PLATINUM-MODIFIED DNA: SOLUTION STRUCTURE
AND PROTEIN-BINDING PREFERENCES OF
1,2-INTRASTRAND d(GpG) ADDUCTS

by

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ABSTRACT

In the first chapter, the current knowledge relating the structural role of the antitumor agent cisplatin and its chemotherapeutic mechanism will be outlined.

The second chapter describes the solution NMR structure determination of a platinum-modified duplex oligonucleotide. Modification of an undecamer, d(CTCTCGGTCTC), with the paramagnetic cisplatin analog cis-[Pt(NH₃)(4AT)Cl₂] afforded two orientational isomers of the bifunctional d(GpG) chelate. Following duplex formation with the complementary strand, the 3' orientational isomer was investigated by using paramagnetic NMR methods and 99 long range electron-proton distance restraints were determined from the loss in NOESY intensity due to the presence of the unpaired electron. These long range restraints provide direct NMR-derived evidence that the modified duplex is bent substantially toward the major groove. Refinements of this duplex with either conventional interproton restraints or a combination of the electron-proton and interproton restraints afforded the same local but different global duplex structures. Both refinements resulted in duplexes which deviated from canonical B-DNA with widened minor grooves. Addition of the long-range restraints, however, allowed for the refinement of duplex structure with marked similarity to the tertiary structure of a cisplatin-modified dodecamer duplex (RMSD for all backbone atoms = 1.98 Å). The contribution of electron-proton distance restraints to the NMR-based refinement of duplex oligonucleotide structures is assessed, and the implications of the resulting solution structures are discussed.

The third and final chapter describes the interactions between HMG-domain proteins and platinated oligonucleotides containing a single 1,2-intrastrand
d(GpG) cross-link. A cisplatin-modified duplex oligonucleotide 15 base pairs in length was sufficient for specific recognition by isolated HMG domains from several structure-specific proteins. The presence of a bulky amine in a cisplatin analog did not affect this specific interaction. HMG domains were, however, unable to recognize cisplatin-modified DNA-RNA hybrids, revealing the need for a deoxyribose sugar backbone for specific complex formation. The molecular basis for the specificity of binding was investigated for the two isolated domains of HMG1 with a series of 15-bp oligonucleotides, d(CCTCTCN₁G*G*N₂TCTTC)-(GAAGAN₃CCN₄GAGAGG), where asterisks denote N7-modification of guanosine with cisplatin. Alteration of the nucleotides flanking the platinum lesion modulated HMG1domA recognition in this series by over 2 orders of magnitude and revealed an unprecedented preference for N₂ = dA > T > dC. The same preference was observed for HMG1domA and full length HMG1 recognition of a site-specifically platinated 159-bp duplex. The flanking nucleotide preference for HMG1domB interaction with the 15-bp oligonucleotide series was less pronounced and had a 20-fold range of binding affinities. Protein-DNA contacts which may account for these observed binding preferences are proposed, and potential implications for the biological processing of cisplatin-DNA adducts are discussed.

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to my steve
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ABBREVIATIONS

