Cisplatin Interstrand Cross-linking of Defined Sequence Nucleosomal DNA

Erin Elizabeth Wilkes
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Cisplatin Interstrand Cross-linking of Defined Sequence Nucleosomal DNA

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May 2000
Cisplatin Interstrand Cross-linking of Defined Sequence Nucleosomal DNA

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A thesis submitted to the Chemistry Department in partial fulfillment of the requirements for graduation with HONORS IN RESEARCH in Chemistry

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VITAE

Erin Elizabeth Wilkes was born in Ellsworth, Maine on December 6, 1977. Her father, Michael P. Wilkes, is a contractor and her mother, Karin M. Wilkes, is a graphic artist. She has one younger sister, Katherine L. Wilkes, born in 1982, who is aspiring to be an opera singer. She graduated from Ellsworth High School in 1996 and began attending Colby College in the fall of that same year.

Prior to Colby and during, Erin held internships at both the Mount Desert Island Biological Laboratory and the Jackson Laboratory that sparked her interest in scientific research. Erin majored in Chemistry and Classics at Colby but occupied her time with many things. She was heavily involved in Colby Dancers, choreographing and dancing in many pieces during her time at Colby. She was a founding member, chair, and trainer for the Project Ally Committee, as well as actively involved with the Student Government Association. She spent the summer after her sophomore year in Rome studying spoken Latin with an officer of the Vatican, and the next summer at Merck in West Point, Pennsylvania researching hepatitis C. Erin tried hard to pursue her research interests under Dr. Paul Greenwood for two years, but eventually joined and prospered in the Millard lab her senior year. She graduated in May of 2000 with hopes of pursuing a career in medicine.
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ABSTRACT

DNA interstrand cross-linking agents have been recognized for many years to be powerful antitumor chemotherapeutics. While the interactions between free DNA and many anticancer drugs have been explored extensively, there is less information available about these interactions with nucleosomal DNA. Nitrogen mustards have been reported to exhibit relatively similar cross-linking sites and efficiencies of binding in free and nucleosomal DNA, but mitomycin C exhibits markedly reduced cross-linking near the center of nucleosomal DNA. This current study focuses on cisplatin interstrand cross-linking of the 154 base pair 5S rRNA gene fragment of *Xenopus borealis* reconstituted with histones from chicken erythrocytes. The results showed that nucleosomal structure positively affects the efficiency of interstrand cross-linking of DNA by cisplatin at one site on the gene, believed to be at a site three-helical turns from the dyad. Nevertheless, nucleosomal structure does not determine the sites at which cisplatin can bind to DNA. The similarity in cross-linking patterns between free and nucleosomal DNA supports a locally dynamic model of the nucleosome. Additional reconstitution experiments also demonstrate that a cisplatin-modified DNA molecule cannot be precisely incorporated into a nucleosome, indicating that cross-linking of the DNA molecule somehow impedes adoption of nucleosomal structure.
INTRODUCTION

DNA cross-linking agents have a wide variety of effects on cells. While the nitrogen mustards, mitomycin C, and cisplatin are known antitumor agents, other cross-linkers such as some of the diepoxides are carcinogens (Hopkins et al., 1991; Ehrenberg and Hussain, 1981). The mechanisms by which these agents have such opposite effects is still largely unclear. However, the structure of the cross-link and the resulting distortion in DNA structure that cross-linking induces are believed to play key roles in cytotoxic and carcinogenic properties. The in vitro aspects of interactions between DNA and cross-linkers have been well studied, but a large portion of the DNA in the cell is complexed with histone proteins in order to form chromatin. The incorporation of DNA into chromatin alters the dynamic and structural properties of the DNA and, thus, may influence reactivity and repair of the DNA (Millard, 1996). Studies incorporating nucleosomal structure might, therefore, provide further insight into the carcinogenicity and cytotoxicity of cross-linkers. Relatively few studies have been done, but most have found that nucleosomal structure has little impact on site preference of cross-linking with some effect on efficiency of interstrand cross-linking at particular sites (Millard et al., 1998). The focus of this study was to investigate the impact of nucleosomal structure on interstrand cross-linking of DNA by cisplatin. In order to more closely mimic in vivo conditions, we made use of the 154 base pair (bp) EcoR1/Rsal restriction fragment of the 5S rRNA gene of Xenopus borealis reconstituted with core particles isolated from chicken erythrocytes.

The antitumor activity of cis-diaminedichloroplatinum (II), also called cisplatin, was discovered in 1969 (Rosenberg et al., 1969), and the Food & Drug Administration approved it for use in clinical treatments of testicular cancer, ovarian cancer, and cancers of the head and
neck in 1979 (Borman, 1995). Cisplatin consists of a centrally oriented platinum bound to two chlorides and two amino groups in a cis conformation (Figure 1).

\[
\text{Cl} \\
\begin{array}{c}
\text{H}_3\text{N} \\
\text{Pt} \\
\text{Cl} \\
\text{NH}_3
\end{array}
\]

**Figure 1.** The structure of cis-diaminedichloroplatinum (II).

In an aqueous environment, the molecule loses the chlorides and forms an aqua species which can then react with nucleophilic groups on nucleic acids (Chu, 1994). Cisplatin first covalently binds to the N-7 position on a purine (guanine is preferred) to form a monoadduct (Figure 2).

![Image of guanine with binding site](image)

**Figure 2.** The site of cisplatin binding at the N-7 position on guanine.

This monoadduct can then (1) react with an external nucleophile such as the solvent or a protein, (2) react with a second purine on the same strand to form an intrastrand cross-link, or (3) react with a second guanine on the opposite strand to form an interstrand cross-link at 5'GC sequences (Figure 3) (Hopkins et al., 1991).
Figure 3. The structure of different types of cisplatin adducts. The solid lines represent complementary strands of DNA and the dashed lines represent the cross-linking agent. a) monoadduct; b) intrastrand cross-link; c) interstrand cross-link occurring at 5'GC sequences

About 90% of the original monoadducts result in 1,2- intrastrand cross-links (Eastman, 1986). While interstrand cross-links are the minor product, making up less than 5% of the original monoadducts, it is still debated as to which type of cross-links are the most biologically relevant (Malinge and Leng, 1999).

