I-linearized M13mp19 replicative form produced a heteroduplex with a six-base gap in the minus strand. The modified hexamer [5′-^{32}P]d-[GCT(ϵA)GC], after 5′-phosphorylation, was ligated into this gap by using bacteriophage T4 DNA ligase to generate a singly adducted genome with ϵAde at minus strand position 6274. Introduction of the radiolabel provided a useful marker for characterization of the singly adducted genome, and indeed the label appeared in the anticipated fragments when digested by several restriction endonucleases. Evidence that ligation occurred on both 5′ and 3′ sides of the oligonucleotide also was obtained. The adduct was introduced into a unique *Nhe*I site, and it was observed that this restriction endonuclease was able to cleave the adducted genome, albeit at a lower rate compared to unmodified DNA. The M13mp19-*Nhe*I genome containing ϵAde will be used as a probe for studying mutagenesis and repair of this DNA adduct in *Escherichia coli*.

Vinyl chloride is a widely used industrial chemical and a known human carcinogen (Singer & Grunberger, 1983; IARC Monographs, 1979; Vainio et al., 1985). It is metabolized by microsomal cytochrome P-450 dependent monooxygenases to chloroethylene oxide, which rearranges nonenzymatically to chloroacetaldehyde (Guengerich et al., 1979). Other common chemicals such as chloroethanol and dichloroethane also are suspected to generate chloroacetaldehyde through metabolic activation (McCann et al., 1975). Epidemiologic studies have implicated vinyl chloride as a carcinogen in human populations exposed through industrial contact (Fishbein, 1979), and chloroethylene oxide has been shown to initiate skin tumors in mice (Zajdela et al., 1980). Chloroacetaldehyde was inactive when tested for carcinogenic activity in mice (Van Duuren et al., 1979), although it was shown to be mutagenic in *Salmonella typhimurium* as well as in chinese hamster V79 cells (McCann et al., 1975; Huberman et al., 1975).

Chloroacetaldehyde reacts with adenine and cytosine, and at a lower rate with guanine, to form a group of adducts with an additional five-membered ring system; the initial major product is an hydroxyethane derivative, which slowly dehydrates at neutral pH to form a planar aromatic ring system (Kochetkov et al., 1971; Barrio et al., 1972; Sattsangi et al., 1977; Kusmierek & Singer, 1982). These so-called etheno derivatives are fluorescent and are useful probes for certain biochemical reactions (Leonard, 1984). Chloroethylene oxide also forms etheno adducts; in this case, however, it is not known whether all of the etheno adduct formation involves direct reaction of DNA with chloroethylene oxide or with chloroacetaldehyde, its primary rearrangement product (O'Neill et al., 1986). The major DNA modification by chloroethylene oxide was found to be 7-(2-oxoethyl)guanine (Scherer et al., 1981).

A number of the chloroacetaldehyde/chloroethylene oxide–DNA adducts have been tested for their ability to induce errors during either DNA or RNA synthesis in vitro. The 7-(2-oxoethyl)guanine adduct lacks miscoding properties during in vitro replication (Barbin et al., 1985). In contrast, 1,N^6-ethenoadenine (ϵAde)[1] was found to be a miscoding lesion

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[1] Abbreviations: ϵdAdo, 1,N^6-etheno-2′-deoxyadenosine or 3-(2-deoxy-β-D-*erythro*-pentofuranosyl)imidazo[2,1-*i*]purine; ϵAde, the corresponding base, 1,N^6-ethenoadenine; ϵCyt, 3,N^4-ethenocytosine; MSNT, 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole; DMT, 4,4′-dimethoxytrityl; DBU, 1,8-diazabicyclo[5.4.0]undec-7-ene; CPG, controlled pore glass; CD, circular dichroism; bp, base pair; b, base; ds, double stranded; RF, replicative form; GHD, gapped-heteroduplex DNA; M13mp19-*Nhe*I, insertion mutant of M13mp19 containing d(GCTAGC) in the center of a *Sma*I site; M13mp19-*Nhe*I(ϵA), duplex genome of M13mp19-*Nhe*I in which the adenine at positon 6274 in the minus strand has been replaced with ϵAde; IPTG, isopropyl β-D-thiogalactopyranoside; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; DMSO, dimethyl sulfoxide; THF, tetrahydrofuran; EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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during in vitro transcription by DNA-dependent RNA polymerase, where it was found to direct misinsertion of A > U > C (Spengler & Singer, 1981). The early literature also suggested that this adduct was significantly mutagenic during in vitro replication by *Escherichia coli* DNA polymerase I, which incorporated dGMP (Barbin et al., 1981). However, more recent studies indicate that the in vitro mutagenic potential of ϵAde is actually much lower than originally believed (Singer et al., 1984). 3,N^4-Ethenocytosine (ϵCyt), in contrast, clearly has been demonstrated to induce errors at a high frequency during both in vitro replication and transcription. During in vitro transcription in the presence of Mn^{2+}, Spengler and Singer showed that ϵCyt in RNA copolymers with unmodified nucleosides directed misincorporation of U ≥ A ≫ C (Spengler & Singer, 1981; Singer & Spengler, 1986). Using chloroacetaldehyde or chloroethylene oxide treated poly(dC) templates, Barbin et al. (1981) demonstrated that *E. coli* DNA polymerase I incorporated dTMP at a significant frequency. Using this polymerase to copy a d(C,ϵC) template, Singer and Spengler (1986) also observed a high rate of misincorporation of this nucleotide. In further work, the hydrated form of ϵCyt was not detected to be a miscoding lesion during replication, although it was capable of inducing errors during transcription (Singer & Spengler, 1986).

Only in one instance, and after prolonged vinyl chloride administration, has ϵAde been detected in mammalian tissues (Green & Hathaway, 1978). Moreover, no experimental evidence has been reported that would implicate ϵAde in mutagenesis in vivo. As a first step toward evaluating the possible genetic relevancy of this DNA adduct in vivo, in the work described here we have synthesized and characterized a short piece of DNA containing a single ϵAde adduct. Also described is the development of an insertion clone of *E. coli* phage M13mp19 containing an *amber* codon in the *lacZ* α-fragment. In the presence of a β-galactosidase indicator dye, this phage produces a colorless-plaque phenotype in *Su*⁻ strains of *E. coli*. Finally, the singly adducted oligonucleotide was incorporated into this M13 derivative so that a duplex genome was created with a single ϵAde at the *amber* codon. This genome can be used as a probe to investigate the genetic fate of ϵAde in *E. coli*.

Experimental Procedures

Materials. Protected monomers and support for oligonucleotide synthesis were purchased from Cruachem, Inc., as were 1-(mesitylenesulfonyl)-3-nitro-1,2,4-triazole (MSNT), 1-methylimidazole, *syn*-2-nitrobenzaldoxime, 1,2,4-triazole, 4,4′-dimethoxytrityl chloride, and 2-chlorophenyl phosphorodichloridate. 1,8-Diazabicyclo[5.4.0]undec-7-ene (DBU), 4-(dimethylamino)pyridine, and phenyl isocyanate were products of Aldrich Chemical Co. Chloroacetaldehyde was obtained from Fluka. Nucleosides, snake venom phosphodiesterase (*Crotalus adamenteus*), and Sepharose CL-4B were obtained from Sigma Chemical Co. Restriction endonucleases, bacterial alkaline phosphatase, bacteriophage T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. [γ-^{32}P]ATP was either from Amersham or from New England Nuclear. Solvents and other reagents of highest purity were purchased from standard suppliers. Replicative form (RF) M13mp19 DNA and the tetramer d(GCGC) were from Pharmacia. *E. coli* MM294A, GW5100, and CSH50 were obtained from K. Backman (Biotechnia, International, Cambridge, MA), G. Walker (Biology Department, MIT, Cambridge, MA), and T. Kunkel (National Institute of Environmental Health Sciences, Research Triangle Park, NC), respectively.

