Characterization of a DNA Damage-Recognition Protein from Mammalian Cells That Binds Specifically to Intrastrand d(GpG) and d(ApG) DNA Adducts of the Anticancer Drug Cisplatin†

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ABSTRACT: A factor has been identified in extracts from human HeLa and hamster V79 cells that retards the electrophoretic mobility of several DNA restriction fragments modified with the antitumor drug *cis*-diamminedichloroplatinum(II) (cisplatin). Binding of the factor to cisplatin-modified DNA was sensitive to pretreatment with proteinase K, establishing that the factor is a protein. Gel mobility shifts were observed with probes containing as few as seven Pt atoms per kilobase of duplex DNA. By competition experiments the dissociation constant, K_d, of the protein from cisplatin-modified DNA was estimated to be (1–20) × 10^{-10} M. Protein binding is selective for DNA modified with cisplatin, [Pt(en)Cl_2] (en, ethylenediamine), and [Pt(dach)Cl_2] (dach, 1,2-diaminocyclohexane) but not with chemotherapeutically inactive *trans*-diamminedichloroplatinum(II) or monofunctionally coordinating [Pt(dien)Cl]Cl (dien, diethylenetriamine) complexes. The protein also does not bind to DNA containing UV-induced photoproducts. The protein binds specifically to 1,2-intrastrand d(GpG) and d(ApG) cross-links formed by cisplatin, as determined by gel mobility shifts with synthetic 110-bp duplex oligonucleotides; these modified oligomers contained five equally spaced adducts of either *cis*-[Pt(NH_3)$_2$d(GpG)] or *cis*-[Pt(NH_3)$_2$d(ApG)]. Oligonucleotides containing the specific adducts *cis*-[Pt(NH_3)$_2$d(GpTpG)], *trans*-[Pt(NH_3)$_2$d(GpTpG)], or *cis*-[Pt(NH_3)$_2$(N3-cytosine)d(G)] were not recognized by the protein. The apparent molecular weight of the protein is 91 000, as determined by sucrose gradient centrifugation of a preparation partially purified by ammonium sulfate fractionation. Binding of the protein to platinum-modified DNA does not require cofactors but is sensitive to treatment with 5 mM $MnCl_2$, $CdCl_2$, $CoCl_2$, or $ZnCl_2$ and with 1 mM $HgCl_2$. This protein, alone or in conjunction with other cellular constituents, could be of general importance in the initial stages of processing of mammalian DNA damaged by cisplatin or other genotoxic agents and may belong to a wider class of such cellular damage-recognition proteins (DRPs).

cis-Diamminedichloroplatinum(II) (*cis*-DDP[1] or cisplatin) is a clinically important anticancer drug used mainly to combat ovarian and testicular malignancies (Loehrer & Einhorn, 1984). The major cellular target for *cis*-DDP is generally accepted to be DNA (Roberts & Thomson, 1979), although it is not yet certain whether antitumor efficacy is a consequence of impaired replication or transcription (Sorenson & Eastman, 1988a,b). Covalent coordination of the hydrolysis products of *cis*-DDP (Lee & Martin, 1976; Lim & Martin, 1976) to the DNA bases inhibits DNA synthesis in vitro (Pinto & Lippard, 1985b) and in vivo (Harder & Rosenberg, 1970; Howle & Gale, 1970) and causes mutagenesis (Burnouf et al., 1987). The major DNA adducts of *cis*-DDP are d(GpG) and d(ApG) 1,2-intrastrand cross-links (Sherman & Lippard, 1987), representing 65% and 25% of all adducts, respectively (Fichtinger-Schepman, 1985; Eastman, 1986). Minor products include 1,3-intrastrand cross-links at d(GpNpG) sequences, where N can be any nucleotide, interstrand cross-links, and protein–DNA cross-links. Platinum adducts form at the N7 atoms of guanine and adenine.

trans-Diamminedichloroplatinum(II), the geometric isomer of *cis*-DDP in which the ammine and chloride ligands are in mutually trans positions, is ineffective as a chemotherapeutic agent (Connors et al., 1972). *trans*-DDP will block replication at doses equitoxic to those of *cis*-DDP. It has been postulated that differential repair may be responsible for the chemotherapeutic effectiveness of *cis*-DDP compared to *trans*-DDP (Ciccarelli et al., 1985). The *trans*-DDP reaction products with DNA include monofunctional adducts, intrastrand cross-links, interstrand cross-links, and protein–DNA cross-links (Pinto & Lippard, 1985b; Eastman & Barry, 1987; Eastman et al., 1987; Bancroft et al., submitted for publication). *trans*-DDP cannot form intrastrand cross-links between adjacent nucleotides, and this observation has led to the suggestion that the d(GpG) and d(ApG) adducts formed uniquely by *cis*-DDP are responsible for its antitumor activity (Caradonna & Lippard, 1983; Pinto & Lippard, 1985a). This hypothesis is supported by the observation that most chemotherapeutically effective platinum compounds have chloride ligands in cis positions and are believed to form a spectrum

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[1] Abbreviations: DRP, damage-recognition protein; bp, base pair; ds, double stranded; ss, single stranded; CTAB, (hexamethyldecyl)trimethylammonium bromide; dach, 1,2-diaminocyclohexane; DDP, diamminedichloroplatinum(II); dien, diethylenetriamine; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; en, ethylenediamine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid–NaOH; r_b, bound drug/nucleotide ratio; RF, replicative form; SDS, sodium dodecyl sulfate; TE, 10 mM Tris-HCl, pH 8, and 1 mM Na_2EDTA.

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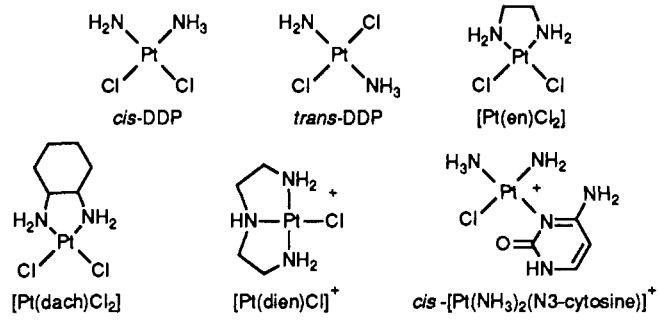
of DNA adducts similar to those of *cis*-DDP, i.e., 1,2-intrastrand cross-links (Lippard et al., 1983). Covalent modification of adjacent nucleotides in DNA is not an absolute prerequisite for antitumor activity of all platinum compounds, however, since recent work has shown that some platinum compounds that form only monofunctional adducts can also be effective antitumor agents (Hollis et al., 1989). The mechanism of action of these latter drugs has yet to be established.



