cis- and *trans*-Diamminedichloroplatinum(II) Interstrand Cross-Linking of a Defined Sequence Nucleosomal Core Particle[†,‡]

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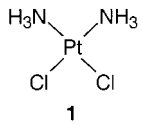
ABSTRACT: Interstrand cross-linking studies with the antitumor drug *cis*-diamminedichloroplatinum(II) and its clinically inactive isomer, *trans*-diamminedichloroplatinum(II), were performed on a fragment of the 5S rRNA gene of *Xenopus borealis* in the free and nucleosomal state. 5S nucleosomes were formed via histone octamer exchange from chicken erythrocyte core particles. Native polyacrylamide gel electrophoresis was used to probe the ability of platinated DNA to reconstitute into core particles. Both isomers negatively impacted reconstitution when histones were present during incubation with the drug. When histones were not present during the drug treatment, platinated DNA was successfully reconstituted into core particles. These results suggest that platination of histones impedes reconstitution of free DNA. However, already-formed core particles were not disrupted upon platination. Sites of interstrand cross-linking were probed through denaturing polyacrylamide gel electrophoresis and quantitative phosphorimagery. We found both site-specific enhancement and depression of *cis*-diamminedichloroplatinum(II) cross-linking in the nucleosomal samples relative to free DNA at both drug concentrations that were tested (0.01 and 0.0025 mM). *trans*-Diamminedichloroplatinum(II) exhibited no detectable differences in the interstrand cross-linking of free and nucleosomal samples.

A number of anticancer drugs target DNA, forming covalent lesions in the cell. For example, nitrogen mustards, originally synthesized for gas warfare, alkylate the N7 position of deoxyguanosine residues (*1*). Post-mortem examination of unfortunate mustard victims during World War I revealed profound diminishing of the white cells in bone marrow, suggesting the possible benefits of mustards in the treatment of certain cancers (*2*, *3*). Likewise, the antitumor potential of *cis*-diamminedichloroplatinum(II) [cisplatin[1] (**1**)] was also discovered fortuitously when Rosenberg observed inhibition of bacterial cell division in the presence of an electrical field, which was later attributed to the presence of this compound, formed during electrolysis of the platinum electrodes (*4*). Cisplatin also targets the N7 position of guanine residues in DNA, forming monoadducts that can then convert to intra- or interstrand cross-links (for a review, see ref *5*). Interestingly, *trans*-diamminedichloroplatinum(II) [*trans*-DDP (**2**)] does not demonstrate useful anticancer properties (*6*).



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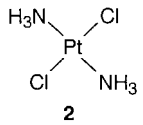

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The focus of this study is the interstrand cross-linking of nucleosomal DNA by cisplatin and *trans*-DDP. While a great deal is known about the interactions between these two isomers and free DNA, less work has been done on nucleosomal DNA, which comprises the bulk of eukaryotic nuclear DNA. Compaction of DNA occurs through packaging with histone proteins to form chromatin, which consists of nucleosomal cores separated by nucleosomal linkers (for reviews, see refs *7* and *8*). The core particles consist of 146 bp of DNA wrapped one-and-three-quarter times around a histone octamer (two copies each of histones H2A, H2B, H3, and H4), while the linkers consist of a variable number of base pairs complexed to histone H1 (and H5 in avian erythrocytes). The incorporation of DNA into chromatin alters the dynamic and structural properties of the DNA and, therefore, the reactivity of the DNA toward a variety of external agents (for a review, see ref *9*).

Previous studies comparing the binding of **1** and **2** to nucleosomes indicate that the *trans* isomer preferentially targets the histones, forming protein−protein and DNA−protein cross-links, but that the *cis* isomer preferentially targets the DNA, forming protein cross-links only after long incubation times (*10*−*12*). Moreover, the level of cisplatin binding to core particle DNA is reduced relative to that of the linker at low binding ratios ($r_b < 0.05$, where r_b equals the number of moles of bound cisplatin per mole of DNA) (*13*−*15*). The preference for linker DNA has been attributed

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[‡] This paper is dedicated to the late Dr. Ben Millard, physical chemist and gentleman.

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); r_b, moles of bound drug per mole of DNA base pairs; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; TE, 10 mM Tris buffer and 1 mM EDTA (pH 7.5); PMSF, phenylmethanesulfonyl fluoride; r_f, moles of drug reacted per mole of DNA base pairs; dPAGE, denaturing polyacrylamide gel electrophoresis.

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FIGURE 1: Sequence of the *Eco*RI−*Rsa*I restriction fragment of the *X. borealis* 5S RNA gene and 5′-flanking region. The numbering is from 1 to 154 in the 5′−3′ direction of the top strand; this corresponds to positions −78 to 75 on the 5S RNA gene. Position 16 in our sequence shows a C → G transversion from the wild-type sequence. 3′-End radiolabeling is of the bottom strand and results in the incorporation of A3′ and A4′ (italics) and the associated ^{32}P. Putative cisplatin cross-linking sites at 5′-GC sequences are shown in bold.

to constraints on the core DNA, blocking the necessary structural changes for cisplatin binding. At higher drug levels (r_b = 0.1−0.2), this preference for linker DNA is not observed (*16*). These studies all examined the total spectrum of platinum adducts with genomic DNA, whereas we have focused our attention on interstrand cross-linking of DNA in defined sequence nucleosome core particles.

Cisplatin forms interstrand cross-links between deoxyguanosine residues at 5′-GC sequences in free DNA (*17*, *18*), whereas *trans*-DDP forms interstrand cross-links between base-paired guanine and cytosine residues at the GN7 and CN3 positions (*19*). While interstrand cross-links are a relatively minor component of the DNA lesions of cisplatin (∼5−10% of the total adducts), the relative contributions of the various products to cytotoxicity remain unclear (*20*). Although recent evidence supports a key role for the 1,2-d(GpG) intrastrand cross-links, which are recognized by a wide range of proteins (*5*, *21*), cisplatin resistance of human ovarian cancer cells is associated with improved repair of interstrand cross-links, suggesting that they may also be involved in cytotoxicity (*22*, *23*). In contrast, interstrand cross-links dominate the bifunctional reaction products of *trans*-DDP (*24*), although the rate of the closure of monoadducts to cross-links is slower than for cisplatin (*20*).