1,2-d(GpG)  cis-[Pt(NH₃)(NH₂R)[d(GpG)-N7(1),-N7(2)]]
1,2-d(ApG)  cis-[Pt(NH₃)₂[d(ApG)-N7(1),-N7(2)]]
1,3-d(GpTpG) cis-[Pt(NH₃)₂[d(GpTpG)-N7(1),-N7(3)]]
4AT        4-aminoTEMPO
bp         base pair
BSA        bovine serum albumin
cisplatin  cis-diaminedichloroplatinum(II)
cpm        counts per minute
COSY       correlation spectroscopy
CyNH₂      cyclohexylamine
d₄-TSP     sodium 3-trimethylsilylpropionate-2,2,3,3,-d₄
DMF        N’,N’-dimethylformamide
DNA        deoxyribonucleic acid
E. coli    Eschericia coli
EDTA       ethylenediaminetetraacetic acid
EPR        electron paramagnetic resonance
HEPES      (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid])
HMG        high mobility group
HMG1       high mobility group protein 1
HMG1domA   domain A of HMG1
HMG1domB   domain B of HMG1
HPLC       high performance liquid chromatography
hSRY       human sex-determining region Y
hUBF       human upstream binding factor
K_d        apparent dissociation constant
NMR        nuclear magnetic resonance
NOE        nuclear Overhauser effect
NOESY      nuclear Overhauser effect spectroscopy
PBS        phosphate buffered saline
rMD        restrained molecular dynamics
RMSD       root mean squared deviation
RNA        ribonucleic acid
TEMPO      2,2,6,6,-tetramethylpiperidinyloxy
TEMPOL     4-hydroxyl-2,2,6,6,-tetramethylpiperidinyloxy
TRIS       (tris[hydroxymethyl]aminomethane)
tsHMG      testis-specific HMG protein
tsHMGdomA  domain A of tsHMG
Chapter I
An Introduction
A simple inorganic compound, $\text{cis-}[\text{Pt(NH}_3\text{)}_2\text{Cl}_2]$, known as Peyrone's chloride was synthesized in 1845 and its square planar structure was first proposed by Alfred Werner (Werner, 1893). More than a half-century later, inhibition of $E. \text{coli}$ cell division by this compound was discovered serendipitously (Rosenberg et al., 1965). The compound, now known as cisplatin (Figure 1.1), has antitumor activity (Rosenberg et al., 1969) and was approved by the FDA for use against several human carcinomas. Although it has been an effective chemotherapeutic for almost two decades, the mechanism by which cisplatin kills tumor cells is not fully known (Pil & Lippard, 1997).

Several characteristics required of platinum compounds for antitumor activity have been identified (Cleare & Hoeschele, 1973). The resulting structure-activity relationships (SARs) for antitumor agents in the cisplatin family include the presence of two cis amine ligands ($\text{NHR}_2$), two cis leaving groups, and overall charge neutrality. Although subsequent investigations have identified exceptions to these general rules (Reedijk, 1996), the current FDA-approved platinum chemotherapeutic compounds adhere strictly to the original SARs. Examples of clinically effective platinum antitumor agents, as well as inactive compounds are given in Figure 1.2.

Whereas cisplatin interacts with many biological components, including RNA, DNA and proteins (Pil & Lippard, 1997), substantial data suggest that DNA is the target responsible for its antitumor activity (Bruhn et al., 1990). A variety of studies strongly support this conclusion. They reveal a correlation between cisplatin-DNA adduct formation and drug response (Reed et al., 1990, Reed et al., 1987, Reed et al., 1993, Reed et al., 1986), as well as

Cisplatin forms a spectrum of covalent monofunctional and bifunctional adducts on DNA (Bruhn et al., 1990). In vivo (Fichtinger-Schepman et al., 1987) as well as in vitro (Eastman, 1983, Eastman, 1986, Fichtinger-Schepman et al., 1985), up to 90% of these cross-links are bifunctional 1,2-intrastrand adducts at d(GpG) or d(ApG) sites. Selective platinum binding in the major groove of the DNA helix occurs at the nucleophilic N7 positions of adjacent purine bases (Figure 1.3).

