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Replication Inhibition and Translesion Synthesis on Templates Containing Site-Specifically Placed *cis*-Diamminedichloroplatinum(II) DNA Adducts†

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ABSTRACT: A series of site-specifically platinated, covalently closed circular M13 genomes (7250 bp) was constructed in order to evaluate the consequences of DNA template damage induced by the anticancer drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP). Here are reported the synthesis and characterization of genomes containing the intrastrand cross-linked adducts *cis*-[Pt(NH_3)$_2${d(ApG)-*N*7(1),-*N*7(2)}], *cis*-[Pt(NH_3)$_2${d(GpCpG)-*N*7(1),-*N*7(3)}], and *trans*-[Pt(NH_3)$_2${d(CpGpCpG)-*N*3(1),-*N*7(4)}]. These constructs, as well as the previously reported M13 genome containing a site-specifically placed *cis*-[Pt(NH_3)$_2${d(GpG)-*N*7(1),-*N*7(2)}] adduct, were used to study replication in vitro. DNA synthesis was initiated from a position approximately 177 nucleotides 3′ to the individual adducts, and was terminated either by the adducts or by the end of the template, located approximately 25 nucleotides on the 5′ side of the adducts. Analysis of the products of these reactions by gel electrophoresis revealed that, on average, bypass of the *cis*-DDP adducts occurred approximately 10% of the time and that the *cis*-[Pt(NH_3)$_2${d(GpG)-*N*7(1),-*N*7(2)}] intrastrand cross-link is the most inhibitory lesion. The *cis*-[Pt(NH_3)$_2${d(GpCpG)-*N*7(1),-*N*7(3)}] adduct allowed a higher frequency of such translesion synthesis (ca. 25%) for two of the polymerases studied, modified bacteriophage T7 polymerase and *Escherichia coli* DNA polymerase I (Klenow fragment). These enzymes have either low (Klenow) or no (T7) associated 3′ to 5′ exonuclease activity. Bacteriophage T4 DNA polymerase, which has a very active 3′ to 5′ exonuclease, was the most strongly inhibited by all three types of *cis*-DDP adducts, permitting only 2% translesion synthesis. This enzyme is therefore recommended for replication mapping studies to detect the location of *cis*-DDP–DNA adducts in a heterologous population. The major replicative enzyme of *E. coli*, the DNA polymerase III holoenzyme, allowed <10% adduct bypass. Postreplication restriction enzyme cleavage studies established that the templates upon which translesion synthesis was observed contained platinum adducts, ruling out the possibility that the observed products were due to a small amount of contamination with unplatinated DNA. The effects on in vitro replication of a recently characterized adduct of *trans*-DDP [Comess, K. M., Costello, C. E., & Lippard, S. J. (1990) *Biochemistry 29*, 2102-2110] were also evaluated. This adduct provided a poor block both to DNA polymerases and to restriction enzymes. The properties of this adduct in the M13 genome were investigated by postreplication sequence analysis of the translesion synthesis product. Taken together, these studies demonstrate that polymerases can traverse through all of the major bifunctional cisplatin adducts formed in vitro and in vivo and strengthen the hypothesis that adduct-induced mutagenesis may occur through replication bypass.

The antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DPP),[1] as well as the clinically inactive isomer *trans*-diamminedichloroplatinum(II) (*trans*-DDP), form bifunctional covalent adducts with the nucleobases of DNA. These interactions are believed to contribute to cytotoxicity by inhibiting replication or transcription [general reviews: Fichtinger-Schepman et al. (1986), Umapathy (1989), and Bruhn et al. (1990)]. Studies of the biological responses to platinum(II) adducts have been restricted primarily to the full spectrum of adducts produced by platination of a heterogeneous population of DNA sequences, previously termed "global

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[1] Abbreviations: *cis*-DPP, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); NMR, nuclear magnetic resonance; RP-HPLC, reversed-phase high-performance liquid chromatography; RE, restriction endonuclease; RF, replicative or double-stranded form of M13 genomes; ss, single-stranded or viral form of M13 genomes (this strand is always the plus strand); EDTA, ethylenediaminetetraacetate dianion; PNK, polynucleotide kinase; ATP, adenosine triphosphate; TE, 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA; HAP, hydroxylapatite; T7 pol, modified bacteriophage T7 DNA polymerase (Sequenase 2.0); Taq pol, *Thermus aquaticus* DNA polymerase; T4 pol, bacteriophage T4 DNA polymerase; pol I, *Escherichia coli* DNA polymerase I large fragment; pol III, *Escherichia coli* DNA polymerase III holoenzyme.

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Oligonucleotide	Platinum Adduct	Restriction Site	Nomenclature: Oligonucleotide	Nomenclature: M13 Genome
d(TCTAGGCCTTCT)	none	d(AGGCCT) (StuI)	un-Pt-GG	M13-un-Pt-GG, M13-StuI
" "	*cis*-[$Pt(NH_3)_2${d(GpG)-*N*7(5), -*N*7(6)}]	StuI	*cis*-Pt-GG	M13-*cis*-Pt-GG
d(TCTCTAGTACTC)	none	d(AGTACT) (ScaI)	un-Pt-AG	M13-un-Pt-AG, M13-ScaI
" "	*cis*-[$Pt(NH_3)_2${d(ApG)-*N*7(6), -*N*7(7)}]	ScaI	*cis*-Pt-AG	M13-*cis*-Pt-AG
d(TCTACGCGTTCT)	none	d(ACGCGT) (MluI)	un-Pt-GCG	M13-un-Pt-GCG, M13-MluI
" "	*cis*-[$Pt(NH_3)_2${d(GpCpG)-*N*7(6), -*N*7(8)}]	MluI	*cis*-Pt-GCG	M13-*cis*-Pt-GCG
" "	*trans*-[$Pt(NH_3)_2${d(CpGpCpG)-*N*3(5), -*N*7(8)}]	MluI	*trans*-Pt-CGCG	M13-*trans*-Pt-CGCG

FIGURE 1: Dodecanucleotide complexes and M13 genomes used in these studies. The alternative nomenclature given for unplatinated M13 genomes refers to material created through clonal insertion of the duplexed unplatinated oligonucleotides. These altered genomes are used in the construction of sequencing ladders, as described in Figure 4.

platination" (Pinto et al., 1986). The most abundant adducts (~90%) formed by *cis*-DDP are *cis*-[$Pt(NH_3)_2${d(GpG)-*N*7(1),-*N*7(2)}] and *cis*-[$Pt(NH_3)_2${d(ApG)-*N*7(1),-*N*7(2)}] intrastrand cross-links (Sherman & Lippard, 1987). These adducts also form in cultured cells in leukocytes from cancer patients undergoing cisplatin chemotherapy (Fichtinger-Schepman et al., 1987). The spectrum of DNA adducts of *trans*-DDP is less well characterized (Pinto & Lippard, 1985; Eastman et al., 1988). Interstrand cross-links comprise only a small fraction (~5%) of adducts formed by the two platinum isomers.

Biological studies involving single, site-specifically placed adducts have also been performed. An M13 viral genome containing a single *cis*-[$Pt(NH_3)_2${d(GpG)-*N*7(1),-*N*7(2)}] intrastrand cross-link (Pinto et al., 1986) reduced survival in a wild type *Escherichia coli* host by approximately 90% (Naser et al., 1988). A cis-[$Pt(NH_3)_2${d(ApG)-*N*7(1),-*N*7(2)}] adduct has been placed site-specifically into a plasmid and its mutagenicity evaluated (Burnouf et al., 1990). A mutation frequency of 1–2% was observed following introduction of this plasmid into *E. coli* cells in which the SOS system had been induced. In addition, polymers of site-specifically platinated oligonucleotides have been used to study transcription inhibition (Corda et al., 1991), bacterial repair (Page et al., 1990) and interaction by mammalian damage-specific DNA-binding proteins (Donahue et al., 1990).

In the present paper we report the construction and characterization of several new M13 genomes containing the following site-specifically placed platinum adducts: *cis*-[$Pt(NH_3)_2${d(ApG)-*N*7(1),-*N*7(2)}], *cis*-[$Pt(NH_3)_2${d(GpCpG)-*N*7(1),-*N*7(3)}], and *trans*-[$Pt(NH_3)_2${d(CpGpCpG)-*N*3(1),-*N*7(4)}]. These genomes have been used, in conjunction with a previously reported genome containing a site-specifically placed *cis*-[$Pt(NH_3)_2${d(GpG)-*N*7(1),-*N*7(2)}] adduct (Pinto et al., 1986; Naser et al., 1988), to assess the ability of DNA polymerases of varied function, fidelity, and processivity to block and/or bypass the adducts.

Materials and Methods

Materials. Oligodeoxyribonucleotides were prepared on a Biosearch 8000 series DNA synthesizer employing β-cyanoethyl phosphoramidite chemistry and 15-μmol scale 500-Å controlled pore glass supports from Biosearch Inc. (San Rafael, CA). Alternatively, oligodeoxyribonucleotides were purchased from Operon Technologies Inc. (Alameda, CA) or New England Biolabs (reverse sequencing primer no. 1233) (Beverly, MA). Restriction enzymes, except for *Hin*dIII, were purchased from Boehringer Mannheim. *Hin*dIII at high concentration (20 units/μL) was obtained from New England Biolabs, as were T4 polynucleotide kinase (PNK) and T4 DNA ligase. Two cell lines were used, *E. coli* GW5100 (JM103 P1⁻, from G. Walker, MIT), and *E. coli* MM294A (from K. Backman, Biotechnia International). Cloned T7 DNA polymerase, genetically modified to inactivate the 3′ to 5′ exonuclease activity (T7 pol), was purchased from U.S. Biochemicals (Sequenase 2.0). Taq DNA polymerase from *Thermus aquaticus* BM (Taq pol) and T4 DNA polymerase (T4 pol) were obtained from Boehringer Mannheim. *E. coli* DNA polymerase I large (Klenow) fragment (pol I) was obtained from New England Biolabs. *E. coli* DNA polymerase III holoenzyme (pol III) was provided as a gift by C. S. McHenry.

Platination Reactions. The oligonucleotides (Figure 1) were synthesized, cleaved from the solid support with 15 N NH_4OH and then deprotected by heating for 15–18 h at 55 °C in 15 N NH_4OH. After lyophilization, the samples were desalted and protecting groups were removed by Sephadex G-10 chromatography. The desired products were purified by ion-exchange chromatography on a DE-52 column (Whatman LabSales) with a linear gradient of 0.075–0.75 M triethylammonium bicarbonate/20% ethanol; detection was with an ISCO (Lincoln, NE) Model UA-5 absorbance monitor set to

254 nm. Further purification was carried out by using C18 reversed-phase HPLC (RP-HPLC) (Waters μ-Bondapak) with linear gradients of acetonitrile in 0.1 M ammonium acetate.

The *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ cation was prepared by stirring a solution of *cis*-$[Pt(NH_3)_2Cl_2]$ (5.0 mg, 0.017 mmol) with 1.98 equiv of $AgNO_3$ overnight in the dark. Precipitated AgCl was removed by centrifugation and the supernatant was diluted to a final concentration of 1×10^{-4} M *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ in water. The unplatinated oligomer, un-Pt-GCG (Figure 1), was dissolved in water to a concentration of 5×10^{-4} M strand and allowed to react with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ at a formal platinum/strand ratio (r_f) of 1.5 at room temperature for 2.5 h. Under the RP-HPLC conditions indicated above, the major product (*cis*-Pt-GCG) eluted at 22.8 min and was isolated in large scale (ca. 2–3 μmol) for characterization. Un-Pt-AG was most effectively platinated with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$ at 5×10^{-4} M strand, $r_f = 1.2$ at 47 °C, also in unbuffered aqueous solution (pH ~ 6.5). After 45 min most of the starting material has been consumed and three products were visible at RP-HPLC retention times of 25.8, 30, and 34 min. The principal product (*cis*-Pt-AG), eluting at 25.8 min, was isolated on a large scale and characterized. The *cis*-Pt-GG complex and 1,4-intrastrand cross-linked isomer of the *trans*-Pt-CGCG complex were synthesized by using *cis*-DDP or *trans*-DDP, respectively, and were purified as described previously (Pinto et al., 1986; Comess et al., 1990).

