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Chemical and Biological Studies of the Major DNA Adduct of *cis*-Diamminedichloroplatinum(II), *cis*-[$Pt(NH_3)_2${d(GpG)}], Built into a Specific Site in a Viral Genome†

Lisa J. Naser, Ann L. Pinto, Stephen J. Lippard,* and John M. Essigmann*
Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139
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ABSTRACT: A duplex *Escherichia coli* bacteriophage M13 genome was constructed containing a single *cis*-[$Pt(NH_3)_2${d(GpG)}] intrastrand cross-link, the major DNA adduct of the anticancer drug *cis*-diamminedichloroplatinum(II). The duplex dodecamer d(AGAAGGCCTAGA)·d(TCTAGGCCTTCT) was ligated into the *Hin*cII site of M13mp18 to produce an insertion mutant containing a unique *Stu*I restriction enzyme cleavage site. A genome with a 12-base gap in the minus strand was created by hybridizing *Hin*cII-linearized M13mp18 duplex DNA with the single-stranded circular DNA of the 12-base insertion mutant. The dodecamer d(TCTAGGCCTTCT) was synthesized by the solid-phase phosphotriester method and platinated by reaction with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ (yield 39%). Characterization by pH-dependent ^1H NMR spectroscopy established that platinum binds to the N7 positions of the adjacent guanosines. The platinated oligonucleotide was phosphorylated in the presence of [γ-^{32}P]ATP with bacteriophage T4 polynucleotide kinase and incorporated into the 12-base gap of the heteroduplex, thus situating the adduct specifically within the *Stu*I site in the minus strand of the genome. Approximately 80% of the gapped duplexes incorporated a dodecanucleotide in the ligation reaction. Of these, approximately half did so with the dodecanucleotide covalently joined to the genome at both 5′ and 3′ termini. The site of incorporation of the dodecamer was mapped to the expected 36-base region delimited by the recognition sites of *Xba*I and *Hind*III. The *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link completely inhibited *Stu*I cleavage, which was fully restored following incubation of the platinated genome with cyanide to remove platinum as $[Pt(CN)_4]^{2-}$. Gradient denaturing gel electrophoresis of a 289-base-pair fragment encompassing the site of adduction revealed that the presence of the *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link induces localized weakening of the DNA double helix. In addition, double- and single-stranded genomes, in which the *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link resides specifically in the plus strand, were constructed. Comparative studies revealed no difference in survival between platinated and unmodified double-stranded genomes. In contrast, survival of the single-stranded platinated genome was only 10–12% that of the corresponding unmodified single-stranded genome, indicating that the solitary *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link is lethal to the single-stranded bacteriophage.

Most chemicals that bind to DNA form a spectrum of adducts, some or all of which can serve as lethal and/or premutagenic lesions (Miller, 1978). Genotoxicity has been exploited by development of chemotherapeutic agents that kill rapidly dividing cells by binding to DNA and inhibiting replication. One of the most effective antitumor drugs to date is *cis*-diamminedichloroplatinum(II) (*cis*-DDP).[1] This bifunctional electrophilic compound reacts with DNA to form a variety of intra- and interstrand cross-links [for a review, see Sherman and Lippard (1987)]. The principal adducts are the *cis*-[$Pt(NH_3)_2${d(GpG)}] and *cis*-[$Pt(NH_3)_2${d(ApG)}] intrastrand cross-links. Minor products include *cis*-[$Pt(NH_3)_2${d(GpNpG)}] intrastrand cross-links (where N is any intervening nucleotide), interstrand cross-links, and monoadducts. In all of these cases, the N7 atoms of the purine bases have replaced the chloride ligands in the *cis*-DDP coordination plane. The clinically inactive geometric isomer of *cis*-DDP, *trans*-diamminedichloroplatinum(II) (*trans*-DDP), also binds

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* Correspondence should be addressed to these authors.

[1] Abbreviations: bp, base pair; DDP, diamminedichloroplatinum(II); ds, double stranded; EthBr, ethidium bromide; EDTA, disodium salt of ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; nt, nucleotide; RF, replicative form; ss, single stranded; TAE, 40 mM Tris–acetate (pH 7.5) and 1 mM EDTA; TE, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

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to DNA to form cross-links, although these have not been as well characterized as those of *cis*-DDP. For stereochemical reasons *trans*-DDP cannot form [$Pt(NH_3)_2${d(GpG)}] or [$Pt(NH_3)_2${d(ApG)}] intrastrand cross-links, and these adducts may therefore be responsible for the unique biological activity of the cis isomer.

The *cis*-[$Pt(NH_3)_2${d(GpG)}] intrastrand cross-link constitutes >65% of the platinum adducts formed in DNA in vitro (Fichtinger-Schepman et al., 1985) and >45–65% of adducts formed in vivo (Plooy et al., 1985; Fichtinger-Schepman et al., 1987). Several lines of evidence have correlated its presence with the biological effectiveness of *cis*-DDP. First, *cis*-DDP inhibits DNA replication in bacterial and mammalian cells [see Pinto and Lippard (1985a) for a review], and in vitro replication on templates treated with *cis*-DDP is arrested primarily opposite G_n sites, where $n \geq 2$ (Pinto & Lippard, 1985b). Second, antibodies that selectively recognize the *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link bind to the peripheral white blood cell DNA of patients treated with *cis*-DDP (Poirier et al., 1985; Reed et al., 1987; Fichtinger-Schepman et al., 1987), and the extent of binding correlates with the clinically observed response to drug treatment (Poirier et al., 1985; Reed et al., 1987).

cis-DDP is carcinogenic in the mouse and rat (Leopold et al., 1979). Moreover, the appearance of second malignancies in patients treated with *cis*-DDP has been noted (Johnson, D. C., et al., 1980; Mead et al., 1983), leading to speculation that these tumors may have been the result of *cis*-DDP treatment. Like many electrophilic carcinogens, *cis*-DDP is a mutagen in bacterial (Beck & Brubaker, 1975; Benedict et al., 1977) and in mammalian (Johnson, N. P. et al., 1980) cells. *cis*-DDP is predominantly a base-pair (bp) substitution mutagen in bacteria (Beck & Brubaker, 1975; Beck & Fisch, 1980). The exact nature of these mutations has been studied in several forward mutation assays with conflicting results. Brouwer et al. (1981), using the *Escherichia coli lacI* gene as a target, found that mutations due to *cis*-DDP treatment of cells arise primarily at GAG and GCG sequences. In contrast, more than 90% of the mutations observed by Burnouf et al. (1987) occur at AG and, to a lesser extent, GG sequences in an assay where the tet^R gene of pBR322 was treated in vitro with *cis*-DDP and then transferred into *E. coli* for replication in vivo.

In order to understand at the molecular level the toxic and mutagenic effects of *cis*-DDP-platinated DNA in vivo, we are constructing genomes containing the various *cis*-DDP adducts at specific sites. This paper describes in detail the synthesis and characterization of a dodecanucleotide containing the *cis*-[$Pt(NH_3)_2${d(GpG)}] instrastrand cross-link and its use in the construction of a site-specifically platinated genome. The characterization of the genome and the effect the adduct has on its physical and biological properties are described. Preliminary results of a part of this work were previously communicated (Pinto et al., 1986).

Experimental Procedures

Materials. Oligonucleotide synthesis reagents were purchased from Cruachem. Dodecamer d(AGAAGGCCTAGA) was purchased from the Department of Chemistry at Yale University. All enzymes, except where noted, were purchased from New England Biolabs. The cell lines used were the following: *E. coli* GW5100 (JM103 $P1^-$, from Graham Walker, MIT), *E. coli* DL7 (AB1157 *lac* Δ*U169*, from Dana Lasko, MIT), and *E. coli* MM294A (*lac*$^+$, from K. Backman, Biotechnia International).

Oligonucleotide Synthesis. Dodecamer d(TCTAGGCCTTCT) (12-T)[2] was synthesized by the solid-phase phosphotriester method (Sproat & Gait, 1984) on a polyacrylamide/Kieselguhr support (250 mg). Loading of the 3′ monomer (dT) on the resin was 76.4 μmol/g as determined by spectrophotometric quantitation (495 nm) of the dimethoxytrityl (DMT) protecting group released upon treatment with perchloric acid in EtOH. Deprotection was performed by established procedures (Sproat & Gait, 1984). The product dodecanucleotide was purified by anion-exchange chromatography on DE-52 (Whatman) using a linear gradient of 0.075–0.75 M triethylammonium bicarbonate in 20% EtOH. The sample was then desalted by chromatography on Sephadex G-15 (Sigma) and converted to the NH_4^+ form on Dowex AG-50X8 (Bio-Rad). The yield after purification was 470 OD_{260} units (24%) or approximately 18 mg of oligonucleotide ($\epsilon_{d(TCTAGGCCTTCT)}$ = 102 900 M^{-1} cm^{-1}) (Fasman, 1975).