General Methods. DNA synthesis was done on a Cruachem manual module. Absorption spectra were recorded on a Hitachi 557 spectrophotometer. ^{1}H NMR was performed on a Bruker Model NML500 (500-MHz) spectrometer. Fluorescence spectra were obtained from a Farrand MK1 spectrofluorometer. Circular dichroism studies were done on a Jasco Model J-500 spectropolarimeter. HPLC was performed on a Beckman Model 322 gradient liquid chromatograph equipped with two 100-A pumps, a 420 microprocessor system controller, and a Waters 440 UV absorbance detector operated at 254 nm. Anion-exchange HPLC was carried out with a Whatman Partisil SAX column (10-μm particle size; 4.6-mm i.d. × 25 cm, column dimensions) eluted with a linear gradient of 1–500 mM aqueous potassium phosphate buffer (pH 5.3). Nucleoside composition analysis and the stability studies on ϵdAdo and the oligonucleotides were conducted by reversed-phase HPLC with a Macherey-Nagel C_{18} Nucleosil column (10 μm) or a Beckman C_{18} Ultrasphere ODS column (5 μm) by using a gradient of 0–50% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.8).

ϵdAdo was synthesized according to the literature procedure and was identified by its fluorescence, UV, and NMR spectra (Secrist et al., 1972). This material was chromatographically and spectroscopically identical with ϵdAdo supplied by Sigma.

Restriction enzyme digestions, gel electrophoresis, and autoradiography were done essentially as described by Lasko et al. (1987). Denaturation of DNA fragments was done by heating at 100 °C for 3 min followed by rapid cooling to 0 °C. To prevent renaturation, 25% (v/v) formamide was added prior to heating and gel electrophoresis was performed at 4 °C.

5′-O-(Dimethoxytrityl)-3-(2-deoxy-β-D-erythro-pentofuranosyl)imidazo[2,1-i]purine (5′-DMT-ϵdAdo). ϵdAdo (726.5 μmol, 200 mg) was dried by evaporation with dry pyridine and was resuspended in pyridine (8 mL). 4,4′-Dimethoxytrityl chloride (1.2 equiv, 298 mg) and 1.4 equiv (138 μL) of triethylamine were added, followed by 0.05 equiv (5 mg) of 4-(dimethylamino)pyridine. The mixture was stirred vigorously in the dark for approximately 2 h. TLC monitoring (silica gel, 5% methanol in methylene chloride) indicated almost complete disappearance of the ϵdAdo and appearance of a nonpolar trityl-positive fluorescent spot. An equal volume of water was added, and the product was extracted with diethyl ether. The ether layer was evaporated and purified through a silica gel column. The yield was 654 mg (90%): UV λ_{max} (ethanol) 263 nm (ϵ 8300), 272 (ϵ 8600), 280 (sh) (ϵ 5600), and 296 (ϵ 3400); ^{1}H NMR (DMSO-d_6) δ 9.22 (s, 1, H_5), 8.44 (s, 1, H_2), 8.09 (s, 1, H_7), 7.57 (s, 1, H_8), 7.33–6.74 (m, 13, Ar), 6.52 ("t", 1, H_1'), 5.44 (m, 1, H_3'), 4.51 (m, 1, H_4'), 4.04 (m, 1, 3′-OH), 3.69 (s, 6, $2CH_3O$–), 3.36 (m, 2, $H_{5',5''}$), 3.24 (m, 2, $H_{2',2''}$).

5′-O-(Dimethoxytrityl)-3-(2-deoxy-β-D-erythro-pentofuranosyl)imidazo[2,1-i]purine 3′-O-(2-Chlorophenyl phosphate) Triethylammonium Salt [5′-DMT-ϵdAdo-3′-ClPh-(P)-TEA]. A mixture of 1,2,4-triazole (138 mg, 2 mmol), 2-chlorophenyl phosphorodichloridate (150 μL, 0.75 mmol), redistilled triethylamine (278 μL, 2 mmol), and freshly distilled dry THF (5 mL) was stirred for 30 min at room temperature in a flask equipped with a drying tube. The mixture was rapidly filtered and added to the 5′-protected ϵdAdo (654 μmol, 377 mg). The solution was stirred for 1 h under argon. Silica gel TLC (5% ethanol in methylene chloride) indicated the reaction to be complete with a trityl-positive fluorescent spot near the base line. The reaction was terminated by the addition of 1 M triethylammonium bicarbonate (5 mL, pH

8.0), and the product was extracted with methylene chloride. The residue obtained by evaporation of the organic solvent was purified through a short silica gel column (10% ethanol and 1% triethylamine in methylene chloride). The purified protected monomer was dried twice by evaporation in vacuo with pyridine and stored in a desiccator at −20 °C. The yield was 395 mg (62%).

General Procedure for Oligonucleotide Synthesis. After the poly(dimethylacrylamide)–Kieselguhr support (100 mg, 7.5 μmol) was dried for 10 min in a small glass column with 1 mL of 10% phenyl isocyanate in pyridine, the protocol for deprotection and washing was followed in sequence: pyridine (3 min), 1,2-dichloroethane (2 min), 3% dichloroacetic acid in dichloroethane (3.5 min), 1,2-dichloroethane (1 min), and pyridine (4 min). The solvent flow rate was approximately 2.5 mL/min. During the 4-min pyridine wash, 75 μmol of the monomer and 90 mg of MSNT were dissolved in 500 μL of anhydrous pyridine, to which was added 50 μL of 1-methylimidazole. After the pyridine wash, the solvent flow was stopped and this activated monomer was injected onto the column over a period of 5 min; then the monomer–support mixture was allowed to stand for another 10 min, following which the wash cycle was resumed. After addition of the last nucleotide, the resin was washed in the column with pyridine and dichloroethane and finally with diethyl ether in a small sintered funnel. Ten percent of the resin-bound oligonucleotide was deprotected by the procedure of Sproat and Gait (1984). The remainder of the oligomer was deprotected by the modified nonaqueous condition developed by Kuzmich et al. (1983). Oligonucleotides were purified by HPLC using a Partisil SAX column and desalted on a C_{18} Sep-Pak cartridge (Waters).

Enzymatic digestion of the oligonucleotides was done as previously described (Fowler et al., 1982). Analysis of nucleoside compositions was performed by HPLC (Utrasphere ODS column, 1 mL/min, 0–25% acetonitrile in 0.1 M ammonium acetate buffer, pH 5.8).

NMR Spectroscopy. For proton magnetic resonance studies, the oligonucleotides were passed through a Chelex (Bio-Rad) column and dried with EDTA (final concentration, 0.5 mM) and tetramethylammonium chloride. The samples were prepared in D_2O (~50 A_{260} units/mL) after an additional drying in the same solvent. The methyl resonance of tetramethylammonium chloride at 3.18 ppm served as an internal standard. Presaturation of the HOD resonance was carried out and NMR data were collected at different temperatures until optimal resolution was achieved.

Construction of M13mp19 Duplex DNA with a Hexanucleotide Insertion in the SmaI Site. One microgram of M13mp19 RF DNA was linearized with 10 units of *Sma*I in 20 mM KCl, 6 mM Tris-HCl (pH 8.0), 6 mM $MgCl_2$, and 6 mM 2-mercaptoethanol at 25 °C for 1 h. The enzyme was heat-inactivated at 60 °C for 10 min, after which the DNA was precipitated with ethanol in presence of polyacrylamide. The linear DNA was mixed with approximately 2–3 μg of nonphosphorylated hexanucleotide, d(5′-$_{HO}$GCTAGC$_{OH}$-3′) and 200 units of T4 DNA ligase in 40 mM Tris-HCl buffer (pH 7.8), 8 mM $MgCl_2$, 16 mM dithiothreitol, and 1 mM ATP in a total volume of 20 μL. The ligation mixture was incubated at 16 °C for 18 h. Electrophoretic analysis of this material in the presence of ethidium bromide on a 0.8% low melting point agarose gel revealed three DNA forms: a large amount of linear-duplex DNA (form III), a smaller amount of relaxed circular duplex DNA (form II), and a trace of supercoiled duplex DNA (form I_0). The linear-duplex DNA band was cut from the gel, melted by heating at 70 °C for 3 min, and then separated from the agarose by forming a hexadecyltrimethylammonium–DNA ion pair, which was extracted to the alcohol layer in a water–butanol biphasic system, leaving the agarose in the aqueous phase (Langridge et al., 1980). The DNA was recovered after dissociating the complex by increasing the salt concentration and precipitated with ethanol.