Formation of cisplatin interstrand cross-links results in a local switch from a right-handed to a left-handed helix and extrusion of the cytosine residues, allowing a 180° rotation of the platinated guanines into the minor groove (*25*, *26*). The overall result is an ∼20−40° bending of the helix axis toward the minor groove and an ∼80° unwinding of the helix. The DNA distortions induced by *trans*-DDP are less severe than those induced by cisplatin. The helix is unwound by ∼12° and bent ∼20° toward the minor groove, with a local conformational distortion extending over about 4 bp (*19*, *27*). The distinct structures of the interstrand cross-links formed by cisplatin and *trans*-DDP have been postulated to be related to the differences in antitumor activity of the two isomers (*28*). Indeed, novel *trans*-dichloroplatinum(II) complexes containing planar aromatic amines demonstrate not only profound cytotoxicity but also the formation of cisplatin-like interstrand cross-links at 5′-GC sequences (*29*). The role of the nucleosome and its tolerance for the structural modifications induced by the cisplatin 5′-GC and the *trans*-DDP G-C interstrand cross-links have remained unexplored to date. Thus, studies of the effects of nucleosomal structure on cisplatin and *trans*-DDP interstrand cross-linking of DNA could provide insight into the relative cytotoxicities of these two isomers, thereby potentially aiding in the rational design of new platinum drugs as well as illuminating aspects of nucleosome structure.

We used a 154 bp fragment of the 5S rRNA gene of *Xenopus borealis* (Figure 1) for these studies because it has been shown previously to exhibit exceptional nucleosomal positioning properties (*30*, *31*). Moreover, the 5S DNA system has been used to examine the reactions of a number of agents with DNA (*32*−*36*). Overall, such studies have provided support for an inhibition of reactivity within the nucleosome, most profound at the center, but a high degree of DNA accessibility to small molecules. There are 14 putative interstrand cross-linking sites for cisplatin (5′-GC) and multiple sites for *trans*-DDP (G-C) in the 154-mer, presented in a variety of rotational (i.e., facing toward or away from the histone octamer) and translational positions (i.e., the distance from the nucleosome center). The core particle center of symmetry, termed the nucleosome dyad, is located approximately at C76 in this sequence (*31*). Because of the substantial structural alterations of nucleosomal relative to free DNA, including curvature of ∼45° per helical turn, sharp bends at several sites, and periodic compression and expansion of the major and minor groove widths (*37*−*41*), differential formation of interstrand cross-links by cisplatin and/or *trans*-DDP within the nucleosome seems likely.

MATERIALS AND METHODS

Purification of Core Particles. Core particles were purified from chicken erythrocytes (Jason's Butcher Shop, Albion, ME) using the method of Libertini and Small (*42*) as described previously (*32*). Eighteen percent SDS−polyacrylamide gel electrophoresis [PAGE (20:1 acrylamide:bisacrylamide ratio)] showed four major protein bands of approximately equal intensities with the same mobilities as a commercially obtained sample of a histone mixture containing histones H3, H4, H2A, and H2B (Boehringer Mannheim). Core particles were quantified through A_{260}.

Preparation of the 154 bp Fragment. pXP-11 (*36*), containing a portion of the 5S rRNA gene of *X. borealis*,

was kindly provided by the Tullius laboratory (Boston University, Boston, MA). Following transformation into *Escherichia coli*, the plasmid was purified from the resulting colonies using Qiagen p-2500 purification columns. Plasmid DNA was doubly digested with *Eco*RI and *Rsa*I under standard conditions, followed by ethanol precipitation and 3′-end radiolabeling with [α-^{32}P]dATP (Amersham Pharmacia Biotech)/Klenow Exo Minus (New England Biolabs) (*43*). Six percent native PAGE (37.5:1 acrylamide:bisacrylamide ratio) and subsequent autoradiography revealed two bands, of which the higher-mobility one was excised from the gel and purified through the crush-and-soak method (*43*).

Reconstitution. Two methods of reconstitution were used to achieve either a mixture of free and nucleosomal DNA or approximately 100% nucleosomal DNA. For the "dilution" method (*44*), lyophilized, radiolabeled 154-mer (∼0.5 μg) in a siliconized microcentrifuge tube was incubated with ∼1 OD_{260} of core particles in 1 M NaCl, TE [10 mM Tris buffer (pH 7.5) and 1 mM EDTA], and phenylmethanesulfonyl fluoride (PMSF; 0.50 mM in 2-propanol) for 1 h on ice (total volume of 20 μL). The salt concentration was then gradually decreased by adding aliquots of TE/PMSF (5 μL for 1 h, an additional 5 μL for 1 h, 170 μL for 15 min, and 200 μL for 15 min). This method resulted in a mixture of free and nucleosomal DNA. For the "dialysis" method (*45*), lyophilized, radiolabeled 154-mer (∼0.5 μg) was mixed with ∼3 OD_{260} of core particles in a TE/PMSF/1 M NaCl mixture (final volume of 100 μL), incubated on ice for 45 min, and then transferred to a slide-a-lyzer (Pierce). The salt concentration was then gradually decreased by dialyzing the mixture at 4 °C for 3 h periods against 200 mL of a TE/PMSF mixture successively containing 0.8, 0.6, and 0.05 M NaCl. This method resulted in almost entirely nucleosomal DNA.

Cisplatin and trans-DDP Cross-Linking Reactions. A 1.0 mM cisplatin or *trans*-DDP (Aldrich) stock (3 mg in 10 mL of H_2O) was prepared with stirring 24 h before incubation. Cisplatin was then diluted to 0.1 mM, whereas *trans*-DDP was first reacted with 2 equiv of $AgNO_3$ overnight and then centrifuged before dilution (*46*). Reactions of 154-mer were with 0.01 mM [$r_f = 0.07$ (where r_f equals the number of moles of drug reacted per mole of DNA bp) for the ∼1:1 mixture of free and nucleosomal DNA] or 0.0025 mM ($r_f = 0.02$ for the ∼1:1 mixture of free and nucleosomal DNA) cisplatin or *trans*-DDP in platination buffer [from a 10× stock of 400 mM sodium perchlorate and 20 mM monobasic potassium phosphate (pH 7.6)] incubated in the dark at 37 °C for 8 h (cisplatin) or 24 h (*trans*-DDP).