The clinical efficacy of *cis*-DDP and other anticancer drugs is determined by the differential toxicity of the drug to tumor cells as compared to the cells of surrounding normal tissues. This toxicity imbalance may be influenced by many biochemical and other pharmacokinetic factors, including the differential ability to remove adducts (Ciccarelli et al., 1985). This same mechanism has been postulated to explain why some cells are resistant and others sensitive to *cis*-DDP (Masuda et al., 1988; Eastman & Schulte, 1988; Sheibani et al., 1989). The pathways by which platinum adducts are removed from DNA have been the subject of detailed investigation in bacteria. In *Escherichia coli*, both genetic evidence and biochemical evidence indicate that the *uvr*ABC-encoded excision repair system plays a role in *cis*-DDP adduct removal (Konishi et al., 1981; Beck et al., 1985). Genetic studies have additionally shown that *dam* mutants of *E. coli* display increased sensitivity to killing by *cis*-DDP (Fram et al., 1985), suggesting that the postreplication mismatch repair system also plays a role in the processing of *cis*-DDP adducts. Interestingly, the adducts of *trans*-DDP are comparatively unaffected by either DNA repair system (Beck et al., 1985; Fram et al., 1985).

By contrast to the extensive literature on platinum adduct repair in prokaryotes, comparatively little information is available about analogous pathways in mammalian cells. It is known that, when mammalian cells are treated with platinum compounds, adducts form and then are removed from DNA by a process that presumably is enzymatic (Ciccarelli et al., 1985; Bedford et al., 1988; Dijt et al., 1988; Eastman & Schulte, 1988; Masuda et al., 1988; Sheibani et al., 1989; Ullah et al., 1989). Furthermore, mammalian cells possessing suspected defects in excision repair, such as human xeroderma pigmentosum and Fanconi's anemia cells, are highly sensitive to *cis*-DDP (Plooy et al., 1985). To date, however, the specific cellular factors catalyzing platinum adduct removal from the mammalian genome have not been isolated.

By analogy to the pathway of nucleotide excision repair in *E. coli* (Myles & Sancar, 1989; Grossman et al., 1989), a reasonable first step in mammalian excision repair would be the recognition of damaged sites by a DNA-binding factor, presumably a protein. Recently Chu and Chang (1988) and our laboratories (Toney et al., 1989) used different experimental approaches to demonstrate the presence of such a *cis*-DDP adduct binding activity in extracts from mammalian cells, although it is not yet certain that the binding factors described in the two studies are identical. Our studies additionally determined the molecular weight of the factor to be approximately 100 000 and reported the molecular cloning of a cDNA encoding a portion of a similar or identical protein. Moreover, by using the cDNA clone, we employed Northern analysis to reveal a message corresponding to a protein of the same size as observed in crude extracts and established that the recombinant and crude cellular proteins bind similarly to platinated DNAs.

In the present report we describe the use of an electrophoretic gel mobility shift assay (Fried & Crothers, 1981), in conjunction with chemically synthesized oligonucleotide probes containing specific adducts, to characterize the structural features of platinated DNA that are recognized by the damage-recognition protein (DRP). The binding constant to platinum-damaged DNA and other properties of this cisplatin DRP are also described.

Materials and Methods

Materials. Restriction endonucleases and polynucleotide kinase were purchased from New England Biolabs. The Klenow fragment of *E. coli* polymerase I and bacteriophage T4 DNA ligase (Boehringer Mannheim Biochemicals), proteinase K and RNase A (Sigma), CTAB (Fluka), and poly(dI-dC)·poly(dI-dC) (Pharmacia) were obtained from commercial sources. The cell lines used were HeLa (from M. Chow, MIT), *cis*-DDP-resistant HeLa, V79 hamster, and *cis*-DDP-resistant V79 cells (from S. L. Bruhn, MIT), and human B cells (RPMI 4265; from H. Singh, MIT).

Cell Extract Preparation. Cytosolic, nuclear, and whole-cell extracts were prepared according to published procedures [Stillman and Gluzman (1985), Dignam et al. (1983), and Wood et al. (1988), respectively]. Protein concentrations were determined by the method of Bradford (1976).

Preparation of Platinum-Modified DNA Probes. *cis*-DDP, *trans*-DDP, [Pt(en)Cl_2], [Pt(dach)Cl_2], and [Pt(dien)Cl]Cl were prepared as described (Johnson, 1966; Dhara, 1970; Watt & Cude, 1968; Lippard et al., 1983). Restriction fragments, a 274-bp *Cla*I–*Sma*I fragment generated from pSTR3 (Couto et al., 1989) and a 422-bp *Ava*I fragment generated from bacteriophage M13mp18 DNA, were purified on low melting point agarose electrophoresis gels followed by phenol extraction (Maniatis et al., 1982) or butanol extraction in the presence of CTAB (Langridge et al., 1980). The DNA fragments were suspended in 1 mM sodium phosphate buffer, pH 7.4, containing 3 mM NaCl (buffer B) or in TE at a DNA nucleotide concentration of $\sim 10^{-4}$ M. A portion of the DNA was allowed to react with the appropriate platinum complex at a variety of formal drug/nucleotide ratios at 37 °C for 12–24 h. An identical volume of buffer B or TE was added to control, unmodified DNA and incubated in parallel with the modified DNA fragment. Unbound platinum was removed by ethanol precipitation, followed by several washes with 80% ethanol. DNA concentrations were determined by UV spectroscopy with the relation 1 OD_{260} = 50 μg/mL. Bound levels of Pt to DNA were measured on a Varian AA-1475 atomic absorption spectrometer equipped with a GTA-95 graphite furnace. DNA fragments were labeled with [α-^{32}P]dCTP (>5000 Ci/mmol, New England Nuclear) by the Klenow fragment of DNA polymerase I. Labeled fragments were purified on native polyacrylamide gels (Maniatis et al., 1982) and resuspended in TE to 5000 cpm/μL.

Construction of Oligonucleotides Containing Specific Platinum–DNA Adducts. Oligonucleotides 22 bases in length containing single, 1,2-intrastrand d(GpG) or d(ApG) or 1,3-intrastrand d(GpTpG) adducts of *cis*-DDP, the 1,3-intrastrand d(GpTpG) adduct of *trans*-DDP, or the monofunctional N7-

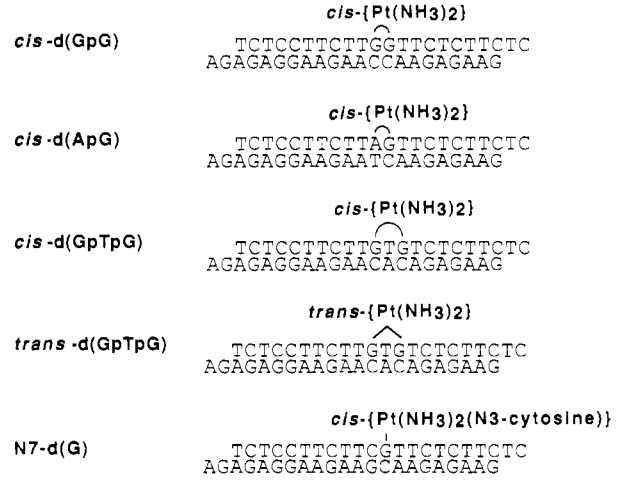


FIGURE 1: Duplex oligonucleotides containing specific platinum adducts. The 22-base oligonucleotides containing specific platinum adducts and designated as Top strands are shown with their complementary Bottom strands. The oligonucleotides contain a single 1,2-intrastrand d(GpG) or d(ApG) or a 1,3-intrastrand d(GpTpG) adduct of *cis*-DDP, 1,3-intrastrand d(GpTpG) adduct of *trans*-DDP, or monofunctional N7–d(G) adduct formed by $[Pt(NH_3)_2(N3\text{-cytosine})Cl]Cl$.