The linear M13 DNA was 5′-phosphorylated by incubation at 37 °C with 10 units of polynucleotide kinase and 1 nmol of ATP in 50 mM Tris-HCl buffer (pH 7.8), 10 mM $MgCl_2$, and 20 mM dithiothreitol, in a total volume of 10 μL. After 45 min, the enzyme was inactivated by heating at 65 °C for 10 min. The linear DNA was allowed to circularize overnight at 16 °C in presence of 400 units of T4 DNA ligase and 1 mM ATP in the ligation buffer described above. The DNA was precipitated and redissolved in 10 μL of H_2O and subsequently treated with 10 units of *Sma*I and appropriate salts at 25 °C for 1 h to linearize any DNA that escaped the blunt-end ligation.

The modified form I_0 DNA was used to transfect competent *E. coli* MM294A cells in 30 mM $CaCl_2$ and 10 mM Tris-HCl (pH 8.0) by incubating for 60 min at 0 °C, followed by a heat shock at 42 °C for 1.5 min. After chilling to 0 °C, 1 mL of Luria broth (LB) medium was added and the mixture was incubated at 37 °C for 2.5 h. The cells were pelleted by centrifugation at 15600*g* for 10 min. The supernatant (phage stock) was serially diluted and plated with *E. coli* GW5100 cells in the presence of IPTG and X-Gal.

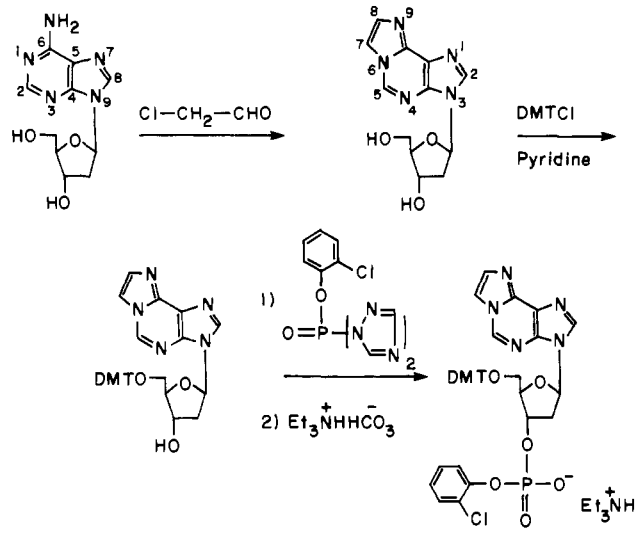
As a control, a portion of M13mp19 DNA was taken through all of the above steps with the exception of the addition of oligonucleotide. As expected, this material did not generate any phage, whereas samples in which the hexanucleotide was included produced a large number of bacteriophage displaying light blue plaques on GW5100 plates.[2] A single light blue plaque was selected, and RF DNA was isolated. After characterization by restriction endonuclease digestion, this DNA was further used to transfect and produce single-strand (ss) DNA (Messing, 1983) as well as more RF (Maniatis et al., 1982).

The circular ss DNA, thus produced, was hybridized with 10% (w/w) *Sma*I linearlized M13mp19 RF to form a heteroduplex with a six-nucleotide gap specifically in the minus strand. Electrophoretic analysis indicated a major band of gapped-heteroduplex DNA (GHD) possessing electrophoretic mobility indistinguishable from open-circular duplex. A trace amount of the renatured linear duplex DNA and a large excess of ss DNA were also present. The ss DNA was removed on a hydroxylapatite column (~2 g) in 0.14 M potassium phosphate buffer (pH 7.0) preequilibrated with the same buffer; finally, the GHD was eluted with 0.4 M potassium phosphate buffer (pH 7.0). The pooled GHD fractions were dialyzed against 10 mM Tris-HCl buffer (pH 8.0) and 1 mM EDTA and precipated with ethanol.

Phosphorylation of Deoxyhexanucleotides and Ligation into GHD. The hexanucleotides d[GCT(εA)GC] and d-(GCTAGC) (100 ng of each) were phosphorylated with [γ-^{32}P]ATP by using T4 polynucleotide kinase, and each product was ligated into the GHD according to Lasko et al. (1987). Each ligation mixture included 1.5 μg of GHD and 0.1 μg of hexanucleotide, in a total volume of 50 μL. After 16 h of incubation at 16 °C, the crude ligation mixtures were drop-

[2] Approximately 2–5% dark blue plaques also appeared. Analysis indicated these plaques to be the wild-type M13mp19.

Scheme I



dialyzed at room temperature for approximately 2 h; this step eliminated >95% of the radioactivity from unincorporated [^{32}P]ATP. Final purification of the ligation products was carried out on a Sepharose 4B column (15 × 0.75 cm) pre-equilibrated with 10 mM Tris-HCl buffer (pH 7.8), 1 mM EDTA, and 0.1 M NaCl. The ligation product eluted in the excluded volume.

The specific activities of the 5′-phosphorylated oligonucleotides were determined by analyzing a small portion of the polynucleotide kinase reaction mixtures by HPLC and associating the amount of radioactivity in the phosphorylation products (which eluted 2–3 min earlier than their unphosphorylated precursors) with their absorbance at 260 nm. The ligation efficiency was determined by relating the incorporation of radioactivity into the M13 DNA to the amount of GHD used.

Results

Synthesis of a Protected Deoxyhexanucleotide Containing ϵAde. Oligonucleotide synthesis was performed with a manual DNA synthesis module using a solid-phase phosphotriester method. The suitably protected ϵdAdo monomer necessary for the solid-phase protocol was unavailable commercially, so it was synthesized according to Scheme I. Although long-chain alkylamine controlled pore glass (CPG) is generally considered the support of choice for preparation of long oligonucleotides (Atkinson & Smith, 1984), we experienced unacceptable variation in the quality of the high-loading CPG available commercially. For relatively large-scale synthesis of short oligonucleotides, we successfully used a poly(dimethylacrylamide)–Kieselguhr support. We also have noticed that the acid wash by 10% trichloroacetic acid in dichloroethane can be replaced by 3% dichloroacetic acid in dichloroethane which, in principle, should cause less depurination; the use of dimelthylformamide also can be omitted.

Using this altered protocol, we synthesized the protected modified hexamer d[GCT(ϵA)GC]. For the sake of comparison, the unmodified counterpart d(GCTAGC) was also synthesized with the same batch and quantity of support, monomers, and solvents. Trityl analysis indicated excellent coupling efficiency (average ~98%) for both oligomers.

Deprotection of the Protected Deoxyhexanucleotide Containing ϵAde. ϵAde has been reported to be unstable under alkaline conditions (Secrist et al., 1972). Accordingly, we performed a series of studies on the stability of ϵdAdo with the conventional deprotecting reagents used for oligonucleotide synthesis. This modified base was found to be unstable both to oximate in dioxane/water and in aqueous NH_3, the standard deblocking agents. While overnight treatment with 2-nitrobenzaldoximate of 1,1,3,3-tetramethylguanidine in 50% aqueous dioxane decomposed >70% of this adduct, only 1–2% ϵdAdo could be recovered after NH_4OH treatment at 50 °C for 16 h.

The product generated by aqueous alkali has been reported to be a biimidazole derivative (Yip & Tsou, 1973). A nucleophilic attack at C5 of ϵAde followed by ring opening and subsequent deformylation would form this compound (Tsou et al., 1974). Experiments in our laboratory revealed, however, that a large proportion of this intermediate, which exhibits a λ_{max} of 269 nm, has the ability to regenerate the etheno derivative upon careful neutralization or isolation. This indicates that under mild conditions deformylation of the base-induced intermediate does not take place.