Cross-Linked-Then-Reconstituted Experiments. A 15 μL dilution reconstitution was prepared with radiolabeled free 154-mer and chicken histones in a 1× platination buffer/TE/PMSF mixture in the absence of 1 M NaCl. Cisplatin or *trans*-DDP was added at either 0.01 mM ($r_f = 0.003$) or 0.0025 mM ($r_f = 0.0007$) and allowed to react as described above. At the end of the appropriate incubation period at 37 °C, 5 μL of 4 M NaCl was added, and the mixture was incubated at 4 °C for 1 h with subsequent additions of a TE/PMSF mixture to decrease the salt concentration as described previously. Alternatively, free 154-mer was reacted with cisplatin or *trans*-DDP at 0.01 or 0.0025 mM in the absence of histones ($r_f = 12$ and 4, respectively). Following the appropriate incubation time, the samples were ethanol precipitated. Lyophilized samples were then reconstituted via the dialysis method described previously.

Separation of Free and Nucleosomal DNA. Free and nucleosomal 154-mer were separated by 6% native PAGE (37.5:1 acrylamide:bisacrylamide ratio) containing 5% glycerol run in 0.5× TBE at 4 °C and 250 V. Autoradiography revealed two bands: a higher-mobility band for the free 154-mer and a lower-mobility band for the nucleosomal 154-mer. Both were excised, and the DNA was purified through the crush-and-soak method (*43*).

Separation of Cross-Linked DNA. Lyophilized, cisplatin- or *trans*-DDP-treated free and nucleosomal 154-mer samples were resuspended in 10 μL of 5 M aqueous urea and 0.1% xylene cyanol and run on a 41 cm × 37 cm, 0.35 mm thick, 6% denaturing formamide/urea polyacrylamide gel (19:1 acrylamide:bisacrylamide ratio, 30% formamide, 42% urea) at ∼55 W and ∼55 °C. Gels were dried under vacuum for 1 h (Drygel Sr. model SE 1160, Hoefer Scientific Instruments) and autoradiographed or phosphorimaged (Bio-Rad GS-505 Imaging System with Molecular Analyst version 2.1.2). Alternatively, cross-linked DNA was located via autoradiography and excised for purification from wet gels.

Mapping of Cross-Linked DNA. Individual cisplatin−cross-linked DNA bands were recovered from formamide/urea gels by the crush-and-soak procedure (*43*). These samples were hydroxyl-radical cleaved prior to loading onto a sequencing gel.

Hydroxyl Radical Cleavage. Reactions were run as previously described by Dixon et al. (*47*). Stock solutions of 500 μM ferrous ammonium sulfate/1 mM EDTA, 0.3% hydrogen peroxide, and 10 mM sodium ascorbate were made fresh. To 70 μL of a dialysis reconstitution mixture or a cross-linked sample (in H_2O) was added 10 μL of each of these reagents at the side of a microcentrifuge tube. The reaction was initiated by brief centrifugation. After 1 min, reactions were stopped by the addition of thiourea to a final concentration of 0.2 mM and EDTA to 10 mM. Samples were phenol−chloroform extracted (*43*) prior to analysis on a sequencing gel.

Sequencing Gel Analysis. Samples were dissolved in 10 μL of 5 M urea/0.1% xylene cyanol and loaded onto an 8% denaturing gel (19:1 acrylamide:bisacrylamide ratio, 50% urea) run at ∼55 W and ∼55 °C followed by drying and autoradiography. Bands were assigned via reference to Maxam−Gilbert G-lanes (*43*).

RESULTS

A radiolabeled 154 bp restriction fragment of the 5S RNA gene of *X. borealis* was incubated with varying amounts of purified chicken erythrocyte core particles under conditions of high salt to disassemble the chicken DNA−histone interactions. The salt concentration was then lowered through either dilution or dialysis to reconstitute a defined sequence, radiolabeled nucleosomal core particle. Successful incorporation of the 154-mer into the core particle has been shown previously to result in both a native gel mobility shift and a 10−11 bp periodicity pattern upon cleavage with Fe(II)-EDTA (*31*, *44*). We generally observed two major bands on native gels for our reconstitutions: one band that comigrated with the free 154-mer and a second lower-mobility band attributed to incorporation of the 154-mer into nucleosomal

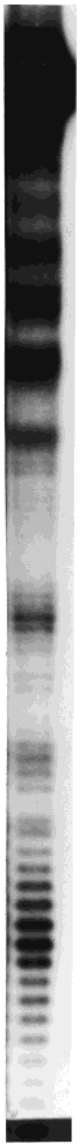

FIGURE 2: Autoradiogram of the hydroxyl radical cleavage pattern of the 3′-radiolabeled *Eco*RI−*Rsa*I restriction fragment as nucleosomal DNA. The 10−11 bp periodicity of the nucleosomal sample suggests that the histone octamer is present in both defined translational and rotational settings on the DNA.

DNA. The amount of low-mobility product was dependent on the histone:DNA ratio, with higher ratios leading to more of the presumed nucleosomal DNA. The Fe(II)EDTA cleavage pattern of the low-mobility product did indeed demonstrate the 10−11 bp periodicity indicative of a core particle with precise rotational and translational positioning (Figure 2). Free 5S DNA shows some nonuniformity in its hydroxyl radical cleavage patterns, which has been attributed to bending of this sequence, but in the nucleosomal state these variations are exaggerated to yield patterns such as those seen here (*31*, *44*).