Platination Reaction. cis-[$Pt(NH_3)_2Cl_2$] was synthesized by a modification of the method of Dhara (1970) and recrystallized from 0.1 N HCl. *cis*-[$Pt(NH_3)_2(H_2O)_2]^{2+}$ was prepared by stirring a solution of *cis*-[$Pt(NH_3)_2Cl_2$] (18.1 mg, 6×10^{-5} mol) with 1.98 equiv of $AgNO_3$ (20.2 mg) in a total volume of 2.5 mL of H_2O overnight in the dark. The precipitated AgCl was then removed by repeated centrifugation. One equivalent of the supernatant, 20 mM *cis*-[$Pt(NH_3)_2(H_2O)_2]^{2+}$, was immediately allowed to react in the dark at 37 °C for 5 h with dodecamer d(TCTAGGCCTTCT) (Na^+ form; 2 mL of a 0.9 mM solution). The initial pH of the solution was 6.0. *cis*-[$Pt(NH_3)_2${d(TCTAGGCCTTCT)}] (Pt-12-T) was separated from the starting material by high-performance liquid chromatography (HPLC) using a C-18 reversed-phase column and a gradient of 17–35% B over 18 min, where A = 0.1 M NH_4OAc (pH 6.5) and B = 1:1 A/CH_3CN. The appropriate HPLC fractions were pooled, lyophilized, desalted on Sephadex G-15, and converted to the Na^+ form on Dowex AG-50X8. The yield of purified Pt-12-T was 74 OD_{260} units (39%). Platinum analysis was performed on a Varian AA-1475 atomic absorption spectrophotometer equipped with a GTA-95 graphite furnace.

Characterization of Unplatinated and Platinated d-(TCTAGGCCTTCT). pH titrations of the nonexchangeable base protons of 12-T and Pt-12-T were performed to identify the platinum binding sites. Dodecamer 12-T was converted to the Na^+ form and desalted as before. Trace paramagnetic impurities were removed by passing the sample through a small column of Chelex resin (Na^+ form, Bio-Rad). The sample was dissolved in D_2O (99.8%, Aldrich), lyophilized twice, and finally dissolved in 350 μL of D_2O (99.996%, Kor) to yield a strand concentration of 6 mM. EDTA was present at a concentration of 0.5 mM; [$N(CH_3)_4$]Cl was used as a temperature- and pH-independent internal standard (δ 3.18). Spectra (4K real data points) were recorded at 65 °C on the 500-MHz NMR spectrophotometer at the Francis Bitter National Magnet Laboratory. Presaturation (170-ms duration) of the residual HDO peak was employed to reduce its intensity. After data accumulation, convolution difference methods were employed to enhance resolution. The pH* of the sample was measured both before and after each spectrum was recorded and was changed by the addition of small amounts of NaOD or DCl (Kor) in D_2O. Seventeen spectra

[2] Nomenclature: d(TCTAGGCCTTCT) and d(AGAAGGCCTAGA) are abbreviated 12-T and 12-A, respectively; Pt-12-T is 12-T containing the *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link; M13-12A and M13-12T are the products of insertion of 12-A and 12-T, respectively, into the plus strand of M13mp18 at the *Hin*cII site; M13-12A-Pt(−) and M13-12A-u(−) are M13-12A with Pt-12-T and 12-T, respectively, built into the minus strand; (+) designates genomes in which the stated dodecanucleotide resides in the plus strand.

in the pH* range 3.3–10.9 were recorded.

Pt-12-T was prepared for NMR pH titration studies by the same procedure. The NMR parameters were unchanged except that the spectral size was increased to 8K real. The concentration of the oligonucleotide was 2.1 mM. Eleven spectra in the pH* range 4.1–9.6 were recorded. After the titrations were completed, the samples were once again purified by reversed-phase HPLC, desalted, and converted to the Na^+ form for use in subsequent experiments.

Construction of Precursor Genomes. Replicative form (RF) and single-stranded (ss) DNAs were prepared as described by Lasko et al. (1987). A blunt-end ligation reaction between 1 μg of *Hin*cII-linearized M13mp18 DNA and a 450-fold molar excess of the unphosphorylated duplex d-(AGAAGGCCTAGA)·d(TCTAGGCCTTCT) was carried out in the presence of 66 mM Tris-HCl buffer (pH 7.6), 10 mM $MgCl_2$, 15 mM dithiothreitol, 1 mM ATP, and 800 units of bacteriophage T4 DNA ligase in a reaction volume of 20 μL at 16 °C overnight. The ligation products were then electrophoresed through a 1% low melting point agarose gel containing 0.5 μg/mL EthBr. The band migrating at the position of a linearized genome was excised and the DNA isolated by heating and phenol extraction (Maniatis et al., 1982). This DNA was phosphorylated in the presence of 1 mM ATP, 66 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 15 mM dithiothreitol, and 40 units of T4 polynucleotide kinase, in a total volume of 10 μL at 37 °C for 45 min. The reaction volume was brought to 20 μL, maintaining the same ATP and buffer concentrations, and 800 units of T4 DNA ligase was added to catalyze intramolecular ligation. The ligation products were precipitated with EtOH, resuspended, and treated with *Hin*cII to linearize any residual starting material. Competent *E. coli* MM294A cells were transformed with the restriction digest mixture (Maniatis et al., 1982). Two to three hours after the heat-shock treatment, cells were pelleted by centrifugation, leaving the progeny phage in the supernatant. Phage were screened for dodecanucleotide insert presence and orientation by sequencing according to the method of Sanger et al. (1977), using the M13 pentadecamer primer from New England Biolabs and DNA polymerase I (Klenow fragment, Boehringer Mannheim Biochemicals). This method produced the two insertion genomes M13-12A[2] and M13-12T.

Insertion of Platinated and Unplatinated Dodecanucleotides into the (−) Strand of Duplex Genomes. Gapped duplex formation, using formamide denaturation, and purification were performed as described by Lasko et al. (1987). Briefly, hydroxylapatite-purified M13-12A ss viral DNA and *Hin*cII-linearized M13mp18 RF were hybridized at a 20:1 molar ratio of ss (+) to linearized (−) strand to produce a 12-base gap specifically in the (−) strand.

Phosphorylation of Pt-12-T and 12-T prior to ligation into the gapped duplexes was carried out according to the method of Green et al. (1984) on 126 pmol of the dodecanucleotide, in the presence of 200 μCi of [γ-^{32}P]ATP (3000 Ci/mmol, Amersham). Unlabeled ATP was added to the reaction to bring the ATP:dodecamer ratio to 10:1. HPLC conditions employed to determine extent of phosphorylation of the dodecamers were the same as described above, with the exception that a 30-min gradient of 15–45% B was used.

The ligation was performed on 1 μg of gapped duplex DNA, with a 600:1 molar excess of ^{32}P-phosphorylated dodecanucleotide in a reaction volume of 40–80 μL under conditions described in Green et al. (1984), without the addition of bovine serum albumin. T4 DNA ligase was added to the reaction mixture twice in 800-unit portions, and the reaction was allowed to proceed for ≥6 h. Excess ATP and dodecanucleotide were separated from higher molecular weight ligation products by drop dialysis (Silhavy et al., 1984) against 1 L of TE buffer for 1–3 h, followed by Sepharose CL-4B chromatography using TE containing 100 mM NaCl as eluant. Three-drop fractions were collected, and the radioactivity was quantitated by Cerenkov counting. Peak fractions eluting at and around the void volume were pooled and stored at 4 °C; these fractions contained the genomes that had incorporated Pt-12-T and 12-T, designated M13-12A-Pt(−)[2] and M13-12A-u(−), respectively.

Experiments in which the site-specifically modified genome was replicated in cells did not utilize a ^{32}P label and omitted the drop dialysis and Sepharose chromatography steps. Instead, these DNA samples were extracted with phenol, precipitated with EtOH, and then resuspended in TE.