The cisplatin interstrand cross-link has very severe effects on the structure of the helix. Cisplatin interstrand cross-links cause the helix to locally switch from the right-handed helical form to the left-handed helical form (Huang et al., 1995; Kasparkova et al., 1996). In addition, the interstrand cross-links unwind the helix about 79° and bend the axis of the phosphodiester backbone 49° toward the minor groove (Malinge and Leng, 1999). The cytosine residues, no longer paired with the guanines, are extruded from the double helix allowing the 180° rotation of the platinated guanine that brings the structure into the minor groove. The resulting electrostatic interactions between the oxygens of the phosphate groups on the platinated guanine residues and the square planar Pt(II) atom lead to a pseudo-octahedral geometry around the metal ion. This interaction and the stacking of both platinated guanines with the adjacent base pairs contributes to the stabilization of the structure (Malinge and Leng, 1999). Since these resulting distortions are believed to be linked to the drug's cytotoxicity, the question then follows: does the incorporation of DNA into chromatin affect the ability of cisplatin to cross-link the DNA?
order to investigate this question, we developed a more biologically relevant model. The model includes the 154 bp EcoR1/Rsal restriction fragment of the 5S rRNA gene of *Xenopus borealis* that is reconstituted with histones isolated from chicken erythrocytes.

The 154 bp restriction fragment (Figure 4) of the 5S rRNA gene of *Xenopus borealis* was chosen because it has been previously shown to exhibit exceptional nucleosomal positioning properties (Hayes *et al.*, 1990).

![Figure 4](image)

**Figure 4.** The sequence of the 154 bp EcoR1/Rsal restriction fragment of the 5S rRNA gene of *Xenopus borealis* used in this study. Numbering is from 1 to 154 in the 5'-3' direction. The fourteen d(GC) sites for potential interstrand cross-linking are underlined, bolded, and numbered with the 5'residue.

Nucleosomal structure consists of repeating subunits called nucleosomes. Nucleosomes include 165-246 bp of DNA complexed through ionic interactions with histone proteins (van Holde, 1989). The nucleosome can be divided into two regions, the core particle and the linker region.

The core particle consists of 146 bp of DNA wrapped one and three-quarters times around a histone octamer containing two copies of H2A, H2B, H3, and H4 proteins (Kornberg and Klug, 1991). Within this structure, the octamer is composed of two dimers of the H2A and H2B histones and a tetramer of the H3 and H4 histones. The linker region is 20-70 bp of DNA in length, and it contains binding sites for histone proteins H1 and H5 (in avian erythrocytes) as
The incorporation of DNA into the above structure of proteins has several structural implications, including a curvature of about 45° per helical turn and various bends, which result in periodic narrowing and widening of the minor groove (Millard, 1996).

The 14 putative binding sites of cisplatin interstrand cross-linking in the 154-mer, given the 5'-GC sequence preference, are presented in a variety of rotational and translational conformations when the 154-mer is incorporated into the nucleosomal core particle. The rotational positioning refers to the orientation of the site with respect to the histone core (i.e. facing toward or away from the histone molecules). The translational positioning refers to the number of base pairs a site is located from the dyad (C76 in this sequence) (Pruss and Wolffe, 1993). The periodic compression and expansion of the minor and major grooves giving rise to varying distances between the guanine N-7 atoms, the distortion of the DNA molecule by the core particle, and the site concealment by DNA-protein contacts could all be sources of potential differential site specificity between free and nucleosomal DNA for cisplatin interstrand cross-linking.

Previous work on interstrand cross-linking of DNA by cisplatin using nucleosomal calf thymus DNA has shown that reactivity varies depending on the level of binding. At higher \( R_r \) values of 0.1-0.2 (moles of drug/moles of nucleotide base pairs), the binding sites in free DNA, chromatin, and nucleosomal DNA show similar reactivity, suggesting no preference for any particular region (Houssier et al., 1983). However, at lower levels of platinum \( (R_r < 0.03) \) the amount of bound platinum is reduced for core particles relative to chromatin and free DNA, indicating preferential binding to linker DNA (Foka and Paoletti, 1986). Moreover, it was found that micrococcal nuclease digestion of chromatin was inhibited at \( R_r < 0.05 \). Normally, when
micrococcal nuclease is incubated with chromatin, the result is rapid cleavage of linker DNA to produce polynucleosomes and ultimately core particles (Noll, 1974). Thus, inhibition of micrococcal nuclease digestion of chromatin by cisplatin at these low Rf values is also suggestive that cisplatin binds preferentially to linker DNA at such Rf values (Millard, 1996). The preference of cisplatin for linker DNA is attributed to histone-induced constraint on the core particle (Millard, 1996). We wished to further investigate the effects of nucleosomal structure on cisplatin by narrowing our focus to look specifically at interstrand cross-linking of cisplatin within the model described above to determine whether similar tendencies in cross-linking existed.

Interestingly, the stereoisomer of cisplatin, trans-diaminedichloroplatinum (II) (transplatin), is a clinically inactive compound that has little to no antitumor activity (Paquet et al., 1999), but there has recently been some controversy over this point (Malinge and Leng, 1999). Due to the vastly different effects seen from a slight change in structure of the drug, we thought that a parallel study of transplatin in conjunction with cisplatin might provide some additional insight into the mechanisms relating to cisplatin's cytotoxicity. Thus, we also investigated the effect of nucleosomal structure on the interstrand cross-linking of DNA by transplatin.

Similar to cisplatin, transplatin does bind to the N-7 position of guanine to form a monoadduct. However, unlike cisplatin, transplatin cannot form 1,2- intrastrand cross-links because of steric hindrances and instead forms interstrand cross-links with a cytosine on the opposite strand, along with some 1,3- intrastrand cross-links with a second guanine. The 1,3 adducts have been shown, however, to frequently rearrange to form the interstrand cross-links (Paquet et al., 1999). In addition, transplatin has a much higher reactivity with glutathione, thus,
potentially leading to a lower level of interstrand cross-links (Malinge and Leng, 1999). The kinetics of the reactions of the two isomers are very different with the transplatin reactions being much slower (Paquet et al., 1999). In addition, the distortions in the DNA induced by transplatin are much less severe than those induced by cisplatin. The helix is unwound by only about 12° and the axis of the phosphate-sugar backbone is bent 26° toward the minor groove (Malinge and Leng, 1999). Since these differences might be linked to the inability of transplatin to be used as an effective antitumor agent, the effects of nucleosomal structure on both cisplatin interstrand cross-linking and transplatin interstrand cross-linking of DNA could provide very interesting information about the mechanisms of cytotoxicity.
MATERIALS & METHODS

Purification of Core Particles

Core particles were generously purified by Jake Conklin using a method similar to Millard, Spencer, and Hopkins (1998), from chicken erythrocytes (Jason’s Butcher Shop; Albion, ME). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was then performed using an 18% Tris-glycine gel to ensure the purity of the histone samples (Sambrook et al., 1989). Core particles were quantified on a Shimadzu UV-160 spectrophotometer by their DNA using the convention that 50 μg/mL DNA is equivalent to 1 OD at 260 nm (Sambrook et al., 1989).