d(G) adduct of $\{Pt(NH_3)_2(N3\text{-cytosine})\}^{2+}$ were prepared as previously reported (Rice et al., 1988; Bellon & Lippard, 1990; Bellon and Lippard, unpublished results). These oligonucleotides are designated as "Top" strands. Unmodified Top strands were also constructed as controls. Complementary oligonucleotides designated as "Bottom" strands were constructed such that, when annealed to the adducted single-stranded fragments, they formed duplexes containing two-base 3′-overhangs at both ends (Figure 1). The Bottom oligonucleotides were 5′-end labeled with [γ-^{32}P]ATP (<3000 Ci/mmol, New England Nuclear) by polynucleotide kinase and purified from unincorporated ATP on a Nensorb-20 column (New England Nuclear). Adducted and control Top oligonucleotides were 5′-end phosphorylated with nonradioactive ATP and also purified on Nensorb-20 columns. Top and Bottom strands were mixed at a mole ratio of 4:3, heated at 90 °C, and then cooled slowly to 4 °C to allow the two strands to anneal. High-concentration T4 DNA ligase (10000 units/mL) was added, and the samples were incubated overnight at 13 °C. Double-stranded oligonucleotides of 44, 66, 88, and 110 bp in length were then purified from native polyacrylamide gels as described above.

Preparation of UV-Damaged DNA Probes. The 422-bp DNA fragment derived from *Ava*I digestion of M13mp18 was purified by electrophoresis through a low-melting agarose gel followed by butanol extraction in the presence of CTAB. DNA fragments were labeled with [α-^{32}P]dCTP and purified as above. The labeled DNA fragments were then irradiated with a General Electric 15-W germicidal lamp (maximum output at 254 nm) calibrated with a UVX digital radiometer at a flux of 5 J/(m^2·s) and a final dose of 1500 J/m^2.

Electrophoretic Gel Mobility Shift Assay. The study of protein–DNA binding complexes with the use of gel electrophoresis was carried out as described by Carthew et al. (1985) with minor modifications. End-radiolabeled DNA restriction fragments [(1–5) × 10^3 cpm; ~0.2 ng) that were either unmodified or modified with the various platinum compounds were incubated in the presence of crude extracts, typically 5–10 μg of protein, and 6 μg of poly(dI-dC)·poly(dI-dC) for 15 min at 37 °C in binding buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5 mM Na_2EDTA, 5% glycerol, and 1 mM DTT) in a final volume of 10–50 μL. Protein–DNA complexes were then resolved on a 4% polyacrylamide gel [29:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)]. Gels were preelectrophoresed in Tris–glycine buffer (50 mM Tris-HCl, pH 8.5, 380 mM glycine, 2 mM Na_2EDTA) for >1 h at 25 mA. Samples subsequently were electrophoresed for ~4 h at 30 mA. Gels were dried and autoradiographed overnight at −120 °C with an intensifying screen.

Competition Assays. Competition assays were performed by adding various amounts of unlabeled competitor DNA to the binding reactions of the gel mobility shift assay before the 15-min incubation step. The competitor DNA was either a restriction fragment or M13mp18 RF DNA that was either unmodified or modified with *cis*-DDP or UV.

Determination of the Binding Constant of the cis-DDP DRP. The binding constant of the protein to platinated DNA was estimated as described by Müller (1983). A competition assay was performed in which radiolabeled 274-bp fragment modified with *cis*-DDP at an r_b of 0.036 was incubated in the presence of increasing amounts of unlabeled 274-bp fragment modified with *cis*-DDP to the same extent. Binding reactions were done in triplicate for each level of competitor DNA. The amount of labeled platinated DNA bound to the protein was estimated by scintillation counting of the free and bound labeled DNA excised from dried gels.

Ammonium Sulfate Precipitation of the cis-DDP–DNA DRP from Crude Cellular Extracts. Saturated ammonium sulfate was added dropwise to HeLa cytosolic extracts to a final concentration of 40%. The mixture was stirred on ice for 30 min and centrifuged at 11000 rpm in a Sorvall SM24 rotor for 30 min. The protein in the supernatant was precipitated with ammonium sulfate added as above to a final concentration of 65%. The 40–65% fraction was resuspended in buffer H (25 mM HEPES, pH 7.5, 150 mM KCl, 0.1 mM Na_2EDTA, 1 mM DTT, and 10% glycerol) and dialyzed extensively against the same buffer.

Sucrose Gradient Ultracentrifugation. A portion of the ammonium sulfate fraction representing 1 mg of protein was centrifuged through a 0–15% sucrose gradient for 18 h at 43600 rpm ($\omega t^2 = 1.34 \times 10^{12}$, 170000*g*) in a Beckman SW 50.1 rotor. Fractions were removed from the top of the gradient and dialyzed extensively against buffer H. Fractions were assayed for *cis*-DDP–DNA binding activity by the gel mobility shift assay. Protein standards were centrifuged in parallel as molecular weight markers. Fractions from this gradient were precipitated with methanol/chloroform (3:1) and resuspended in SDS loading dye (0.3 M Tris base, pH 9.0, 50% glycerol, 5% SDS, 5% 2-mercaptoethanol, 0.0025% bromphenol blue). The fractions were then electrophoresed through a 12% SDS–polyacrylamide gel, and the gel was stained with Coomassie blue R-250 to detect protein.

Proteinase K and Ribonuclease A Pretreatment of Cellular Extracts. Extracts were incubated in the presence of proteinase K at 100 μg/mL or RNase A at 20 μg/mL for 1 h at 37 °C in 10 mM Tris-HCl, pH 7.4, containing 1 mM Na_2EDTA. Pretreated extracts were then examined for Pt–DNA binding by the electrophoretic gel mobility shift assay.