Construction of ds and ss Genomes in which the Pt Adduct Resides Specifically in the (+) Strand. Heteroduplex genomes were prepared according to the heating method of Green et al. (1984) by hybridizing *Hin*cII-linearized M13mp18 RF DNA with dephosphorylated *Bgl*II-linearized M13-12T RF DNA. Phosphorylation and ligation of Pt-12-T and 12-T into the gapped duplexes were carried out as described above with the exception that the ATP concentration was 1 mM and no radiolabeled ATP was used. Ligation efficiency was determined by monitoring the amount of DNA converted from nicked (form II) to linear (form III) upon digestion with *Stu*I [after cyanide reversal, vide infra, of platinum binding in the case of M13-12T-Pt(+)]. Single-stranded M13-12T-Pt(+) and ss M13-12T-u(+) were produced by heat denaturation (100 °C for 3 min, followed by cooling on ice) of their respective ds genomes. For the survival experiments, samples were denatured immediately prior to *E. coli* transformation.

Characterization of Site-Specifically Adducted Genomes. The following methods were employed to study M13-12A-Pt(−) and M13-12A-u(−). Restriction digests were typically performed on 300–1000 cpm of the ligation products by using the three-buffer system of Maniatis et al. (1982), with the addition of 0.1 μg of unlabeled M13-12A RF DNA as an internal control. Agarose dye gels contained 0.8% agarose and 0.5 μg/mL EthBr. In all cases digestion was complete as judged by visualization of carrier M13-12A RF DNA by EthBr fluorescence. Autoradiography was performed overnight at room temperature.

Removal of the platinum adduct as $[Pt(CN)_4]^{2-}$ was accomplished by incubating the ligation products for 3 h at 37 °C in the presence of 0.3 M NaCN (pH 8.0). The pH of the cyanide solution was adjusted with HCl in a fume hood. In another experiment, M13-12A-Pt(−) was incubated with 50 mM thiourea at 37 °C for 1 or 4 h. After incubation, the DNA samples were precipitated with EtOH, digested with *Stu*I, and electrophoresed and autoradiographed as described.

Ligation efficiency was determined by a densitometric method. A portion of each ligation mixture was electrophoresed through an agarose dye gel. The gel was photographed with type 55 Polaroid positive/negative land film, dried, and then autoradiographed with preflashed Kodak X-Omat AR film at room temperature. The lanes on the photographic negative and the autoradiogram were scanned by using a Bio-Rad Model 620 video densitometer, and the relative amounts of nicked circular (form II) and covalently closed circular (form I_o) material for each lane were compared between the negative and the autoradiogram.

The effect of the Pt–DNA adduct on duplex DNA stability was examined by denaturing gradient gel electrophoresis

(Fischer & Lerman, 1983; Lerman et al., 1984). A 12.5% polyacrylamide gel [37.5:1 acrylamide/*N,N'*-methylenebis-(acrylamide)] containing a linear gradient of 35–55% denaturant (100% denaturant: 7 M urea, 40% formamide) was poured with a two-chamber gradient mixer. The gel was submerged in an aquarium filled with TAE buffer equilibrated at 60 °C and preelectrophoresed at 150 V for 1 h. The DNA samples [≈1000 cpm each of an *Eco*RI/*Mst*II digest of M13-12A-Pt(–) and M13-12A-u(–)] were loaded and run at 150 V for 12 or 14 h. The gel was then soaked in 40% methanol and 5% glycerol for 1 h and dried. Autoradiography was performed overnight at –70 °C with an intensifying screen.

Survival Studies of Site-Specifically Adducted ss and ds Genomes. *E. coli* MM294A and DL7 cells were made competent for DNA uptake by $CaCl_2$ (Maniatis et al., 1982) or by a modification of this protocol that utilizes 50 mM $MnCl_2$, respectively. For some transformations, DL7 cells were used because they, unlike MM294A, are *lac*$^-$ and therefore do not produce a blue color when plated in the presence of isopropyl β-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal); they are otherwise a wild-type cell line. Transformed cells were plated in the presence of *E. coli* GW5100, IPTG, and X-Gal as described by Messing (1983) and incubated at 37 °C overnight. If necessary, cells were diluted with an appropriate amount of culture medium before plating.

RESULTS

Oligonucleotide Synthesis and Characterization. Dodecanucleotide d(TCTAGGCCTTCT) was synthesized in high yield by the solid-phase phosphotriester method. Platination with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ resulted in the formation of one major product, which eluted by HPLC 4.5 min after the starting material and accounted for more than half of the eluted material absorbing at 260 nm. The drug-to-nucleotide ratio was 1.0 as determined by platinum atomic absorption and UV spectroscopy, thereby ruling out the possibility that interstrand cross-linked products, which would have a 1:2 stoichiometry, might be present.

An expanded plot of the downfield region of the 500-MHz ^{1}H NMR spectrum of 12-T is presented in Figure 1a. Farthest upfield are the five thymine H6 resonances. From the relative peak intensities, the peak at 7.61 ppm is assigned to the overlapping resonances of two thymine H6 protons. These resonances were not resolved under any conditions of pH or temperature investigated. The four cytosine H6 doublets, as indicated in Figure 1a, were identified by their characteristic coupling constant, $J_{H5,H6}$ = 7.3 Hz. The remaining resonances correspond to the purine H8 and H2 protons. During the course of the pH titration, the intensities of the purine resonances, except the one at 8.10 ppm, decreased due to deuterium exchange. This result indicates that the resonance at 8.10 ppm corresponds to adenine H2. By comparison with the spectrum of $[d(AGGCCT)]_2$ (Caradonna et al., 1982), it is likely that the resonance farthest downfield is the adenine H8 proton. The downfield region of Pt-12-T is given in Figure 1b. It is very similar to that of 12-T, except that one of the two guanine resonances has been shifted far downfield. Such a shift is routinely observed in spectra of oligonucleotides containing *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$ adducts and corresponds to the H8 proton of the 3′ guanosine residue involved in the cross-link (Neumann et al., 1984; den Hartog et al., 1985; Caradonna & Lippard, 1988).

The pH titration data are presented in Figure 2. Protonation of cytosine N3 is clearly observed as a downfield shift centered at approximately pH* 4.5 (Izatt et al., 1971); pro-

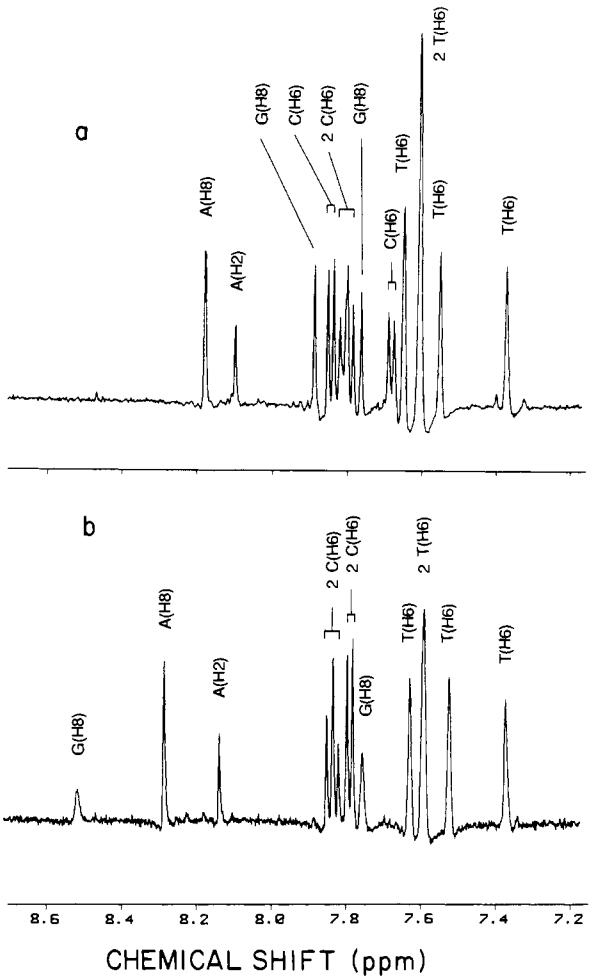

FIGURE 1: 500-MHz ^{1}H NMR spectrum of the downfield nonexchangeable base protons of (a) 12-T and (b) Pt-12-T. Spectra were recorded at 65 °C, pH* 9.6.

tonation of adenine N1 (pK_a = 3.7) is evident as well. Deprotonation of thymine N3 is indicated by a slight upfield shift at high pH (pK_a = 10.0). Both guanines and one thymine exhibit chemical shift changes at low pH, although no transition occurs for these bases in the pH* range from 3 to 6. This behavior is probably the result of secondary effects due to base destacking induced by protonation or deprotonation at an adjacent base; all guanine and thymine residues in the sequence are adjacent to at least one base (adenine or cytosine) that titrates at acidic pH. The important information obtained from this titration is that there are no transitions of any sort observed in the neutral pH range (pH* 6.0–9.0). This result is in accordance with expectations based on previous work (Sherman & Lippard, 1987).