We also found that ϵdAdo was stable to some organic bases under nonaqueous conditions. Although 1,1,3,3-tetramethylguanidine slowly degraded ϵdAdo to a more polar material even in anhydrous dioxane, it was quite stable to triethylamine and DBU in dioxane, THF, or methanol. As a result, the deprotection protocol used by Kuzmich et al. (1983) (16-h treatment of 2-nitrobenzaldoxime in DBU followed by 3-day incubation with methanol in DBU at room temperature) was found to be particularly useful. Only a total of 4–5% decomposition of ϵdAdo was observed after exposure to this deblocking protocol. Because of these observations, the oligonucleotides were deblocked by the nonaqueous conditions of oximate, DBU, and methanol. Both the modified and unmodified hexamers were cleanly deprotected by this protocol. After purification by Partisil SAX chromatography, the overall yield of the modified hexamer was 50%, in comparison to 56% for the unmodified oligonucleotide. (It is noteworthy that a small portion of the protected ϵdAdo-containing hexamer was also treated with the conventional deblocking agents and no hexanucleotide peak could be detected after the reaction.)

Figure 1A shows the anion-exchange elution profile of the crude deblocked d[GCT(ϵA)GC]; the collected major peak (24 min) exhibited a single peak by reversed-phase HPLC (Figure 1B). It was retained slightly longer than the unmodified hexamer d(GCTAGC). Small portions of the purified hexamers were digested with snake venom phosphodiesterase and bacterial alkaline phosphatase, and the nucleoside compositions were analyzed by reversed-phase HPLC (Figure 2) (Fowler et al., 1982). The identity of each peak was further confirmed by its absorption spectrum.

Stability of d[GCT(ϵA)GC]. Both modified and unmodified hexamers (~0.2 A_{260} unit) were incubated at pH 2.0, 3.5, 13, and 14 in a 100-μL aqueous solution for 4 h at 37 °C, and their stability over time was analyzed by HPLC. Each showed minimal degradation at pH 3.5, in that >97% of the starting material was unchanged after 4 h, as seen by HPLC analysis. At pH 2.0, however, a substantial portion (>8%) of both oligonucleotides was found to have depurinated, releasing Gua, Ade, or ϵAde. No difference in the rate of release of Ade or ϵAde was observed. These data suggest that at low-pH conditions ϵdAdo in DNA probably possesses a glycosidic bond of comparable stability to that of dAdo. In contrast, the ϵdAdo-containing hexamer was unstable to both of the alkaline pH conditions tested, whereas the unmodified hexamer remained unaffected. A late eluting peak gradually developed as the d[GCT(ϵA)GC] peak was reduced in size in a time-dependent manner. At pH 14 total conversion to the breakdown product was complete in 90 min, whereas at pH 13 the

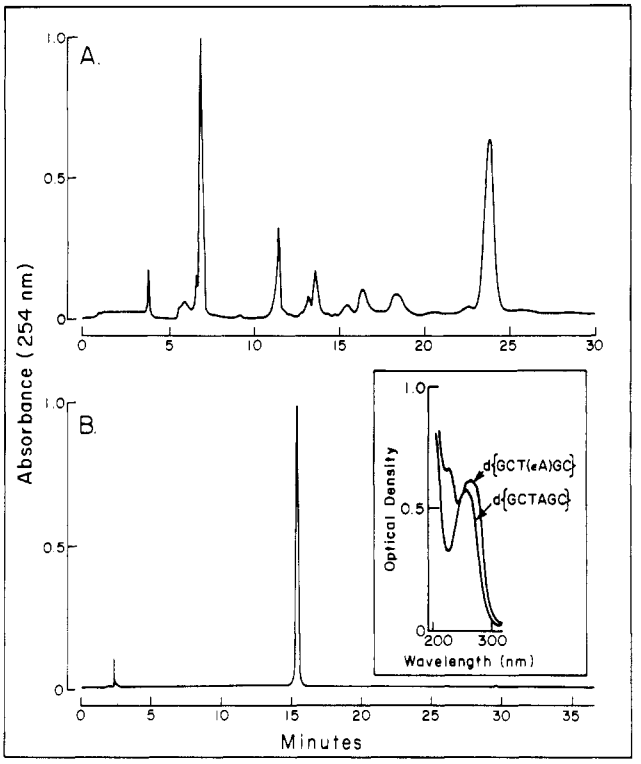


FIGURE 1: HPLC profiles of crude and purified d[GCT(εA)GC] and comparison of the absorption spectrum of the latter with the corresponding unmodified hexanucleotide. Frame A displays the preparative HPLC profile of the deblocked crude d[GCT(εA)GC] on a Whatman Partisil SAX column. Chromatographic conditions: linear gradient of 1–500 mM KH_2PO_4 buffer (pH 5.3) over a period of 30 min at a flow rate of 1.5 mL/min. The peak at 24 min was collected. (The large peak at approximately 7 min was generated from the deblocking reagent 2-nitrobenzaldoxime in DBU.) Frame B shows the analytical reversed-phase HPLC profile of the collected peak from the anion-exchange column on a Macherey-Nagel Nucleosil C_{18} column. Chromatographic conditions: 0–50% acetonitrile in aqueous 0.1 M ammonium acetate buffer (pH 5.8) over a period of 30 min with a flow rate of 1.5 mL/min. The inset in frame B compares the absorption spectrum of the purified εAde-containing hexanucleotide (15-min peak) with that of the unmodified hexanucleotide.

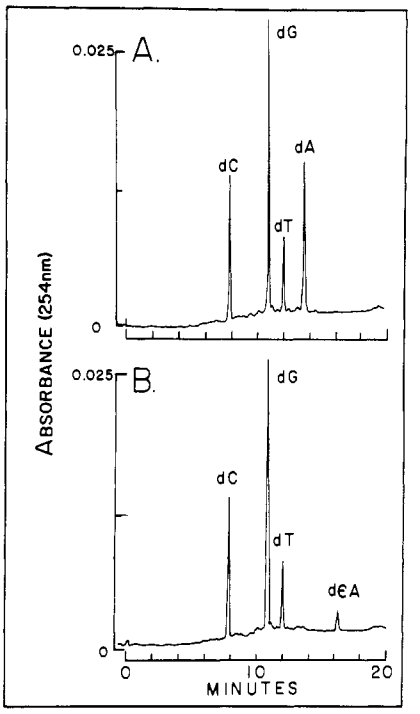


FIGURE 2: Reversed-phase HPLC profiles of the snake venom phosphodiesterase and bacterial alkaline phosphatase digestion products from (A) d(GCTAGC) and (B) d[GCT(εA)GC]. Chromatographic conditions: 0–25% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.8) over a period of 30 min with a flow rate of 1 mL/min. The column used was Ultrasphere ODS (C_{18}; 5 μm; Beckman). In both profiles, the relative proportions of the nucleosides were consistent with the calculated peak heights at 254 nm.

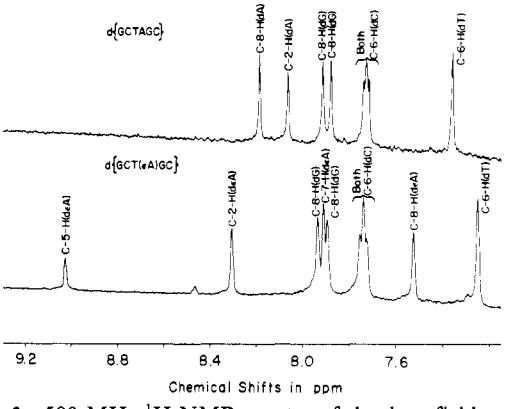


FIGURE 3: 500-MHz ^1H NMR spectra of the downfield region of d(GCTAGC) and d[GCT(εA)GC] at 60 °C.

half-time for degradation was approximately 1 h. When reinjected into the HPLC, the new alkali-induced peak was observed to have partially equilibrated to a product chromatographically identical with the εAde oligomer. In further support of our above-mentioned studies on the alkaline hydrolysis of εdAdo, these results also indicate the interconvertibility of the biimidazole or related compounds and εdAdo depending on the pH of the medium. The εAde-containing hexamer, however, was found to be reasonably stable at neutral pH. Even when the temperature of the medium was raised to 70 °C, the hexamer was virtually unaffected after 2 h. In addition, d[GCT(εA)GC] is stable in polynucleotide kinase, DNA ligase, and endonuclease buffers commonly used for genetic engineering techniques.