RESULTS

DNA Binding Characteristics of a Cellular Protein That Recognizes Platinum-Modified DNA. A cellular protein (DRP) that binds selectivity to cisplatin-modified DNA was readily detected by the electrophoretic mobility shift assay (Figure 2). The DNA binding protein retarded the migration of radiolabeled platinum-modified DNA through the gel, enabling the DRP-modified DNA complex to be visualized in nuclear extracts from human HeLa and Chinese hamster V79

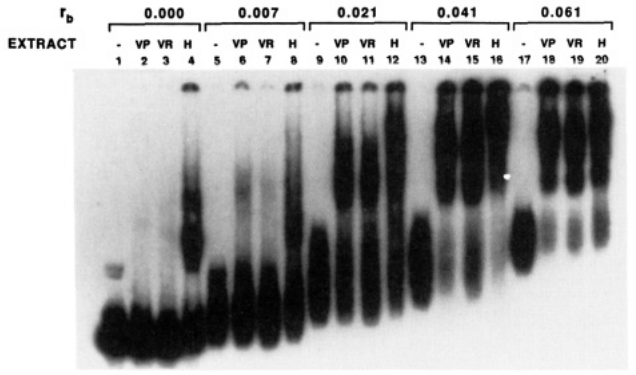


FIGURE 2: Binding of cellular factors to platinum-modified DNA at different bound ratios of Pt/nucleotide (r_b). End-labeled 422-bp DNA restriction fragments containing bound *cis*-DDP levels of 0 (lanes 1–4), 0.007 (lanes 5–8), 0.021 (lanes 9–12), 0.041 (lanes 13–16), and 0.061 (lanes 17–20) were incubated in the absence (lanes 1, 5, 9, 13, and 17) or presence of crude nuclear extract prepared from V79 parental (VP) (lanes 2, 6, 10, 14, and 18), V79 *cis*-DDP-resistant (VR) (lanes 3, 7, 11, 15, and 19), or HeLa (H) (lanes 4, 8, 12, 16, and 20) cell lines. Migration of unbound DNA is retarded with increasing levels of modification (lanes 1, 5, 9, 13, and 17) owing to increased positive charge and increased structural alterations of the DNA as a result of *cis*-DDP binding (Sherman & Lippard, 1987). Note also that cellular factors from HeLa nuclear extracts bind to unmodified DNA (lane 4). The binding is reproducible, independent of the oligonucleotide probe, and currently of unknown origin. A second band also appears with the unmodified DNA probe (lane 1) and probably represents denatured probe DNA.

parental and *cis*-DDP-resistant (adapted to 15 μg/mL, ~30-fold resistant; S. L. Bruhn, D. E. Housman, and S. J. Lippard, unpublished results) cell lines. Selectivity for platinated DNA was demonstrated by the correlation between the extent of binding and the level of DNA platination. An estimated minimum modification level of ~0.007 Pt/nucleotide was required to observe binding of the protein to labeled modified DNA, whereas, at a modification level of 0.06 Pt/nucleotide, nearly all labeled DNA was complexed. For probes of higher r_b, two bands were observed in the gel (lanes 18–20), possibly indicating the binding of two protein molecules to those DNA fragments having higher numbers of damaged sites. In other experiments (data not shown), *cis*-DDP-specific DNA DRPs were found in cytosolic extracts and whole-cell extracts prepared from HeLa cells and in nuclear extracts from human B cells. Cytosolic and whole-cell extracts from this latter source were not examined. It has not yet been definitively established that the protein found in cytosolic extracts is the same protein as that found in nuclear extracts. Both proteins have similar specificites of binding to DNAs modified with various platinum compounds, however. Furthermore, both proteins are precipitated with 40–65% ammonium sulfate.

A series of competitive binding experiments was performed to assess the specificity and affinity of the DRP for *cis*-DDP-treated DNA. As shown in Figure 3, binding to the labeled 274-bp fragment platinated at 0.036 Pt/nucleotide was effectively competed by increasing quantities of unlabeled fragment modified to the same extent (lanes 6–20). By contrast, unplatinated DNA did not compete with the labeled platinated DNA for binding of the cellular factor (data not shown). Competition for binding was complete when a 100-fold excess of unlabeled platinated DNA was added to the binding reaction mixture (lanes 18–20). Binding of protein to labeled, platinated DNA was inhibited by 50% at a 3-fold excess of unlabeled platinated DNA. From these results the affinity constant of the *cis*-DDP DRP could be estimated. It was assumed that bands 1–3 in each lane represented one, two, and three bound protein molecules, respectively. DNA in the

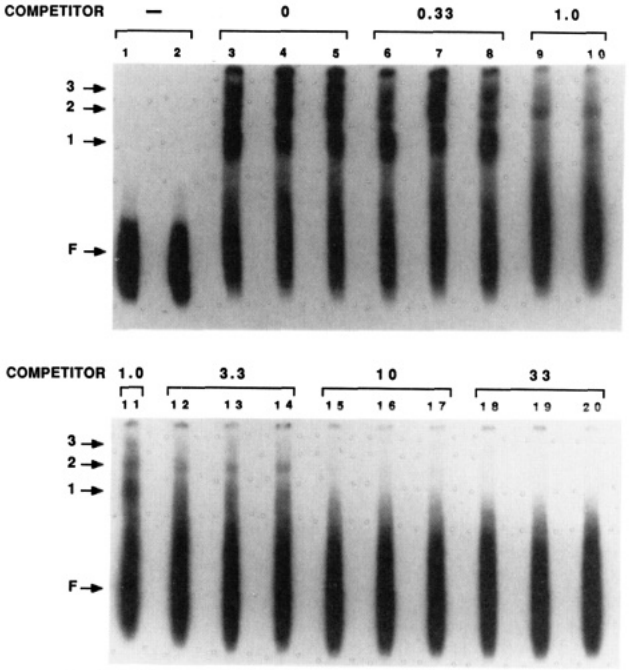


FIGURE 3: Binding to labeled platinum-modified DNA can be competed with unlabeled platinum-modified DNA. End-labeled 274-bp fragment of pSTR3 DNA (~0.33 ng) modified with *cis*-DDP at r_b = 0.036 was incubated in the presence of 24 μg of cytosolic extract from HeLa cells and 0.33–33 ng of unlabeled 274-bp fragment modified with *cis*-DDP at r_b = 0.036 (lanes 6–20). Unbound 274-bp fragment modified at r_b = 0.036 is shown in lanes 1 and 2, and binding of the cellular factor to this fragment in the absence of competitor DNA is shown in lanes 3–5.

well of each lane was also assumed to contain bound protein. From these data the extent of inhibition of binding due to the competitor DNA could be calculated. The affinity constant was determined from the equation derived by Müller (1983):

$$K = 8/\{3([I_t] - [T_t])\}$$

where $[I_t]$ represents the concentration of unlabeled platinated DNA that results in 50% inhibition of binding and $[T_t]$ represents the concentration of labeled platinated DNA. The dissociation constant (K_d) is the reciprocal of the binding constant (K). From the results of this competition experiment, K_d was estimated to be $\sim 1 \times 10^{-10}$ M. This estimate, which is a lower limit, was made by assuming one binding site for each molecule of DNA. Bands 2 and 3, however, suggest that more than one protein can bind per molecule of DNA. Both the radiolabeled and unlabeled competitor DNA fragments contained an average of 20 platinum adducts. Since the *cis*-DDP DRP binds only to the 1,2-d(GpG) and -d(ApG) adducts formed by cisplatin, comprising 90% of all platinum adducts, it was assumed that each molecule of competitor DNA contained ~18 potential binding sites. When the concentrations of unlabeled and labeled binding sites were used in the above equation, the upper limit of the dissociation constant was calculated to be 2×10^{-9} M. The true value of the dissociation constant, therefore, lies in the range of $(1–20) \times 10^{-10}$ M. Competition assays with purified protein and probes containing single, site-specific platinum adducts will ultimately be used to determine the dissociation constant more accurately.