Enzymatic Degradation and pH-Dependent ^1H NMR Studies. Digestions to yield mononucleosides and platinated nucleosides for subsequent RP-HPLC analyses were carried out by using DNase I (Sigma, from bovine pancreas), nuclease P1 (Boehringer Mannheim, from *Penicillium citrinum*), and alkaline phosphatase (Boehringer Mannheim, from calf intestine) as previously described (Eastman, 1986; Comess et al., 1990). Digested platinated and unplatinated oligonucleotides were analyzed by RP-HPLC. *cis*-$[Pt(NH_3)_2\{d(GpG)$-*N*7-G(1),*N*7-G(2)$\}]$, *cis*-$[Pt(NH_3)_2\{d(ApG)$-*N*7-A(1),*N*7-G(2)$\}]$, and *cis*-$[Pt(NH_3)_2\{d(Guo)_2$-*N*7-G(1),*N*7-G(2)$\}]$ dinucleotide standards for coinjection were provided by S. F. Bellon and M. V. Keck of our laboratory.

Platinum binding sites were determined by pH-dependent ^1H NMR (500 MHz) studies as described (Caradonna et al., 1982; Comess et al., 1990). Experiments were performed on a Varian VXR-500 NMR spectrometer and on the 500-MHz spectrometer constructed and located at the Francis Bitter National Magnet Laboratory. *cis*-Pt-GCG (800 nmol) was lyophilized twice from D_2O (99.8 atom %) and then dissolved in 0.5 mL of 99.96 atom % D_2O. The pH was adjusted between 2 and 11 with NaOD or DCl and was not corrected for the deuterium isotope effect (Glasoe & Long, 1960). EDTA (500 μM) was added to prevent line broadening due to paramagnetic impurities in the DCl or NaOD. The solution pH was recorded before and after each NMR spectrum. To minimize the likelihood of acid-catalyzed depurination followed by base-promoted strand cleavage, individual samples were titrated initially from pH 5 to 11; the pH was then lowered back to 5 and the titration continued from pH 5 to 2. Chemical shifts were referenced to tetramethylammonium chloride (Aldrich) at 3.18 ppm. The residual water peak was minimized by selective presaturation and the spectra were recorded at 55 °C to sharpen the signals and prevent oligonucleotide base pairing and aggregation. The chemical shifts of the nonexchangeable base proton resonances were plotted as a function of pH, and the pK_a values of the associated endocyclic amines were determined graphically.

Titrations on the platinated *cis*-Pt-AG oligomer were performed in a similar fashion. The oligonucleotide complex was titrated in the range from pH 2 to 12.

Preparation of Heteroduplex DNA with a (−)-Strand Gap. M13 genomes containing a single *cis*-$[Pt(NH_3)_2\{d(ApG)$-*N*7(1),-*N*7(2)$\}]$, *cis*-$[Pt(NH_3)_2\{d(GpCpG)$-*N*7(1),-*N*7(3)$\}]$, or *trans*-$[Pt(NH_3)_2\{d(CpGpCpG)$-*N*3(1),-*N*7(4)$\}]$ intrastrand cross-link were prepared according to the strategy previously employed for the site-specific placement of a *cis*-$[Pt(NH_3)_2\{d(GpG)$-*N*7(1),-*N*7(2)$\}]$ adduct into the (−) strand (Pinto et al., 1986; Naser et al., 1988). Duplex viral genomes M13-*Sca*I and M13-*Mlu*I, containing un-Pt-AG or un-Pt-GCG insertions, respectively, were provided by L. J. N. Bradley (see Figure 1 for nomenclature). The synthetic duplex dodecadeoxynucleotides were cloned into the *Hin*dII recognition site (position 6264) in M13mp18 by blunt-end ligation. Large-scale (2-L) growths of the appropriate clones M13-*Sca*I and M13-*Mlu*I were then carried out. Closed circular M13-*Sca*I or M13-*Mlu*I ss viral DNA was isolated by poly(ethylene glycol) precipitation and purified by hydroxylapatite (HAP) chromatography (DNA-grade Bio-Gel HTP, Bio-Rad). M13mp18 RF DNA was also isolated from large-scale preparations and was purified by two successive equilibrium centrifugations in cesium chloride–ethidium bromide gradients. A 20-fold molar excess of ss DNA was mixed with *Hin*dII-linearized M13mp18 RF DNA. The mixture was dialyzed against decreasing amounts of formamide to denature the M13mp18 RF strands and allow renaturation of the (−) strand to the excess circular viral (+) strands (Lundquist & Olivera, 1982; Pinto et al., 1986). These gapped heteroduplexes were separated from remaining closed and linear single strands by hydroxylapatite chromatography.

Insertion of Platinated and Unplatinated Dodecanucleotides into the (−) Strand of Duplex Genomes. Purified platinated and unplatinated dodecamers were phosphorylated by using polynucleotide kinase (PNK) and [γ-^{32}P]ATP for genome characterization experiments and cold ATP for replication inhibition experiments. Tris-HCl (50 mM, pH 7.6), $MgCl_2$ (10 mM), and dithiothreitol (20 mM) were also present in the buffer. Ligation reactions were performed directly after heat inactivation of the PNK, by using T4 DNA ligase and a 300:1 molar ratio of oligonucleotide to gapped duplex. The reaction buffer contained fresh ATP (1 mM) and dithiothreitol (20 mM). The total Tris-HCl and $MgCl_2$ concentrations were as before. A 1-μg (genome characterization experiments) or 50-μg (replication inhibition experiments) aliquot of the gapped duplexes was used. Reactions were allowed to proceed at 16 °C for 12–16 h. Radioactively labeled samples were purified by Sepharose CL-4B size-exclusion chromatography, by eluting with 50 mM NaCl in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (TE buffer). Large-scale preparations with cold oligonucleotides were used for replication experiments directly after extractions with buffer-saturated phenol, buffer-saturated phenol/chloroform/isoamyl alcohol (50:48:1), and chloroform/isoamyl alcohol (24:1) (Sambrook et al., 1989) and precipitation to remove unincorporated ATP, enzymes, and salt. Alternatively, double-stranded closed circular material was isolated by manual excision from a 0.8% agarose electrophoresis gel run in the presence of ethidium bromide, electroelution (Centrilutor microelectroeluter, Amicon), concentration (Centricon-30 microconcentrators, Amicon), and precipitation by 2.5 volumes of ethanol in the presence of 0.31 M sodium acetate.

^{32}P Localization and Restriction Endonuclease Inhibition Experiments. The ligated genomes were characterized by

restriction endonuclease digestion and analysis on 0.8% agarose gels containing 0.5 μg/mL ethidium bromide. All digests were carried out on material containing 100–150 cpm of the ligation products at 37 °C for 90 min with use of the recommended restriction enzyme buffers. Carrier DNA (0.1 μg) was added to each reaction as an internal control in order to demonstrate complete digestion when the sample was visualized by ethidium bromide fluorescence. Identification of the labeled samples was achieved by autoradiography after the gels were dried at 60 °C for 2 h in a vacuum gel dryer (Hoefer Scientific Instruments).

For some experiments the platinum adducts were removed from site-specifically modified genomes by displacement with cyanide ion (Bauer et al., 1978). NaCN solution (1 M) was freshly prepared in 100 mM Tris-HCl (pH 7.5) and the pH was adjusted to approximately 8.5 by the addition of concentrated HCl in a fume hood. The modified genomes were incubated in 0.3 M cyanide for 5 h at 37 °C, precipitated, and washed to remove any traces of residual cyanide.

Preparation of Templates for Replication Inhibition Experiments. Portions (1.5–3 μg) of the site-specifically modified platinated and unplatinated genomes were linearized with 20 units of *Hin*dIII in a 20-μL volume containing the recommended buffer. After linearization was complete, as verified by ethidium bromide-containing agarose gels, digests were brought to 200 μL with TE buffer and extracted with phenol and phenol/chloroform as above. Finally, the linearized genomes were precipitated with sodium acetate and ethanol. After being washed once with 75% ethanol, samples were reconstituted in the appropriate primer annealing buffer.

Replication Inhibition Experiments. All of the experiments were conducted in a final volume of ca. 20 μL. Extinction coefficients for the primers were calculated according to Fasman (1975), and a molecular weight estimate for the M13 clones of 4.7×10^6 g/mol was used. Denaturation and primer annealing were carried out by heating the linearized templates together with a 1.5-fold molar excess of primer in a boiling water bath for 3 min and then quickly plunging the samples into ice for at least 3 min. Experiments were carried out employing 21-mer or 24-mer primers, the 5′-termini of which lie at positions 6095 or 6185, respectively, of the M13mp18 genome. The primers were either unlabeled or labeled at the 5′-end with [γ-^{32}P]ATP (New England Nuclear, 6000 Ci/mmol, 10.0 mCi/mL) as noted. ^{32}P-Radiolabeled primers were purified and desalted before use by elution from a Nensorb-20 nucleic acid purification cartridge (Dupont NEN).

Enzyme incubation conditions, including the buffer, temperature, and length of time, were adapted from the commercial supplier's instructions (T7 pol, Taq pol, and T4 pol), other published studies (pol I) (Taylor & O'Day, 1990), or personal communication (pol III) (G. Walker laboratory; C. S. McHenry laboratory). Varying the deoxynucleoside triphosphate concentration over a range of 10 to 45 μM for the T7 polymerase- and *E. coli* polymerase I-catalyzed reactions did not affect the results. The recommended dNTP concentrations were used for the other polymerases. For T7 polymerase reactions, the denaturation/annealing step was carried out in buffer containing Tris-HCl, pH 7.5, $MgCl_2$, and NaCl. The buffer was then adjusted after annealing to contain final component concentrations of 25 mM Tris-HCl, 6.5 mM dithiothreitol, 12 mM $MgCl_2$, 30 mM NaCl, 10–45 μM each dNTP and 1–5 units of T7 polymerase. Reactions were allowed to proceed for 12 min at 37 °C, at which point they were stopped by the addition of a solution containing 95% formamide, 20 mM EDTA, and xylene cyanol and bromophenol blue tracking dyes. Stopped reaction mixtures were stored at −80 °C for subsequent analysis. The Taq polymerase reactions were carried out by using 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, and 1 mg/mL gelatin in the denaturation/annealing buffer. The final reaction mixture also contained 200 μM each dNTP and 5 units of Taq polymerase. The replication step was carried out for 10 min at 70 °C, and the reactions were stopped as above. T4 polymerase reaction substrates were denatured/annealed in the presence of Tris-HCl, pH 8.5, $MgCl_2$, EDTA and 2-mercaptoethanol; other buffer components were then added to bring the final incubation conditions to 50 mM Tris-HCl, pH 8.5, 7 mM $MgCl_2$, 0.1 mM EDTA, 10 mM 2-mercaptoethanol, 15 mM ammonium sulfate, 200 μg/mL bovine serum albumin (RIA grade, Sigma), 45 μM each dNTP, and 0.1 unit of T4 polymerase. Reactions were stopped after 30 min at 37 °C and stored as above. *E. coli* pol I reactions were carried out in 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 10–45 μM each dNTP, and 2.5 units of pol I, with the polymerase and dNTPs added after annealing. Reactions were stopped after 30 min of incubation at 37 °C as above. *E. coli* pol III holoenzyme was used on substrates that were denatured/annealed in Na-HEPES and magnesium acetate. The final incubation conditions included 50 mM Na-HEPES, pH 7.75, 10 mM magnesium acetate, 10 mM dithiothreitol, 200 μg/mL bovine serum albumin, 20% glycerol, 50 μM dNTPs, and 0.25 μg/μL *E. coli* single-stranded binding protein (U.S. Biochemicals). Pol III, stored at −80 °C in 20% glycerol, 50 mM imidazole, pH 6.8, 1 mM EDTA, 5 mM dithiothreitol, and 150 mM NaCl, was melted on ice and used immediately without dilution. Seventy units (0.6 μL) was added to the reaction mixtures after the other components. Reactions were stopped after 5 min of incubation at 30 °C. Proteinase K (Boehringer Mannheim) was used to inactivate the single-stranded binding protein (0.5 μL of a 20 mg/mL stock, 65 °C, 20 min) before the samples were loaded on gels.

Sequence Analysis of Translesion Synthesis Products. In one case, involving the M13-*trans*-Pt-CGCG genome, translesion synthesis was sufficiently extensive to permit the oligonucleotide product of adduct bypass to be analyzed by degradative sequence analysis. The replication products produced by extension of end-labeled primers on platinated or unplatinated templates were separated on denaturing polyacrylamide gels, and the bands corresponding to termination at the *Hin*dIII-generated template terminus were excised and electroeluted as described above for agarose gels. The samples were washed with distilled water several times in Centricon-30 microconcentrators (Amicon) to remove all buffers and salts and separated into five tubes each for base-specific chemical reactions. Carrier DNA (1.5 μg of sonicated salmon sperm DNA, Sigma) was added to each tube. Dimethylsulfate, hydrazine in water, hydrazine in 3.75 M NaCl, and potassium permanganate treatment, followed by desalting and treatment with 1 M piperidine, yielded guanine-, pyrimidine-, cytosine-, and thymidine-specific cleavages, respectively, as previously described (Banaszuk et al., 1983; Comess et al., 1990). Potassium tetrachloropalladate(II) (Aldrich) was also employed as an adenine-specific strand-weakening reagent (Iverson & Dervan, 1987). After a final desalting step, the samples were dissolved in 3 μL of formamide loading dye and resolved on 8% polyacrylamide/7 M urea denaturing gels.