Physical Characteristics of d[GCT(εA)GC]. The asborption spectrum of the hexamer d[GCT(εA)GC] in aqueous solution displayed a characteristic maximum for εdAdo at 228 nm (Figure 1B, inset). Also, the peak in the vicinity of 260 nm exhibited a shape unlike those observed for unmodified oligonucleotides. A comparison of fluorescence emission spectra of d[GCT(εA)GC] before and after complete enzymatic digestion indicated a high degree of quenching. Over the broad range of concentrations from 0.2 to 25 μM, the integrated fluorescence of the oligonucleotide was consistently 4–5% of that of the enzymatically hydrolyzed components. These data strongly suggest that the adduct is well stacked in the oligonucleotide, owing to the planar nature of the εAde ring system. Leonard and co-workers (Tolman et al., 1974) have shown similar results with εAde-containing dinucleotide phosphates although the extent of quenching was much larger in the present study, presumably due to the longer sequence under investigation.

Certain physical aspects of the oligonucleotide were also established by ^1H NMR. Figure 3 compares the downfield region of the proton NMR spectrum of the unmodified hexamer with the εAde-adducted hexanucleotide at 60 °C. It was necessary to raise the temperature to resolve all of the nonexchangeable base proton resonances. At 45 °C, for instance, the signals of the C8 protons of two guanines and the C7 proton of εAde overlap and appear as a single peak. Due to the electronic interaction of the new five-membered aromatic ring, the C2 proton of εAde was deshielded slightly more than the respective proton in adenine, leading to the downfield shift of approximately 0.1 ppm. The most remarkable influence,

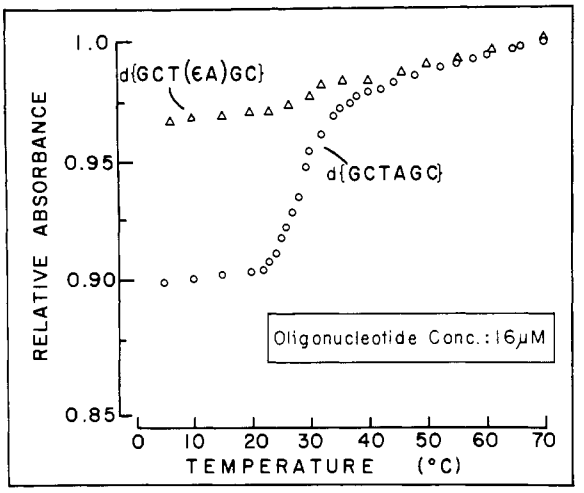


FIGURE 4: Absorbance vs. temperature plots of d(GCTAGC) and d[GCT(ϵA)GC] at 16 μM concentration.

Table I: Hyperchromicity at Different Oligonucleotide Concentrations[a]

	6.4 μM	16 μM	100 μM	160 μM
d(GCTAGC)	8.0	10.0	16.6	ND
d[GCT(ϵA)GC]	2.6	3.4	4.2	5.2
d(GCGC)	2.2	2.4	3.9	4.8

[a] Determined from temperature vs. absorption profiles between 5 and 70 °C. ND, not determined.

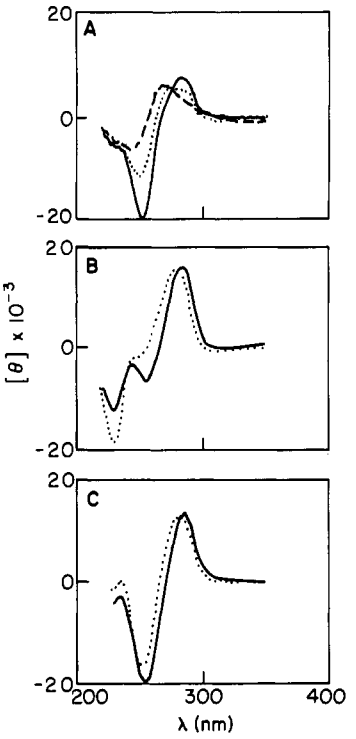


FIGURE 5: CD spectra of (A) d(GCTAGC), (B) d[GCT(ϵA)GC], and (C) d(GCGC). A comparison of spectra at 0 °C (solid line) and at room temperature (dotted line) was provided in each case. The broken line (only in frame A) displays the spectrum at 55 °C.

however, was manifested in the ϵAde C5 proton, which shifted almost 1 ppm downfield compared to the same resonance in the unmodified oligomer. The new etheno ring protons appeared at approximately 7.9 and 7.5 ppm. Each of these four ϵAde proton signals was shifted 0.09–0.12 ppm upfield, as compared to the ϵdAdo spectrum under the same conditions (data not shown). This is indicative of stacking interactions as a consequence of the ring-current effect of the neighboring bases. Among the neighboring base protons, only the thymine C6 proton signal was shifted significantly upfield (~0.1 ppm), suggesting an increased stacking effect with this 5′ base. The small peak at approximately 8.5 ppm is a transiently generated impurity that appeared occasionally with variable intensity in the d[GCT(ϵA)GC] spectra. If the sample was lyophilized and redissolved in fresh D_2O, this impurity signal usually disappeared. Previous literature reports on the susceptibility of ϵAde to alkali (Tsou et al., 1974) and observations in our laboratory suggest that this was due to a pH-dependent generation of a hydrated product at N4–C5 or its subsequent ring-opened form which apparently remains in equilibrium with ϵAde unless the pH of the solution is altered.

A comparison of the absorbance vs. temperature plots indicated a remarkable difference between the modified and the unmodified hexamers. While d(GCTAGC) exhibited a cooperative effect with a hyperchromicity of 9.98% at a concentration of 16 μM, the corresponding plot for d[GCT(ϵA)GC] indicated only partial unstacking of bases with increasing temperature, exhibiting a hyperchromicity of 3.38% (Figure 4). As suggested by the chemical structure of this adenine adduct, the etheno ring joining the N1 and N^6 positions appears to disrupt Watson–Crick hydrogen bonding completely. Indeed, the melting profile of the tetranucleotide d(GCGC) was quite similar to that of d[GCT(ϵA)GC], even though the flanking GC base pairs in the latter molecule are separated by two unpaired bases. As the concentration of the ϵAde-containing oligonucleotide was increased, the hyperchromicity of the molecule was also enhanced (Table I). Base stacking is unlikely to be concentration dependent (Arnott, 1970), and this suggests, therefore, that partial formation of duplex molecules may be occurring at low temperature. The similarity between d(GCGC) and the ϵAde-containing hexamer in terms of hyperchromicity at any given concentration is noteworthy. We also note, however, that even at a concentration of 160 μM, the melting curves for both d[GCT(ϵA)GC] and d(GCGC) were very broad, and a reliable T_m value could not be obtained. A similar observation was reported in the study of melting behavior of the tetraribonucleotide CGCG (Davis et al., 1986).