Because previous reports have suggested that cisplatin and *trans*-DDP react with both DNA and protein, we performed cross-linking experiments on several systems: 5S DNA reconstituted with chicken core particles, free 5S DNA in the presence of histones, and free 5S DNA in the absence of histones (Figure 3). For the latter two cases, treatment with cisplatin or *trans*-DDP was then followed by a reconstitution protocol. The experiment performed with free

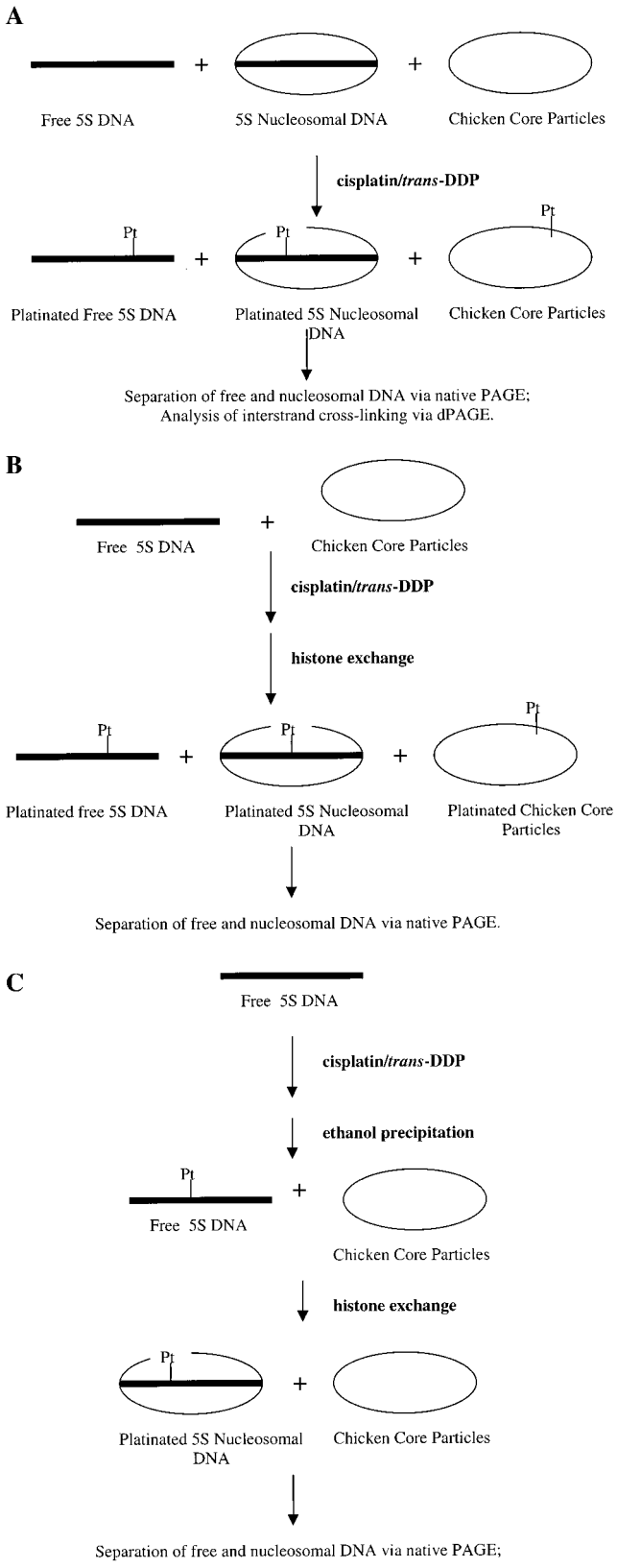

FIGURE 3: Schematic of cross-linking experiments. (A) Reconstituted-then-platinated protocol. (B) Platinated-then-reconstituted protocol. (C) Platinated-then-ethanol-precipitated-then-reconstituted protocol. Histone exchange indicates one of the two reconstitution methods described in Materials and Methods. Chicken core particles are present in excess.

5S DNA and histones present was scaled down so that the reconstitution volume and subsequent conditions would be similar to the reaction mixture that was reconstituted prior

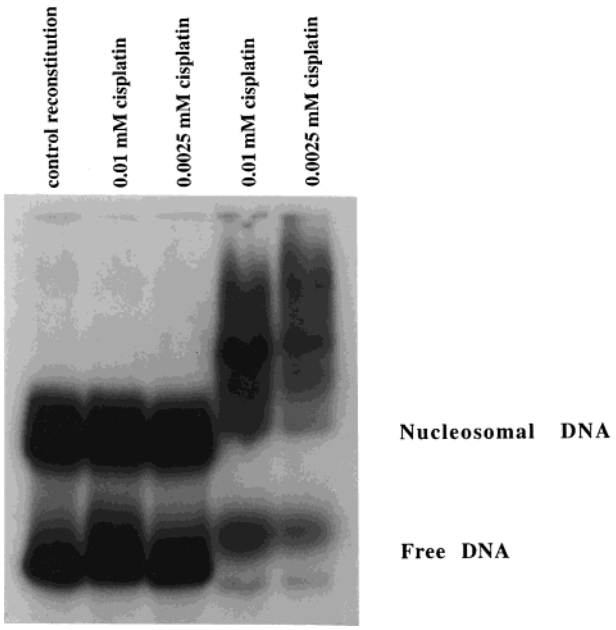

FIGURE 4: Autoradiogram of a 6% native gel separating nucleosomal and free cisplatin-treated DNA. Lanes 2 and 3 contained samples that were first reconstituted with histones and then reacted with cisplatin (see Figure 3A). Lanes 4 and 5 contained samples that were first reacted with cisplatin and then reconstituted with histones (see Figure 3B): lane 1, control reconstitution (no cisplatin); lane 2, 0.01 mM cisplatin, reconstituted first; lane 3, 0.0025 mM cisplatin, reconstituted first; lane 4, 0.01 mM cisplatin, reacted first; and lane 5, 0.0025 mM cisplatin, reacted first.

to cisplatin or *trans*-DDP treatment. Our concentrations of cisplatin were selected so that r_f values for the reconstituted-first experiment would flank the critical r_b value of 0.05 at which this agent shows a preference for linker DNA relative to core: $r_f = 0.07$ for the concentration of 0.01 mM and $r_f = 0.02$ for 0.0025 mM.

A comparison of reconstituted-then-cisplatin-treated samples to cisplatin-treated-then-reconstituted samples showed a cisplatin-induced inhibition of 154-mer incorporation into the nucleosomal core particle (Figure 4). Native gel electrophoresis of the samples that were reconstituted first and then reacted with cisplatin showed two distinct bands of free and nucleosomal DNA similar to a control reconstitution that was not treated with drug, suggesting that cisplatin treatment does not affect the integrity of an already-existing core particle. However, the reacted-then-reconstituted samples showed a smear instead of the defined bands for free and nucleosomal DNA present in the controls, indicating problematic incorporation of the free 154-mer into a defined core particle structure.