cis-DDP–DNA DRP Is Present at the Same Levels in Platinum-Sensitive and Platinum-Resistant Cell Lines. Platinated probes incubated with nuclear extracts from either V79 parental or *cis*-DDP-resistant cell lines were bound to similar extents, suggesting that the *cis*-DDP DRP is present in both cell lines at approximately equal levels. Similar results

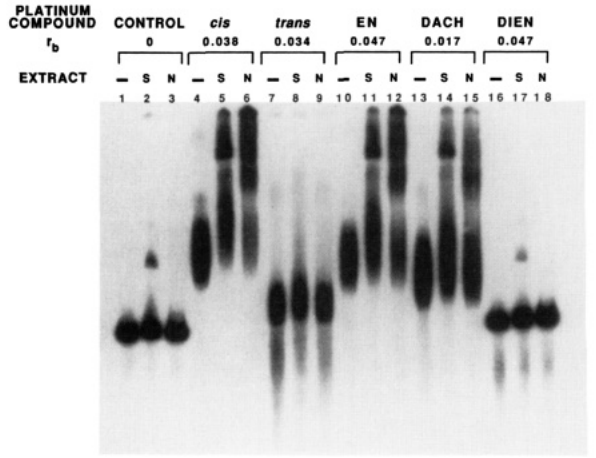


FIGURE 4: Selectivity of the *cis*-DDP–DNA binding factor for DNA modified with chemotherapeutically active platinum compounds. The 422-bp fragment (Figure 2) derived from M13mp18 was modified with *cis*-DDP at r_b = 0.038 (lanes 4–6), *trans*-DDP at r_b = 0.034 (lanes 7–9), [Pt(en)Cl]Cl at r_b = 0.047 (lanes 10–12), [Pt(dach)Cl_2] at r_b = 0.017 (lanes 13–15), or [Pt(dien)Cl]Cl at r_b = 0.047 (lanes 16–18). Unmodified 422-bp fragment appears in lanes 1–3. DNA fragments were incubated in the absence (–) (lanes 1, 4, 7, 10, 13, and 16) or presence of cytosolic (S) (lanes 2, 5, 8, 11, 14, and 17) or nuclear extracts (N) (lanes 3, 6, 9, 12, 15, and 18) prepared from HeLa cells.

were obtained with parental and ~50-fold *cis*-DDP-resistant HeLa cell extracts (data not shown). Hence, in these cell lines the amount of DRP does not seem to be associated with an acquired resistance to *cis*-DDP.

Protein Specifically Binds to DNAs Modified with Platinum Compounds Forming Intrastrand d(GpG) and d(ApG) Cross-Links. DNA probes modified by five different platinum complexes were used to determine the binding specificity of the DRP. As revealed in Figure 4, the cellular protein binds selectively to DNA modified with *cis*-DDP, [Pt(en)Cl_2], and [Pt(dach)Cl_2] but not to DNA modified with either *trans*-DDP or [Pt(dien)Cl]Cl. The latter two platinum compounds are unable to link adjacent nucleotides in DNA, whereas the former three can do so.

Further identification of the *cis*-DDP adducts to which the protein binds was approached by preparing a series of duplex oligonucleotides containing known, specifically placed platinum–DNA adducts (Figure 1). These uniquely modified DNAs were used as probes in the electrophoretic gel mobility shift assay (Figure 5). There is substantial nonspecific binding to these probes, as evidenced by the presence of slower migrating bands seen in the cases where the oligonucleotides were not modified with platinum (Figure 5A,B, lanes 2–8). Specific binding was observed, however, to DNA probes containing the 1,2-intrastand d(GpG) and d(ApG) cross-linked adducts of *cis*-DDP (Figure 5A,B, lane 10). Binding of the DRP occurred to oligonucleotides 88 or 110 bp in length but not to probes that were 44 or 66 bp long (data not shown). This probe size limitation presumably reflects a minimum requirement for a flanking nucleic acid domain in order for protein binding to occur. Binding was not observed with randomly modified DNA fragments at r_b values of less than 0.007, suggesting that a minimum level of modification is required for binding of the DRP in crude extracts. The band representing specific binding to platinated oligonucleotides of 110 bp could be competed away with an ~340-fold excess of unlabeled M13mp18 RF DNA modified with *cis*-DDP at a bound drug to nucleotide level of 0.041 (lane 16) but not with unlabeled unplatinated M13mp18 DNA (lane 13) at the same ~340-fold excess (Figure 5A,B). No specific binding occurred in cases where the DNA probes contained the d(GpTpG) 1,3-intrastrand cross-linked adducts of *cis*-DDP and *trans*-DDP or the monofunctional d(G)-N7 adduct of $\{Pt(NH_3)_2(N3\text{-cytosine})\}^{2+}$ (Figure 5C).

The 1,2-intrastrand d(GpG) and d(ApG) DNA adducts formed by *cis*-DDP bend the helix (Rice et al., 1988; Bellon & Lippard, 1990) and possibly produce a localized ss region in the vicinity of the platinum lesions. Such a ss motif was detected by antinucleoside antibodies (Sundquist et al., 1986). This result suggested that the DRP might recognize a single-stranded domain near the site of platinum binding on DNA. To address this possibility, a competition assay was performed in which nuclear extracts from HeLa cells were incubated in the presence of 5000 cpm (0.2 ng) of a 274-bp ds fragment of DNA modified with *cis*-DDP at 0.040 Pt/nucleotide and 0.2–100 ng of unlabeled ss M13mp18. The ss DNA did not compete for binding of the DRP (data not shown), suggesting that the protein is not simply responding to ss domains.

DRP Does Not Recognize DNA Lesions Induced by UV Light. A factor has been reported in nuclear extracts prepared from HeLa cells that binds specifically to DNA damage induced by UV irradiation (Chu & Chang, 1988). In order to determine if this factor is related to the platinum DRP described here, a series of competition experiments with UV-damaged DNA was carried out (Figure 6). Binding of labeled DNA modified with *cis*-DDP at an r_b of 0.050 could be competed with a 100-fold excess of unlabeled M13mp18 RF DNA modified with *cis*-DDP at an r_b of 0.041. It was not competed, however, by a 1000-fold excess of M13mp18 RF DNA treated with UV at 1500 J/m^2, which corresponds to a calculated level (Spivak et al., 1988) of ~5.7 cyclobutane dimers per kilobase. Similarly, the binding of a cellular factor to labeled DNA modified with UV at 1500 J/m^2 could be competed with a 1000-fold excess of unlabeled UV-irradiated M13mp18 DNA but not with a 1000-fold excess of DNA platinated with *cis*-DDP. The factor that recognized the UV-treated DNA was found only in nuclear extracts, in agreement with the results of Chu and Chang (1988). These data indicate that the *cis*-DDP–DNA DRP is not the factor that recognizes UV-treated DNA.