Isolation of DNA Fragment

pXP-11 (Kuduvalli et al., 1995), containing a portion of the 5S rRNA gene of Xenopus borealis, was kindly provided by the Tullius laboratory (John Hopkins University). Transformations into Eschericia coli were graciously performed by Jake Conklin, and the plasmid was further purified from those colonies using Qiagen p-2500 purification columns. For two 500 mL cultures of 2 colonies, the yield was approximately 8 mg of DNA at 1 μg/μL. DNA was quantified on the Shimadzu UV-160 spectrophotometer using the convention that 50 μg/mL is equivalent to 1 OD at 260 nm (Sambrook et al., 1989).

For each experiment a varying amount of DNA was required, and thus the numbers of digestion replicates were varied. Digestions were performed under standard conditions (Sambrook et al., 1989). For each 400 μL digestion replicate, 20 μg of DNA were digested with 0.06 units/reaction EcoRI and 0.03 units/reaction RsaI in 1X Reaction Buffer 1 (from New England Biolabs; 10X Reaction Buffer 1 diluted in sterile H2O). The reactions were incubated at
37°C for at least 2 hr and then ethanol precipitated using 1/10 volume of 3 M sodium acetate (NaOAc) (pH 5.0) and 2 volumes of chilled 95% ethanol. The mixtures were next incubated at -20°C for at least 20 minutes and then centrifuged in an Eppendorf model 5402 refrigerated microcentrifuge at 11,600 x g for 15 minutes at 4°C. The supernatant was removed and discarded, and the pellets were washed in 1 mL of chilled 80% ethanol. Washes also consisted of an incubation at -20°C for at least 20 minutes and centrifugation in the above manner. The pellet was dried in a Labconco Centrivap and then 3'-end radiolabeled with 32P under standard conditions (Sambrook et al., 1989). Each labeling reaction consisted of 0.005 units of Klenow fragment, 1X Eco Pol buffer (from New England Biolabs; 10X Eco Pol Buffer), 25 μCi 32P-dATP (Amersham) in a total volume of 26 μL. The reactions were incubated at room temperature for 15 minutes, and then 20 μL of native dye (0.1% xylene cyanol / 50% glycerol) were added to each tube.

In order to purify the desired 154-mer, the reactions were run on a 19 cm x 16 cm 6% native PAGE gel [8 mL 30% acrylamide (37.5:1 acrylamide:bisacrylamide), 8 mL 5X TBE, 400 μL 20% APS, 20 μL TEMED] in 1X TBE at 250 V at 4°C for ~2 hr or until the dye had migrated 10 cm. The gel was visualized using autoradiography which consistently revealed two bands with the 154-mer at a much higher mobility near the dye front (Figure 5).
Figure 5. The separation of the 154 bp EcoR1/Rsa1 restriction fragment of the 5S rRNA gene of *Xenopus borealis* from the rest of the pXP-11 plasmid. This is an autoradiograph of a 6% native PAGE gel containing identically ethanol precipitated and 32P radiolabeled *EcoR1/Rsa1* digests of pXP-11 plasmid. The lower mobility bands represent the rest of the pXP-11 plasmid and the higher mobility bands the 154-mer.

The band representing the 154-mer was next excised from the gel and the DNA was eluted using the crush-and-soak procedure (Sambrook *et al.*, 1989). Each gel band was crushed in a microcentrifuge tube containing 800 μL of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, 0.1% SDS) and incubated at 37°C for at least 4 hr. Then the elution buffer, now containing the 154-mer, was removed, spin filtered (0.45 micron spin filters obtained from Altech), and ethanol precipitated (including the 80% wash) as previously described. If reconstitution was to follow, the buffer was spin filtered into siliconized, sterile microcentrifuge tubes. In order to ensure sufficient recovery of DNA, the bands were then incubated at 37°C for an additional 2-4 hr in 600 μL of fresh elution buffer which was subsequently removed, spin filtered, and ethanol precipitated. The pellets were dried in the
Labconco Centrivap, 50 μL of H₂O was added to each one in order to dissolve the DNA, and the replicates were then consolidated as necessary. Finally, the 154-mer was re-precipitated in 1 mL of 80% ethanol, which was chilled, pelleted, and dried as above.

Reconstitution

Two methods of reconstitution were employed. For the first method, herein called the Wolfe method (Hayes et al., 1991), the dried, radiolabeled 154-mer from above (in a siliconized, sterilized microcentrifuge tube) was placed in 0.325 OD₂₆₀ or 0.975 OD₂₆₀ of core particles and 1 M NaCl (5 μL of 4 M NaCl). The final volume was brought up to 20 μL with 100:1 of TE [10 mM Tris (pH 7.5) and 1 mM EDTA]: phenylmethylsulfonyl fluoride (PMSF) (50 mM PMSF in isopropanol). The mixture was incubated for 1 hr on ice. The salt concentration was then gradually decreased by adding aliquots of TE/PMSF (5 μL for 1 hr, 5 μL for 1 hr, 170 μL for 15 minutes, 200 μL for 15 minutes) (Hayes, et al., 1991). For the second method, herein called the Smerdon method (Suquet and Smerdon, 1993), the dried, radiolabeled 154-mer was mixed with 2.93 OD₂₆₀ or 4.88 OD₂₆₀ of core particles, 1 M NaCl (5 μL 4 M NaCl), and the final volume was brought to 100 μL with TE/PMSF. The mixture was incubated on ice for 45 minutes and then transferred to a 0.5 mL slide-a-lyzer. The salt concentration was then gradually decreased by dialyzing the mixture at 4°C for 3 hr periods in 200 mL TE/PMSF containing 0.8 M NaCl, 0.6 M NaCl, and 0.05 M NaCl. The contents of the slide-a-lyzer were then removed and placed in a microcentrifuge tube. Reconstitutions were stored at 4°C.

Reconstitutions were analyzed and free and reconstituted nucleosomal 154-mer were separated using 6% native PAGE. The gels (19 x 16 cm, 8 mL 30% 37.5:1 acrylamide: bisacrylamide, 4 mL 5X TBE, 4 mL 50% glycerol, 200 μL 20% APS, 16 μL TEMED) were run
in 0.5X TBE at 4°C for ~3 hr or until the dye front had migrated about 10 cm. If samples were to be reacted with cisplatin or transplatin, separation of reconstituted and free 154-mer by PAGE was delayed until after the platinum-modification reactions had taken place in order to ensure that both the free and nucleosomal DNA were subjected to the same reaction conditions.