The major DNA adducts of cisplatin distort the structure of the double helix. Macroscopic changes in helical structure of such cisplatin-DNA adducts have been characterized by gel mobility shift and footprinting assays. The distortions include bending by 32-34° toward the major groove (Bellon & Lippard, 1990), unwinding by ~13° (Bellon et al., 1991), and an increase in minor groove accessibility (Visse et al., 1991). The structural details which produce these helical changes have been investigated by X-ray diffraction and solution NMR methods. The crystal structures of cisplatin-modified single-stranded di- and trinucleotides (Admiraal et al., 1987, Sherman et al., 1985, Sherman et al., 1988) revealed (i) head-to-head orientation and destacking of the coordinated purine bases, (ii) anti conformations of all nucleoside residues, (iii) hydrogen-bonding between a platinum ammine ligand and a backbone phosphate of the DNA, and (iv) a C₂'- to C₃'-endo change in the conformation of the deoxyribose sugar ring in the 5' platinum-coordinated residue (Figure 1.4).
Solution NMR studies of DNA duplexes containing a cisplatin 1,2-intrastrand d(GpG) cross-link (Gelasco & Lippard, 1997, Yang & Wang, 1996) indicate platinum-induced destabilization arising, at least in part, from disruption of hydrogen-bonds and destacking of base pairs at the platinum-coordination site. As observed in the short single-stranded model compounds, the 5' coordinated guanosine residue exhibits an A-type, C₃'-endo sugar conformation in these duplexes. Moreover, evidence was reported in one study for C₃'-endo sugar puckers at other residues near the platinum coordination site (Herman et al., 1990), which may reflect DNA sequence-dependent conformational changes surrounding the platinated d(GpG) sites. NMR-based evidence has not been reported for hydrogen-bond formation between an exogenous platinum ligand and the phosphate backbone, although molecular mechanics studies have indicated that such interactions are possible in solution (Herman et al., 1990, Kozelka et al., 1987, Kozelka et al., 1985, Kozelka et al., 1986). Early NMR data predicted a kinked duplex structure (den Hartog et al., 1985). An extensive NMR/molecular mechanics study (Herman et al., 1990) and an NMR-based structure refinement (Yang et al., 1995, Figure 1.5B) generated duplex structures which are bent toward the major groove with angles ranging from 40-70°. Although the range of structures determined by these NMR methods accommodates characteristics of the macroscopic bending and unwinding of the cisplatin-DNA duplex, it is unclear whether localized NMR observables and current computational methods can accurately address overall duplex shape. Since tertiary structure is essentially a free parameter in NMR-based structure
refinement of duplex oligonucleotides (Brünger, 1992), a unique set of structural parameters may not result from such refinements.

The recent crystal structure of (CCTCTG*G*TCTCC)-(GGAGACCA-GAGG), where asterisks denote cisplatin-modification at the N7 positions, revealed an unusual A-B DNA junction (Takahara et al., 1996, Figure 1.5A). More than half of this dodecamer duplex is an A-form helix with corresponding residues adopting A-type C3'-endo sugar conformations. This duplex exhibits a wide and shallow minor groove and is bent significantly (≈40°) toward the major groove. Difficulties are encountered, however, when quantitating bend angles for short DNA duplexes and direct comparisons of reported duplex bend angles may not be appropriate (Takahara et al., 1996). In addition, the extent of the structural contributions from crystal packing forces (Luxon & Gorenstein, 1995) and the dehydrating, high ionic strength conditions required for crystallization (Hartmann & Lavery, 1996) are not known for this dodecamer. Such forces have been reported to ‘unbend’ DNA (Harvey et al., 1995) and cause discrepancies between duplex structure in the solid versus the solution state (Clark et al., 1990, Gao et al., 1995, Robinson & Wang, 1996, Xu et al., 1993).

The platinum-induced structural distortions of the DNA helix are specifically recognized by cellular proteins (reviewed in Whitehead & Lippard, 1996) which include components of DNA repair complexes and members of the HMG-domain protein family. Many DNA repair mechanisms are active in mammalian cells and proteins in both the mismatch (Mello et al., 1996) and the nucleotide excision repair (NER) pathways (Jones & Wood, 1993) bind to cisplatin-modified DNA. Competent
repair of platinum-modified DNA has been detected with a combination of the excision and recombination repair mechanisms (Whitehead & Lippard, 1996).

Nearly all proteins in the HMG-1/-2 family bind specifically to the major cisplatin 1,2-intrastrand cross-links in DNA (Whitehead & Lippard, 1996). Proteins in this family contain at least one copy of a mildly conserved region of ~80 amino acids known as the HMG domain (Grosschedl et al., 1994, Landsman & Bustin, 1993). HMG-domain proteins exhibit the common characteristics of binding to distortable, usually bendable, motifs such as four-way junction DNA with little or no DNA sequence preference (Bianchi et al., 1989, Read et al., 1995). Members of one class of HMG-domain proteins, including HMG1, HMG2 and the human upstream binding factor (hUBF), contain two or more consecutive HMG domains and exhibit structure-specific DNA interactions (Read et al., 1995). Another class, which comprises many tissue-specific transcription factors such as the lymphoid enhancer-binding factor 1 (LEF-1) and the testis-determining factor (SRY), has the additional ability to recognize a specific DNA sequence and induce sharp bends (>100°) in the duplex (Grosschedl et al., 1994, Landsman & Bustin, 1993, Read et al., 1995).