DNA Sequencing. Sequencing ladders, constructed for use as molecular weight standards, were generated through double-stranded sequencing of M13-*Stu*I, M13-*Sca*I, and M13-*Mlu*I RF templates by the chain-termination method (Sanger

et al., 1977); the DNA primers were the same as those used for replication experiments. Sequence analysis of the precursor genomes M13-*Sca*I ss and M13-*Mlu*I ss was also carried out by the chain-termination method, but the M13 (-40) sequencing primer (U.S. Biochemicals) was used.

Electrophoresis. The products of replication and sequencing reactions were resolved on 6% or 8% polyacrylamide/7 M urea denaturing gels and were fixed (10% methanol/10% acetic acid), dried, and autoradiographed for analysis. Analytical experiments were conducted by using 0.4-mm-thick, 35-cm × 43-cm gels on an STS 45 DNA sequencing apparatus (International Biotechnologies, Inc.). Samples (2–6 μL) were loaded immediately after being boiled for 3 min, and the gels were run approximately 3.5 h at 100–110 W constant power (ca. 1900 V). Samples were resolved for excision of the translesion synthesis band by loading 20–30 μL on 1.5-mm-thick preparative gels. Autoradiograms were prepared from wet gels (covered with Saran wrap) both before and after excision, initially to locate translesion synthesis bands and then to confirm that they had been properly excised.

Data Analysis. Dried analytical gels were analyzed by using Molecular Dynamics storage phosphor screens and a Model 400 Phosphorimager with Imagequant 3.0 software (Molecular Dynamics, Sunnyvale, CA). Translesion synthesis was quantitated by summing the integrated areas for fragments terminated at the 5′ end of the oligonucleotide insert, a consequence of incomplete ligation, and at the *Hin*dIII end of the template. Total synthesis was taken as the sum of the integrated areas in a given lane due to translesion synthesis as well as platinum-induced stops. The percent translesion synthesis was then calculated by dividing translesion synthesis by the total synthesis and multiplying by 100. Other fragments due to contaminating M13mp18 DNA (vide infra) were not incorporated into the calculation.

Results

Synthesis and Characterization of Platinated Oligonucleotides. The synthetic dodecanucleotides (Figure 1) were extensively purified by DE-52 anion-exchange chromatography and RP-HPLC before the platination reactions were carried out. They were allowed to react with *cis*- or *trans*-DDP or with *cis*-$[Pt(NH_3)_2(H_2O)_2]^{2+}$, and the products were repurified by RP-HPLC or anion-exchange chromatography. Platination of the un-Pt-AG oligomer yielded more undesired side products than did platination of the un-Pt-GCG oligomer. The ease of obtaining platinum coordination at d(ApG) sites appears to be more dependent on sequence context than for d(GpG) sites. This observation is underscored by the fact that *cis*-$\{Pt(NH_3)_2L_2\}^{n+}$ (where L = H_2O and n = 2 or L = Cl and n = 0) reacts with DNA tetranucleotides to form the adducts *cis*-$[Pt(NH_3)_2\{(CpGpGpT)\text{-}2,3\}]^-$, *cis*-$[Pt(NH_3)_2\{(TpGpGpC)\text{-}2,3\}]^-$, and *cis*-$[Pt(NH_3)_2\{(CpApGpT)\text{-}2,3\}]^-$ in 70–80% yield after purification, whereas the adduct *cis*-$[Pt(NH_3)_2\{(TpApGpC)\text{-}2,3\}]^-$ forms in less than 20% yield under a variety of conditions of temperature, buffer, and formal drug:nucleotide ratio (A. Plaziak, C. E. Costello, K. M. Comess, & S. J. Lippard, unpublished results). Both platinated and unplatinated oligonucleotides were chromatographed at least twice by RP-HPLC to afford material of >99% purity.

The *cis*-Pt-GCG and *cis*-Pt-AG oligonucleotides were characterized by pH-dependent proton NMR titrations and complete enzymatic degradations followed by RP-HPLC analysis to identify the platinum binding sites. Figure 2 shows the pH dependence of the downfield nonexchangeable base proton resonances for the two platinated oligomers. The thymine H6 resonances in both complexes titrate above pH

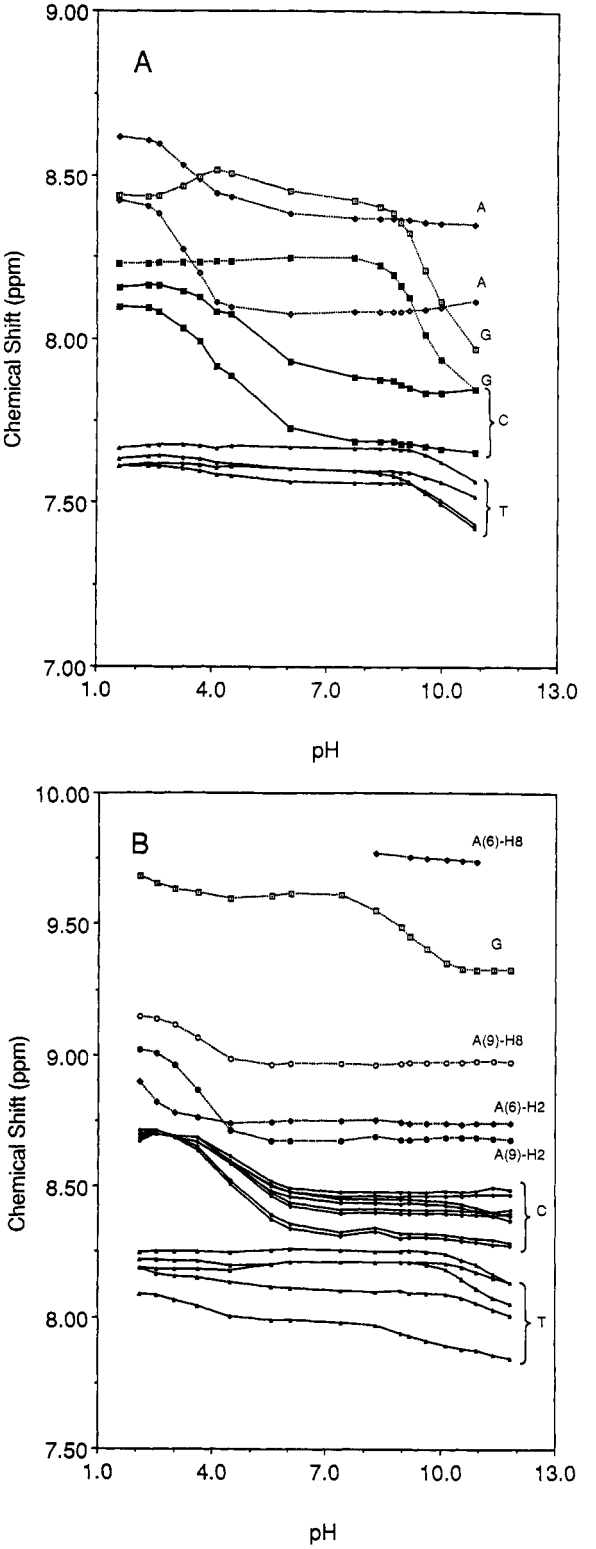


FIGURE 2: pH dependence of the downfield nonexchangeable base proton resonances of (A) *cis*-$[Pt(NH_3)_2\{d(TCTACGCGTTCT)\text{-}N7\text{-}G(6),N7\text{-}G(8)\}]$ (*cis*-Pt-GCG) and (B) *cis*-$[Pt(NH_3)_2\{d(TCTCTAGTACTC)\text{-}N7\text{-}A(6),N7\text{-}G(7)\}]$ (*cis*-Pt-AG). NMR spectra were recorded at 55 °C. The cytidine resonances are plotted as a group in panel A.

~ 10 due to removal of the N3 proton (Izatt et al., 1971). The group of cytosine H6 resonances undergoes a shift below pH 4.5 owing to protonation of the cytosine N3 atoms. The H2 and H8 resonances of the single adenine residue in *cis*-Pt-GCG and of the unplatinated adenine of *cis*-Pt-AG titrate with pK_a values of 3.7, as expected for protonation of their N1 atoms. The farthest downfield resonance in the *cis*-Pt-AG

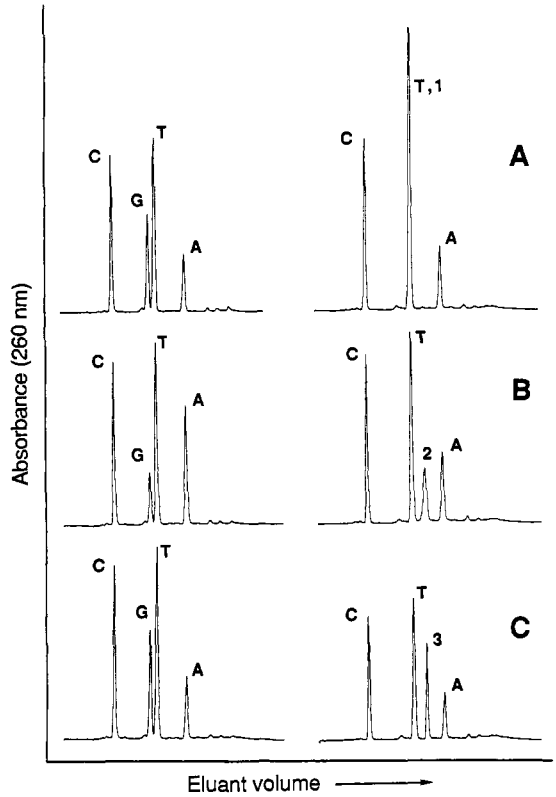


FIGURE 3: Enzymatic digestion analysis of unplatinated and platinated oligonucleotides. Samples were completely digested with DNase I, nuclease P1, and alkaline phosphatase and resolved by RP-HPLC as described in the text. (A) un-Pt-GG and *cis*-Pt-GG; (B) un-Pt-AG and *cis*-Pt-AG; (C) un-Pt-GCG and *cis*-Pt-GCG (see Figure 1 for abbreviations). Peak C is deoxycytidine, peak G is deoxyguanosine, peak T is thymidine, peak A is deoxyadenosine, peak 1 is *cis*-$[Pt(NH_3)_2\{d(GpG)\}]^+$, peak 2 is *cis*-$[Pt(NH_3)_2\{d(ApG)\}]^+$, and peak 3 is *cis*-$[Pt(NH_3)_2\{d(Guo)_2\}]^{2+}$.

spectrum rapidly exchanges with bulk D_2O and is no longer observed below pH 8. Both the downfield chemical shift and the rapid exchange rate of this resonance allow us to assign it as H8 of a platinated adenine (Kong & Theophanides, 1974; Lepre et al., 1987; van Hemelryck et al., 1987). The H2 resonance of this base begins to shift below pH 4 with an apparent $pK_a < 3$; the movement of this peak is not complete at pH 2 where the titration was stopped. The titration of this resonance at such a low pK_a value is consistent with protonation at N1 of an adenine base platinated at N7. The remaining two curves in the *cis*-Pt-GCG titration and one in the *cis*-Pt-AG titration arise from shifts in the H8 resonances of guanine bases platinated at N7. These plots reveal no pH-dependent behavior in the pH 2.3 region, the pK_a value of unplatinated guanosine N7, and have titration midpoints for removal of the N1 proton that are lower than the measured N1 pK_a values (≥ 11) for un-Pt-GCG (data not shown). Both observations are consistent with coordination of platinum to the guanosine N7 position (Chu et al., 1978; Lepre et al., 1987, and references therein).