**Cisplatin Cross-linking Reaction**

A 1.0 mM cisplatin (Aldrich) stock (3 mg of cisplatin in 10 mL H₂O) was prepared 24 hr before incubation. The stock was diluted 1:10 with H₂O, and thus the appropriate amount of cisplatin for each reaction was taken from a 0.1 mM cisplatin solution in order to increase the accuracy of the final solution concentration. Two concentrations of cisplatin were used to cross-link DNA: 0.01 mM and 0.0025 mM. The first set of experiments had final volumes of 150 μL. They contained 100 μL of a Wolffe reconstitution or 40 μL of a Smardon reconstitution, 1X platination buffer [15 μL of 10X stock containing 400 mM sodium perchlorate (NaClO₄), 20 mM monobasic potassium phosphate (H₂KPO₄) (pH 7.6)], and 0.01 mM cisplatin or 0.0025 mM cisplatin that was brought up to final volume with H₂O. Subsequent experiments were scaled down to a 90 μL final volume using 70 μL of Wolffe reconstitution. This smaller volume was used so that the entire reaction could be loaded onto one gel lane. All reactions were incubated in the dark at 37°C for 8 hr. A 10 μL portion of native dye was then added to each tube, and the free and reconstituted nucleosomal 154-mer were separated by 6% native PAGE as described in the Reconstitution section above. For the first set of reactions with final volumes of 150 μL, the 0.01 mM cisplatin reaction had an Rᵣ = 0.081, and the 0.0025 mM cisplatin reaction had an Rᵣ = 0.020. For the reactions with final volumes of 90 μL, the 0.01 mM cisplatin reaction had an Rᵣ = 0.070, and the 0.0025 mM cisplatin reaction had an Rᵣ = 0.017.
Generally two bands, both near the dye front, were observed: the band of higher mobility representing the free 154-mer and the band of lower mobility representing the reconstituted, nucleosomal 154-mer. Both were excised and the DNA was eluted as described above.

**Separation of Cross-linked DNA**

Lyophilized, cisplatin-treated free and nucleosomal 154-mer samples were resuspended in 10 µL of 5 M aqueous urea/0.1% xylene cyanol and run on a 41 cm x 37 cm, 0.35 mm thick, 6% denaturing formamide/urea polyacrylamide gel [15 mL 40% 19:1 acrylamide:bisacrylamide, 20 mL 5X TBE, 30 mL formamide, 42 g urea, 200 µL 20% APS, 35 µL of TEMED brought up to a final volume of 100 mL with H₂O] at 55 watts at 37°C for ~3 hr or until the dye had migrated ~10 cm from the bottom. For this procedure a Hoefer thermojacketed Poker Face gel stand was used. A negative control containing unmodified DNA was also run on each gel.

**Determination of Cross-linking Pattern**

If the above large gels were to be analyzed for cross-linking patterns, the gels were dried under a vacuum for one hour with 20 minutes of heating at 55°C using a Drygel Sr. Model SE 1160 (Hoefer Scientific Instruments). The gels were then autoradiographed by placing them under an intensifying screen and exposing them to film at -80°C.

Once a revealing autoradiograph was obtained, the gels were phosphorimaged using a BioRad GS-505 Imaging System (Sample Exposure Platform, Screen Eraser and Molecular Imager) with the software program Molecular Analyst version 2.1.2. Profiles were taken across the cross-linking bands in order to determine the percentage of total cross-linking that each band
represented. In addition, profiles were taken across the entire lane in order to determine the percentage of total DNA that was interstrand cross-linked.

**Mapping of Cisplatin Cross-linking Sites**

In order to map the cross-linking sites, the large denaturing gel from above, while still on the small glass plate, was autoradiographed. Based on the autoradiograph, sections revealing cross-linking were excised from the gel, and DNA was eluted from these samples as described above. Maxam-Gilbert G protection assays were then performed on the lyophilized, cross-linked DNA samples in a manner similar to standard (Sambrook et al., 1989). A negative control consisting of non cisplatin-reacted DNA was also subjected to the following G reaction and piperidine cleavage. A 190 μL portion of dimethyl sulfate (DMS) buffer [50 mM sodium cacodylate (pH 7.0), 1 mM EDTA (pH 8.0)] was added to the dry pellet of DNA and the mixture was chilled to 0°C. A 5 μL portion of 10% DMS was added, the cap was immediately closed, and the contents were thoroughly mixed by vortexing for 10-15 seconds. The mixture was then incubated at room temperature for 5 minutes. Next, 50 μL of DMS stop solution [1.5 M sodium acetate (pH 7.0), 1 M β-mercaptoethanol, 250 μg/mL yeast tRNA] were added to each reaction and the mixture was cooled to 0°C. The samples were then ethanol precipitated in the following manner. A 750 μL portion of chilled ethanol was added to each tube, and the tubes were incubated at -20°C for at least 20 minutes and then centrifuged in an Eppendorf model 5402 refrigerated microcentrifuge at 11,600 x g for 15 minutes at 4°C. The supernatants were removed and discarded, and 300 μL of 0.3 M NaOAc (pH 5) and 900 μL of chilled ethanol were added to each pellet. The tubes were incubated at -20°C for at least 20 minutes and then centrifuged as previously described. The supernatants were removed and discarded and the pellets were washed.
in 1 mL of chilled 80% ethanol. Washes also consisted of an incubation at −20°C for at least 20 minutes and centrifugation in the above manner. After the wash, the pellets were dried in the Labconco Centrivap.

Cisplatin was then removed with the use of cyanide (Naser et al., 1988). The lyophilized pellets were resuspended in 100 µL of 0.3 M NaCN (pH 8.0) and the mixture incubated at 37°C for 3 hr. This reaction was ethanol precipitated as described in the sections above. The DNA was then piperidine cleaved in the following manner. The lyophilized pellets were resuspended in 100 µL of 1 M piperidine. The mixtures were incubated at 90°C for 15 minutes, cooled to room temperature, and dried in the Labconco Centrivap. To ensure the removal of all piperidine, the pellets were resuspended in 40 µL of H2O, vortexed for 30 seconds, and dried in the centrifuged. This process was repeated once, and then 10 µL of 5 M aqueous urea/0.1% xylene cyanol were added to each pellet. The tubes were vortexed in order to re-dissolve the DNA and briefly centrifuged to deposit all of the fluid at the bottom of the tubes.

The samples were loaded onto a 41 cm x 37 cm, 0.35 mm thick, 6% denaturing formamide/urea polyacrylamide gel [15 mL 40% 19:1 acrylamide:bisacrylamide, 20 mL 5X TBE, 30 mL formamide, 42 g urea, 200 µL 20% APS, 35 µL of TEMED brought up to a final volume of 100 mL with H2O] and run at 55 watts at 37°C until the dye had migrated 10 cm from the top. The sequencing gel was then dried, autoradiographed, and phosphorimaged as described above.