The results with *trans*-DDP were somewhat more dramatic. A DNA−histone mixture that was treated with *trans*-DDP and then taken through the reconstitution process showed only a trace of incorporation of the radiolabeled 154-mer into the core particle when analyzed on a native gel (Figure 5). The bands corresponding to free and nucleosomal *trans*-DDP-treated DNA were both excised and eluted. Analysis of these samples via denaturing PAGE (dPAGE) showed no interstrand cross-links (data not shown) even after overexposure, suggesting that the effect of the *trans*-DDP was primarily at the level of the histones rather than the DNA.

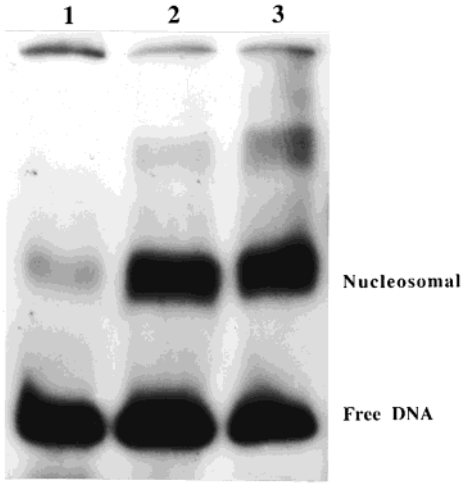

FIGURE 5: Autoradiogram of a 6% native gel separating nucleosomal and free *trans*-DDP-treated DNA: lane 1, 0.01 mM *trans*-DDP, reacted first (see Figure 3B); lane 2, 0.01 mM *trans*-DDP, reconstituted first (see Figure 3A); and lane 3, control reconstitution (no *trans*-DDP).

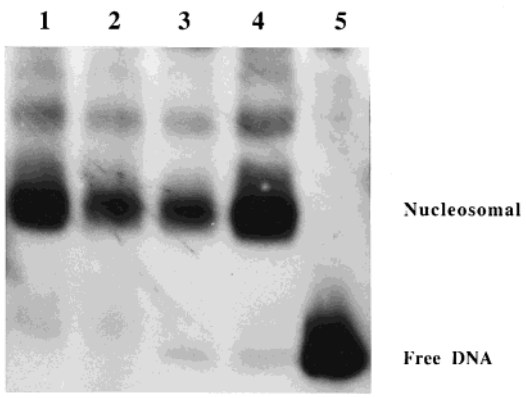

FIGURE 6: Autoradiogram of a 6% native gel separating nucleosomal and free DNA first treated with cisplatin or *trans*-DDP and then ethanol precipitated prior to reconstitution (see Figure 3C): lane 1, 0.01 mM cisplatin; lane 2, 0.0025 mM cisplatin; lane 3, 0.01 mM *trans*-DDP; lane 4, 0.0025 mM *trans*-DDP; and lane 5, control free 154-mer.

To determine conclusively whether cisplatin and *trans*-DDP were negatively affecting the protein rather than the radiolabeled DNA component in the mixture during reconstitution, free DNA was treated with cisplatin or *trans*-DDP, ethanol precipitated, and then reconstituted with chicken core particles under conditions designed to yield approximately 100% nucleosomal DNA. In this case, both cisplatin- and *trans*-DDP-treated DNA were incorporated cleanly into the core particle structure (Figure 6). These results suggest that the adverse impact of these agents on the formation of nucleosomal DNA was indeed primarily at the level of the histones rather than the DNA.

We then carried out a series of dilution reconstitutions to achieve an approximately 1:1 mixture of free and nucleosomal DNA for further cross-linking studies. Following reconstitution, samples were treated with cisplatin or *trans*-DDP at 0.01 and 0.0025 mM. Drug treatment of the reconstitution mixture prior to native gel purification of free and nucleosomal DNA ensured identical reaction conditions for these two samples. After reaction with the platinum agent, free and nucleosomal 154-mer were separated via 6% native PAGE, purified from the gel, and run on a 6% denaturing

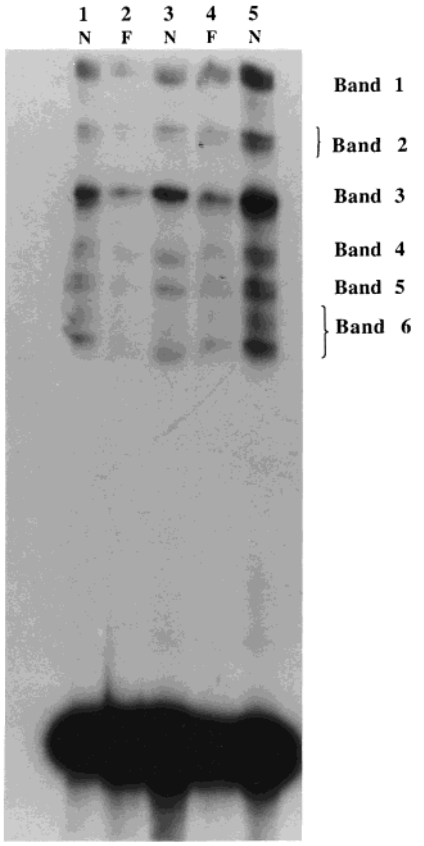

FIGURE 7: Autoradiogram of the cisplatin interstrand cross-linking patterns of free and nucleosomal DNA following dPAGE. Samples in lanes 1−4 were reconstituted by the dilution method; the sample in lane 5 was reconstituted by the dialysis method: lane 1, nucleosomal, 0.01 mM cisplatin; lane 2, free, 0.01 mM cisplatin; lane 3, nucleosomal, 0.0025 mM cisplatin; lane 4, free, 0.0025 mM cisplatin; and lane 5, nucleosomal, 0.01 mM cisplatin.

formamide/urea gel to separate interstrand cross-links. Autoradiography indicated a family of approximately six bands of reduced mobility relative to single strands, corresponding to interstrand cross-linking at different sites. Similar positions of cross-linked bands were observed for free and nucleosomal DNA treated with cisplatin (Figure 7). Included on the cisplatin gel was one nucleosomal sample obtained through the dialysis method to verify that the cross-linking pattern was independent of the reconstitution method (see lane 5). The cross-linking pattern of *trans*-DDP was different from that of cisplatin, although again, the positions of these bands in the free and nucleosomal DNA samples were similar for this agent (Figure 8). However, there was a band of noticeably increased intensity in the 0.01 mM *trans*-DDP-treated nucleosomal sample (marked with an arrow in Figure 8) that migrated just above single-stranded DNA and presumably corresponded to an intrastrand cross-linked product. While there appeared to be more interstrand cross-linked bands than for the cisplatin samples, the resolution of these bands suggests that there may not have been cross-linking at every possible G-C site, which would likely have led to a smear of low-mobility products.