Several independent studies, each using a single HMG-domain protein and/or its isolated domain (Chow et al., 1995, Farid et al., 1996, Kane & Lippard, 1996, Locker et al., 1995, McA’Nulty et al., 1996, Ohndorf et al., 1997, Treiber et al., 1994, Trimmer, 1997), have characterized such protein interactions with cisplatin-modified DNA. The wide variety of conditions used in these experiments, however, renders quantitative comparisons
difficult. One gel mobility study, which surveyed recognition of cisplatin-modified DNA by several HMG-domain proteins (Chow et al., 1994), revealed that the bend angles induced upon protein binding to the same cisplatin-modified probe range from \(-50^\circ\) to \(90^\circ\), depending on the protein used. Since the natural functions of many of these HMG-domain proteins remain unclear (Read et al., 1995) and the sequence identity between HMG domains is relatively low (\(-30\%\)), it is not unlikely that variations in both the protein and DNA composition may affect HMG-domain protein interactions with cisplatin-modified DNA.

The details described above and other information form the basis for a current view of the antitumor mechanism of cisplatin, illustrated in Figure 1.6. Cisplatin, being a small, neutral compound, passively diffuses into cells (1). In the cytoplasm the chloride ion concentration is lower than in plasma which facilitates hydrolysis to form the activated monoaqua species (2). This cationic species migrates to and forms covalent cross-links with DNA (3), inducing structural distortions in the double helix (4). These structural distortions interfere with normal biological processing of the genetic material and trigger cellular responses, ultimately leading to cell death. HMG-domain and other proteins which specifically recognize cisplatin-modified DNA are believed to play a key role in these cellular events (5).

Evidence from several studies (Brown et al., 1993, Huang et al., 1994, McA’Nulty et al., 1996, Treiber et al., 1994, Zamble et al., 1996) supports two different but compatible mechanisms by which HMG-domain proteins can mediate cisplatin-antitumor activity. These mechanisms (Lippard, 1993) are illustrated in Figure 1.7 and are not mutually exclusive. In the top of Figure
cisplatin-DNA adducts divert HMG-domain proteins away from their natural targets, disrupting required cellular function and resulting in lethality. At the bottom of the figure, HMG-domain proteins bind to cisplatin-DNA adducts, shielding them from recognition by the cellular repair machinery. Lack of repair leads to persistence of platinum adducts on the genome which, in turn, can facilitate the blockage of DNA and RNA synthesis and trigger apoptosis.

The work in this thesis focuses on the two latter steps (4 & 5) of the proposed antitumor mechanism of cisplatin (Figure 1.6). In the next chapter we address the global structure of a platinum-modified DNA duplex in solution by using paramagnetic methods to determine long range distance restraints for NMR-based structure refinement. The principal goals of this study were to investigate (i) the ability of short interproton distance restraints to characterize the overall shape of a DNA duplex containing a 1,2-intrastrand d(GpG) adduct of a cisplatin analog, (ii) the contributions from long range distance restraints in NMR-based structure refinement of such a duplex, and (iii) by comparison of final structures, those features of the crystal structure which may be influenced by crystal packing forces. In the final chapter, HMG-domain protein recognition of short platinum-modified duplexes is investigated. This work addresses how HMG-domain protein recognition of cisplatin-modified DNA depends upon (i) the exogenous ligands of the platinum compound, (ii) the composition of the protein, (iii) DNA length and helical form, and (iv) the DNA sequence context surrounding a cisplatin 1,2-intrastrand d(GpG) adduct. We hope the results will facilitate future high resolution structure studies of HMG-domains complexed with cisplatin-
modified DNA. Ultimately, understanding the molecular basis of the platinum-induced DNA structure distortions and their recognition by cellular proteins could lead to the design of new platinum-based therapies for the treatment and cure of cancer.
References


Lippard, S. J. (1993) in Proc. Robert A. Welch Foundation 37th Conf. on Chemical Research, 40 Years of the DNA Double Helix pp 49-60, Houston, TX.


Figure 1.1. Cisplatin or cis-diamminedichloroplatinum(II).