Preliminary Characterization of the cis-DDP–DNA DRP. The size of the *cis*-DDP–DNA DRP was determined by sucrose gradient sedimentation. Prior to the sedimentation study, a cytosolic extract prepared from HeLa cells was fractionated by stepwise ammonium sulfate precipitation. The platinum–DNA specific binding activity was found in the 40–65% ammonium sulfate fraction. One milligram of this fraction was centrifuged through a linear 0–15% sucrose gradient (Johns & Stanworth, 1976). The profile of the gradient is shown in Figure 7. By use of gel electrophoretic mobility shift assays with the *cis*-DDP-platinated 422-bp restriction fragment as a probe, the DRP was located in fractions 7–12, with the peak of activity in fraction 9. The sedimentation coefficient was calculated to be 5.6, which corresponds to an apparent molecular weight of 91 000 for a globular protein.

The gel mobility shift assay was also used to assess the possible cofactor and metal ion requirements for binding of the DRP to *cis*-DDP-modified DNA. The factor in crude extracts required neither ATP nor divalent cations such as Mg^{2+} and was insensitive to EDTA at concentrations up to 100 mM. Binding activity was sensitive, however, to some metal ions. Binding to platinum-modified DNA was completely inhibited in the presence of 5 mM $ZnCl_2$, $MnCl_2$, $CoCl_2$, or $CdCl_2$ and by 1 mM $HgCl_2$. The protein binds to

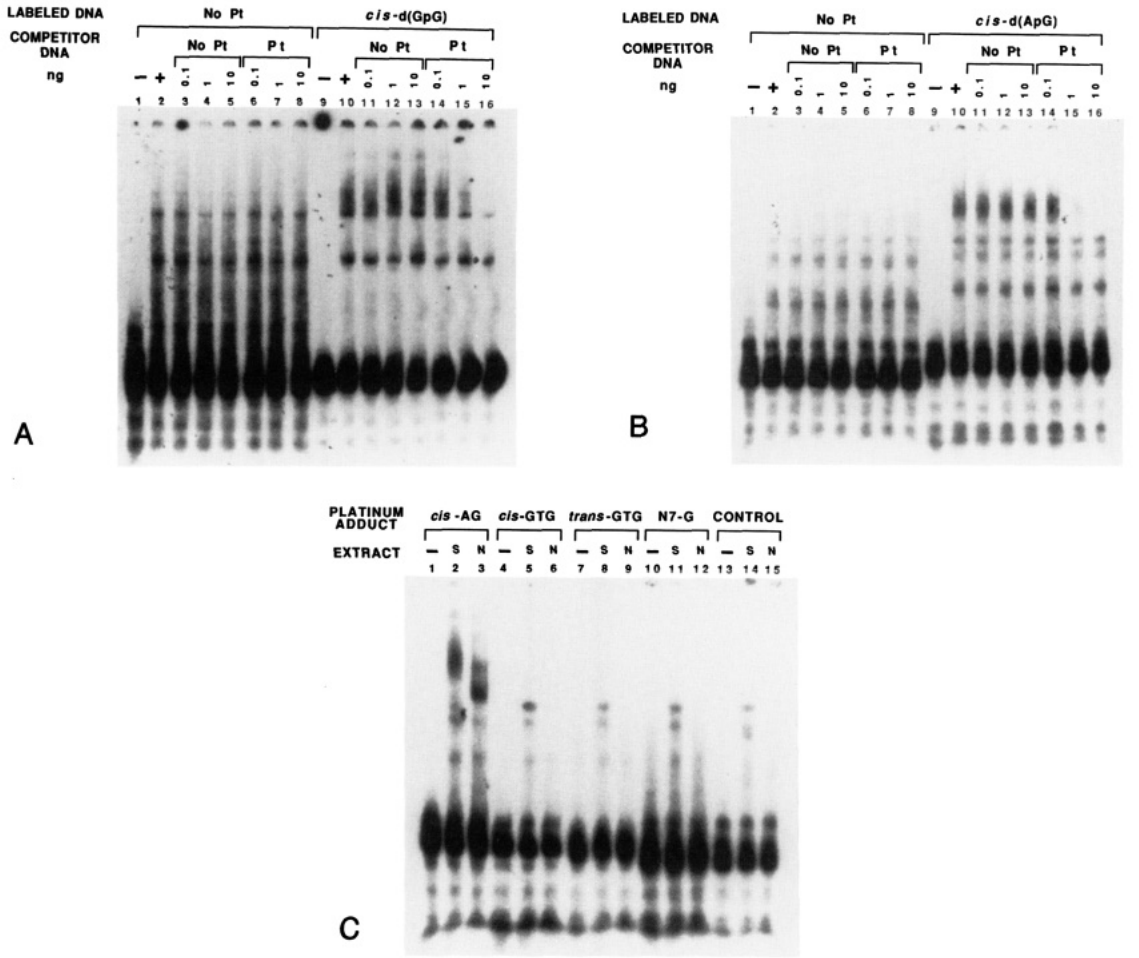


FIGURE 5: Specificity of the *cis*-DDP–DNA binding factor for the d(GpG) and d(ApG) 1,2-intrastrand adducts formed by *cis*-DDP (see Figure 1). (A) Competition assay with the 1,2-intrastrand d(GpG) adduct. DNA fragments, 110 bp in length, were prepared from oligonucleotides 22 bases in length that were either unmodified (lanes 1–8) or contained the d(GpG) adduct formed by *cis*-DDP (lanes 9–16). These oligonucleotides were incubated in the absence (–) (lanes 1 and 9) or presence of 20 μg of HeLa cytosolic extract (lanes 2–8 and 10–16). Unmodified, unlabeled M13mp18 DNA was used as a nonspecific competitor at 0.1, 1, and 10 ng per reaction (lanes 3, 4, 5, 11, 12, and 13). Unlabeled M13mp18 DNA modified with *cis*-DDP at $r_b = 0.041$ was used as a specific competitor at 0.1, 1, and 10 ng per reaction (lanes 6, 7, 8, 14, 15, and 16). Binding in the absence of competitor (+) is seen in lane 2 for the unmodified oligonucleotide and in lane 10 for the modified oligonucleotide. (B) Competition assay for the 110-bp DNA fragment containing a specific 1,2-intrastrand d(ApG) adduct (Figure 1). Lane designations as in panel A. (C) The *cis*-DDP–DNA binding factor does not bind to the d(GpTpG) 1,3-intrastrand cross-links formed by *cis*- or *trans*-DDP or a monofunctional adduct formed by $\{Pt(NH_3)_2(N3\text{-cytosine})\}^{2+}$ (Figure 1). DNA fragments, 110 bp in length, constructed from oligonucleotides containing the d(GpTpG) cis adduct (lanes 4–6), the d(GpTpG) trans adduct (lanes 7–9), or the N7-d(G) adduct of $[Pt(NH_3)_2(N3\text{-cytosine})]^+$ (lanes 10–12) were incubated in the presence of 20 μg of HeLa cytosolic extract (S) (lanes 5, 8, and 11) or 10 μg of HeLa nuclear extract (N) (lanes 6, 9, and 12). DNA probes incubated in the absence of extract (–) are seen in lanes 4, 7, and 10. Nonspecific binding to the unmodified 110-bp fragments is shown in lanes 13–15, and specific binding to the *cis*-d(ApG) adduct is shown in lanes 1–3.