Circular dichroism studies confirmed and complemented the results obtained from other experiments. At low temperature and low salt concentration, the unmodified hexamer d(GCTAGC) displayed a CD profile typically observed for B-DNA, and at elevated temperature, it existed primarily in ss form (Figure 5A). At room temperature, d(GCTAGC) was only partially ss, whereas the modified hexamer d[GCT(ϵA)GC] was entirely ss with a trough at 230 nm and a peak at 280 nm (Figure 5B). The nucleoside ϵdAdo exhibited only a minimum at 230 nm although in the oligonucleotide the magnitude of this minimum was increased due to effective stacking interactions with neighboring bases. At 0 °C, a new minimum at 250 nm appeared and the magnitude of the trough at 230 nm significantly decreased due to partial duplex formation (Figure 5B). From the relative intensity of the peaks, we estimate that, at 160 μM concentration, approximately one-third of the oligonucleotide existed in duplex form at 0 °C. It is noteworthy that the modified hexamer maintained a regular B form despite the central unpaired dinucleotide unit. The tetramer d(GCGC) also attained partial duplex character at 0 °C (Figure 5C). The relative proportion of duplex character of the hexamer d(GCTAGC) compared to the modified hexamer d[GCT(ϵA)GC] or the tetramer d(GCGC) was easily noticeable from the differences in magnitude of the bands at two different temperatures. None of the oligo-

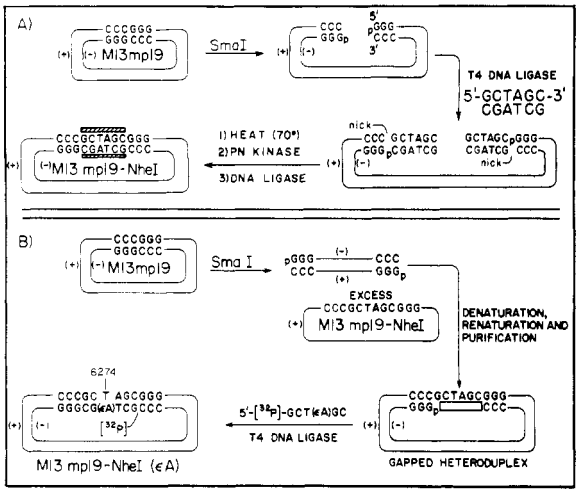


FIGURE 6: Frame A: Protocol for insertion of a hexanucleotide in M13mpl9, generating the *amber* mutant M13mpl9-*Nhe*I. Frame B: Site-specific modification of the M13mpl9-*Nhe*I genome to introduce a single εAde at position 6274 in the (−) strand. See Experimental Procedures for details.

nucleotide spectra were significantly altered in solutions of high salt concentration.

Construction of an M13 Genome with a Single εAde Residue within an amber Codon. The first step in the protocol for construction of an adducted oligonucleotide insertion site in M13mpl9 DNA is outlined in Figure 6A. M13mpl9 RF DNA was digested with *Sma*I to form a blunt-end linear duplex, which then was mixed with a 600–900-fold molar excess of nonphosphorylated, self-complementary hexanucleotide d(GCTAGC) and incubated for 18 h in the presence of T4 DNA ligase. This blunt-end ligation resulted in the formation of a linear M13mpl9 genome with duplex d(GCTAGC) segments on each end; it is noteworthy that only the 5′-terminal phosphates of the linear genome formed phosphodiester bonds with the hexamer duplexes. Mild heat treatment (70 °C) denatured and removed the two noncovalently attached hexamer residues, generating a product with 5′ complementary overhanging ends. Following phosphorylation with polynucleotide kinase, ligation of the ends produced M13mpl9-*Nhe*I, which had a unique recognition site [d(GCTAGC)] for *Nhe*I in the center of what was originally the *Sma*I site of M13mpl9. The ligation mixture was again treated with *Sma*I to linearize any residual M13mpl9 DNA that might have escaped the blunt-end ligation. Following transfection into *E. coli*, the resultant phage population was plated for inspection. The insertion of d(GCTAGC) generated an in-frame *amber* codon (underlined) within the polylinker insert of the *lacZ* α-fragment of the phage. This insertion clone produced light blue plaques when plated on GW5100 cells (a *sup*E strain) in the presence of X-Gal and colorless plaques on CSH50 (*Su*⁻). The RF DNA isolated from the M13mpl9-*Nhe*I clone had the following characteristics: sensitivity to *Nhe*I, resistance to *Sma*I, sensitivity to *Kpn*I and *Bam*HI (restriction enzymes that cleave parts of the *Sma*I site) indicating that the flanking base sequences were not altered, and as expected, identical behavior toward all other restriction endonucleases tested as compared to M13mpl9. The primary structure of the genome in the polylinker region was further confirmed by DNA sequencing[3] (Sanger et al., 1977; Mizusawa et al., 1986). An analogous approach has been used in our laboratory to introduce a unique site suitable for insertion of a dodecanucleotide containing a *cis*-diammineplatinum(II)–GpG cross-link (Pinto et al., 1986).

Circular ss M13mpl9-*Nhe*I DNA was mixed with *Sma*I-linearized M13mpl9 RF DNA, heated, and renatured as previously described (Green et al., 1984) to produce a circular heteroduplex DNA with a six-nucleotide gap in the (−) strand (indicated by the box in Figure 6B). 5′-Phosphorylation of d[GCT(εA)GC] with [γ-^{32}P]ATP followed by ligation into the GHD generated a duplex genome with a single εAde in the (−) strand at position 6274; this site is in the *amber* codon. For purposes of description, this genome is defined as M13mpl9-*Nhe*I(εA). A radiolabeled phosphodiester linkage between nucleotides 6277 and 6278 provided an easy marker for characterization. As a control, the unmodified hexamer, d(GCTAGC), was introduced into the GHD in a similar manner; this genome is denoted as M13mpl9-*Nhe*I. HPLC analysis indicated a high specific activity of both the oligonucleotides (>10^8 cpm/μg). Ligation efficiency was approximately 30% with the εAde-adducted oligonucleotide, while it was somewhat higher (45%) with the unmodified hexamer. Electrophoresis followed by autoradiography of these genomes indicated two major bands due to supercoiled circular duplex (form I_0) and nicked circular duplex (form II) DNAs (Figure 7A, lanes a and f). Formation of form I_0 DNA indicated that both sides of the oligonucleotide had ligated into the genome, whereas form II DNA could have arisen either from introduction of nicks nonspecifically or from ligation only on one end of the synthetic DNA fragment.

Characterization of Site Specifically Modified Genome. Physical mapping experiments were conducted to ascertain the genomic location of the radiolabel at the 5′ end of the inserted oligonucleotide. To ensure complete restriction endonuclease digestion and to visualize the bands by ethidium bromide staining, approximately 100 ng of carrier DNA (M13mpl9 RF or M13mpl9-*Nhe*I RF) was added, and an excess of the restriction endonuclease was used. *Bgl*II cleaved the DNA only at one site and thus formed a 7256-bp-long duplex linear DNA (Figure 7A, lanes b and g). *Cla*I cut twice, generating fragments of 4361 and 2895 bp. Electrophoretic separation of the fragments indicated the radiolabel to be localized in the larger fragment (Figure 7A, lanes c and h). Similarly, *Fok*I cleaved the genome to produce four fragments, with the ^{32}P label exclusively localized in the 2820-bp DNA segment (Figure 7A, lanes d and i). Furthermore, in a double digestion with *Bgl*II and *Bam*HI, the radiolabel was retained in a fragment with a 673-bp duplex core flanked by 4-b ss overhangs (total length of DNA = 677 b; Figure 8A, lane a). This fragment ran significantly faster than the larger fragment. Likewise, when digested with *Bal*I and *Kpn*I, the label was in the 1197-bp DNA segment (Figure 8B, lane a). Together, these data indicated that both the modified and unmodified hexanucleotides were specifically introduced into the area of the genome containing the six-base gap.

The behavior of the endonuclease *Nhe*I toward the site-specifically altered M13 genome was somewhat surprising. At 37 °C for 1 h, 4 units of *Nhe*I digested M13mpl9-*Nhe*I and carrier DNA (~0.1 μg) almost completely; the same amount of enzyme cleaved a portion of the εAde-adducted DNA as well (Figure 7A, lanes e and j). These results are contrary to those obtained with either O^6-methylguanine (Green et al., 1984) or the C8 guanine adduct of 4-aminobiphenyl (Lasko et al., 1987), where nearly complete resistance to cleavage by *Pst*I was observed when these adducts were built into a *Pst*I site. The present observation that *Nhe*I can digest the modified

[3] The dideoxy chain-termination method of DNA sequencing using deoxy-7-deazaguanosine triphosphate in place of dGTP was performed to avoid GC compression in this region.