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Chapter II
Solution NMR Studies of a Platinated DNA Duplex
Introduction

High resolution structures of cisplatin-modified duplex DNA are available, but they have their limitations in addressing solution tertiary structure. An X-ray crystal structure of a cisplatin-modified dodecamer duplex, solved to 2.6 Å resolution, revealed details of the platinum d(GpG) cross-link and a double helix with an unusual A-B junction (Takahara et al., 1996, Takahara et al., 1995). Both end-to-end (B-B) and end-to-groove (A-A) packing interactions occur in the crystal, accounting for the heterogeneous character of the duplex (Takahara et al., 1996). In solution, where such crystal packing forces do not occur, the same platinated DNA has primarily a B-type structure (Gelasco & Lippard, 1997). The solution structure of a metastable cisplatin-modifed octamer duplex has also been reported (Yang et al., 1995). Both of these NMR-derived structures were refined with short range NOE restraints, however, which accurately address local geometric features but may not adequately characterize more global features such as the details of DNA duplex bending or unwinding.

NMR spectroscopy is the method of choice for investigating the structure and dynamics of biological macromolecules in solution. The primary NMR-derived restraints used in solution structure refinements arise from the nuclear Overhauser effect (NOE). An NOE is the result of a dipolar interaction between two magnetic nuclei and exhibits a $1/r^6$ dependence, where $r$ is the internuclear distance. The magnitude of the proton magnetic moment limits the interproton NOEs to distances of $\leq 5$ Å. In globular macromolecules such as proteins, interproton distances of $\leq 5$ Å are present within a residue, between sequential adjacent residues and, most important
for characterizing the tertiary structure, between non-sequential but proximal residues. NMR-derived restraints for extended polypeptides and, in particular, for duplex oligonucleotides are highly localized with no tertiary structure to provide NOE restraints between non-sequential residues. The predominance of nearest-neighbor restraints leaves NMR structures of duplex oligonucleotides underdetermined and makes characterization of the overall shape or bend of the duplex a significant challenge (Brünger, 1992, Goljer & Bolton, 1994, Wüthrich, 1995).

In the present investigation we have addressed this problem by introducing a localized paramagnetic moiety into a platinum-modified DNA duplex. The platinum compound employed, cis-[Pt(NH₃)(4AT)Cl] (Dunham & Lippard, 1995), contains an unpaired electron on the organic nitroxide moiety of the 4AT (4-aminoTEMPO) ligand. The platinum compound is a structural analog of cis-[Pt(NH₃)(CyNH₂)Cl₂], the active metabolite of oral platinum(IV) antitumor agents (Barnard et al., 1996, Hartwig & Lippard, 1992, Kelland et al., 1992). The unpaired electron of a nitroxide spin label, which can couple with magnetic nuclei by a dipolar mechanism, has previously been used to determine long range (10 - 30 Å) distances in proteins (Girvin & Fillingame, 1994, Kosen, 1989, Kuntz & Schmidt, 1984, Yu et al., 1994). Since the magnetic moment of the electron is more than 500 times that of a proton, dipolar coupling of the electron spin with a proton nucleus is effective over distances much greater than the 5 Å limit of NOEs. Previously we used the paramagnetic properties of the 4AT ligand to provide structural information about cis-[Pt(NH₃)(4AT){d(GpG)}]+ (Dunham & Lippard, 1995). Here we extend its application to quantitate long range electron-proton distance
restraints and assess their contribution to NMR-based structure refinements of a related platinum-modified DNA undecamer duplex (Figure 2.1).

Materials and Methods

Synthesis, Platination and Purification of Oligonucleotides. The deoxyribonucleotides d(CTCTCGGTCTC), tsll, and d(GAGACCGAGAG), bsll, were synthesized in micromole quantities on a Cruachem PS250 DNA synthesizer by using phosphoramidite chemistry on a solid support. Deprotected oligonucleotides were initially purified by size exclusion chromatography (G25 Sephadex, Pharmacia). The unmodified oligonucleotide bsll was purified either by preparative ion exchange HPLC (DIONEX, NucleoPac PA-100 9 x 250 mm column with a linear NaCl gradient in 0.025 M NH₄OAc, pH 6.0, 10% acetonitrile) or C₄ reversed phase HPLC (VYDAC, 22 x 250 mm column, with a nonlinear acetonitrile gradient in 0.1 M NH₄OAc, pH 6.0). Following reversed phase HPLC purification and prior to platinum modification, oligonucleotides were converted to their sodium forms by using cation exchange chromatography (DOWEX, Aldrich).