platinated DNA at both 37 and 0 °C, but heating of the extracts at 42 °C for 15 min before the binding assay was carried out resulted in complete loss of activity. The binding activity was also inhibited at high salt concentrations, such as 500 mM KCl.

In an effort to determine the chemical nature of the cellular factor, cytosolic extracts were treated with either proteinase K at 100 μg/mL or RNase A at 20 μg/mL. Pretreatment of crude extracts with proteinase K resulted in loss of binding activity, confirming that the factor is a protein. Pretreatment of crude extracts with RNase A also resulted in loss of activity, but this sensitivity disappeared after partial purification of the *cis*-DDP–DNA binding factor by ammonium sulfate fractionation and ion exchange chromatography (B. A. Donahue, S. J. Lippard, and J. M. Essigmann, unpublished results).

Discussion

This work has elucidated several key properties of a mammalian protein that binds selectively to DNA modified with the antitumor drug *cis*-DDP. The platinum damage-recognition protein may be part of a DNA repair complex or it may be a cellular constituent that responds to structural elements that occur naturally in the genome. It is impossible at present to distinguish between these two possibilities. Since it is unlikely that biological systems would evolve a protein to complex with cisplatin adducts specifically, the *cis*-DDP–DNA DRP probably recognizes a structural motif common both to certain platinum–DNA adducts and to other types of DNA damage or possibly sequences that form tertiary DNA structural domains that are the sites of specific protein–DNA interactions.

Some insight into the nature of the genetic target for the DRP is afforded by the specificity of the protein for the 1,2-intrastrand d(GpG) and d(ApG) adducts formed by *cis*-DDP. The *cis*-DDP–DNA DRP does not recognize the 1,3-intrastrand d(GpTpG) adducts formed by *cis*- and *trans*-DDP nor a monofunctional adduct formed by $\{Pt(NH_3)_2(N3\text{-cytosine})\}^{2+}$ at the N7 position of deoxyguanosine. Interstrand cross-links formed by both *cis*- and *trans*-DDP were not examined. The 1,2-intrastrand d(GpG) adduct of *cis*-DDP produces a bend in the helix of DNA by 32–34° directed toward the major

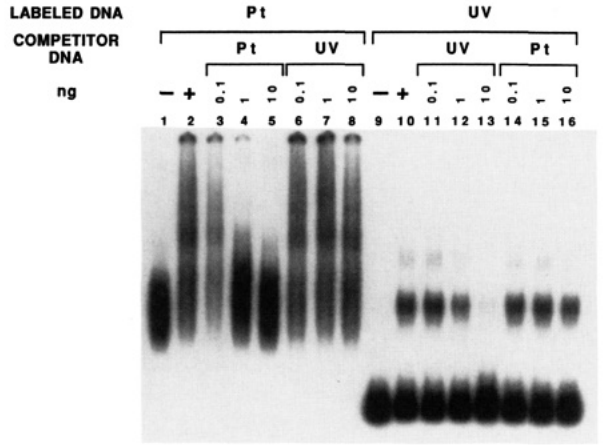


FIGURE 6: Binding of platinum-modified DNA cannot be competed with UV-irradiated DNA. End-labeled 422-bp fragment modified with *cis*-DDP at $r_b = 0.038$ was incubated in the presence of 10 μg of HeLa nuclear extract and 0.1–10 ng of unlabeled M13mp18 modified with either *cis*-DDP at $r_b = 0.041$ (lanes 3–5) or UV at 1500 J/m^2 (lanes 6–8). Binding in the absence of competitor is shown in lane 2, and DNA incubated in the absence of extract is shown in lane 1. End-labeled 422-bp fragment treated with UV at 1500 J/m^2 was used in lanes 9–16. Unlabeled UV-modified M13mp18 was added to the reactions in lanes 11–13 and unlabeled M13mp18 modified with *cis*-DDP was added to the reactions in lanes 14–16. Binding of the UV-modified DNA binding factor is shown in lane 10, and the labeled UV-modified 422-bp fragment incubated in the absence of extract is shown in lane 9. Binding reactions in this experiment were performed in 50 mM Na_2EDTA, which has been shown to be optimal for the UV-modified DNA binding factor.

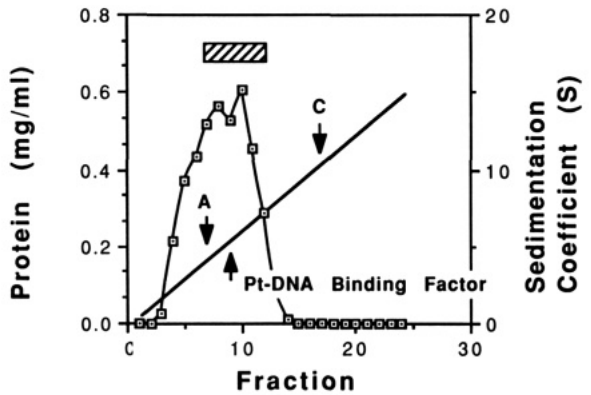


FIGURE 7: Sucrose gradient sedimentation of the *cis*-DDP–DNA binding factor. Conditions of ultracentrifugation are given under Materials and Methods. (⊡) Protein concentration (mg/mL); (—) sedimentation coefficient. Size markers: A = albumin (M_r = 67000); C = catalase (M_r = 232000). Hatched box = *cis*-DDP–DNA binding activity.