The pH titration of the platinated dodecamer (Figure 2) clearly reveals two sharp transitions occurring in the pH* range 7.0–9.0, resulting from reduction in pK_a of both guanine N1 protons due to platinum coordination at N7 (Chu et al., 1978; Inagaki et al., 1979). This result conclusively demonstrates that the platinated product contains an intrastrand cross-link between the N7 atoms of the adjacent guanosine nucleosides. All four cytosine titrations at pH 4.5 are still observed, indicating that Pt has not coordinated to the N3 position of cytosine. The thymine H6 resonances exhibit pH behavior almost identical with that of 12-T; at pH values below 9.6, all five thymine resonances are resolved. *cis*-DDP coordination to thymine does not ordinarily occur at pH 6 and 37 °C (Martin & Mariam, 1979). At low pH, both adenine resonances begin to exhibit chemical shift changes characteristic

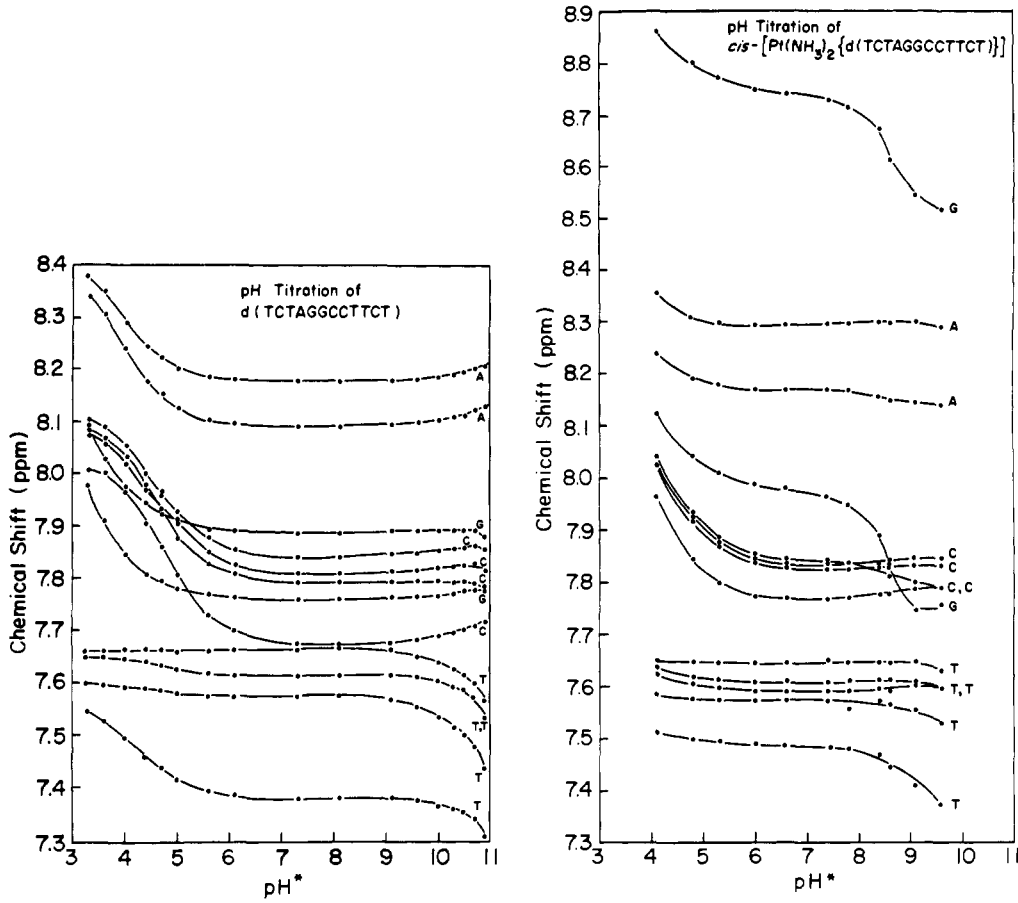

FIGURE 2: pH* dependence of the downfield nonexchangeable base proton NMR resonances of 12-T and Pt-12-T. Spectra were recorded at 65 °C.

of adenine N1 protonation. Spectra were not recorded at pH values below 4.0, however, to avoid possible degradation of the platinated oligonucleotide, which was later employed in biological experiments. If platinum coordination to adenine had occurred to form a *cis*-[$Pt(NH_3)_2${d(ApG)}] cross-link, only one guanine base would exhibit a lowered pK_a. Such an effect was observed in the case of one of several *cis*-[$Pt(NH_3)_2${d(GpApG)}] adducts (van der Veer et al., 1986) but clearly is not occurring here.

Construction of Precursor Genomes. The duplex dodecamer d(AGAAGGCCTAGA)·d(TCTAGGCCTTCT) was inserted into the unique *Hin*cII site of M13mp18 by a blunt-end ligation procedure (see Figure 3) to produce vectors denoted as M13-12A[2] and M13-12T. The excess of dodecamer used in the first ligation reaction produced linear M13mp18 molecules flanked at each end by a duplex dodecamer having only one strand covalently attached via the 5′-phosphoryl groups to the linear genome (note the nicks in Figure 3). The heating step employed during the isolation of these linear DNA molecules from the agarose gel also served to denature noncovalently associated dodecamer strands, resulting in genome-length molecules with 12-base overhanging ends. It should be noted that, owing to the non-self-complementary nature of the dodecamer sequences, the inserts can assume two different orientations at either end of the linear genome; consequently, only half of the resulting molecules have complementary overhanging ends. Only genomes with complementary ends can participate in an intramolecular ligation reaction. The genomes produced by this protocol were sensitive to *Stu*I cleavage and refractory to *Hin*cII, indicating correct placement of the inserts. Insert orientation was established by DNA sequencing. Both orientations, M13-12A and M13-12T, were found in the first 10 insert genomes sequenced.

Preparation and Characterization of the ds Genome Containing the Pt–DNA Adduct Specifically in the (−) Strand. Figure 4 outlines the strategy employed to situate Pt-12-T and 12-T within a gap in a heteroduplex genome. The phosphorylation efficiency of both Pt-12-T and 12-T was approximately 95%. The ATP:dodecamer ratio used in the polynucleotide kinase reaction optimized both the amount of label incorporated and the extent of phosphorylation, as determined by HPLC analysis (data not shown). Under the HPLC conditions employed, the phosphorylated dodecamer, whether platinated or unplatinated, eluted approximately 1 min before its corresponding unphosphorylated form. Ligations using 1 μg of gapped duplexes typically yielded (5–7) × 10^4 cpm of high molecular weight material. The products of one such ligation, M13-12A-Pt(−) and M13-12A-u(−), are shown in Figure 5 (lanes a).

Incorporation of a ^{32}P radiolabel in the fifth phosphodiester bond 5′ to the site of platination in the dodecamer (Figure 4) facilitated characterization of the adducted genome. To determine whether the dodecamer had ligated into the correct area of the genome, the position of the ^{32}P label was physically mapped by using pairs of restriction endonucleases. Following double digests with *Xba*I and *Sna*BI or with *Hin*dIII and *Sna*BI (Figure 5, lanes d and e, respectively), the radiolabel migrated with the fragment encompassing the polylinker region. Thus the position of the dodecamer was localized to the 36-base region delimited by the cleavage sites of *Xba*I and *Hin*dIII (Figure 3), confirming and extending preliminary results presented previously (Pinto et al., 1986).