The compound cis-[Pt(NH₃)(4AT)ClII] was synthesized from the potassium or tetraphenylphosphonium salt of [Pt(NH₃)Cl₃]⁻ (Giandomenico et al., 1995) and 4-aminoTEMPO (4AT) (Aldrich) as previously described (Dunham & Lippard, 1995). Oligonucleotides containing a single d(GpG) site for platinum modification were allowed to react with 1.0 to 1.5 equiv of the paramagnetic, doubly activated platinum species, cis-[Pt(NH₃)(4AT)X₂]ⁿ⁺ (₁), where X is DMF (n=2) or NO₃⁻ (n=0) (Dunham & Lippard, 1995). Reactions were carried out in 10 mM sodium phosphate buffer, pH 6.0, at DNA strand
concentrations of $\geq 10 \, \mu M$ for 2-20 h at 22 °C. Platination of \textit{tssI} was monitored by reversed phase HPLC (C$_4$, VYDAC, 4.6 x 250 mm column with linear gradients of acetonitrile in 0.1 M NH$_4$OAc, pH 6.0). Two major products eluted sequentially from the column, \textit{ts11A} and \textit{ts11B}, respectively, and were separated and isolated by preparative HPLC (C$_4$, VYDAC, 22 x 250 mm column) with optimized gradient conditions.

Concentrations of \textit{ts11} and \textit{bs11} were determined from optical absorbance readings at 260 nm using estimated extinction coefficients of 90,000 M$^{-1}$cm$^{-1}$ and 118,700 M$^{-1}$cm$^{-1}$, respectively (Borer, 1975). The ratio of bound platinum per oligonucleotide was determined by optical spectroscopy and atomic absorption spectroscopy, the latter on a Varian 1475 graphite furnace spectrometer, operating in peak height mode at 265.9 nm.

Annealed oligonucleotide duplexes were prepared by titrating \textit{bs11} into a buffered (100 mM NaCl, 10 mM sodium phosphate, pH 6.8) aqueous solution of \textit{ts11}, \textit{tss1A} or \textit{tss1B} followed by moderate heating (37 - 45 °C for 5 to 15 min) and cooling over 5-10 h to room temperature or 4 °C. Duplex formation was monitored by ion exchange HPLC (DIONEX NucleoPac PA-100 4 x 250 mm column, with a linear NaCl gradient in 0.025 M NH$_4$OAc, pH 6.0, 10% acetonitrile). Paramagnetic duplexes (\textit{ds11A$_{para}$} and \textit{ds11B$_{para}$}) were formed by annealing the isolated paramagnetic strands (\textit{ts11A$_{para}$} or \textit{ts11B$_{para}$}) with 1.0 equivalent of \textit{bs11}. Diamagnetic oligonucleotides (\textit{ts11A$_{dia}$}, \textit{ts11B$_{dia}$}, \textit{ds11A$_{dia}$}, \textit{ds11B$_{dia}$}) were prepared by ascorbic acid reduction (Figure 2.1), which converts the 4AT ligand from its paramagnetic nitroxide to the diamagnetic hydroxyamine form (4ATH) (Dunham & Lippard, 1995, Kosen, 1989). Following reduction, ascorbic acid was removed
either by size exclusion or reversed phase chromatography. Anaerobic conditions were required to maintain the modified DNAs in their reduced forms in buffered aqueous solution at room temperature.