Quantitative phosphorimagery of cisplatin gels revealed similar cross-linking efficiencies for free and nucleosomal DNA (∼13% for the 0.01 mM samples and ∼5% for the 0.0025 mM samples). The pattern of interstrand cross-linking was consistently six or seven bands, depending on the

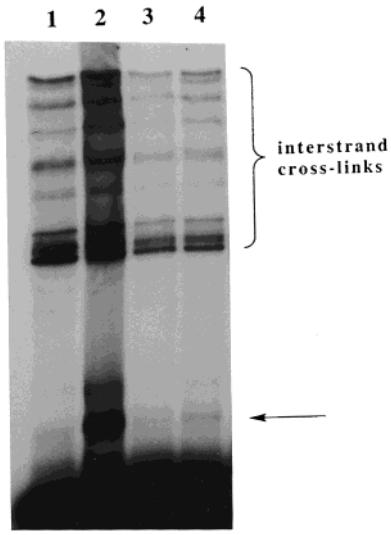

FIGURE 8: Autoradiogram of the *trans*-DDP interstrand cross-linking patterns of free and nucleosomal DNA following dPAGE: lane 1, free, 0.01 mM *trans*-DDP; lane 2, nucleosomal, 0.01 mM *trans*-DDP; lane 3, free, 0.0025 mM *trans*-DDP; and lane 4, nucleosomal, 0.0025 mM *trans*-DDP.

resolution of the particular gel. Two-dimensional profile scanning of each gel lane was performed for a quantitative comparison of the relative intensities of each band for the free and nucleosomal DNA, as represented by the 0.0025 mM sample (Figure 9). Integration data were averaged for three experiments and normalized to account for differences in the amount of radioactivity loaded across lanes (Table 1). These data showed that the intensity if band 3 (from the top) was elevated significantly (Analysis of Variance) by ca. 30% in the nucleosomal samples while the intensity of band 1 was decreased significantly in the nucleosomal samples by ca. 20%.

Quantitative phosphorimagery of *trans*-DDP gels again revealed similar cross-linking efficiencies for free and nucleosomal DNA (∼6% for 0.01 mM samples and ∼2% for 0.0025 mM samples). Profile scanning of the *trans*-DDP interstrand cross-linked bands showed no detectable differences between free and nucleosomal DNA in the 0.0025 mM samples. The only significant difference between the free and nucleosomal DNA in the 0.01 mM samples was an increase in the level of presumed intrastrand cross-linked product noted with an arrow in Figure 8.

To assign the sites within the 5S DNA that were elevated and depressed in the cisplatin nucleosomal samples, we performed hydroxyl radical cleavage assays on individual cross-linked bands. After separation of interstrand cross-links via 6% dPAGE, individual gel bands were located by autoradiography, purified, subjected to hydroxyl radical cleavage, and then analyzed via 8% dPAGE. Phosphorimagery revealed enhanced cleavage relative to that of free DNA at specific deoxyguanosine residues at 5′-GC sites presumably because of the structural alterations resulting from cross-linking. For example, band 4 showed elevated levels of cleavage at G25 and G30 relative to free DNA (Figure 10). Our results suggest that the least mobile band corresponds to the most centrally cross-linked isomers (cross-linking at G75 and G79). This is consistent with previous findings for mitomycin C in short DNA oligomers (*48*) and for mustards within this same fragment from *X. borealis* (*49*).

Table 1: Normalized Ratios of Nucleosomal to Free DNA Band Intensities for the Six Major Families of Cisplatin Interstrand Cross-Links[a]

band 1	band 2	**band 3**	band 4	band 5	band 6	average
0.78 ± 0.084	0.92 ± 0.080	**1.3 ± 0.15**	0.97 ± 0.052	0.99 ± 0.023	0.97 ± 0.11	0.99

[a] Data from three experiments were averaged. Band 1 represents the band of lowest mobility and band 6 the band of highest mobility. Ratios that differ significantly (analysis of variance; $p = 0.0012$) from the average are shown in bold.

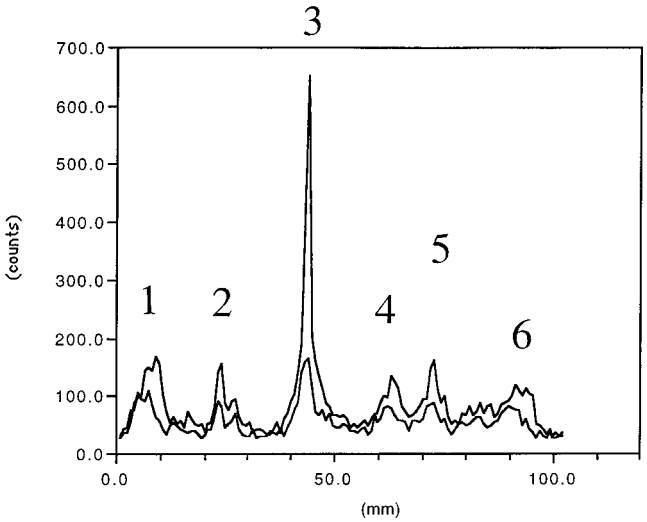

FIGURE 9: Two-dimensional profile scans of the cisplatin (0.0025 mM) interstrand cross-linked DNA in the free and nucleosomal states. The trace with the elevated peak at band 3 corresponds to lane 3 (nucleosomal DNA) of the gel shown in Figure 7; the other trace corresponds to lane 4 (free DNA).