Digestion of M13-12A-u(−) with *Stu*I yielded linear DNA, whereas M13-12A-Pt(−) was insensitive to cleavage (Figure 5, lanes b). This result indicates the presence of the platinum adduct within the recognition sequence of the enzyme, since

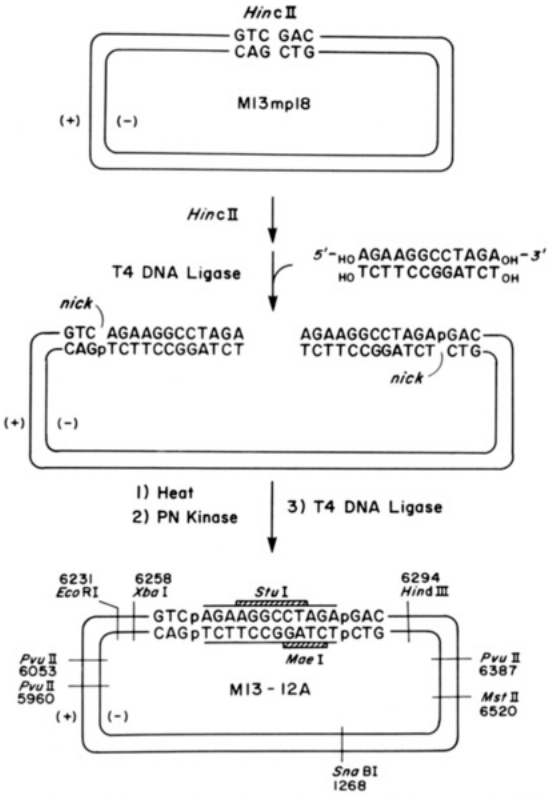

FIGURE 3: Scheme for insertion of dodecanucleotide d-(TCTAGGCCTTCT)·d(AGAAGGCCTAGA) into the *Hin*cII site of M13mp18 to produce the insertion mutant M13-12A as described in the text; a second insertion mutant, M13-12T, was also produced (not shown here), in which the duplex dodecanucleotide was ligated in the opposite orientation. Relevant restriction enzyme recognition sites are indicated on the M13-12A map.

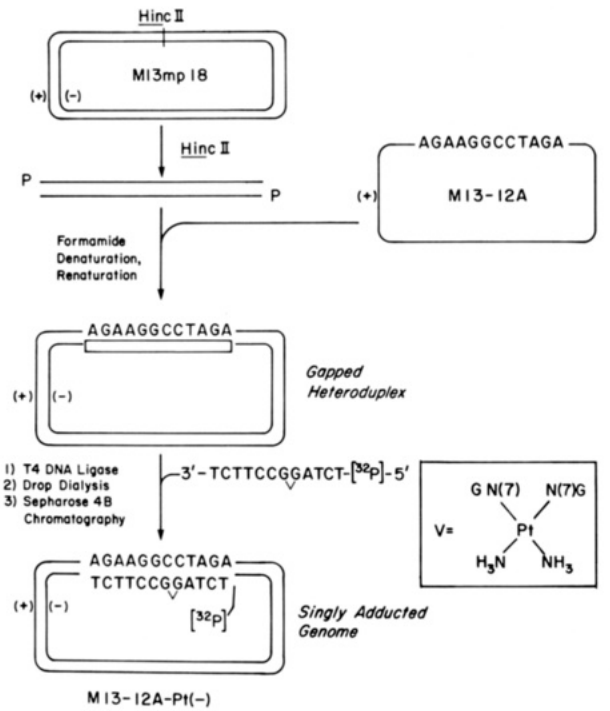

FIGURE 4: Scheme for gapped heteroduplex synthesis and subsequent ligation of the adducted dodecanucleotide to produce the site-specifically platinated ds genome M13-12A-Pt(-), as described in the text.

cis-DDP-platinated DNA is known to inhibit restriction enzyme digestion (Ushay et al., 1981). The platinum adduct did not directly inhibit the activity of the enzyme, as shown by the complete digestion of the nonradioactive, unplatinated

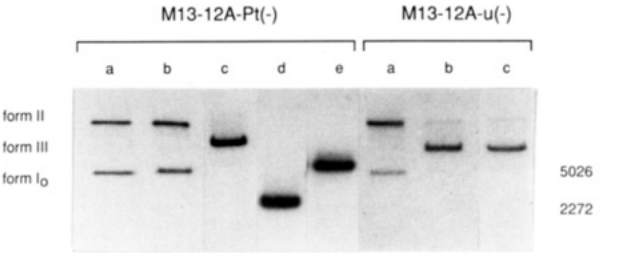

FIGURE 5: Characterization of M13-12A-Pt(−) and M13-12A-u(−) by restriction enzyme digestion. Shown is an autoradiogram of a 0.8% agarose gel containing 0.5 μg/mL EthBr. Lanes a: Uncut ligation products. Lanes b: *Stu*I-digested ligation products. Lanes c: Ligation products treated for 3 h with 0.3 M NaCN (pH 8.0) and digested with *Stu*I, as described in the text. Lane d: Ligation products digested with *Xba*I and *Sna*BI. Lane e: Ligation products digested with *Hin*dIII and *Sna*BI. All digestions were judged complete by EthBr fluorescence visualization of unlabeled M13-12A RF added to the digestions as an internal standard. The sites of migration of DNA forms I_o and III were determined by EthBr visualization of control M13-12A RF.

M13-12A RF DNA added as an internal control (data not shown) and by the fact that other restriction enzymes can digest M13-12A-Pt(−) (Pinto et al., 1986). Incubation of M13-12A-Pt(−) with cyanide released the platinum from DNA and rendered the molecule cleavable by *Stu*I (Figure 5, lanes c). Thiourea (50 mM for 1 or 4 h) did not remove the intrastrand *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link as evidenced by the retention of *Stu*I insensitivity (data no shown).

Determination of the Extent of Ligation at the 5′ and 3′ Ends of the Dodecamer. On the basis of the foregoing experiments, it was concluded that specific ligation of the platinated dodecamer into the 12-base gap had occurred at the desired position within the genome. We next determined whether the dodecanucleotide had ligated at both its 5′ and 3′ termini. Ligation products migrated as both form I_o and form II DNAs in an agarose dye gel (Figure 5, lanes a); both forms were visible in the dye gel itself and on the corresponding autoradiogram. Bands appearing on the autoradiogram represent DNA that has, by virtue of the ^{32}P radiolabel, incorporated a dodecamer and is therefore denoted M13-12A-Pt/u(−). Form I_o DNA is relaxed, covalently closed circular DNA that comigrates with the supercoiled genome in an agarose dye gel. The presence of labeled form I_o DNA indicates conclusively that the dodecanucleotide ligated into the genome at both ends. The form II (nicked circular) DNA visible in the autoradiogram could have arisen either from incomplete dodecamer ligation or from random nicks present elsewhere in the genome. These two possibilities were distinguished by the following experiment.

The M13-12A-Pt(−) and M13-12A-u(−) ligation products were digested with the enzyme *Pvu*II to produce a 334-base ds fragment containing the dodecanucleotide (Figure 6). The fragment was denatured with formamide and heat and electrophoresed on a denaturing 5% polyacrylamide gel, which was then autoradiographed. The presence of a single band corresponding to the 334-nt ss fragment indicates ligation occurred on both sides of the dodecanucleotide. Ligation exclusively at the 5′ terminus of the dodecamer leaves a nick on the 3′ side such that the radiolabel would be contained within a 223-nt ss fragment (Figure 6a). Conversely, if ligation occurred only on the 3′ terminus, the radiolabel would migrate with a 123-nt fragment. The results in Figure 6b show that, in both the platinated and unmodified genomes, approximately half of the label is associated with a 334-nt fragment and the remainder is equally distributed between the 123- and 223-nt fragments. Therefore, in half of the genomes the dodecamer