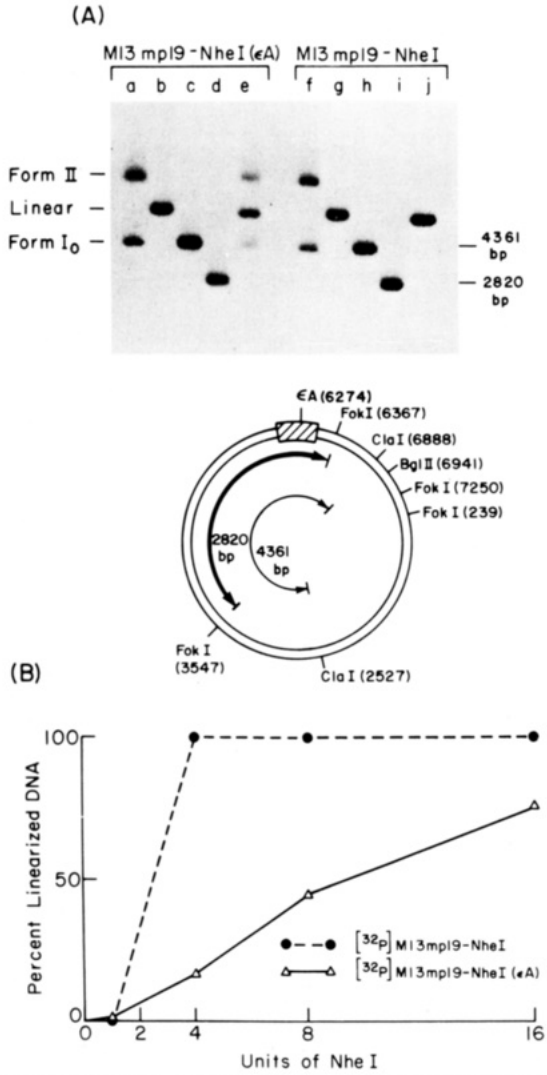


FIGURE 7: Physical mapping experiments to ascertain the genomic location of the inserted radiolabeled oligonucleotides, and digestion pattern of adducted and unadducted genomes with *Nhe*I. Frame A: Autoradiogram of the purified ligation products of gapped heteroduplexes with [5′-^{32}P]d[GCT(ϵA)GC] (lane a) and [5′-^{32}P]d-(GCTAGC) (lane f) [M13mp19-*Nhe*I(ϵA) and M13mp19-*Nhe*I, respectively]. Lanes b, c, d, and e show the material in lane a digested with *Bgl*II, *Cla*I, *Fok*I, and *Nhe*I, respectively, and lanes g–j are the corresponding endonuclease digestions of the product in lane f. (The numbers associated with restriction sites refer to the 5′ base in the recognition sequences.) Frame B: Quantitation of the *Nhe*I digestion pattern of ϵAde-modified and unmodified genomes. Percent linearized DNA was determined from the autoradiogram by a scanning densitometer and was plotted against the number of units of the endonuclease used.

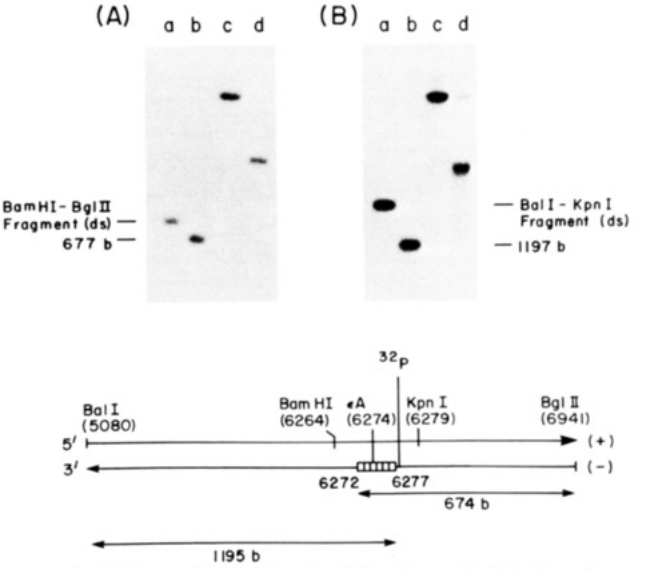


FIGURE 8: Evidence that both ends of the oligonucleotides have been ligated into the gapped-heteroduplex genome. Frame A: Autoradiogram providing evidence for 3′-end ligation of the oligomer. Lane a shows the radiolabeled 673-bp duplex core flanked by 4-b ss overhangs generated by double digestion of M13mp19-*Nhe*I(ϵA) with *Bgl*II and *Bam*HI. (The nonradioactive larger fragment runs more slowly in the gel and was seen by ethidium bromide staining.) Lane b displays the faster migrating 677-b long ss DNA fragment formed by heat denaturation of the double-digestion product in lane a. Lane c shows the *Bgl*II-digested linear genome, whereas lane d is the same after denaturation. It is significant that no specific band of 674-b-long ss DNA fragment can be detected in lane d. If a part of the oligomer did not ligate on the 3′ end, a radiolabeled band corresponding to 674-b ss DNA should appear. Frame B: Autoradiogram providing evidence for 5′-end ligation of the oligomer. Lane a shows a 1197-bp fragment from M13mp19-*Nhe*I(ϵA) formed by double digestion with *Bal*I and *Kpn*I. Lane b shows 1197-b-long ss DNA made by denaturation of the double-digestion product in lane a. Lane c shows the *Bal*I-digested linear genome. Lane d displays material in lane c after denaturation. No specific band of 1195-b-long ss DNA was found.

restriction site, albeit somewhat more slowly than the unmodified sequence, was confirmed by using a series of increasing concentrations of the endonuclease. As shown in Figure 7B, the results indicate that, with a large excess of the enzyme, it is indeed possible to completely digest the adducted *Nhe*I site of M13mp19-*Nhe*I(ϵA). Interestingly, we recently have observed that O^6-methylguanine in the context of a *Pst*I site (CTGCAG) prevented cleavage by the *Pst*I enzyme, whereas the same adduct only partially inhibited cleavage by *Pvu*I when situated in a *Pvu*I site (CGATCG; Dogliotti, Ellison, Basu, and Essigmann, unpublished results). This situation is similar to what was observed in the present work with ϵAde situated in the *Nhe*I site. It may be a mechanistically related phenomenon that certain type II restriction enzymes can cleave recognition sites containing mismatched bases (Jiricny & Martin, 1986).

A substantial proportion of the M13 DNA into which ligation had occurred was in the nicked-circular form (Figure 7A). One possible reason for the appearance of this material was failure of the oligomer to be ligated on both sides of the gap. To test the possibility that ligation failed to occur on the 3′ end of the oligonucleotide, the ϵAde-containing genome was digested with *Bgl*II, the DNA was denatured by heating, and the resultant ss DNA fragment(s) was (were) electrophoresed. *Bgl*II cleaves the (−) strand at position 6945, which is 674 or 668 b away from the ligation sites that flank the 3′ and 5′ ends of the hexanucleotide, respectively. If a part of the oligomer did not, for example, ligate on the 3′ end, a radioactive band of ss DNA corresponding to 674 b should appear, in addition to genome-length DNA. The size standard for the shorter fragment was generated by double digestion with *Bgl*II and *Bam*HI, which generated a ss fragment of 677 b. The results of this experiment are shown in Figure 8A. A complementary experiment tested whether ligation occurred on the 5′ side of d[GCT(ϵA)GC]. This was done by digesting the genome with *Bal*I, which should generate a 1195-b fragment if any portion of the oligonucleotide failed to ligate on the 5′ end. In this case, the size standard was created by a double digestion with *Bal*I and *Kpn*I (Figure 8B). In both experiments, the radioactivity appeared in the larger DNA fragment. The absence of any detectable radioactive short fragment indicated clearly that ligation of the modified and unmodified hexanucleotides into the six-base gap had occurred on *both* the 5′ and 3′ ends. However, a smear of radioactivity was seen in both of the d lanes (Figure 8A and 8B), suggesting that the form II DNA

was almost entirely generated due to nicks distributed nonspecifically throughout the genome.