Figure 3 shows the RP-HPLC analysis of the products of enzymatic degradation of the *cis*-DDP-platinated and unplatinated dodecanucleotides. The DNA was digested to nucleosides and platinum-containing nucleosides and dinucleotides. The mononucleoside peaks are well resolved in these chromatograms and reflect the proper proportions of the various species when integrated and normalized by their extinction coefficients. Digestion of the platinated samples results in the loss of both deoxyguanosine peaks (Figure 3A,C) or the deoxyguanosine peak and half of the deoxyadenosine peak (Figure 3B). In addition, peaks appear corresponding to the appropriate platinated nucleoside pair or dinucleoside monophosphate species (labeled 1, 2, and 3 in Figure 3), as confirmed by coinjection of model compounds (not shown). The relative absorbances (peak areas) of the deoxycytidine and thymidine peaks remain unchanged after platination.

Construction and Characterization of Platinated Genomes. The strategy used to build the site-specifically modified genomes is described under Materials and Methods and shown schematically in Figure 4 for the M13-*cis*-Pt-GG genome. The gapped duplexes were purified by HAP chromatography before insertion of the platinated or unplatinated oligonucleotides. This purification step separates double-stranded material,

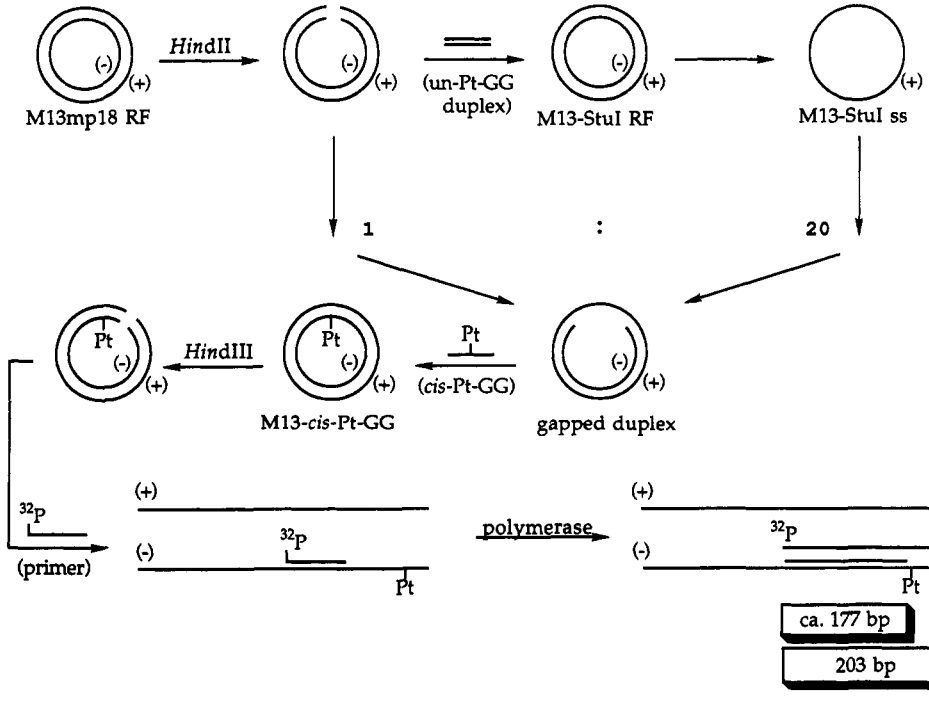


FIGURE 4: Scheme for construction of the site-specifically modified genome M13-*cis*-Pt-GG [after Naser et al. (1988)] and replication inhibition experiments, as described in the text. The other platinated and unplatinated genomes were constructed in analogous fashion.

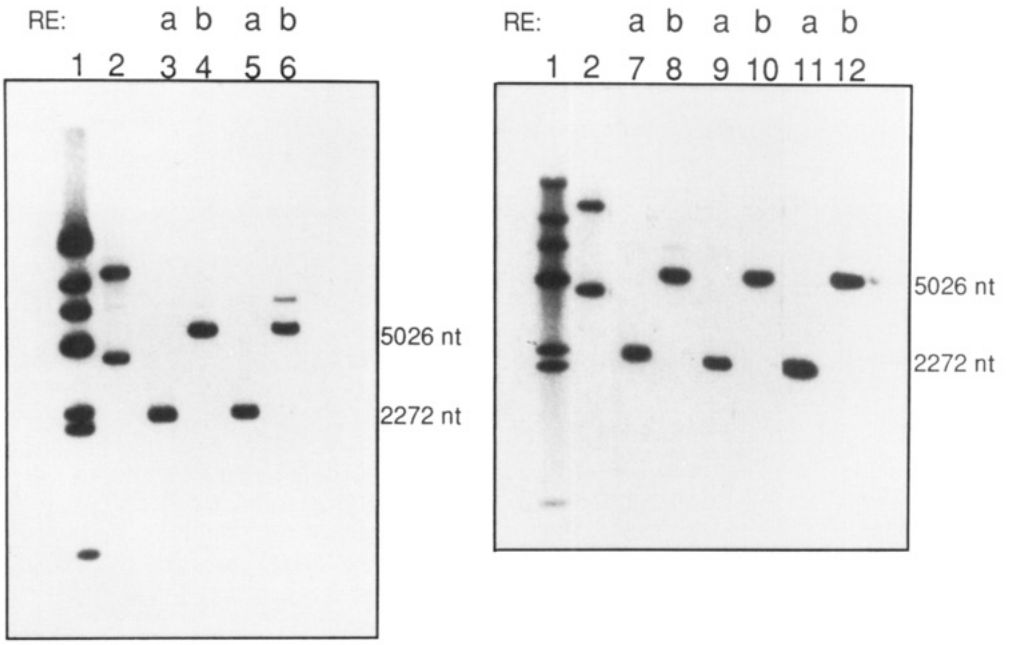


FIGURE 5: Restriction endonuclease localization of the ^{32}P-end-labeled oligomers un-Pt-AG (lanes 3 and 4), *cis*-Pt-AG (lanes 5 and 6), un-Pt-GCG (lanes 7 and 8), *cis*-Pt-GCG (lanes 9 and 10), and *trans*-Pt-CGCG (lanes 11 and 12) in M13 genomes doubly digested with *Xba*I and *Sna*BI (RE a) (lanes 3, 5, 7, 9, and 11) or *Hin*dIII and *Sna*BI (RE b) (lanes 4, 6, 8, 10, and 12) restriction endonucleases. Ethidium bromide-containing 0.8% agarose gels were run, dried, and autoradiographed for analysis. (Lane 1) ^{32}P-Labeled molecular weight ladder (*Hin*dIII digest of λ DNA). (Lane 2) Undigested genome showing the positions of closed circular and nicked circular double-stranded DNA. The extra band in lane 6 migrates with linear M13 (ca. 7250 bp) and is also present in the digested carrier DNA (M13-*Sca*I RF); it therefore signifies incomplete digestion by one of the enzymes, *Hin*dIII or *Sna*BI. All other digestions were judged to be complete by ethidium bromide fluorescence visualization of unlabeled carrier DNA, which was added to each sample as an internal standard.

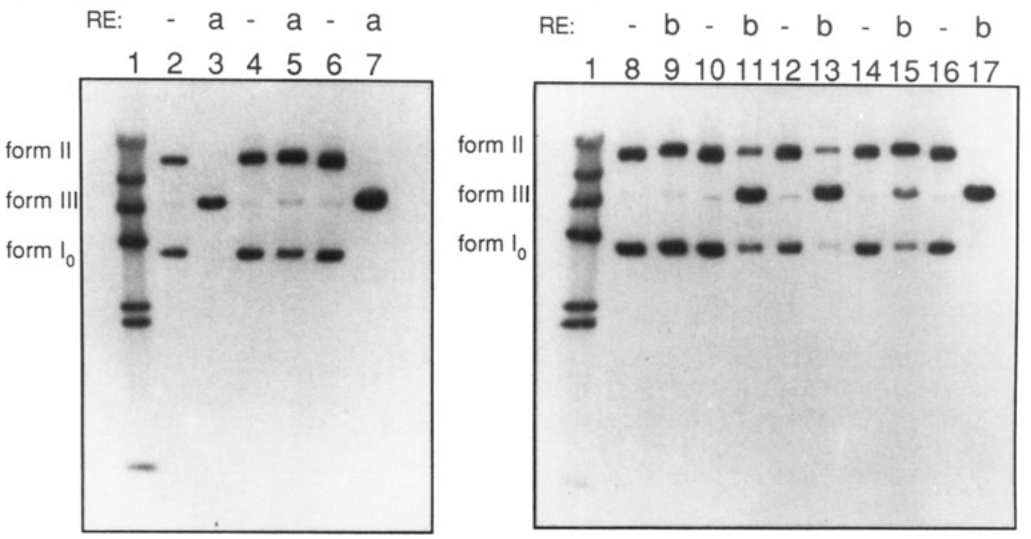


FIGURE 6: Restriction endonuclease characterization of site-specifically modified genomes. The presence of the platinum adducts within unique RE recognition sites was confirmed by digestion with the enzyme *Sca*I (RE a) (lanes 3, 5, and 7) or *Mlu*I (RE b) (lanes 9, 11, 13, 15, and 17), followed by electrophoresis on 0.8% agarose gels containing 0.5 μg/μL ethidium bromide and autoradiography. All digests were judged to be complete by ethidium bromide fluorescence visualization of digested carrier DNA added as an internal control to each lane (not shown). (−) refers to control lanes in which no RE was added. (Lanes 2 and 3) M13-un-Pt-AG. (Lanes 4 and 5) M13-*cis*-Pt-AG. (Lanes 6 and 7) M13-*cis*-Pt-AG treated with sodium cyanide prior to RE digestion (see text). (Lanes 8 and 9) M13-*cis*-Pt-GCG. (Lanes 10 and 11) M13-*cis*-Pt-GCG treated with sodium cyanide prior to RE digestion. (Lanes 12 and 13) M13-un-Pt-GCG. (Lanes 14 and 15) M13-*trans*-Pt-CGCG. (Lanes 16 and 17) M13-*trans*-Pt-CGCG treated with sodium cyanide prior to RE digestion. Form I_0 refers to relaxed, covalently closed circular DNA that comigrates with supercoiled M13 DNA in the ethidium bromide-containing gel. Form II refers to nicked circular DNA. Form III refers to linearized M13 DNA.

including gapped duplexes as well as linearized M13mp18 that did not denature and reanneal to M13-*Mlu*I ss or M13-*Sca*I ss DNA, from excess single strands (Sambrook et al., 1989; Lasko et al., 1987). In the next step, platinated and unplatinated oligonucleotides were phosphorylated at their 5′-ends and ligated into the gapped duplexes. Labeled ATP was used for characterization experiments. The position of the γ-^{32}P label could then be determined by double RE digests (Pinto et al., 1986). The enzymes *Xba*I, *Sna*BI, and *Hin*dIII allowed the label to be mapped to a 36-base region defined by the *Xba*I (position 6258) and *Hin*dIII (position 6282 + 12-base insert) sites (Figure 5).

Next, a series of restriction digests by the appropriate *Sca*I or *Mlu*I enzyme was carried out to determine the presence or absence of the platinum atom coordinated at the proper site in the construct (Pinto et al., 1986; Naser et al., 1988) (Figure 6). The M13-*cis*-Pt-AG and M13-*cis*-Pt-GCG genomes were resistant to digestion with *Sca*I and *Mlu*I, respectively, whereas the M13-un-Pt-AG and M13-un-Pt-GCG genomes were linearized by treatment with these enzymes. Incubation of

M13-*cis*-Pt-AG and M13-*cis*-Pt-GCG with NaCN restored *Sca*I or *Mlu*I sensitivity by removing platinum from the DNA as $[Pt(CN)_4]^{2-}$ (Bauer et al., 1978). Interestingly, the adduct in the M13-*trans*-Pt-CGCG genome was only partially resistant to cleavage by the *Mlu*I restriction enzyme (Figure 6, lane 15). Other stable, covalently-modified DNA substrates can be cleaved by restriction endonucleases, albeit usually at a much reduced rate. Examples include DNA containing adducts of 4-aminobiphenyl, thymine glycol, 1,N^6-ethenoadenine, and O^6-methylguanine (Green et al., 1984; Basu et al., 1987; Lasko et al., 1987). The *trans*-DDP adduct studied here can undergo a linkage isomerization reaction at its 5′ coordination site to afford a 1,3-intrastrand cross-linked isomer (Comess et al., 1990). This kinetic lability may facilitate endonuclease cleavage through the transient formation of a monofunctional adduct. As discussed below, there is strong evidence that the partial cleavage of the M13-*trans*-Pt-CGCG genome by *Mlu*I is not the consequence of deplatination of the DNA.