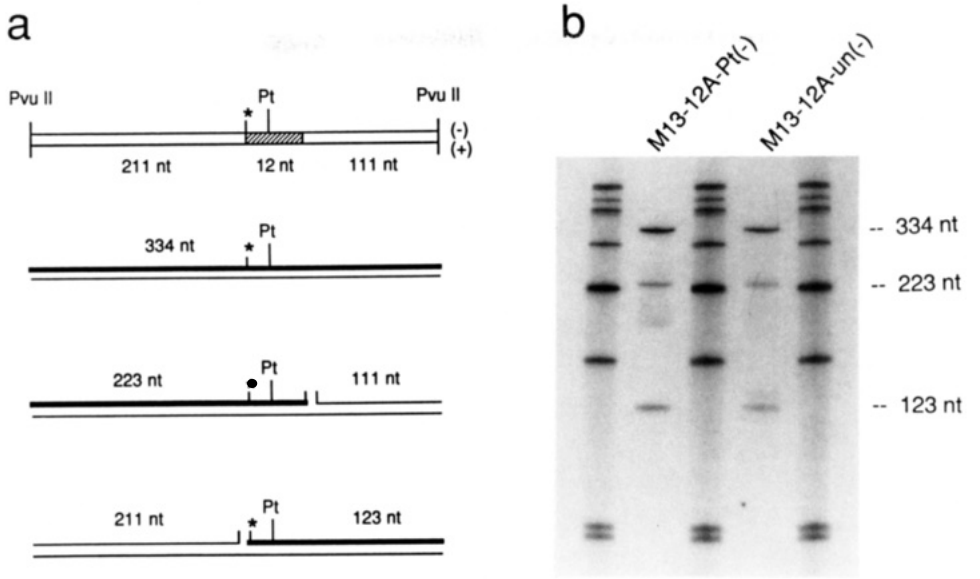

FIGURE 6: Determination of the extent of ligation at 5′ and 3′ termini of the platinated and unmodified dodecanucleotides into the gapped duplex M13-12A genome. Frame a: Map of the 344-bp *Pvu*II fragment encompassing the inserted dodecanucleotide. Bold lines in the lower three maps denote ss fragment sizes produced by ligation at both the 5′ and 3′ termini, on the 5′ terminus only, or on the 3′ terminus only. Pt denotes the site of platinum binding, if present; *, the site of the ^{32}P label; and nt, nucleotide. Frame b: Autoradiogram of a 5% denaturing polyacrylamide gel. Ligation products were digested with *Pvu*II, denatured with formamide and heat, and electrophoresed. Unlabeled lanes are ss molecular weight standards.

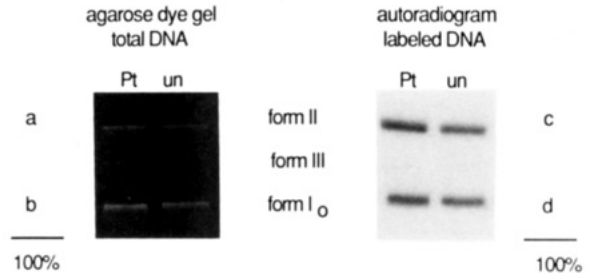

FIGURE 7: Agarose dye gel and corresponding autoradiogram used for ligation efficiency determinations, as described in the text. Pt is M13-12A-Pt(−), and un is M13-12A-u(−).

ligated on both the 5′ and 3′ sides. In the other half, ligation occurred only on one side of the dodecamer.

Determination of Ligation Efficiency. Although, by definition, all DNA visible in the autoradiogram has incorporated labeled dodecamer, the same need not be true for DNA visible in the agarose dye gel (see Figure 7). If the ligation efficiency, defined as the percent of gapped duplexes having a dodecamer (either fully or partially ligated) incorporated into the 12-base gap, were less than 100%, then residual gapped duplexes would appear as unlabeled form II DNA. Densitometry was used to determine the ligation efficiency, i.e., whether all DNA visible in the form II band in the agarose dye gel had incorporated a dodecamer.

The method relies on the assumption that all DNA visible as form I_o in the agarose dye gel is M13-12A-Pt(−)/u(−) (Figure 7). Work in our laboratory has shown that a ligation event can occur between the 5′ and 3′ ends of the (−) strand of a four- or six-base gapped duplex molecule (termed "gapped duplex ligation") and that the resultant DNA migrates with form I_o in an agarose dye gel (E. L. Loechler and A. K. Basu, unpublished observations). To test whether such a ligation occurred with a 12-base gap, a mock ligation using only gapped duplex DNA and no dodecamer was carried out, and the products were electrophoresed through an agarose dye gel. No form I_o material was detected, even when the gel was intentionally overloaded (data not shown). It is concluded, therefore, that ligation does not detectably occur across the 12-base gap. This result thus establishes that all DNA appearing as form I_o in the dye gel is M13-12A-Pt(−)/u(−). Therefore, form I_o bands visible in the dye gel and autoradiogram represent identical DNA molecules.

To determine whether form II DNA in the agarose dye gel bands was solely M13-12A-Pt(−)/u(−) or a mixture of ligation products and residual gapped duplexes, relative band intensities for forms II and I_o in corresponding lanes in the agarose gel and autoradiogram were determined by densitometry and normalized such that the intensities of form II + form I_o = 100 in each lane (Figure 7). The ratio of band intensities of form II and form I_o in the autoradiogram (c/d using the designations in Figure 7) defines the ratio of these forms of M13-12A-Pt(−)/u(−), both labeled and unlabeled, in the ligation products. Therefore, this same ratio should obtain for DNA present in the agarose dye gel if the ligation efficiency were 100% (i.e., $a/b = c/d$). Any increase in the form II:form I_o ratio for the agarose dye gel versus the autoradiogram would be due to excess form II DNA present in the former, which could only result from the presence of residual gapped duplex DNA. Denoting this excess by x gives $(a - x)/b = c/d$. Since x is the percent of gapped duplexes that did not incorporate a dodecamer, then $100 - x$ is the ligation efficiency. According to this method it was shown that the ligation efficiency for both M13-12A-Pt(−) and M13-12A-u(−) was ≈80% (average of three determinations).

Stability of a Platinated Duplex Fragment As Measured by Gradient Denaturing Gel Electrophoresis. A series of denaturing gradient gel experiments (Fischer & Lerman, 1983; Lerman et al., 1984) was performed in order to assess the effects of a single *cis*-[$Pt(NH_3)_2${d(GpG)}] adduct on the melting behavior of DNA. Figure 8 displays an autoradiogram of a 35–55% denaturant containing gel of the 298-bp *Eco*RI/*Mst*II fragment of M13-12A-Pt(−) and M13-12A-u(−). The platinated fragment clearly melts at a lower denaturant concentration than the unmodified fragment. Localized melting of the duplex around the *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link could provide a nucleation site for the cooperative melting transition that is not found in the unplatinated DNA. Observation of weakened duplex stability due to platinum

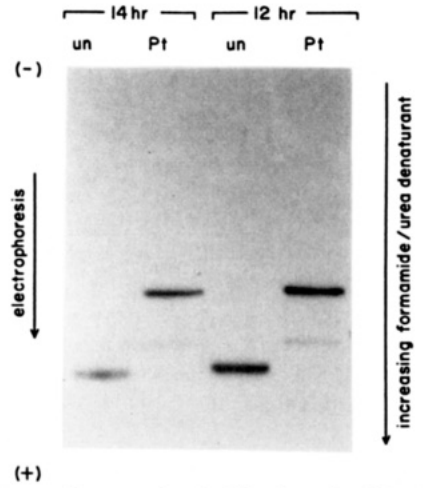

FIGURE 8: Autoradiogram of a 12.5% polyacrylamide gel containing a gradient of 35–55% denaturant (100% denaturant = 7 M urea, 40% formamide) parallel to the direction of electrophoresis. The 289-bp *Eco*RI/*Mst*II fragments of M13-12A-u(−) (un) and M13-12A-Pt(−) (Pt) were electrophoresed at 150 V for 12 or 14 h as indicated.

coordination is consistent with anti-nucleoside antibody experiments (Sundquist et al., 1986) and NMR studies of the imino protons of duplex oligonucleotides (den Hartog et al., 1984; van Hemelryck et al., 1984). This difference in mobility is clearly a melting phenomenon and not the result of charge or some other effect, as evidenced by the fact that no difference in migration of the DNA fragment occurs between the 12- and 14-h electrophoresis times.