Reconstitution Experiment

In order to assess the ability of cisplatin-treated DNA to be reconstituted with histones, 154-mer was isolated and radiolabeled as described above. The pellets of dried, isolated, free
154-mer were then reacted with cisplatin using reaction conditions and reconstitution conditions similar to those described above. In this case, however, the reaction was scaled down to a 20 µL final volume. Again, two concentrations of cisplatin were used to cross-link DNA: 0.01 mM (R_f = 0.00271) and 0.0025 mM (R_f = 0.000676). Because of the small final volume, an additional 1:5 dilution of the 0.1 mM cisplatin was made when setting up the 0.0025 mM cisplatin reactions. Generally, there are histones present for the cisplatin reaction which tend to react with much of the cisplatin by forming protein to DNA cross-links. Thus, in order to keep the true reaction ratio of cisplatin to 154-mer, the 0.975 OD_{260} of histones were present for the cisplatin reaction part of the experiment. See above for the rest of the required concentrations of constituents. At the end of the 8 hr incubation at 37°C, 5 µL of 4 M NaCl were added, and the mixture was incubated at 4°C for 1 hr. From this point, the reconstitution was performed as above according to the Wolffe method, slowly decreasing the salt concentration over time.

Next, a 6% native PAGE reconstitution gel was run as described above. The samples consisted of 90 µL of these cisplatin-reacted, reconstituted samples and 10 µL of dye. For comparison, samples of the normal reconstituted, cisplatin reacted 154-mer at both concentrations of cisplatin were also run. The gel was then autoradiographed as described above.

**Transplatin Experiments**

To determine the cross-linking pattern of transplatin, 154-mer was isolated, radiolabeled, and reconstituted as described above. Stock solutions, dilutions, and concentrations of transplatin were identical to those used in the cisplatin experiments described above. The reactants and final volumes were also identical to those used in the above cisplatin experiments. The only difference was that the transplatin reactions were incubated at 37°C for 2 weeks. A control reconstitution
(volume 70 μL) along with a control 0.01 mM cisplatin reaction were also incubated at 37°C for 2 weeks.

At the end of the two week period, 10 μL of dye was added to each of the samples and the samples were run on a 6% native PAGE reconstitution gel as described above. Normal 8 hr cisplatin reaction samples of both concentrations, along with a non-reacted reconstitution sample were included on the gel for comparison. These cisplatin-reacted samples were prepared so that the reactions would terminate at the exact end of the two week incubations. All samples, cisplatin, transplatin, and additional controls were derived from the same two reconstitutions that were stored at 4°C.

The gel was then autoradiographed, and the bands were excised and treated as described above. The cross-links were separated as described above and the gel was dried, autoradiographed, and phosphorimaged as described above.

An experiment to determine the ability of transplatin-reacted DNA to reconstitute was also performed. The conditions were exactly the same as those described above in the similar cisplatin experiment, except that the transplatin reaction was incubated for 2 weeks. Similar controls and gels were also run.
RESULTS

The $^{32}$P-3'-end-labeled 154 bp EcoR1/Rsa1 restriction fragment of the 5S rRNA gene of *Xenopus borealis* was incubated with purified chicken erythrocyte histones in order to reconstitute the DNA. In order to obtain the best results for cross-linking experiments, both the free and reconstituted nucleosomal DNA needed to be subjected to the same reaction conditions. Thus, it was desirable to obtain a set of reconstitution parameters that yielded a 1:1 ratio of free:nucleosomal 154-mer. As described above, two methods of reconstitution, the Smerdon and the Wolffe methods, were examined, and the amount of histones was varied. The preferred method based on time required and simplicity of procedure was the Wolffe method. Thus, initially only the Wolffe method was attempted, but these attempts failed because the 6% native PAGE revealed that the samples were being lost during the procedure. At this point, the Smerdon method was pursued, and a sample of 154-mer, reconstituted with the maximal amount of histones according to the Wolffe method, was included for comparison purposes only. However, as can be seen in Figure 6, the sample reconstituted according to the Wolffe method yielded a ratio of ~3:7 for free:nucleosomal 154-mer. The Smerdon method, on the other hand, yielded even higher proportions of nucleosomal 154-mer with ratios approaching 100% nucleosomal 154-mer.
Figure 6. Reconstitution experiment to determine a method that would yield a 1:1 ratio of free:nucleosomal DNA. An autoradiograph of a 6% native PAGE to separate free and nucleosomal DNA. The higher mobility band represents free DNA and the lower mobility band is nucleosomal DNA. Lane 1: Wolffe reconstitution with 0.975 OD\textsubscript{260} histones, 0.01 mM cisplatin; Lane 2: Wolffe reconstitution with 0.975 OD\textsubscript{260} histones, 0.0025 mM cisplatin; Lane 3 and 4: Smerdon reconstitution with 2.93 OD\textsubscript{260} histones, 0.01 mM cisplatin; Lane 5 and 6: Smerdon reconstitution with 2.93 OD\textsubscript{260} histones, 0.0025 mM cisplatin; Lane 7 and 10: Smerdon reconstitution with 4.88 OD\textsubscript{260} histones, 0.01 mM cisplatin; Lane 8 and 9: Smerdon reconstitution with 4.88 OD\textsubscript{260} histones, 0.0025 mM cisplatin.

Because the Wolffe method seemed to work well, it was employed in all future experiments. The Wolffe method was not further optimized, and all reconstitutions were done using 0.975 OD\textsubscript{260} of histones, the maximal amount possible at this concentration of histones.

After successful reconstitution yielding a nearly 1:1 ratio of free:nucleosomal 154-mer, the mixture was then treated with two concentrations of cisplatin in an attempt to obtain approximately one cross-link per DNA molecule. Next, the free and nucleosomal 154-mer were separated via 6% native PAGE, purified from the gel, and run on a 6% denaturing formamide/urea PAGE gel to separate the cross-links. Autoradiographs indicated that the free and nucleosomal 154-mer had similar cross-linking patterns (Figure 7).
Both concentrations of cisplatin also yielded similar patterns. The pattern of interstrand cross-linking consisted of what appear to be six or seven sets of bands, with the third set being the most intense. A large native band was seen near the bottom of the gel along with a few bands of higher mobility at low intensities. Furthermore, the negative control containing no cisplatin yielded no low mobility bands (data not shown), showing only the bottom band representing the native unmodified DNA. Phosphorimagery of these gels and subsequent profile scanning of the

Figure 7. The interstrand cross-linking pattern of both free and nucleosomal DNA generated by cisplatin. An autoradiograph of a formamide/urea denaturing 6% PAGE gel to separate cross-linking. All samples were reconstituted according to the Wolff method using the maximal number of histones (0.975 OD_{260}), except for the sample in lane 5 which was reconstituted according to the Smerdon 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. Lane 5: nucleosomal, 0.01 mM cisplatin.
interstrand cross-linked bands in each of the lanes to visualize the percentage of total of interstrand cross-linking that each band represents gives the distribution in Figure 8.