The incomplete digestion of the genome in lane 11 may indicate that NaCN has not fully removed all the platinum, but since the digestion in lane 13 of the unplatinated M13-un-Pt-GCG genome is also incomplete, it seems more likely that in this experiment the *Mlu*I enzyme was not fully active. In other experiments, the M13-un-Pt-GCG genome was observed to be linearized completely. In each of these analyses, unlabeled carrier DNA (M13-*Sca*I RF or M13-*Mlu* RF) was present as an internal control and was visualized by ethidium bromide fluorescence staining. Carrier DNA, in lanes 3, 5, 7, 9, 11, 13, 15, and 17, was completely linearized (data not shown). Similar studies were also conducted on genomes directly after replication inhibition experiments, in which the label resides in the (+) strand at the 5′-end of an extended primer (vide infra, Figure 9).

Replication Inhibition Experiments. The site-specifically modified genomes were linearized at the single *Hin*dIII RE site (Figure 4). In the case of the genome containing the *cis*-Pt-GG oligomer, there are 25 nucleotides between the *Hin*dIII cleavage site and the platinum atom. This number was 26, 26, and 25 nucleotides, respectively, for genomes containing *cis*-Pt-AG, *cis*-Pt-GCG and *trans*-Pt-CGCG oligomers. *Hin*dIII treatment provided a defined stop site for control experiments employing unadducted genomes and for any products of translesion synthesis using platinated material. The *Hin*dIII site is also far enough from the platination site such that the adduct-induced fragment terminations were not influenced by the template terminus (Clark & Beardsley, 1989). The linearized templates were denatured and annealed to an end-labeled 21-mer DNA primer. The 3′ terminus of the primer was located 151 nucleotides away from the 3′ nucleotide of the inserted dodecanucleotide. Synthesis using unplatinated genomes, as well as translesion synthesis, afforded labeled oligonucleotide products 203 nucleotides in length. For increased resolution, some experiments were carried out with an unlabeled 24-mer primer, the terminus of which was located 58 nucleotides away from the 3′ nucleotide of the inserted oligonucleotide. In these cases, [α-^{35}S]dATP was added to label the synthesized strands.

DNA synthesis was performed under a variety of conditions optimized for the individual polymerases. For a given enzyme, identical conditions were employed for genomes containing the different adducts. The results of several replication inhibition experiments are presented in Figure 7. In Figure 7A are shown representative data for T7 pol replication of all seven platinated and unplatinated constructs. The more closely placed primer was used in this study. A cluster of bands corresponding to replication inhibition across from the site of platinum binding was observed in the M13-*cis*-Pt-GG (*cis*-Pt-GG), M13-*cis*-Pt-AG (*cis*-Pt-AG), M13-*cis*-Pt-GCG (*cis*-Pt-GCG), and M13-*trans*-Pt-CGCG (*trans*-Pt-CGCG) lanes. The exact sites of termination are revealed by comparison with sequencing ladders in the adjacent lanes, produced from the respective M13-*Stu*I, M13-*Sca*I, and M13-*Mlu*I RF genomes. The T, C, A, and G lane designations correspond to bases in the template strand, the 5′ → 3′ sequences of which may be read directly from the top to the bottom of the gel. Fragment termination sites visible 4–6 nucleotides on either side of the platinum position correspond to the ends of the inserted oligonucleotide. These stops result from incomplete ligation of the oligonucleotide on either the 5′-, 3′-, or both (no ligation at all) ends and visually delineate the boundaries of the inserted oligonucleotides in all seven replication lanes. The dark bands near the tops of the lanes appear at the *Hin*dIII restriction site. Their presence indicates that translesion synthesis occurred in all four lanes containing platinum adducts.

Doubled bands are observed at the template termini (edges of gaps and *Hin*dIII-generated terminus), the second band corresponding to a fragment one nucleotide longer than expected. Since these bands occur for both unplatinated and platinated genomes, they are not related to the presence of the metal adduct. Such bands have been observed by others (Michaels et al., 1987). It is possible that these fragments arise either by addition of an extra nucleotide to the 3′ terminus of the blunt-ended fragment (Clark et al., 1987; Clark, 1988) or from a transient misalignment of the strands near the terminus that allows a frameshifting insertion (Streisinger & Owen, 1985; Carroll & Benkovic, 1990; Kunkel, 1990).

The resolution in Figure 7A is quite good, but the frequency of translesion synthesis is difficult to quantitate accurately. In this specific experiment, radiolabel (^{35}S) was introduced into the extended primer via labeled DNA synthesis precursors. Hence, the replication bypass products have more incorporated radioactivity than the shorter, platinum-terminated fragments. Further experiments employed a ^{32}P-phosphate end-labeled primer at the more distant priming site. Panels B and C of Figure 7, shown without a sequencing ladder, reveal that Taq pol and T4 pol, respectively, also are strongly blocked by the *cis*- but not the *trans*-DDP adducts. A variety of fragments corresponding to platinum-induced stops is apparent in the *trans*-Pt-CGCG lane in Figure 7B. Fragment termination due to incomplete ligation of the dodecanucleotide is also evident in Figure 7B,C. A band is seen in all lanes corresponding to a fragment 12 nucleotides shorter than the *Hin*dIII-generated fragment. This band is produced through replication of *Hin*dIII-linearized M13mp18, as discussed below.

In Figure 7D are shown the results for replication experiments utilizing *E. coli* pol I. For this experiment, form I_0 DNA was purified by using ethidium bromide-containing agarose gels, which greatly reduced contributions from inefficient ligation of the 5′ and 3′ ends of the inserts. The *Hin*dIII-12 band was still present, however, suggesting that the source of this material might be a small amount of contaminating M13mp18 RF DNA that was linearized by *Hin*dIII along with the ligated gapped duplexes. The size of full-length DNA synthesized from this template would be 12 nucleotides less than full-length DNA obtained from the cloned genomes, since it lacks the dodecamer insert. Such a contaminating M13mp18 RF DNA would be carried through the gapping procedure (Figure 4) and religated at the *Hin*dII RE

Table I: Frequency of Replication Bypass on Site-Specific Adducts of *cis*- and *trans*-$[Pt(NH_3)_2]^{2+}$

DNA polymerase	*cis*-$[Pt(NH_3)_2$-{d(GpG)-1,2}]	*cis*-$[Pt(NH_3)_2$-{d(ApG)-1,2}]	*cis*-$[Pt(NH_3)_2$-{d(GpCpG)-1,3}]	*trans*-$[Pt(NH_3)_2$-{d(CpGpCpG)-1,4}]
T7 pol[a]	3 (1)[b]	6 (4)	25.0 (0.3)	86 (7)
Taq pol[c]	3	19	5	49
T4 pol[c]	2	2	2	39
pol I[d]	3.3 (0.4)	14 (5)	27 (2)	90 (2)
pol III[a]	6 (1)	9 (2)	4 (1)	54 (2)

[a] Average of two experiments. [b] Translesion synthesis frequency, reported as mean percent (±standard deviation). [c] One experiment. [d] Average of four experiments.

site. Since this parental M13 DNA effectively comigrates with the form I_0 material containing the dodecanucleotide insert, it would be present in the gel-purified genomes used for the experiment shown in Figure 7D. Treatment with *Hin*dII prior to gel purification confirmed this hypothesis, as shown for the experiments conducted with pol III (Figure 7E). Since the constructed genomes no longer contain a *Hin*dII site (Figure 4), *Hin*dII pretreatment linearizes only the M13mp18 RF DNA. Gel purification of form I_0 DNA therefore yields only the fully ligated constructs. The data in Figure 7E again demonstrate that *cis*-DDP adducts provide strong, although not absolute, blocks to replication. The one *trans*-DDP adduct studied is a poor inhibitor of this enzyme.

The data for a number of experiments, including those shown in Figure 7B–E, were quantitated by exposure of the dried gels used for autoradiography to phosphor storage screens and analysis as described under Experimental Procedures. Only experiments employing the more distant initiation site and an end-labeled primer were analyzed in this fashion. A summary of the frequency of translesion synthesis found for each type of adduct and polymerase is presented in Table I. Fragments generated from synthesis on *Hin*dIII-linearized M13mp18 RF DNA were ignored in these calculations. A schematic diagram of the adduct-dependent termination points of replication for the different site-specifically platinated genomes is presented in Figure 8. The data represent the composite of several experiments for the most part and were reproducible.

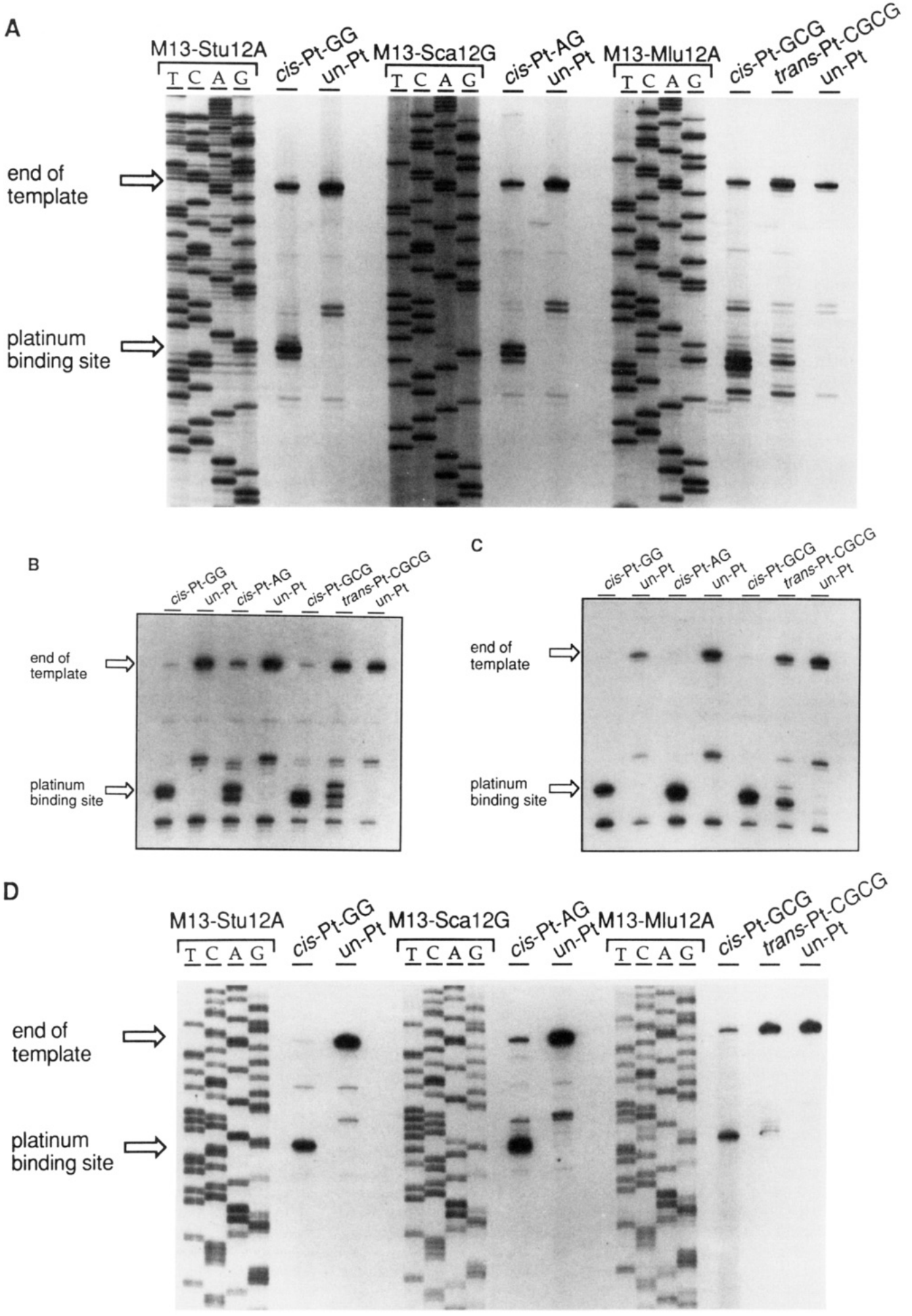
Determination of Adduct Stability during Template Preparation. Since some fragments were observed that correspond to DNA synthesis beyond the sites of platination (Figure 7, Table I), several experiments were performed to examine the possibility that the platinum adduct was labilized during the replication experiment. In one such experiment the products of replication were subjected to treatment with the enzymes *Stu*I, *Sca*I, or *Mlu*I. Appropriate buffer components were added after replication to bring the incubation media close to the recommended conditions for RE cleavage. Figure 9 shows that, whereas *Hin*dIII-terminated control fragments produced by synthesis on unplatinated genomes were cleaved to the expected lengths upon RE treatment, the *Hin*dIII-terminated fragments produced by translesion synthesis on platinated templates (see last step in Figure 4) were not cleaved. This result proves that the full-length fragments were synthesized from templates having platinum at the engineered site, particularly for the major d(GpG) and d(ApG) platinum intrastrand cross-links. Truncated fragments arising from synthesis blocked at the adduct site are not affected in this experiment. Some cleavage is observed for the replicated M13-*cis*-Pt-GCG genome (lanes 9 versus 10), and the M13-*trans*-Pt-CGCG genome only slightly inhibited the *Mlu*I RE (data not shown). The latter result is analogous to that shown in Figure 6 (lane 15).