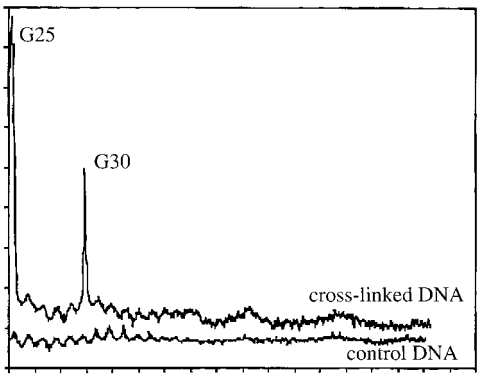

FIGURE 10: Two-dimensional profile scan of the hydroxyl radical cleavage products of cisplatin interstrand cross-linked band 4 and control (not cross-linked) DNA.

We have therefore postulated the composition of the top four cisplatin-cross-linked bands, based on the hydroxyl radical cleavage data, the distance of the 5′-GC sites from the center of the molecule (at T77), and the expected resolution of our denaturing gels (Table 2).

DISCUSSION

Previous studies of the reactivity of the antitumor agent *cis*-diamminedichloroplatinum(II) and its clinically inactive isomer, *trans*-diamminedichloroplatinum(II), with nucleosomes have been limited to overall platination of genomic DNA-containing chromatin and core particles. In general, these studies conclude that cisplatin primarily targets the DNA, with a similar rate of binding for free and nucleosomal core DNA (*10*). *trans*-DDP, however, primarily forms specific cross-links between histone proteins (*10*, *11*). An examination of the distribution of DNA lesions within chromatin suggests that at low ratios of cisplatin ($r_b < 0.05$), linker DNA is preferred over core DNA for targeting (*13*−*15*). We have extended this work by focusing on the interstrand cross-linking of both the *cis* and *trans* isomers within defined sequence core particles, using r_f values close to the critical r_b of 0.05 found previously ($r_f = 0.02$ and 0.07 in our studies). These core particles contained a radiolabeled 154 bp restriction fragment of the 5S rRNA gene of *X. borealis* reconstituted by histone exchange from chicken erythrocyte core particles. This 5S DNA was presented as both free and nucleosomal DNA within the same reaction, thereby ensuring identical reaction conditions for both samples.

Table 2: Predicted Sites of Cisplatin Interstrand Cross-Linking for the First Four Major Low-Mobility Bands (from the top) Seen on Denaturing Polyacrylamide Gels (See Figure 7)[a]

cross-linked site	predicted band	bp from center	rotational position
G75	1	2	away
G79	1	2	facing
G68	2	9	facing
G86	2	9	away
G44	3	33	away
G47	3	30	facing
G105	3	28	away
G25	4	52	neither
G30	4	47	neither
G129	4	52	facing

[a] The numbering reflects that shown in Figure 1. Bands were assumed to result from cross-linking deoxyguanosine residues on opposite strands at 5′-GC sequences (*17*, *18*). Assignments were made through hydroxyl radical cleavage data and by the fact that interstrand cross-links separate on the basis of their distance from the center of the molecule (*48*, *49*), T77 in this DNA. The "rotational position" reflects whether this site is facing away from or toward the histone octamer (*31*).

We first tested whether platinated DNA could be incorporated into core particles. When chicken core particles were included with free radiolabeled DNA, subsequent incorporation of the 154-mer was negatively impacted after reaction with both cisplatin and *trans*-DDP. However, while the reconstituted, cisplatin-treated DNA did form diffuse bands of high-molecular weight products on a native gel suggestive of an ill-defined nucleosome structure, the *trans*-DDP-treated DNA formed only a trace of high-molecular weight products. Isolation of the *trans*-DDP-treated DNA from these native gels followed by dPAGE showed that there were no interstrand cross-links in either the free or nucleosomal DNA. These findings suggest that reaction with histones occurred for both cisplatin and *trans*-DDP, although this reaction was far more complete for the *trans* isomer. This hypothesis was supported by the fact that treatment of the 154-mer with cisplatin or *trans*-DDP followed by ethanol precipitation led to successful incorporation into the core particle structure for the platinated DNA. Although our drug concentrations were identical in the reconstituted-then-platinated samples and the platinated-then-reconstituted samples, the r_f values

were much lower in the latter. Thus, at very low relative levels of platinum, it appears that histones are modified by both agents, although less so by cisplatin than by *trans*-DDP. Such modification impedes subsequent histone octamer exchange with radiolabeled DNA. These findings agree with the previous reports of Lippard and Hoeschele (*10*) and Thompson et al. (*11*), demonstrating the existence of both specific histone−histone (e.g., H3−H2A, H2B−H4, and H3 dimers) and histone−DNA cross-links in *trans*-DDP-treated nucleosomal samples. It is likely that these interactions inhibit disassembly of core particles during histone octamer exchange.

We then created reconstitution mixtures designed to contain approximately equal proportions of free and nucleosomal DNA. Following treatment of these mixtures with cisplatin or *trans*-DDP, free and nucleosomal DNA was purified through native PAGE. These gels indicated that the integrity of the core particle was not affected by platination as the relative proportions of free and nucleosomal DNA did not differ from that of control untreated DNA. We then used dPAGE to separate the different interstrand cross-linked isomers, which appeared as a family of bands with higher molecular weights than single strands. Interestingly, both agents did form interstrand cross-links under our reaction conditions, suggesting that DNA already incorporated into a nucleosomal core particle is indeed a target for platination. We also found that the pattern of cisplatin interstrand cross-links was independent of the reconstitution method (dilution or dialysis). Moreover, the positions of cross-linked bands on these gels were remarkably similar for nucleosomal and free DNA for both agents, suggesting both that access to the major groove is not greatly impaired by the histone core and that core particle DNA tolerates the conformational changes imparted by cross-linking. Phosphorimagery demonstrated that cisplatin was ∼2.5 times more efficient at forming interstrand cross-links than *trans*-DDP at comparable concentrations under our reaction conditions. Furthermore, there were some distinct differences between the free and nucleosomal samples in the intensities of the cisplatin cross-linked bands.