**Figure 8.** Two-dimensional profile scans of the cisplatin interstrand cross-linked DNA in the free and nucleosomal samples of the gel in Figure 7. The line with the larger third peak corresponds to a scan of the interstrand cross-links in lane 3 (nucleosomal DNA) of the gel in Figure 7. The other line corresponds to a scan of the interstrand cross-links in lane 4 of the gel in Figure 7. The labeled peaks represent major bands seen on the autoradiograph in Figure 7.

Profile scanning of each of the entire lanes to determine the percentage of total DNA that was interstrand cross-linked revealed that the highest percentage of interstrand cross-linked DNA in any sample was 23% with the average no more than 15% (Table 1).
Table 1: Total cisplatin interstrand cross-linked DNA as a percentage of the total DNA in free and nucleosomal samples. 2D profile scans were made of the entire lane of each DNA sample from the phosphorimage of denaturing gels. The percentages of interstrand cross-linking and percentages of native DNA were then calculated from the counts calculated by the Molecular Analyst program. The 0.01 mM samples are an average of samples from 2 different gels. The native DNA refers to non-cross linked DNA.

<table>
<thead>
<tr>
<th>[Cisplatin] (mM)</th>
<th>Sample</th>
<th>% Interstrand Cross-linked DNA</th>
<th>% Native DNA</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01</td>
<td>free</td>
<td>11</td>
<td>89</td>
<td>9</td>
</tr>
<tr>
<td>0.01</td>
<td>nucleosomal</td>
<td>15</td>
<td>85</td>
<td>11</td>
</tr>
<tr>
<td>0.0025</td>
<td>free</td>
<td>8</td>
<td>92</td>
<td>—</td>
</tr>
<tr>
<td>0.0025</td>
<td>nucleosomal</td>
<td>2</td>
<td>97</td>
<td>—</td>
</tr>
</tbody>
</table>

In order to confirm the 5'GC sequence preference for interstrand cross-linking of DNA by cisplatin on this particular 154-mer and with the hopes of determining which bands in the gel of Figure 7 correspond to which sites, Maxam Gilbert G-protection assays were performed. After the cross-links were separated using 6% denaturing PAGE, the gel was autoradiographed in an attempt to determine where the interstrand cross-linking bands were in the gel so that they could be separately excised. However, no such autoradiograph has yet been obtained. Instead regions were cut out where the interstrand cross-linked DNA was expected. The DNA was then methylated by using dimethyl sulfate. Next, the cisplatin was removed using sodium cyanide, and then the DNA was piperidine-cleaved into individual nucleotides. Finally, the samples were run on a 6% denaturing formamide/urea PAGE gel as described above, and the gel was autoradiographed. Unfortunately, there are, as yet, no conclusive results. The loss of sample due to the number of gels run and the decay of the radiolabel over time made analysis of the final gel difficult because the signal was too faint even after two weeks in the -80°C freezer with an intensifying screen.
Because the pattern of cross-linking is the same in both free and nucleosomal DNA, we have tried to circumvent this problem by only looking at the G-reaction results for free DNA. In this procedure, the DNA was radiolabeled and the 154-mer isolated. The free DNA was then reacted with cisplatin in a manner similar to the samples in the cisplatin-reacted-then-reconstituted experiment. Thus, the cisplatin was incubated with the same amount of histones included in the original reconstitution reactions in order to try to ensure that conditions remain similar to those in all previous experiments. After the 8 hr incubation period at 37°C, the DNA was ethanol precipitated in an attempt to remove the histones. Next, cross-links were separated using 6% denaturing formamide/urea PAGE, and the gels were then autoradiographed.

Unfortunately, the procedure has not gone further because the results of these autoradiographs show the samples to be trapped in the wells. This is mostly likely due to ineffective removal of histones. One possible solution to this problem would be a phenol:chloroform extraction prior to ethanol precipitation, which is currently being tested.

A reconstitution experiment to determine whether cisplatin-reacted free DNA could be reconstituted with histones was also performed. The cisplatin reaction was scaled down so that the reconstitution volume and subsequent conditions would be similar. For the gel containing both reconstituted and then reacted samples as well as reacted and then reconstituted samples see Figure 9.
Figure 9. Reconstitution experiment to determine whether cisplatin-reacted 154-mer could be incorporated into nucleosomal structure. This is an autoradiograph of a 6% native PAGE gel to separate nucleosomal and free DNA. Lanes 1-3 and 7-9 exhibit the normal 2 band, free and nucleosomal, distribution with the free DNA represented by the higher mobility band and the nucleosomal DNA by the lower mobility band. Lanes 1-3 and 7-9 contain samples that were first reconstituted with histones and then reacted with cisplatin. Lanes 4 and 5 contain samples that were first reacted with cisplatin and then reconstituted with histones. Lanes 1 and 7: no cisplatin; Lane 2, 4, and 8: 0.01 mM cisplatin; Lanes 3, 5, and 9: 0.0025 mM cisplatin.

The samples that were reconstituted first and then reacted with cisplatin contain two distinct lanes of free and nucleosomal DNA, while the reacted-then-reconstituted samples contain a distribution of bands with both higher and lower mobility than the free and nucleosomal bands of the controls.

At this point a few attempts have been made with the transplatin experiments, but few significant results have been attained. The 154-mer was successfully radiolabeled, isolated and reconstituted. All samples were taken from two reconstitutions. The controls and reaction at 0.01
mM were all taken from one reconstitution, and the reactions at 0.0025 mM were taken from the second reconstitution. Reconstitutions were then incubated with two concentrations, 0.01 mM and 0.0025 mM, of transplatin for two weeks at 37°C. In addition, several controls were incubated at 37°C for two weeks. One control consisted of reconstitution reacted with 0.01 mM cisplatin, and another consisted of non-reacted reconstitution. At the end of the two week period, a 6% native PAGE was performed on all samples including a portion of the reconstitution that had been stored at 4°C, as well as control 8 hr reactions of cisplatin with reconstitution at both the 0.01 mM and 0.0025 mM concentrations. The resulting autoradiograph of the gel is shown in Figure 10.
Figure 10. An autoradiograph of a 6% native PAGE to separate free and nucleosomal DNA for the transplatin experiments. Lanes 3-7 represent samples that were first reconstituted and then reacted with cisplatin or transplatin. Lane 9 contains a sample that was first reacted with transplatin and then reconstituted. All incubations were done at 37°C and all transplatin reactions were incubated for a two week time period. Lane 1: non-reacted stored reconstitution; Lane 2: non-reacted reconstitution, incubated for 2 weeks; Lane 3: 0.01 mM cisplatin, incubated for 2 weeks; Lane 4: 0.01 mM cisplatin, incubated for 8 hr; Lane 5: 0.0025 mM cisplatin, incubated for 8 hr; Lane 6: 0.01 mM transplatin; Lane 7: 0.0025 mM transplatin; Lane 9: 0.01 mM transplatin.