Effect of a Single cis-[$Pt(NH_3)_2${d(GpG)}] *Intrastrand Adduct on M13 Viability in Cells.* To investigate the biological consequences of the *cis*-[$Pt(NH_3)_2${d(GpG)}] adduct, a ds genome, in which the adduct is situated specifically in the (−) strand, and comparable genomes containing the cross-link in the (+) strand of ds and ss DNA were constructed. The former was prepared as described above. The latter was made as outlined in Figure 9. Briefly, hybridization between *Hinc*II-linearized M13mp18 RF DNA and dephosphorylated *Bgl*II-linearized M13-12T RF DNA produced a mixed population of gapped duplexes having a 12-base gap in either the (+) or (−) strand. Due to the non-self-complementary nature of the dodecamer sequences, only those genomes having the gap in the (+) strand can successfully incorporate Pt-12-T or 12-T. It was then possible to produce genomes M13-12T-Pt(+) and M13-12T-u(+), in which the dodecamers reside exclusively in the (+) strand. Owing to the presence of a nonligatable nick at the *Bgl*II site in the DNA strand opposite that containing the adduct, ligation products migrate only as form II DNA in an agarose dye gel. Treatment with *Stu*I [after cyanide reversal in the case of M13-12T-Pt(+)] linearized only those genomes that had incorporated a dodecamer. The efficiency of the ligation reaction, determined by the amount of DNA linearized upon *Stu*I digestion, was almost 50%, which is close to the theoretical maximum. As previously discussed, ligation across the 12-base gap does not occur without the incorporation of a dodecamer. This result confirms that no covalently closed material is formed by the fraction of gapped heteroduplex genomes that cannot incorporate an oligonucleotide, i.e., those with a gap in the (−) strand. Therefore, denaturation of heteroduplexes with a gap in the (−) strand produces only ss linear DNA, which is inactive in vivo. Double-stranded M13-12T-Pt(+)/u(+), in contrast, yielded biologically viable circular ss M13-12T-Pt(+)/u(+) and a nonviable linear (−) strand. Examination of a portion of the DNA on an agarose gel confirmed that denaturation was complete.

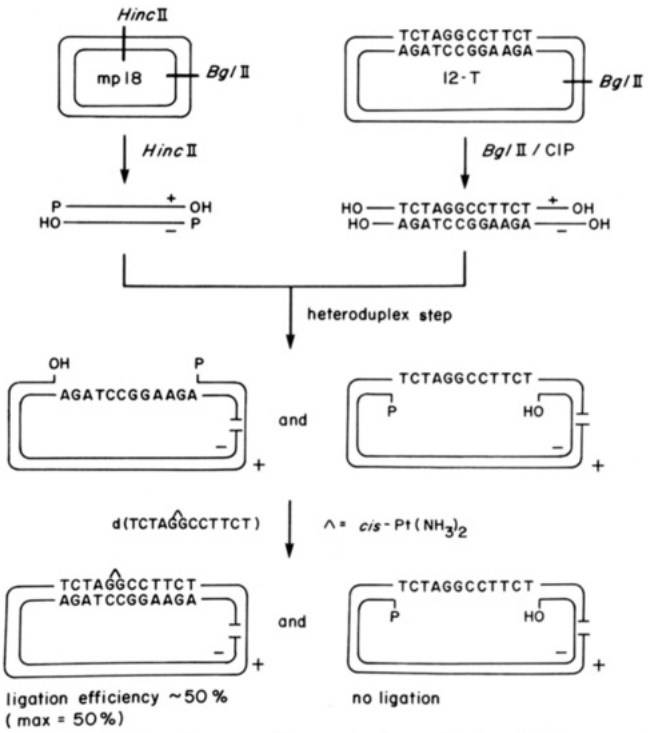

FIGURE 9: Scheme for gapped heteroduplex synthesis and subsequent ligation of the adducted dodecanucleotide to produce the site-specifically platinated ds and ss genomes M13-12A-Pt(+), as described in the text.

Table I: Survival of Singly Adducted Single-Stranded Phage DNA in *E. coli*

expt	no. of infective centers		% survival
	ss M13-12T-u(+)	ss M13-12T-Pt(+)	
I	2905	335	11.5
II	6291	655	10.4
III	5103	552	10.8

The genotoxic potential of the *cis*-[$Pt(NH_3)_2${d(GpG)}] intrastrand adduct in ds and ss site-specifically platinated genomes was evaluated in wild-type *E. coli*. There was no difference in survival between ds M13-12T-Pt(−) and ds M13-12T-u(−) as assayed in competent MM294A cells, nor between ds M13-12T-Pt(+) and ds M13-12T-u(+) as assayed in competent DL7 cells. There was markedly reduced viability of the platinated ss genome M13-12T-Pt(+), however, as compared with ss M13-12T-u(+) in DL7 cells (Table I). In three separate experiments, the number of infective centers produced by transformation with ss M13-12T-Pt(+) DNA was only 10–12% of that produced by transformation with the same amount of ss M13-12T-u(+).

Discussion

Construction of viral genomes containing the major *cis*-DDP–DNA adduct, the *cis*-[$Pt(NH_3)_2${d(GpG)}] intrastrand cross-link, was accomplished by ligation of a dodecanucleotide containing the adduct at a specific site into a gapped duplex M13 genome. A dodecanucleotide was chosen for several reasons. Insertion of the 12-base sequence into the cloning region of M13mp18 preserves the *lacZ* reading frame, ensuring a functional β-galactosidase protein. It was anticipated that a short (four to six base) platinated oligonucleotide, similar to those previously employed (Green et al., 1984; Lasko et al., 1987; Basu et al., 1987), might not efficiently ligate into a gapped genome because of the severe structural distortions imposed on the DNA by *cis*-DDP binding, as evidenced by

helix unwinding (Cohen et al., 1979), reduction in the degree of Watson–Crick base pairing (den Hartog et al., 1984; van Hemelryck et al., 1984; Sundquist et al., 1986), and the 90° dihedral angle between the coordinated guanine bases in a *cis*-[$Pt(NH_3)_2${d(pGpG)}] cross-link (Sherman et al., 1985). A 12-base oligonucleotide was chosen to provide the structural insulation believed necessary for ligation. The sequence was designed to position the *cis*-[$Pt(NH_3)_2${d(GpG)}] target centrally within the dodecamer and to provide a restriction site (*Stu*I) unique to the M13-12A genome. Location of the adduct within the *Stu*I site affords a powerful tool for characterizing the platinated genome, and for mutant selection in forthcoming mutagenicity studies. Pyrimidines were selected to flank the *Stu*I sequence to minimize the number of platination targets. Although both GG and AG sequences are potential platinum binding sites in the d(TCTAGGCCTTCT) dodecamer employed, the *cis*-[$Pt(NH_3)_2${d(GpG)}] adduct is the major product of the reaction. The *cis*-[$Pt(NH_3)_2${d(GpG)}] G(N7)–G(N7) structure of the platinated dodecamer was established by pH-dependent NMR studies. A chemically well characterized platinated dodecanucleotide of high purity was thus created for use in preparing site-specifically modified genomes.

Ligation of platinated and unmodified dodecamers into the 12-base gap was demonstrated by the experiments presented in Figure 5 and in our earlier preliminary communication (Pinto et al., 1986). The *cis*-[$Pt(NH_3)_2${d(GpG)}] adduct is located in the recognition sequence for the restriction enzyme *Stu*I and overlaps a *Mae*I restriction site by one nucleotide, and its presence inhibited cleavage by both of these enzymes. This result is consistent with an earlier study of *Bam*HI cleavage of globally platinated pBR322 DNA in which it was estimated that restriction enzyme activity would be inhibited ±3 base pairs from the site of a platinum cross-link (Ushay et al., 1981). That this inhibition was due to the platinum adduct was demonstrated by removal of the adduct with cyanide, which restored the sensitivity of the genome to both enzymes. Cyanide and thiourea (Filipski et al., 1979) treatments have been described in the literature for removal of DNA-bound platinum. The lower thiourea concentration (50 mM) commonly used to reduce *cis*-DDP toxicity in cell culture experiments was completely ineffective at reversing the intrastrand *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link, whereas removal by cyanide was complete. The ability to remove completely the platinum adduct by cyanide reversal is analogous to the enzymatic removal of O^6-methylguanine by O^6-methylguanine methyltransferase (Green et al., 1984; Couto et al., 1986). This reversibility provides a tool for directly correlating adduct presence with biological activity.

Interestingly, both platinated and unplatinated dodecamers ligated into the gapped duplexes with similar, high efficiencies. The extent of ligation on either end of the dodecamers was also similar; however, only half of the incorporated oligonucleotides formed covalent bonds at both termini. This result is in contrast to previous results from our laboratory using tetranucleotides containing O^6-methylguanine or *N*-(deoxyguanosin-8-yl)-4-aminobiphenyl and a hexanucleotide containing 1,N^6-ethenoadenine (Green et al., 1984; Lasko et al., 1987; Basu et al., 1987). In each case the adducted oligonucleotide ligated into gapped genomes completely (i.e., on both the 5′ and 3′ ends), albeit with overall efficiencies less than half that of the corresponding unmodified oligonucleotide, a result attributed to the presence of the adduct. The reason for incomplete ligation of 12-T and Pt-12-T at their 5′ and 3′ termini is unknown.