groove (Rice et al., 1988; Bellon & Lippard, 1990), suggesting the possibility that this helix kink is a structural feature recognized by the binding factor. It is clear from the present results, however, that not all DNA damage-induced bends are recognized. For example, the 1,3-intrastrand d(GpTpG) adduct of *cis*-DDP, which is not bound by the cellular DRP (Figure 5C), also bends the helix by 34° (Bellon & Lippard, 1990), although it is not known if this bend is directed toward the major groove of the DNA helix. The 1,3-intrastrand *trans*-DDP adduct at d(GpTpG) sites similarly is unrecognized by the DRP, possibly because its bend is more flexible (Bellon & Lippard, 1990). Finally, cyclobutane-type pyrimidine dimers formed by UV irradiation, which are not recognized by the DRP under investigation here (Figure 6), also have been suggested to bend the DNA helix by ~30° (Husain et al., 1988), probably in the direction of the major groove (Pearlman et al., 1985). The *cis*-DDP-DNA DRP, therefore, does not appear to recognize specifically the ~32° kinks in the helix, nor does it respond to ss DNA formed opposite the platinum lesions, as evidenced by the failure of ss DNA to compete with platinum-modified DNA for binding. The protein may, however, recognize a combination of helix axis bending and local unwinding at the site of platination in 1,2-intrastrand *cis*-DDP–DNA cross-links. The 1,3-intrastrand *cis*-d(GpTpG) adduct unwinds the DNA helix to a much greater extent than the 1,2-intrastrand d(GpG) and d(ApG) adducts (S. F. Bellon and S. J. Lippard, unpublished results). This 1,3-intrastrand cross-linked adduct may therefore unwind the helix too much for DRP binding. The possibility that a residue on the DRP binds directly to the platinum atom is unlikely since the protein does not bind to DNA modified with *trans*-DDP, [Pt(dien)Cl]Cl, or the specific monofunctional adduct formed by {Pt$(NH_3)_2$(N3-cytosine)}$^{2+}$ (Figure 5C).

The DRP we describe here and, presumably, that reported by Chu and Chang (1988) may be identical with the protein that binds specifically to DNA platinated with *cis*-DDP recently identified by a modified Western blot analysis (Toney et al., 1989). The latter study also described the isolation from a human cDNA library of two clones that encode a platinum-modified DNA binding protein. Results of the modified Western blot analysis, as well as those of Northern blot analysis using the recombinant DNA as a probe, indicate that the protein we have previously studied has an apparent molecular weight of ~100000. In the present work, sucrose gradient sedimentation analysis of the *cis*-DDP–DNA DRP observed in the electrophoretic gel mobility shift assay reveals an apparent molecular weight of 91000. The similarity of these values suggests that the two proteins identified by two independent techniques are probably the same. Both proteins also have the same binding specificities for DNA modified with different platinum compounds. Further work is required, however, to ascertain whether or not they are indeed the same protein.

The *cis*-DDP–DNA DRP is sensitive to treatment with proteinase K, and when present in crude cellular extracts, the protein does not appear to require any specific cofactors to bind to platinum-damaged DNA. Binding is inhibited, however, by addition of metal ions that have an affinity for sulfur donor ligands, suggesting that thiols may be involved at or near the sites on the protein responsible for its binding to platinum-modified DNA.

The function of the *cis*-DDP–DNA DRP is unknown although, as indicated earlier, one possible role is to recognize sites of DNA damage as a signaling event for DNA repair. A current model for recognition of DNA damage by the *E. coli* ABC excision system is that UvrA forms a complex with UvrB, either in solution or after it has bound to DNA at a site of damage (Orren & Sancar, 1989). UvrA then dissociates from DNA, and UvrB, in conjunction with UvrC, excises an oligonucleotide encompassing the damage. The resulting gap is then filled in with the correct nucleotides by DNA polymerase. On the basis of this model, a reasonable role for the platinum DRP would be to serve as the mammalian analogue of UvrA in *E. coli*.

It is noteworthy that the *cis*-DDP–DNA DRP binds to DNA modified with chemotherapeutically active platinum compounds but not with two inactive platinum compounds. Moreover, the specific adducts recognized comprise ~90% of all cisplatin–DNA structures formed in vivo. If the protein were a component of a repair complex, it is possible that it facilitates the antitumor effectiveness of cisplatin. If tumor cells were deficient, relative to nontumor cells, in their ability

to repair platinum-damaged DNA, the platinum drug would be selectively lethal to tumor cells. Repair-proficient surrounding cells would remove platinum adducts from their DNA and hence survive. Platinum–DNA adducts not recognized by the *cis*-DDP–DNA DRP, such as those formed by *trans*-DDP, would not be repaired in either tumor or normal cells and thus would be toxic to both types of cells. One caveat to this model is that cells are generally much less sensitive to *trans*- than to *cis*-DDP (Ciccarelli et al., 1985). *trans*-DDP, however, is much more likely to interact with other cellular constituents prior to reaching the cellular DNA, and hence, it may be less effective than *cis*-DDP because it is less likely to bind to the DNA at equitoxic doses. Another caveat is that the *cis*-DDP–DNA DRP does not recognize the monofunctional adduct formed by $\{Pt(NH_3)_2(N3\text{-cytosine})\}^{2+}$, which is effective as an antitumor drug (Hollis et al., 1989). The latter compound, however, may be chemotherapeutically effective by a mechanism different from that of *cis*-DDP.

Alternatively, the factor may not be involved in DNA repair at all. The *cis*-DDP–DNA DRP may actually impede DNA repair by binding to the 1,2-intrastrand d(GpG) and d(ApG) adducts of *cis*-DDP, thereby shielding these adducts from the DNA repair machinery. DNA adducts not recognized by the *cis*-DDP–DNA DRP would be accessible to repair and would be less lethal. A third possibility is that the normal function of the DRP is to regulate tumor cell gene expression. Platinum adducts, by affording structures that mimic those of the natural regulatory sequences of such genes, would titrate the DRP away from its normal cellular receptors and thus selectively compromise the welfare of the tumor cell. Normal cells, in which such gene expression might be less critical, would be less vulnerable to the drug.

A number of other DNA DRPs have been reported that bind DNA modified with UV light (Feldberg & Grossman, 1976; Tsang & Kuhnlein, 1982) and *N*-acetyl-2-aminofluorene (Moranelli & Lieberman, 1980), DNA containing apurinic sites (Deutsch & Linn, 1979), and DNA containing G-T mismatches (Jiricny et al., 1988). None of these proteins were found to have exonucleolytic or endonucleolytic activity, and none appear to be the *cis*-DDP–DNA DRP on the basis of molecular weight, binding specificity, or other requirements.

In summary, the experiments described here have led to the characterization of a mammalian cellular protein that selectively recognizes and binds DNA containing cisplatin 1,2-intrastrand cross-links. The protein may be involved in initial recognition of damaged DNA as part of a repair event. Alternatively, the protein may be a part of the cellular response to stress, may be involved in maintaining the tertiary structure of DNA, or may initiate or suppress a DNA-directed function at a specific tertiary structure. It should be emphasized that the *cis*-DDP–DNA DRP occurs and produces approximately the same band shift in all cell lines tested; hence, it may be ubiquitous to all mammalian cells.

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Registry No. *cis*-[$Pt(NH_3)_2$d(GpG)], 97333-55-6; *cis*-[$Pt(NH_3)_2$d(ApG)], 119637-81-9.

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