All of the samples that were incubated for two weeks seem to contain mainly free 154-mer, while the control taken from the same reconstitution contains a more equal portion of nucleosomal and free 154-mer. Thus, the nucleosomal DNA is somehow being converted back to free DNA over the two week period at 37°C.

The experiment to test the ability of transplatin-reacted DNA to reconstitute was successful. The 154-mer was radiolabeled, isolated, and reacted with transplatin at a
concentration of 0.01 mM. The conditions of the transplatin reaction were scaled down to have a final volume of 20 μL, so that after the two week incubation period at 37°C, the DNA could be reconstituted in a manner as similar as possible to previous reconstitution conditions. After reconstitution, the free and nucleosomal DNA were then separated using 6% native PAGE. The autoradiograph (Figure 10, lane 9) shows a series of 4 bands, including bands representing the normal free and reconstituted nucleosomal 154-mer. In addition to that, there seems to be some dinucleosome forming (lowest mobility) and a structure that migrates between the free and nucleosomal.

No successful 6% denaturing PAGE have been run on transplatin-modified DNA at this time.
DISCUSSION

Cisplatin has been previously determined to interstrand cross-link duplex DNA at 5'GC sequences (Hopkins et al., 1991). For the purposes of this experiment we used the EcoRI/RsaI 154 bp restriction fragment of the 5S rRNA gene of Xenopus borealis. We attempted to establish the interstrand cross-linking pattern of cisplatin for this fragment and to reconstitute the fragment with histones isolated from chicken erythrocytes in order to determine whether nucleosomal structure impacted the ability of cisplatin to interstrand cross-link DNA. Additional goals were to confirm the 5'GC sequence preference of cisplatin within this DNA and to test the ability of cisplatin-reacted DNA to reconstitute with histones. We also attempted to look at differences in interstrand cross-linking between cisplatin and its clinically inactive sterioisomer transplatin.

After successful 3'-end labeling and isolation of the 154-mer, a reconstitution procedure was established. For the free vs. nucleosomal comparison studies of cisplatin cross-linking, a reconstitution procedure that yielded a 1:1 ratio of free to nucleosomal DNA was desired to ensure that both types of DNA were subjected to the same reaction conditions. After experimentation with two methods and a variety of histone concentrations, the Wolffe method of reconstitution using maximal histone concentration was determined for use in all subsequent experiments. These conditions yielded an approximately 3:7 ratio of free:nucleosomal 154-mer. However, as time was short the procedure was not further optimized. Initial experiments with this reconstitution method showed that the samples were being lost along the way, and the only change in the procedure associated with a change in results was the switch from microcentrifuge tubes siliconized and sterilized in lab to commercially purchased sterilized, siliconized microcentrifuge tubes. Thus, it seems as though our method of siliconization was not adequate and resulted in loss of desired product.
Previous studies indicated that reconstitution of the 154-mer with histones consistently produces uniquely and precisely positioned core particles that place the fourteen potential sites of cisplatin interstrand cross-linking in a variety of rotational and translational positions that could make cross-linking in certain positions by cisplatin quite difficult (Millard et al., 1998).

Autoradiography of reconstitution gels that separated free and nucleosomal DNA typically revealed 2 bands: 1 of higher mobility representing the free DNA and one of lower mobility representing the nucleosomal DNA (Figure 6 and 9). There were no additional bands present that would represent dinucleosome formation or other undesirable arrangements of reconstituted histones, indicating that the histones appear to be consistently reconstituting in the same manner.

Further confirmation of the normal precise positioning is, however, required in the form of a hydroxyl radical cleavage study of the reconstituted 154-mer. Hydroxyl radical cleavage analysis of this 154-mer complexed with histones from chicken erythrocytes has been performed in the Millard lab before, yielding results that confirmed the precise rotational and translational positioning of the core particle on this DNA fragment (Millard et al., 1998). However, the cleavage analysis has not been done on these particular samples of DNA and histones. The additional study would, therefore, serve to confirm the consistency of the cleavage pattern with this particular sample of DNA and histones.

Despite some uncertainty about the positioning of histones, once the reconstitution procedure was established, reconstitutions were reacted with two concentrations of cisplatin, free and nucleosomal 154-mer were separated, and the samples were subjected to denaturing PAGE to separate the cross-links. Autoradiography revealed a consistent interstrand cross-linking pattern of what look like 6 or 7 families of bands, identical in both free and nucleosomal 154-mer (Figure 7). In addition, very few intrastrand cross-links were visible in most samples. Intrastrand
cross-links are represented by bands with lower mobility than native DNA. Although intrastrand cross-links are the majority product of cross-links in vitro, it is not surprising that not many are seen in these experiments because the conditions of the reaction have been adjusted to maximize interstrand cross-linking. The consistency in pattern of cross-linking between nucleosomal and free DNA, despite the difficulties in cross-linking that certain sites would seem likely to experience based on the conformation that the nucleosome imposes on the DNA, seems to point to a locally dynamic model of the nucleosome.

Phosphorimagery and 2D profiling of each of the lanes of interstrand cross-links indicated that although there were no pattern differences between interstrand cross-linking of free and nucleosomal 154-mer, there were some differences in intensity of interstrand cross-linking at particular sites (Figure 8). Profile scans of additional gels and concentrations yield similar results to those seen in Figure 8 (data not shown). The size of the peak corresponding to the third family of bands is much larger in the nucleosomal sample than in the free sample. Other differences seem relatively minor including differences based on concentration of cisplatin. However, it is interesting that the nucleosomal structure in the one case actually significantly increases the ability of cisplatin to cross-link. Other cross-linkers that have been studied including mitomycin C and the nitrogen mustards have been found to produce the same pattern of cross-linking in nucleosomal DNA that they do in free DNA (Millard et al., 1998). When differences in intensity of cross-linking at particular sites have been characterized, as in the case of mitomycin C, the nucleosomal structure has been shown to inhibit the ability of the reagent to cross-link (Millard et al., 1998). This seems more likely as the nucleosome would seem to impose some structural conformations on the DNA that would be quite unfavorable for reagents to cross-link. For example, some of the conformations have both binding sites turned in towards
the histones, seemingly making them unavailable for cross-linking. However, if the dynamic model of nucleosomal structure is correct, then the nucleosomes could be transiently exposing the DNA, thereby periodically providing access to regulatory proteins. Then, it does not seem surprising that little differences due to nucleosomal structure is found.