Genetic Rationale for Using M13mp19-NheI(ϵA) in Site-Specific Mutagenesis Studies. The strategy underlying detection of mutants issuing from biochemical processing of ϵAde has as its key feature the fact that the adduct is situated in an in-frame *amber* codon. Because of its location in the M13 phage genome, the nonsense codon conferred the colorless-plaque phenotype upon phage plated on a Su^- host such as *E. coli* CSH50. Reversion of the *amber* trait by mutation at the ϵAde site will restore the Lac^+ phenotype, as evidenced by blue plaques on IPTG/X-Gal-containing medium. A second method for isolating mutant phage will take advantage of the fact that the ϵAde adduct is located in the unique recognition site for *Nhe*I. Mutations at any of the six bases of this sequence will render the duplex M13 genome insensitive to cleavage and therefore amenable to selection. This strategy for selection has been used successfully in the past for isolation of mutants induced by the DNA adduct O^6-methylguanine (Loechler et al., 1984).

Discussion

Structural alteration of DNA by chemical carcinogens and radiation is believed to be a necessary prelude to mutagenesis and, ultimately, carcinogenesis (Miller, 1978a). Formation of DNA adducts and other structural modifications could trigger alterations in gene expression, replication, or repair, ultimately resulting in permanent heritable changes in DNA. Like most DNA-damaging agents, vinyl chloride generates a multitude of nucleic acid adducts through its metabolically activated derivatives. These include several etheno adducts, their hydrated derivatives, 7-(2-oxoethyl)guanine, apurinic sites, and several recently discovered acyclic adducts (Singer & Grunberger, 1983; O'Neill et al., 1986; Barbin et al., 1986). Despite our knowledge of the DNA-bound forms of vinyl chloride, the mechanism of its induction of mutagenesis and carcinogenesis is not clearly understood. This is primarily due to the difficulty in assigning which adduct or lesion in a population of structural modifications of DNA is responsible for critical mutations or other genotoxic manifestations. The situation is further complicated by the sequence-dependent covalent binding of certain chemicals (Muench et al., 1983; Chen, 1985) and the related fact that some local sequences may be more prone to mutation than others (Miller, 1978b).

In a previous study of the major DNA adduct of the antitumor drug *cis*-diamminedichloroplatinum(II) (Pinto et al., 1986) a general strategy was described for inserting lesions into circular genomes in any local sequence context. Here, we have taken advantage of the ability to customize the genetic environment of an adduct by building ϵAde into an *amber* codon to facilitate detection of targeted mutations. The suppressed light blue plaque of the *amber* phage in GW5100 should turn dark blue by a point mutation at the *amber* site, whereas a frame shift would result in a colorless phenotype. The mutant phage population can also be enriched by restriction endonuclease (*Nhe*I) digestion, which spares the mutant genome.

We have chosen the chloroacetaldehyde modification ϵAde as the representative lesion to adequately establish this model system. Etheno bridging of adenine changes the base electronic structure remarkably; the mesomeric effect within the pyrimidine ring is severely impaired although the partial positive charge at the 2-position of adenine is still retained. Also, unlike the unmodified base, Ade, the alkali-induced anion in ϵAde is very effectively delocalized, with the ultimate result of lability at alkaline pHs. We have been able to demonstrate, however, that the molecule is sufficiently stable at neutral pH to enable its site-specific incorporation into a viral genome for genetic studies.

The physical studies on the hexanucleotide containing the ϵAde adduct revealed data consistent both with predictions based on chemical structure and with literature reports on ribodinucleotide monophosphates (Tolman et al., 1974). The planar nature of the adduct suggested an efficient stacking interaction with the neighboring bases. Experimentally, this was evident both from the quenching of fluorescence and from the enhancement of the minimum in circular dichroism. Proton NMR studies as well provided evidence of the stacked conformation of ϵAde. Interestingly, however, of its neighboring bases, only the 5′-thymine residue experienced an increased stacking effect due to formation of the etheno ring on the adjacent adenine. By use of calculated isoshielding curves and dimerization shift NMR data, it was shown at the ribodinucleotide monophosphate level that ϵApG and ApG have comparable right-handed stacked (g^-g^-) populations, while introduction of ϵAde either 5′ or 3′ to a pyrimidine base causes an increase in g^-g^- conformers (Dhingra et al., 1978). Our data, albeit involving a longer deoxyoligonucleotide, are in agreement with these observations.

The chemical structure of the synthetic hexamer also indicated a loss of hydrogen bonding between ϵAde and its complementary base. This was evident in the melting studies as well as from the CD profiles. It would be of interest to obtain further information concerning the noncovalent interaction between ϵAde and the complementary base in the opposite strand. Our data suggest that the energy of this interaction is small, but an accurate assessment of ΔH and ΔS values preferably should be provided from a longer sequence.

Ligation of the ϵAde-adducted hexamer into the gapped heteroduplex did not pose any problem. The modified genome thus prepared has been characterized to ascertain whether ligation took place at the desired gap and, if so, whether it occurred on both ends of the oligonucleotide. The results of both these lines of experimentation were satisfactory, substantiating insertion of the oligonucleotide into the correct position and showing that ligation indeed did occur at the anticipated sites. The presence of the adduct did not protect the sequence in which it was embedded from cleavage by *Nhe*I, although a distinctly reduced rate of cleavage of the adducted *Nhe*I site was observed, presumably due to the presence of the adduct.

When ϵAde-containing random DNA polymers were used, significant misincorporation by polymerases during in vitro DNA synthesis prompted Barbin et al. (1981) to suggest mispairing schemes for the protonated form of ϵAde and deoxyguanosine similar to an A_{syn}–G_{anti} (enol–imino) base pair. This base pair and those between protonated ϵAde and adenine and, especially, cytosine were suggested to form during DNA synthesis in vitro by Revich and Beattie (1986). While the data of these studies are not in question, the involvement of the protonated form of ϵAde is unlikely, because the pK_a values of ϵAde nucleosides indicate that only the nonprotonated species are available at the neutral pH conditions under which most of these biological experiments are usually performed (Secrist et al., 1972). It is indeed intriguing that ϵAde could induce misincorporation even though it does not possess the ability to hydrogen bond to any significant extent. The mechanism of misincorporation of bases might actually be far more complex than a straightforward mispairing scheme. Using the site-specifically modified genome described here, we shall attempt to characterize the biochemical details of

mutagenesis by ϵAde in vivo. Through these studies we anticipate characterization of the type of mutation induced in vivo by ϵAde, if indeed it is mutagenic in cells, and definition of the genetic requirements for mutagenesis. Complemented by information on the mutational spectrum produced by the electrophilic precursors of ϵAde and its sister adducts, these studies should enable prediction of the extent to which ϵAde could be responsible for the mutations induced by vinyl chloride and other compounds that generate ϵAde subsequent to their reaction with DNA.

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Registry No. ϵdAdo, 68498-25-9; DMT chloride, 40615-36-9; 5′-DMT-ϵdAdo, 109584-91-0; 5′-DMT-ϵdAdo-3′-ClPh(P)-TEA, 109553-55-1; d[GCT(ϵA)GC], 109553-56-2; [5′-^{32}P]d[GCT(ϵA)GC], 109553-57-3; 1,2,4-triazole, 288-88-0; 2-chlorophenyl phosphorodichloridate, 15074-54-1.

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