Cisplatin has been previously determined to form interstrand cross-links between deoxyguanosine residues in duplex DNA at 5′-GC sequences (*17*, *18*), while *trans*-DDP forms cross-links between base-paired deoxyguanosine and deoxycytosine residues (*19*). The 14 5′-GC cisplatin sites and the multiple *trans*-DDP sites are present in a variety of translational and rotational positions along the core particle (Figure 1). For example, translationally, the 5′-GC sites at G75 and G79 are very close to the nucleosomal dyad at C76, whereas the sites at G8 and G144 are close to the ends of the core particle. Rotationally, the site at G75 is facing away from the histone octamer and contains a compressed major groove, whereas the site at G79 is facing toward the histone octamer and contains a compressed minor groove (*31*). Bulky alkylating agents are sensitive to translational positioning (*33*−*35*), whereas cleavage by hydroxyl radical (*31*) and some enediynes (*36*, *50*) and the formation of cyclobutane pyrimidine photodimers (*51*, *52*) are dependent on rotational positioning.

Our findings for cisplatin were a reduction in the efficiency of cross-linking at band 1 (∼20%) but an elevation at band 3 (∼30%) in the nucleosomal samples relative to the free DNA at both concentrations. Previous work has shown that the relative position of an interstrand cross-linked band on denaturing polyacrylamide gels is correlated with its distance from the center of the duplex, with the most centrally cross-linked isomer having the lowest mobility (*48*, *49*). Hydroxyl radical cleavage data supported this assignment for cisplatin cross-links in the 5S DNA. Band 3 exhibited the major difference in efficiency of interstrand cross-linking between the free and nucleosomal samples. We believe that this band comprises three isomers cross-linked at G44, G47, and G105 (see Table 2). These sites are all approximately three helical turns from the nucleosome dyad. G44 and G105 both face away from the histone octamer and contain a compressed major groove (*31*, *36*). On the other hand, G47 faces toward the histone octamer and contains an expanded major groove. Given the constraints of our methodology, it is impossible to predict whether elevation occurs at one, two, or all three of these sites. Although rotational positioning may be involved in mediating cisplatin cross-linking within the nucleosome, it is unlikely to be the sole cause of the observed elevation as it does not correlate with enhancement of cross-linking in all cases. Moreover, agents such as hydroxyl radical that show a clear rotational dependence for DNA binding within the nucleosome appear to react quickly on the time scale of the exposure of DNA by the nucleosome (*32*). Cisplatin, which forms both monoadducts and interstrand cross-links relatively slowly [$t_{1/2} \sim 3$ and 6 h, respectively (*20*)], is consistent with these findings in that it appears to demonstrate no clear rotational dependence for cross-linking.

The reduction of the intensity of band 1, the least mobile cisplatin-cross-linked band, in the nucleosomal sample suggests that cross-linking is impaired at the nucleosomal dyad. These findings are consistent for previous findings with bulky monoalkylators and for interstrand cross-linking by mitomycin C, which shows a 5−10-fold level of reduction at the nucleosomal dyad (*32*). Therefore, the relatively small cisplatin appears to behave like bulky alkylating agents in that its cross-linking of nucleosomal DNA demonstrates a small but significant translational dependence. Formation of the relatively large five-coordinate transition state may account for this observation. In contrast, our findings for *trans*-DDP showed no such reduction at the nucleosomal dyad. Whereas size cannot account for the difference between these two isomers, the kinetics of *trans*-DDP interstrand cross-link formation are slower than for the *cis* isomer [$t_{1/2} \sim 12$ h for closure of *trans*-DDP monoadducts (*20*)], so it is likely that kinetics also play a role in the translational dependence of nucleosome binding.

Cisplatin interstrand cross-linking induces profound structural changes in DNA. However, our findings suggest that there are only relatively modest differences in the attack of nucleosomal and free DNA with the largest effect actually being an enhancement of cross-linking at one or more sites approximately three helical turns from the dyad. The site more then three helical turns from the dyad (G105) is at a region of close contact with histone H3 (*53*), which may lead to a structural alteration in the local area that is ripe for enhanced cisplatin cross-linking. A previous "hot spot" at −1 turns from the helical dyad has been noted previously for cleavage by calicheamicin γ_1^I (*36*) with a concomitant

inhibition of cross-linking by some nitrogen mustards at this site (*32*).

It is possible that during the isolation of nucleosomal DNA, monoadducts could convert to cross-links when histones are removed. Our experimental protocols do not allow verification of similar patterns of platinum monoadducts for the free and nucleosomal DNA samples. However, previous studies have verified that for nitrogen mustards there is no obvious loss of monoalkylation that might suggest conversion to cross-links during electrophoresis and purification in the nucleosomal samples (*32*). Moreover, the extremely denaturing conditions under which cross-links are isolated make it unlikely that duplexes containing monoadducts would remain double-stranded to allow conversion to interstrand cross-links.

In conclusion, we have found that cisplatin and *trans*-DDP form interstrand cross-links with nucleosomal DNA that are remarkably similar to those in free DNA. Moreover, cisplatin shows a decrease in the level of cross-linking at the nucleosomal dyad and a larger increase at a site three helical turns from the dyad. Rotational positioning does not clearly correlate with cross-linking efficiency. Although both agents also react with histones, which negatively impacts nucleosome formation, platinated DNA can indeed be incorporated into nonplatinated chicken core particles, and already-formed nucleosomes are not disrupted upon platination. Our findings are further support for the accessibility of DNA within the nucleosome, which appears to be a relatively minor inhibitor of DNA interstrand cross-linking reactions despite relatively large structural changes accompanying cross-linking. The kinetics of reaction seem to play a larger role than the size of reactant in dictating the sites targeted within the nucleosome for interstrand cross-linking. Evidence suggests that nucleosomes transiently expose DNA, thereby providing access to regulatory proteins (*54*). Reactions with, for example, hydroxyl radical or UV light may occur quickly on the time scale of exposure, therefore providing a "snapshot" of nucleosomal structure (*9*). On the other hand, reagents that react more slowly on the time scale of site exposure, such as cisplatin and *trans*-DDP, appear to produce a time-averaged picture of nucleosomal structure. Further studies will require the use of longer DNA fragments, linker histones, and nonhistone nucleosomal binding proteins to approximate more closely the physiological target of these agents.

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