In order to investigate the stability of the *trans*-DDP adduct to removal and to confirm the stability of the *cis*-DDP adducts, the platinated and unplatinated single-stranded dodecanucleotides used in the ligation of gapped duplexes were end-labeled with [^{32}P]ATP and sequentially subjected to the conditions employed in all steps (Figure 4) used to construct and replicate the genomes. In this experiment, pol I was employed. The buffers, incubation times, and temperatures were the same as used for ligation reactions, RE digests, template denaturation/primer annealing reactions, and polymerization reactions, and all extractions and precipitations were also carried out. Unlabeled, unplatinated M13mp18 RF DNA was added as a carrier after incubation with DNA ligase. Both the platinated and unplatinated dodecanucleotides remain unchanged following the lengthy treatment protocol, as demonstrated by electrophoresis on 20% denaturing polyacrylamide gels (data not shown). Quantitation revealed that bands migrating at the position of unplatinated oligonucleotides in the platinated oligomer lanes increased by no more than 0.7% of the total integrated counts at the end of the procedure. The highest amount found at the start was 0.3%.

Isolation and Sequence Analysis of the M13-trans-Pt-CGCG Translesion Synthesis Products. The 5′-end-labeled 203-base products of replication bypass produced by pol I synthesis through the M13-*trans*-Pt-CGCG genome were excised and subsequently electroeluted from preparative polyacrylamide denaturing gels. Products from the M13-un-Pt genome were obtained in an identical fashion. The collected material was subjected to direct sequence analysis (Banaszuk et al., 1983; Iverson & Dervan, 1987). A single alteration in the sequence is observed, as can be seen by comparing the chemical cleavage patterns for DNA synthesis using the M13-*trans*-Pt-CGCG and M13-un-Pt-CGCG templates (lanes 6–10 and 1–5, respectively, of Figure 10). An additional fragment appears in the thymidine-specific lane for M13-*trans*-Pt-CGCG (lane 8) at a position corresponding to the location of the adduct.

Discussion

Preparation and Characterization of M13 Genomes Containing Single cis-$[Pt(NH_3)_2${*d(ApG)-N7(1),-N7(2)*}]*, cis-*$[Pt(NH_3)_2${*(GpCpG)-N7(1),-N7(3)*}]*, and trans-*$[Pt(NH_3)_2$-{*d(CpGpCpG)-N3(1),-N7(4)*}] *Intrastrand Cross-Links at a Unique Site.* The strategy for inserting platinum at a local site into viral genomes (Pinto et al., 1986; Naser et al., 1988) was utilized to construct M13 DNA site-specifically modified with several adducts of *cis*- and *trans*-DDP. The platinated, single-stranded dodecanucleotide complexes *cis*-Pt-GG (Pinto et al., 1986; Naser et al., 1988), *cis*-Pt-AG, *cis*-Pt-GCG, and *trans*-Pt-CGCG (Comess et al., 1990) (Figure 1) were constructed, purified, and characterized before insertion into the viral genomes. These adducts represent the known spectrum of major *cis*-DDP DNA adducts and one *trans*-DDP adduct (Sherman & Lippard, 1987; Eastman et al., 1988). Interstrand cross-links and DNA–Pt(II)–protein or DNA–Pt(II)–glutathione cross-links, which form in vivo and in vitro to a minor extent, were not studied here but are the subject


A
M13-Stu12A
T C A G
cis-Pt-GG
un-Pt
M13-Sca12G
T C A G
cis-Pt-AG
un-Pt
M13-Mlu12A
T C A G
cis-Pt-GCG
trans-Pt-CGCG
un-Pt
end of template
platinum binding site
B
cis-Pt-GG
un-Pt
cis-Pt-AG
un-Pt
cis-Pt-GCG
trans-Pt-CGCG
un-Pt
end of template
platinum binding site
C
cis-Pt-GG
un-Pt
cis-Pt-AG
un-Pt
cis-Pt-GCG
trans-Pt-CGCG
un-Pt
end of template
platinum binding site
D
M13-Stu12A
T C A G
cis-Pt-GG
un-Pt
M13-Sca12G
T C A G
cis-Pt-AG
un-Pt
M13-Mlu12A
T C A G
cis-Pt-GCG
trans-Pt-CGCG
un-Pt
end of template
platinum binding site

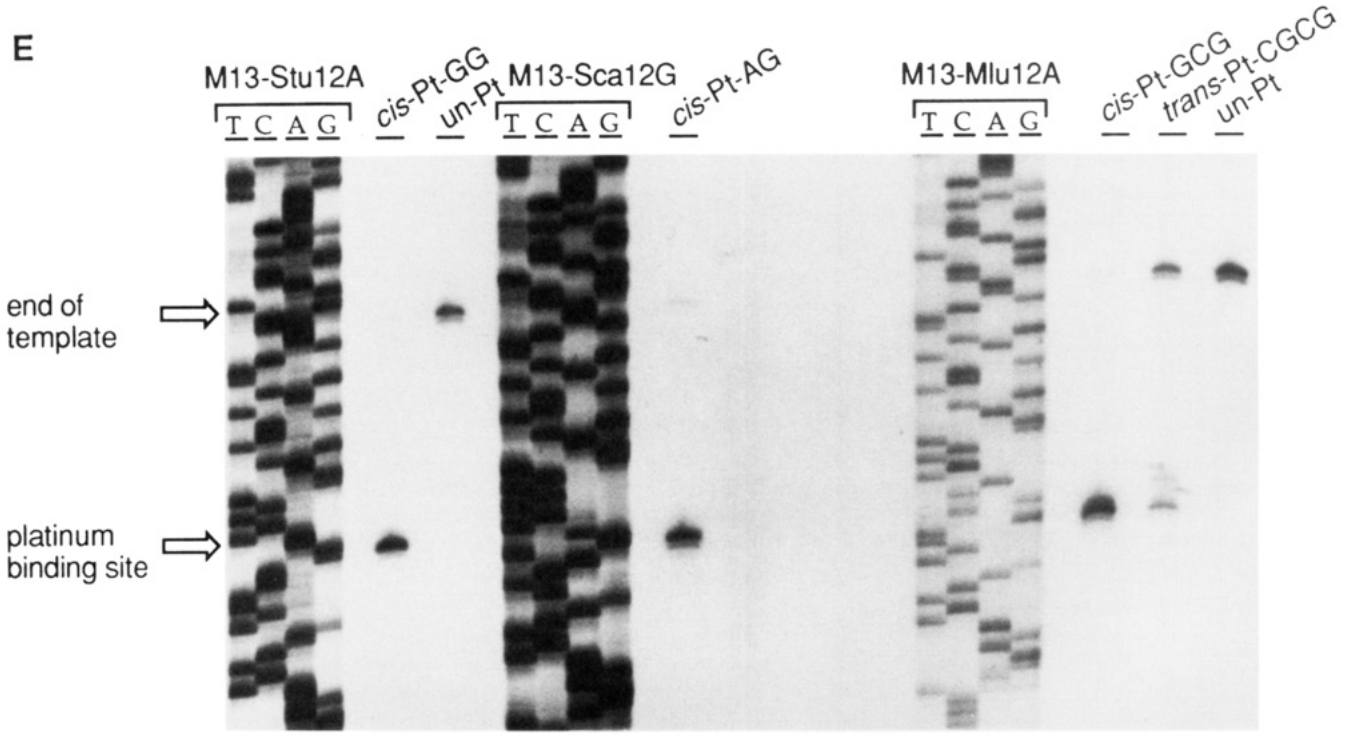


FIGURE 7: Translesion synthesis on site-specifically platinated M13 templates: autoradiograms of high-resolution sequencing gels showing the sites of termination of synthesis, as described in the text. Primers were extended to the site of platinum coordination or ca. 25 nucleotides beyond, to the end of the *Hin*dIII-generated restriction fragment by addition of deoxynucleotide triphosphates and the appropriate DNA polymerase. The resultant mixture of stopped and translesion synthesis products was then resolved by gel electrophoresis and autoradiographed. (*cis*-Pt-GG) M13-*cis*-Pt-GG; (*cis*-Pt-AG) M13-*cis*-Pt-AG; (*cis*-Pt-GCG) M13-*cis*-Pt-GCG; (*trans*-Pt-CGCG) M13-*trans*-Pt-CGCG. The control lanes (un-Pt) contain identical reactions carried out on the unplatinated genomes M13-un-Pt-GG, M13-un-Pt-AG, and M13-un-Pt-GCG. The sequencing ladders, used here as molecular weight standards, were obtained through chain-termination sequencing of M13-*Stu*I, M13-*Sca*I, and M13-*Mlu*I RF templates. (A) T7 pol; (B) Taq pol; (C) T4 pol; (D) Pol I; (E) Pol III. Form I_0 DNA of the modified genomes was gel-purified before the replication experiment, as explained in the text.

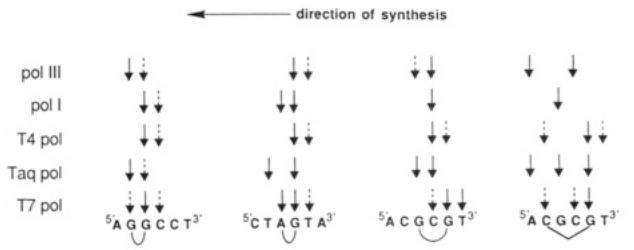


FIGURE 8: Schematic diagram of stop sites obtained by DNA polymerases on site-specifically placed adducts. The positions of the adducts are denoted by a semicircle (*cis*-$\{Pt(NH_3)_2\}^{2+}$ adducts) or a triangle (*trans*-$\{Pt(NH_3)_2\}^{2+}$ adducts) terminating at the coordinated nucleotides. The relative intensities of the stop sites are scored as weak (dashed arrow) or strong (solid arrow).

of a separate investigation. The pH-dependent proton NMR spectra of the newly constructed *cis*-Pt-AG and *cis*-Pt-GCG adducts may be compared with those previously reported for the *cis*-Pt-GG and *trans*-Pt-CGCG complexes (Naser et al., 1988; Comess et al., 1990). The reduction in pK_a of the guanine N1 protons in the *cis*-Pt-GCG complex upon platinum coordination at N7 (Figure 2A) is similar in magnitude to that seen for the *cis*-Pt-GG complex, although the observed pK_a values for both the platinated (*cis*-Pt-GCG) and unplatinated (un-Pt-GCG) genomes were higher by approximately 1 pH unit than seen previously. The reduction in pK_a values upon platination, coupled with the lack of any observable titration in the acidic region (pK_a of guanosine N7 $\sim$ 2.3), convincingly demonstrates that platinum is coordinated to the N7 positions of both guanine bases. Complete enzymatic degradation followed by RP-HPLC analysis of the newly constructed platinated and unplatinated complexes, as well as for the un-Pt-GG and *cis*-Pt-GG complexes, provides further independent evidence for the identity of all three adducts (Figure 3). Both methods of analysis were previously applied in the characterization of the *trans*-Pt-CGCG complex (Comess et al., 1990).

The platinated oligonucleotides were phosphorylated and ligated into gapped duplexes containing a single-stranded 12-base region in their (+) strand complementary to the un-Pt-GG, un-Pt-AG, or un-Pt-GCG sequences (Figure 4). Genome characterization experiments were carried out to assure that the oligonucleotides had been ligated into the expected region of the genome. The 5′ end of each inserted oligonucleotide was mapped to a 36-base-pair restriction fragment representing 0.5% of the total genome (Figure 5). The presence of platinum at the expected sites was demonstrated by restriction endonuclease digestion studies (Figure 6).