The disrupting effect of a single *cis*-[$Pt(NH_3)_2${d(GpG)}] adduct on duplex stability was demonstrated by gradient denaturing gel electrophoresis in which a singly platinated restriction fragment melted at a lower denaturant concentration than did the corresponding unmodified fragment. These data are consistent with the failure of *Stu*I and *Mae*I to cleave the genome, as described above. It is noteworthy, however, that disruption of DNA structure was not so severe as to prohibit DNA restriction at sites farther removed from the adduct nor ligation of the platinated oligonucleotide into the genome.

The survival of singly adducted genomes upon introduction into *E. coli* was studied to evaluate the genotoxic potential of the *cis*-[$Pt(NH_3)_2${d(GpG)}] intrastrand adduct. The number of infective centers produced by transformation with ss M13-12T-Pt(+) was only 10–12% of that produced by the unmodified genome, providing compelling evidence that a solitary *cis*-[$Pt(NH_3)_2${d(GpG)}] cross-link can be a lethal event in a single-stranded bacteriophage. Presumably, the cross-link interferes with conversion of ss to RF DNA in the first stage of the M13 life cycle by presenting a block to the synthesis of the (−) strand. It cannot be ruled out, however, that a cellular activity may have linearized the ss genome in an attempt at repair, thereby rendering the adducted genome biologically inactive. Although *cis*-DDP adducts are substrates for the UvrABC excision repair system (Sancar & Rupp, 1983; Beck et al., 1985), it has been shown that the UvrABC complex is not active on ss DNA (A. Sancar, personal communication). The most likely explanation for the limited survival of the site-specifically adducted genomes is bypass of the lesion by DNA polymerase.

No difference in survival was seen between the platinated and unplatinated ds vectors M13-12A-Pt/u(−) or M13-12T-Pt/u(+). The lack of a difference in survival between singly adducted and unplatinated duplex genomes may be the result of adduct repair. The cells used were uvr^+ and $recA^+$, and both UvrABC and RecA are involved in *cis*-DDP repair (Beck & Brubaker, 1973; Markham & Brubaker, 1980; Konishi et al., 1981). Another possible explanation is that a bias may exist toward replication of the unadducted strand, as shown by Koffel-Schwartz et al. (1987) for replication of genomes containing aromatic amine residues.

Abasic sites are highly lethal to ss bacteriophage (Schaaper & Loeb, 1981). There has been speculation that coordination of platinum to the N7 position of guanine in DNA weakens the glycosidic bond and promotes depurination (Wing et al., 1984). This prediction is unsupported by evidence, however, since *cis*-DDP does not induce depurination in vitro (Royer-Pokora et al., 1981; Ciccarelli and Lippard, unpublished data); moreover, in the present study, Pt-12-T was unaffected by the wide range of pHs used during the NMR titration experiments. Accordingly, unless there is a specific, as yet undescribed, cellular activity that removes platinated bases from DNA in vivo, it is unlikely that abasic sites are key intermediates in the lethal or mutagenic actions of *cis*-DDP.

The multiplicity of DNA adducts formed upon treatment of cells with *cis*-DDP complicates identification of lesions responsible for the genotoxic effects of the drug. For *cis*-DDP and other drugs that act on DNA, it is highly desirable to assess the contribution of each DNA adduct to overall biological activity. In the experiments presented here, the construction of a viral genome containing *cis*-[$Pt(NH_3)_2${d(GpG)}] at a unique site permitted determination of the effect of this specific modification on viral survival and facilitated evaluation of some of the effects of the adduct on DNA structure. These data provide a standard against which the structural and

biological effects of the other *cis*-DDP adducts will be compared. This approach, in combination with forthcoming parallel studies on the other DNA adducts of *cis*-DDP, should help identify the structural features in *cis*-DDP–DNA interactions that are important in antitumor activity.

ACKNOWLEDGMENTS

We thank Engelhard Corp. for a generous loan of K_2PtCl_4 from which *cis*-DDP was synthesized. We are indebted to Neal Cariello for his advice on the gradient denaturing gel experiments.

Registry No. *cis*-$[Pt(NH_3)_2Cl_2]$, 15663-27-1; *cis*-$[Pt(NH_3)_2\{d(GpG)\}]$, 97333-55-6; *cis*-$[Pt(NH_3)_2(H_2O)_2]$, 20115-64-4; d-(TCTAGGCCTTCT), 104833-97-8.

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Interaction of an N-Methylated Polyamine Analogue, Hexamethonium(2+), with NaDNA: Quantitative ^{14}N and ^{23}Na NMR Relaxation Rate Studies of the Cation-Exchange Process†

S. Padmanabhan, B. Richey, C. F. Anderson, and M. Thomas Record, Jr.*
Departments of Chemistry and Biochemistry, University of Wisconsin—Madison, Madison, Wisconsin 53706
Received December 9, 1987; Revised Manuscript Received February 8, 1988

ABSTRACT: The interactions of the divalent hexamethonium (Hex^{2+}) cation with double-helical calf thymus DNA are investigated by means of ^{14}N NMR and, indirectly, by means of ^{23}Na NMR. During a titration of NaDNA with $HexBr_2$, the displacement of Na^+ from DNA by Hex^{2+} is monitored by concurrent measurements of the Lorentzian ^{14}N signals and the bi-Lorentzian ^{23}Na signals. The variations in the quadrupolar relaxation rates of ^{14}N and ^{23}Na are analyzed according to a simple two-state model for the competition between Hex^{2+} and Na^+ associated with DNA. From this analysis parameters characterizing the exchange process are evaluated, and the following conclusions are drawn: (1) The association of one Hex^{2+} displaces 1.7–2.0 sodium ions from the vicinity of the DNA. (2) Cation accumulation near DNA neutralizes approximately half of the phosphate charge at all points in the titration. (3) The exchange coefficient characterizing the displacement of Na^+ by Hex^{2+} is of the same order of magnitude as the exchange coefficients determined by NMR for other divalent cations such as Mg^{2+} and putrescine. These findings imply that the interaction of Hex^{2+} with DNA is primarily electrostatic in character. The transverse and longitudinal relaxation rates observed for ^{14}N are analyzed under the assumption that the quadrupolar relaxation processes of ^{14}N in Hex^{2+} associated with DNA can be characterized by a single-exponential correlation function with correlation time τ_{NB}. The resulting value of τ_{NB}, 7.8 ± 0.8 ns, is 3 orders of magnitude greater than that estimated for Hex^{2+} in the absence of DNA and is only 3–4 times greater than correlation times reported for ^{23}Na and other quadrupolar cations near DNA. These comparisons indicate that the observed enhancements in the relaxation rates of ^{14}N are due mainly to slowing of the motions that modulate its quadrupolar interactions in Hex^{2+} near DNA. The magnitudes of τ_{NB} and of the quadrupolar coupling constant of Hex^{2+} associated with DNA are consistent with the conclusion that this association is primarily electrostatic.

The polyamines putrescine(2+), cadaverine(2+), spermidine(3+), spermine(4+), etc. are important biosynthetic oligocations, which generally are found at high total concentrations (in the millimolar range) in both prokaryotic and eukaryotic cells. Though polyamines are necessary for normal cell growth, little is known about their roles in gene expression and other cellular processes (Cohen, 1971; Tabor & Tabor, 1976, 1984, 1985; Jänne et al., 1978; Morris & Marton, 1981). Electrostatic interactions of these oligocations with the highly charged biological polyanions DNA and RNA must play a major role in the stabilization of native structures and in processes such as DNA collapse/condensation. In addition, competitive ion-exchange processes involving associated polyamines must modulate the affinities of all other ligands, including proteins, that bind to DNA or RNA. The present study focuses on the competitive electrostatic interactions of a divalent polyamine and Na^+ with DNA and provides quantitative information about the competitive function of these oligocations in modulating ligand–nucleic acid interactions.

Equilibrium dialysis studies indicate no specificity in the association of polyamines with double-helical DNA (Hirschman et al., 1967; Shapiro et al., 1969). Furthermore, the magnitudes and salt dependences of the apparent DNA-binding constants are consistent with those expected for predominantly electrostatic interactions (Braunlin et al., 1982). Measurements of the ^1H nuclear Overhauser enhancements of spermine and its complex with DNA indicate that this tetravalent polyamine retains significant motional freedom when associated with DNA (Wemmer et al., 1985). This finding is consistent with delocalized, electrostatic association rather than the formation of a specific complex of the type

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* To whom correspondence should be addressed at the Department of Chemistry.

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