A study that supports the dynamic theory of nucleosomal structure examined the exchange of histones into replication inhibited DNA (Louters and Chalkley, 1985). This study found that while quick exchanges occurred between newly synthesized H1, H2A, and H2B and the histones previously present in the DNA, little to no exchange of H3 and H4 in the core was observed. These observations point to the conclusion that the core is able to bind and unbind the DNA, allowing other molecules access to areas of the DNA that are unavailable in the coiled structure.

2D profile scans of the phosphorimage were also done on each of the entire lanes in order to determine the percent of total DNA that was interstrand cross-linked. It was found that in all samples less than 23% of the DNA was interstrand cross-linked, with the average percentage of interstrand cross-linking being much lower (Table 1). These low percentages indicate that at these cisplatin concentrations, we have single hit conditions in which there is no more than one cisplatin binding per DNA molecule. Cross-linking percentages of up to 35% of the total DNA are indicative of single hit conditions (Goodisman and Dabrowiak, 1985). Thus, both concentrations of cisplatin seem to be optimal for interstrand cross-linking investigation.

The third band that exhibits the major difference in efficiency of interstrand cross-linking between the free and nucleosomal samples is believed to correspond to a site that is three helical turns from the dyad. This prediction is based on a former hydroxyl radical cleavage study of reconstituted 5S rRNA gene of Xenopus borealis (Kuduvalli et al., 1995). In the table below the
sites that have potential for interstrand cross-linking are listed along with their translational and rotational positioning (Table 2).

Table 2: The predicted migratory pattern of cisplatin interstrand cross-linked sites in the 154 bp EcoRI/Rsal restriction fragment of the 5S rRNA gene of Xenopus borealis. The table contains a list of sites that could potentially be interstrand cross-linked by cisplatin, along with their translational and rotational positioning. The translational positioning refers to the number of base pairs that a site is from the dyad, site C76 for this sequence. The rotational positioning refers to the orientation of the site with respect to the histone core particle (i.e. facing toward or away from the histone molecules). The migratory pattern was predicted using the information that the closer a site is to the dyad, the less mobile it will be when interstrand cross-linked. Resolution of the denaturing gel was also taken into account when making the predictions. The bands are given in order of least mobile (1) to most mobile (6).

<table>
<thead>
<tr>
<th>Cross-linking Site</th>
<th>Translational Positioning</th>
<th>Rotational Positioning</th>
<th>Predicted Migratory Band</th>
</tr>
</thead>
<tbody>
<tr>
<td>G8</td>
<td>68</td>
<td>facing</td>
<td>6</td>
</tr>
<tr>
<td>G12</td>
<td>64</td>
<td>away</td>
<td>5</td>
</tr>
<tr>
<td>G25</td>
<td>51</td>
<td>neither</td>
<td>4</td>
</tr>
<tr>
<td>G30</td>
<td>46</td>
<td>neither</td>
<td>4</td>
</tr>
<tr>
<td>G44</td>
<td>32</td>
<td>away</td>
<td>3</td>
</tr>
<tr>
<td>G47</td>
<td>29</td>
<td>facing</td>
<td>3</td>
</tr>
<tr>
<td>G68</td>
<td>8</td>
<td>facing</td>
<td>2</td>
</tr>
<tr>
<td>G13</td>
<td>1</td>
<td>away</td>
<td>1</td>
</tr>
<tr>
<td>G79</td>
<td>3</td>
<td>facing</td>
<td>1</td>
</tr>
<tr>
<td>G86</td>
<td>10</td>
<td>away</td>
<td>2</td>
</tr>
<tr>
<td>G105</td>
<td>29</td>
<td>away</td>
<td>3</td>
</tr>
<tr>
<td>G129</td>
<td>53</td>
<td>facing</td>
<td>4</td>
</tr>
<tr>
<td>G134</td>
<td>58</td>
<td>away</td>
<td>5</td>
</tr>
<tr>
<td>G144</td>
<td>68</td>
<td>away</td>
<td>6</td>
</tr>
</tbody>
</table>

It has been previously shown that the closer a site is to the dyad, the less mobile it will be on a gel when it has been interstrand cross-linked (Millard et al., 1991). We used this piece of information, keeping in mind the resolution of the denaturing gels, to make predictions about the migratory pattern that would be seen on a gel. Of course, later on these predictions will have to be substantiated by Maxam Gilbert protections assay results of these samples. In this
prediction, the third band would correspond to cross-linking at sites G44, G47, and G105. Assuming that there are 10-11 base pairs per helical turn, the site would be approximately three helical turns from the dyad (located at site C76). Of interest is that, at approximately three helical turns upstream from the dyad, histone H4 interacts with the DNA (Pruss and Wolffe, 1993). Perhaps the increase in intensity of cross-linking seen near these sites in nucleosomal DNA is thus due to structural influences of the histone H4 protein.

The reconstitution experiment to determine whether cisplatin- and transplatin-reacted DNA could be reconstituted seems to indicate that the nucleosome as a whole is not a dynamic structure even though it may be locally dynamic. As described earlier in this experiment the cisplatin and transplatin reactions were scaled down and then subjected to conditions quite similar to the previous reconstitutions. When 6% native PAGE was performed on these samples to separate nucleosomal and free DNA, the autoradiographs revealed many additional bands at a variety of molecular weights instead of the normal two band, reconstituted and free, distribution (Figures 9 and 10). This suggests that the cross-linking distorts the structure of the 154-mer to the point where the nucleosome no longer has a single defined translational and rotational positioning. The molecule is too distorted in order to accept the regular histone configuration. A variety of structures can, therefore, be formed with the histones conglomerating onto the DNA in order to accommodate the bends. Thus, the nucleosome does not seem to be quite as flexible in accommodating distorted structures when the nucleosome is not at first present.

Unfortunately, the experiments with transplatin have not yet been completed due to some difficulties with the procedure. As reported in the results, the two week incubation period at 37°C required for optimal interstrand cross-linking somehow, in a manner unrelated to the cross-linker, converts most of the nucleosomal DNA back to free DNA (Figure 10). During the
incubation period, all of the solution in the tube evaporates and re-condenses at the top of the microcentrifuge tube. Some of the solution is probably left in vapor form, thus decreasing the volume of the solution at the top of the tube, and thereby increasing the salt concentration. This change in salt concentration could be drastic enough to disrupt the histones. Spinning the tubes down everyday might be the solution to this problem.

In conclusion, we have found that nucleosomal structure is a rather minor inhibitor of cisplatin interstrand cross-linking reactions with DNA. These data, therefore, support a locally dynamic model of nucleosomal structure. Future studies should, however, include a hydroxyl radical cleavage analysis to confirm precise positioning of the nucleosome. In addition, studies with transplatin, the clinically inactive stereoisomer of cisplatin, in comparison could provide some further insight into cross-linking and its role in relation to antitumor activity.
REFERENCES