Replication Inhibition Experiments. The linearized, site-specifically platinated genomes were replicated as shown in Figure 4. Figure 7 clearly reveals that all of the cis adducts of platinum(II) significantly block replication. Other studies have shown only indirectly that specific adducts block DNA processing enzymes through exonuclease mapping (Tullius & Lippard, 1981) or replication mapping (Pinto & Lippard, 1985; Gralla et al., 1987; Hemminki & Thilly, 1988; Villani et al., 1988; Hoffmann et al., 1989). In one study, specific inhibition by both d(GpG) and d(ApG) adducts was inferred by comparing the kinetics of formation of these bifunctional adducts with the increase in replication inhibition over time (Hoffmann et al., 1989). Whereas the majority of d(GpG) adducts were formed quickly, the number of d(ApG) adducts continued to increase slowly over time. This unsynchronized appearance of the two major *cis*-DDP adducts was found to

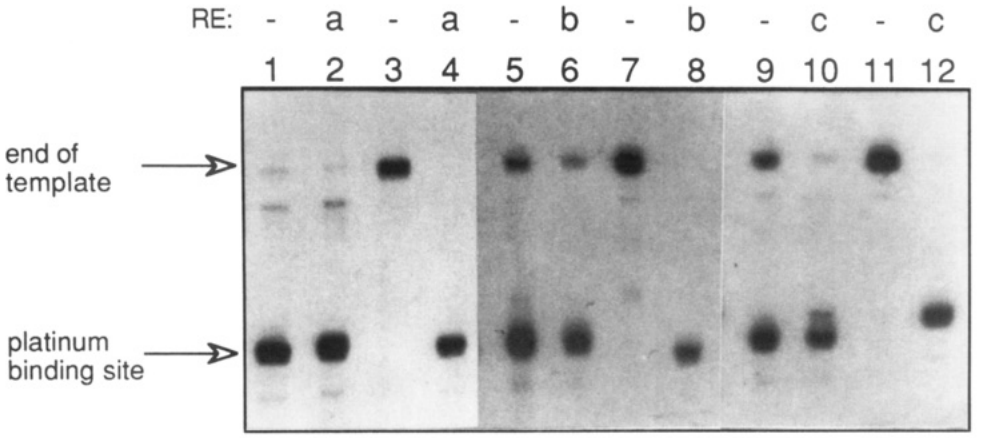


FIGURE 9: Postreplication restriction endonuclease characterization of site-specifically adducted genomes. The presence of site-specific platinum adducts in the template strands after replication bypass was confirmed through treatment of the translesion synthesis products with *Stu*I (RE a), *Sca*I (RE b), or *Mlu*I (RE c) restriction enzymes, followed by electrophoresis and autoradiography, as described in the text. (−) refers to control lanes in which no RE was added. Form I_0 DNA from the modified genomes was isolated and purified before the experiment, and pol I was the polymerase used to extend ^{32}P-end-labeled primers prior to addition of the restriction endonuclease. (Lanes 1 and 2) M13-*cis*-Pt-GG. (Lanes 3 and 4) M13-un-Pt-GG. (Lanes 5 and 6) M13-*cis*-Pt-AG. (Lanes 7 and 8) M13-un-Pt-AG. (Lanes 9 and 10) M13-*cis*-Pt-GCG. (Lanes 11 and 12) M13-un-Pt-GCG.

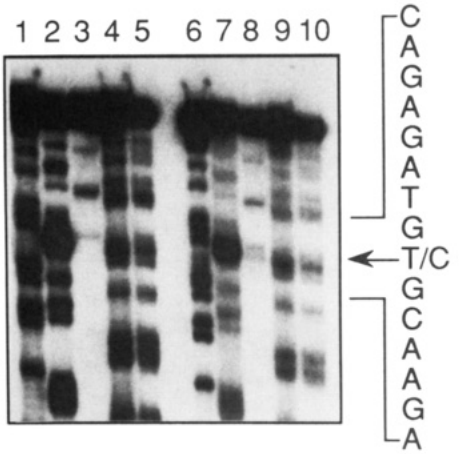


FIGURE 10: Maxam-Gilbert sequence analysis of the products of translesion synthesis on the M13-*trans*-Pt-CGCG genome (lanes 6–10) and the M13-un-Pt-GCG genome (lanes 1–5). The products of translesion synthesis by pol I were excised from a preparative gel and analyzed as described in the text. (Lanes 1 and 6) Deoxyadenosine-specific sequencing reactions. (Lanes 2 and 7) Deoxyguanosine-specific sequencing reactions. (Lanes 3 and 8) Thymidine-specific sequencing reactions. (Lanes 4 and 9) Deoxycytidine-specific sequencing reactions. (Lanes 5 and 10) Pyrimidine-specific sequencing reactions. The arrow points to the region where a difference is observed in lane 3 versus lane 8.

parallel a rapid initial decrease in DNA synthesis, followed by a continued, slowly increasing level of inhibition. For the other studies, adduct identities were inferred from the sequences at enzyme termination sites.

Several other investigations of the replication of site-specifically modified DNA in vitro have been carried out. Comparison of the relative abilities of pol I, T4 pol, HeLa cell polymerase α_2, and AMV reverse transcriptase to replicate a short oligonucleotide template containing a site-specifically placed *cis*-thymine glycol lesion revealed only the reverse transcriptase enzyme, which lacks 3′ to 5′ exonuclease activity, to be capable of synthesizing past the lesion (Clark & Beardsley, 1987). Replication studies of DNA carrying other site-specifically placed adducts, including 2-aminofluorene bound to guanine C8 (O'Connor & Stöhrer, 1985; Michaels et al., 1987), cis–syn thymine dimers (Taylor & O'Day, 1990), synthetic models of abasic sites (Takeshita et al., 1987), and 8-oxo-7-hydrodeoxyguanosine (Kuchino et al., 1987; Shibutani et al., 1991), have all shown some translesion synthesis. With one exception (Michaels et al., 1987), these studies have employed short templates and primers located close to the site of damage. The present work is more relevant to strand elongation, whereas the aforementioned studies model initiation or gap-filling (post-lesion removal) synthesis. The polymerase incubation times employed in the present work were chosen to maximize the amount of replication bypass. Studies of other covalent adducts indicate bypass frequencies within the range of those found here for DNA adducts of *cis*-DDP (Strauss, 1985; Taylor & O'Day, 1990; Shibutani et al., 1991).

The *trans*-DDP adduct in the M13-*trans*-Pt-CGCG genome is an exceptionally poor block to replication (Figure 7, Table I). Previous studies have shown that, in its single-stranded form, this 1,4-d(CNNG) intrastrand cross-linked adduct reversibly isomerizes at its 5′ coordination site to a 1,3-d(GNG) isomer (Comess et al., 1990). The 1,4-intrastrand cross-linked isomer, which is more stable ($K_{eq} \sim 3.0$), was isolated by RP-HPLC just prior to, and used in, the genome construction. The isomeric distribution in the double-stranded M13 genome is not known, however. Preliminary replication inhibition experiments carried out on a genome containing the well-characterized platinated oligonucleotide *trans*-[Pt(NH_3)$_2$[d-(CCTCGAGTCTCC)-*N7*-G(5),*N7*-G(7)}] (Lepre et al., 1990; M. B. Moore and S. J. Lippard, unpublished results) indicate that, for each of the polymerases T7, Taq, T4, and pol I, the frequency of translesion synthesis was greater than for any adduct of *cis*-DDP studied but less than half as much as for the *trans*-DDP adduct in M13-*trans*-Pt-CGCG. The observation that these two *trans*-DDP adducts block replication considerably less effectively than those of *cis*-DDP is seemingly inconsistent with several previous in vitro experiments using globally platinated DNA, which indicated that *trans*-DDP blocks replication as well as *cis*-DDP when the same number and sequence context of adducts per nucleotide are present (Pinto & Lippard, 1985; Ciccarelli et al., 1985; Heiger-Bernays et al., 1990). It is likely that a significant proportion of bifunctional DNA adducts formed by *trans*-DDP do block replication as well as those of *cis*-DDP but that they are not represented in the two adducts examined in the present study. The adduct spectrum produced by *trans*-DDP on heterologous DNA has not been investigated as extensively as for *cis*-DDP, but various experiments indicate that the regioselectivity is much less than for the cis isomer. In particular, *trans*-DDP adducts form between nonadjacent guanines, adenine and

guanine, and cytosine and guanine (Johnson et al., 1985; Pinto & Lippard, 1985; Eastman et al., 1988). Finally, it is important to note that a differential effect of *cis*- and *trans*-DDP on DNA synthesis has been previously reported (Johnson et al., 1978; Alazard et al., 1982). In one such study, T7 DNA required approximately 5 times more bound *trans*-DDP than *cis*-DDP per nucleotide to achieve the same level of replication inhibition in a crude bacterial extract (Johnson et al., 1978). The discrepancy between these results and those of Pinto and Lippard (1985), Ciccarelli et al. (1985), and Heiger-Bernays et al. (1990) is not well understood but may arise from the short platinum incubation time in the earlier study. Very short incubation times would produce a different spectrum of adducts (e.g., more monofunctional adducts). Examination of a wider spectrum of site-specifically modified DNA fragments is clearly warranted.

Previously it has been demonstrated that chlorodiethylenetriamineplatinum(II), which can form only monofunctional adducts, is unimpeded by pol I in a randomly platinated, single-stranded DNA template (Pinto & Lippard, 1985). Replication bypass of bifunctional adducts therefore might occur if the polymerase were to encounter an adduct in a transient monoadducted state. Such a mechanism could hold for both *cis*- and *trans*-DDP adducts and could account for the considerable readthrough observed for the *trans*-DDP intrastrand cross-link studied here.

Translesion Synthesis Is Not Due to Unplatinated DNA. Several methods were used to confirm the presence and stability of the adducts in the platinated dodecanucleotides and genomes. Platinated oligonucleotides were purified by several RP-HPLC cycles until >99% purity was achieved. The purity was monitored by ^{1}H NMR and polyacrylamide gel electrophoresis. The stability of the *cis*- and *trans*-DDP adducts to removal during the course of genome construction and replication experiments was measured indirectly, by assaying for platinum release in parallel studies of the oligonucleotide complexes. In mock ligation/replication experiments with these materials, a very small level (<0.7%) of deplatination did occur, but it was far less than would account for the observed replication bypass. The occurrence of even a low level of deplatination was surprising in view of the previously reported stability of platinum adducts to a variety of harsh conditions of temperature and pH (Royer-Pokora et al., 1981; Johnson et al., 1985). Perhaps platinum removal was effected by 2-mercaptoethanol, dithiothreitol, or other sulfur donor ligands in the medium.

Adduct stability within the *cis*-DDP-platinated genomes was also investigated both before (Figure 6) and after (Figure 9) replication experiments. The former experiments (Figure 6) conclusively demonstrate the successful ligation of the *cis*-DDP adducts into the genomes. Cleavage by the enzymes *Stu*I, *Sca*I, or *Mlu*I (see Figure 1) is completely inhibited in the M13-*cis*-Pt-AG and -GCG genomes (Figure 6, lanes 5 and 9). Sensitivity to these enzymes is obtained, however, after treatment with sodium cyanide, which removes platinum adducts (Bauer et al., 1978). Identical results were previously obtained for the M13-*cis*-Pt-GG adduct (Pinto et al., 1986; Naser et al., 1988). The latter experiments (Figure 9) demonstrate directly that translesion synthesis occurred on platinated templates. If 5′-end-labeled fragments apparently produced by lesion bypass had actually been synthesized from unplatinated material, then cleavage of the extended fragment/template duplex by the appropriate enzyme *Stu*I, *Sca*I, or *Mlu*I would have occurred. That complete RE digestion of duplexes synthesized from primed, unplatinated templates occurs can be seen by comparing the control lanes 3 and 4, 7 and 8, and 11 and 12 in Figure 9. The longer fragments obtained by translesion synthesis from the adducted templates are restricted only to a minor extent (lanes 1, 2, 5, 6, 9, and 10). The small amount of observed cleavage could arise from relaxation of the RE sequence specificity, since the postreplication reaction mixtures could not be adjusted precisely to the composition recommended for the RE incubations. Alternatively, the cleavable fraction of full-length products might correspond to unplatinated DNA or DNA that had been deplatinated during the course of the experiment. The majority of material obtained by bypass, however, is insensitive to RE treatment. Complete restriction of fragments in the control lanes (lanes 4, 8, and 12) strongly supports the assumption that repetitive replication on individual template molecules did not occur. If excess unannealed primers had displaced the annealed, extended primers to initiate additional rounds of synthesis, then not all of the extended primers would have a (−)-strand complement required for cleavage by the added RE. Such single-stranded DNA fragments would be clearly visible in lanes 4, 8, and 12 as uncleaved fragments.

Retention of platinum coordination in the M13-*trans*-Pt-CGCG genome could not be directly demonstrated owing to the surprising sensitivity of this genome to cleavage by *Mlu*I. The single-stranded dodecanucleotide containing this adduct is stable against platinum removal, however, as discussed above. Moreover, although a 1,4-intrastrand cross-linked adduct might be expected to be more distorting than a 1,2- or 1,3-adduct, its ligation efficiency into the M13 gapped duplex host is very similar to that of the corresponding unplatinated oligonucleotide, as well as those of the oligonucleotides modified by cisplatin (data not shown). These comparable ligation efficiencies probably result from the fact that dodecanucleotides were employed. In the *trans*-Pt-CGCG oligomer, four unmodified residues on both sides of the adduct available for base-pairing facilitate ligation into the gapped duplex. Even the platinated hexanucleotide *cis*-[Pt(NH_3)$_2$-{d(AGGCCT)-*N*7(2),-*N*7(3)}], containing only one unmodified nucleotide on the 5′ side of the adduct, ligates efficiently into its corresponding gapped host duplex (K. Yarema and J. M. Essigmann, unpublished results). Finally, the high level of translesion synthesis obtained by replication of the M13-*trans*-Pt-CGCG genome afforded an opportunity for sequence analysis of the bypass products. The results provide further evidence for the presence of the platinum adduct. The autoradiogram shows a base substitution in the thymidine-specific lane in the region spanned by the adduct (Figure 10, arrow). The fact that a difference is observed in the chemical cleavage patterns produced for DNA synthesized on the M13-un-Pt-CGCG and M13-*trans*-Pt-CGCG templates is good evidence that the platinum atom remains bound during the replication experiment. The position and identity of the mutation are interesting and may indicate that targeted mutagenesis by this adduct could occur in vivo.

Comparison of DNA Polymerases. The frequency of translesion synthesis varies for both the adducts studied and the different polymerases employed. The quantitative information summarized in Table I includes translesion synthesis frequency values averaged for as many as four experiments (pol I). The DNA polymerases employed comprise a spectrum of procaryotic enzymes of different origin, structural complexity, processivity, associated exonuclease activity, and in vivo function. For the polymerases T7, Taq, and pol I, the *cis*-[Pt(NH_3)$_2$-{d(GpG)-*N*7(1),-*N*7(2)}] adduct (in M13-*cis*-Pt-GG) inhibits replication more effectively than any other

type of damage. This result is interesting in view of survival and mutagenesis studies conducted on the M13-*cis*-Pt-GG genome (Naser et al., 1988; L. J. N. Bradley, S. J. Lippard, and J. M. Essigmann, unpublished results) and on a similar plasmid genome containing a site-specifically placed *cis*-[Pt-$(NH_3)_2$\{d(ApG)-*N*7(1),-*N*7(2)\}] adduct (Burnouf et al., 1990). The present results support the hypothesis that the 11% survival for the M13-*cis*-Pt-GG genome transfected into *E. coli* cells could be the consequence of replication bypass. Since the single-stranded template used in that experiment is not a viable substrate for excision repair, translesion synthesis was cited as the most likely explanation for the limited survival (Naser et al., 1988).

Recent studies have revealed that the *cis*-[$Pt(NH_3)_2$\{d-(ApG)-*N*7(1),-*N*7(2)\}] adducts are ca. 5 times more mutagenic than those of *cis*-[$Pt(NH_3)_2$\{d(GpG)-*N*7(1),-*N*7(2)\}], an effect attributable to errors introduced during replication bypass under SOS-induced conditions (Burnouf et al., 1987, 1990; L. J. N. Bradley, S. J. Lippard, and J. M. Essigmann, unpublished results). The observation that the *cis*-[$Pt(NH_3)_2$-\{d(GpG)-*N*7(1),-*N*7(2)\}] adduct provides a more effective block to DNA synthesis in vitro than the *cis*-[$Pt(NH_3)_2$\{d-(ApG)-*N*7(1),-*N*7(2)\}] adduct for the enzymes T7 pol, Taq pol, and pol I is consistent with the mutagenic frequency of an adduct in vivo being correlated with replication bypass frequency in vitro. In vivo, the presumably relevant enzyme is pol III (Staudenbauer et al., 1978), however, and SOS induction is required for mutagenesis (Bridges & Woodgate, 1985; Lu et al., 1986; Schwartz et al., 1988). The expectation that translesion synthesis by pol III should be ca. 5 times greater for the M13-*cis*-Pt-AG genome than for the M13-*cis*-Pt-GG genome is not met here, either because replication bypass efficiency is not the only determinant of mutagenic potential in this case or because SOS induction alters the template specificity of pol III (Walker, 1985). In one recent study, SOS induction raised the replication efficiency of DNA containing trans–syn T–T cyclobutane dimers from 14% to 29% and the mutation frequency from 4% to 11% (Banerjee et al., 1990).

It is also possible that the distance between the primer and the adduct is not long enough to simulate the in vivo activity of pol III. The data in Table I were compiled by using a primer, the 5′ end of which is located approximately 177 nucleotides from the platinum adduct. The various subunit assemblies of pol III holoenzyme can bind a primed template and initiate synthesis, but only the highly processive holoenzyme is likely to reach an adduct that is distant from the initiation site (McHenry, 1988). The primer used in the present study may not be placed far enough to ensure that only the holoenzyme reaches the adducts; a distance of ca. 1000 nucleotides may be necessary (J. Battista and D. Burnouf, personal communication). The distance of 177 nucleotides was selected in order to afford comparative information with the less processive enzymes pol I and T4 pol and to facilitate single-base resolution of the synthesized fragments on a polyacrylamide gel.

Replication is best inhibited by all four adducts when T4 polymerase is used (Table I, Figure 7C). This result may derive from the comparatively higher level of 3′ to 5′ exonuclease activity for this enzyme (Huang & Lehman, 1972; Kornberg, 1980; Scheuermann & Echols, 1984) and is consistent with the currently understood mechanism of DNA synthesis by pol I, in which exonucleolytic proofreading competes with chain extension. At a mismatched primer terminus, polymerization activity is stalled as the 3′ to 5′ exonuclease activity attempts to excise the mismatch, in this case any base(s) positioned across from the platinum-modified nucleotide(s) (Kuchta et al., 1988; Carroll & Benkovic, 1990). Thus the greater exonucleolytic capability of T4 polymerase may enable it to discriminate more effectively against incorporation of a base across from the platinum lesion and to block extension past it. Consistent with this model is the observation that T4 polymerase has a higher fidelity in copying DNA than pol I or Taq polymerase (Loeb & Kunkel, 1982).

Adduct Termination Sites. The sites where individual *cis*- and *trans*-DDP adducts terminate DNA synthesis are readily identified by comparison with the products of sequencing reactions using the same primer. For every polymerase and adduct studied, replication was inhibited at or beyond the first platinated nucleotide encountered, as approached from the 3′-side of the adduct site (Figure 8). An intense band arising from termination one nucleotide prior to the first platinated base was produced in only one case, the replication of the M13-*cis*-Pt-GCG genome with T7 pol. These findings are in accord with the results of several replication mapping studies of globally platinated DNA (Pinto & Lippard, 1985; Hemminki & Thilly, 1988; Villani et al., 1988). In one other case, however, DNA synthesis was consistently found to be arrested one to two nucleotides to the 3′-side of the first of a run of guanines (Gralla et al., 1987), an anomaly that warrants further investigation. The observation that stops do not occur until after a nucleotide is inserted across from the 3′-end of the platinum adduct is consistent with X-ray, molecular mechanics modeling, NMR, and chemical nuclease cleavage studies of well-defined *cis*-DDP adducts, all of which reveal the DNA distortion at the 5′-side of intrastrand platinum cross-links to be greater than that to the 3′ side (Sherman & Lippard, 1987; Marrot & Leng, 1989; Schwartz et al., 1989). In addition, mutations occur almost exclusively at the 5′-side of *cis*-[$Pt(NH_3)_2$\{d(GpG)-*N*7-G(1),*N*7-G(2)\}] and *cis*-[Pt-$(NH_3)_2$\{d(ApG)-*N*7-A(1),*N*7-G(2)\}] adducts (Burnouf et al., 1987, 1990; L. J. N. Bradley, S. J. Lippard, and J. M. Essigmann, unpublished results). The most uncomplicated and consistent adduct termination pattern is found for T4 pol (Figure 8). For this reason, as well as its greater ability to block replication described above, this polymerase is recommended as the one best suited for replication mapping studies.

The pattern of termination sites for adduct-induced Taq polymerase and pol III stops extends beyond the platinum cross-link site (Figure 8). Perhaps the higher temperature employed for Taq polymerase (70 °C) and the addition of *E. coli* single-stranded DNA binding protein to pol III reactions, both of which enhance the single-stranded nature of the templates, are responsible for this behavior. Possibly the stringency of the base-pairing requirement for polymerization is also relaxed, leading to the insertion of extra nucleotides. There is no obvious explanation, however, for the absence of a concomitant increase in the frequency of translesion synthesis.

DNA fragments obtained both from unplatinated templates and from readthrough of the platinated templates terminate at the same position. This result demonstrates that translesion synthesis does not produce frameshifts. Such products would migrate one or two nucleotides faster or slower than the control fragments. This finding is consistent with the *cis*-DDP-induced mutation spectrum in *E. coli*, where a very low level of frameshift mutants was scored (Burnouf et al., 1987).

Summary and Conclusions

Several new, site-specifically platinated genomes have been prepared and characterized. These, and a previously described

construct, were used as substrates for a variety of DNA polymerases to demonstrate that *cis*- and *trans*-DDP adducts do not provide an absolute block to in vitro DNA synthesis. The observation of replication bypass is consistent with other studies on the survival of, and targeted mutagenesis by, site-specifically platinated genomes. Site-specific damage was engineered into a biologically viable viral genome and primer extension was initiated more than 150 nucleotides away from the site of damage. This distance provides a good model for in vivo elongation of DNA strands and allows for more complete assembly of the *E. coli* polymerase III holoenzyme. Several direct and indirect methods were employed to assure that platinum coordination was retained on the genome templates both prior to and following replication and that translesion synthesis actually occurred on these templates. The newly prepared genomes are currently being employed in further studies on the survival, mutagenesis, and repair of the site-specifically placed adducts (Naser et al., 1988; L. J. N. Bradley, S. J. Lippard and J. M. Essigmann, unpublished observations). The present results indicate T4 polymerase to be the most effective for replication mapping of new platinum complexes developed as antitumor drugs. It has the most consistent pattern of adduct termination sites and allows the lowest frequency of translesion synthesis for the four adducts and five DNA polymerases examined.

Acknowledgments

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On the Molecular Mechanism of Light-Induced D1 Protein Degradation in Photosystem II Core Particles†

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ABSTRACT: The mechanism of D1 protein degradation was investigated during photoinhibitory illumination of isolated photosystem II core preparations. The studies revealed that a proteolytic activity resides within the photosystem II core complex. A relationship between the inhibition of D1 protein degradation and the binding of the highly specific serine protease inhibitor diisopropyl fluorophosphate to isolated complexes of photosystem II was observed, evidence that this protease is of the serine type. Using radiolabeled inhibitor, it was shown that the binding site, representing the active serine of the catalytic site, is located on a 43-kDa polypeptide, probably the chlorophyll *a* protein CP43. The protease is apparently active in darkness, with the initiation of breakdown being dependent on high light-induced substrate activation. The proteolysis, which has an optimum at pH 7.5, gives rise to primary degradation fragments of 23 and 16 kDa. In addition, D1 protein fragments of 14, 13, and 10 kDa were identified. Experiments with phosphate-labeled D1 protein and sequence-specific antisera showed that the 23- and 16-kDa fragments originate from the N- and C-termini, respectively, suggesting a primary cleavage of the D1 protein at the outer thylakoid surface in the region between transmembrane helices D and E.

Photoinhibition of photosynthesis is targeted to photosystem II (PSII)[1] and seems to be an inevitable consequence of the complicated redox chemistry involved in light-driven water–plastoquinone oxidoreduction (Powles, 1984; Andersson & Styring, 1991; Barber & Andersson, 1992; Prasil et al., 1992). Photoinhibition leads not only to impairment of electron transport but also to irreversible damage to the reaction center of PSII. This damage is rectified by a repair mechanism involving high turnover of the D1 protein and to some extent

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[1] Abbreviations: Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane; CP, chlorophyll protein; DFP, diisopropyl fluorophosphate; FTIR spectroscopy, Fourier transform infrared spectroscopy; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PSII, photosystem II; PVDF, poly(vinylidine difluoride); SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TCA, tricarboxylic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine.

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