**Enzymatic Digestions.** Extreme caution should be used when working with cyanide ion which is a particularly hazardous substance.**

Aliquots (1-5 nmol) of platinated single-stranded oligonucleotides were allowed to react with 25 units of P1 nuclease (GIBCO BRL) for 30 h at 37 °C in 100 mM NaOAc, pH 5.3. One-quarter of this solution (30 μL) was diluted 10-fold in 100 μM EDTA, 50 mM Tris-HCl pH 8.5, and digested further with 4 units of alkaline phosphatase (Boehringer Mannheim) for 24 h at 37 °C. After enzymatic digestion, 1000 to 2000 equiv of sodium cyanide were allowed to react with an aliquot of the digested DNA solutions at 37 °C for several hours. All digested, cyanide reversed, and standard samples were analyzed by reversed phase HPLC under identical conditions (C18 4.6 x 250 mm column, VYDAC, with a linear acetonitrile gradient in 0.1 M NH4OAc, pH 6.0).

**EPR and UV-Vis Spectroscopy.** EPR spectra were recorded at 25 °C on an X-band Bruker ESP-300 spectrometer with the following parameters: 64 - 256 scans, 100 G sweep width centered at 3472 G, 1.0 G modulation amplitude, 100 KHz modulation frequency, 1.28 ms time constant and 2 mW microwave power. Samples were dissolved in 80 μL of water, transferred to a 100 μL glass capillary in a 5 mm quartz tube for measurements, and externally referenced to a 1 mM aqueous sample of TEMPOL (Aldrich), g = 2.006.

Melting and cooling curves for the DNA duplexes (0.1 to 1.0 OD of DNA in 100 mM NaCl, 10 mM sodium phosphate buffer pH 6.8) were obtained by optical absorption spectroscopy at 260 nm on an AVIV Model
14DS UV-vis spectrophotometer. Data were collected in 1 or 2° intervals between 5.5 and 55.5 °C with 2 min equilibration time and 1 s averaging at each step.

**NMR Spectroscopy.** Duplex oligonucleotides (0.5 to 3.0 μmol) were dissolved in 600 μL of 100 mM NaCl, 10 mM sodium phosphate, pH 6.8, with 0.1 mM d₄-TSP (Aldrich) as an internal chemical shift standard. After repeated lyophilization from 99.996% D₂O (Cambridge Isotopes Laboratories), samples were reconstituted in a 600 μL volume. For experiments involving exchangeable protons, samples were lyophilized and redissolved in 90% H₂O/10% D₂O.

1D proton NMR spectra of duplexes in D₂O buffer were acquired at 500 MHz on a Varian VXR spectrometer. Paramagnetic samples were studied at duplex concentrations below 1 mM in order to minimize intermolecular relaxation effects (Dunham & Lippard, 1995). Longitudinal (T₁) and transverse (T₂) relaxation times were measured at 23 °C with the inversion recovery (Vold et al., 1968) and CPMG (Meiboom & Gill, 1958) pulse sequences, respectively. The intensity for each resolved proton signal as a function of delay time was fit to an exponential expression (VNMR version 5.1) to determine relaxation time values.

2D magnitude COSY spectra were also acquired at 500 MHz with 1024 complex points in t₂, 256 points in t₁, and presaturation of the residual water signal. All NOESY data were acquired on a 591 MHz home-built NMR spectrometer (Francis Bitter Magnet Laboratory). A series of phase sensitive NOESY spectra in D₂O buffer were acquired at 23 °C with 4096 complex points in t₂, 512 points in t₁, and 6000 Hz sweep width in each dimension. Data were
collected with five mixing times (80, 100, 150, 200, and 400 ms) for a 3-4 mM sample of $\text{ds11B}_{\text{dia}}$ and with two mixing times (200 and 400 ms) for a $\leq 1$ mM sample of $\text{ds11B}_{\text{para}}$. NOESY spectra in H$_2$O buffer were recorded at 10 °C on a 591 MHz spectrometer with 50, 100 and 300 ms mixing times, WATERGATE (Sklenar et al., 1993) minimization of the water signal, 4096 complex points in $t_2$, 512 points in $t_1$, and 12000 Hz sweep width in each dimension. Data were transferred to a Silicon Graphics workstation and processed in Felix (version 95.0, Biosym Technologies). NOESY data were processed with 5 Hz exponential line broadening in both dimensions and data in the $t_1$ domain were zero-filled to 4096 points. For all spectra, the first data point was divided by two so as to reduce $t_1$ noise, and a polynomial base line correction was applied in the $t_2$ domain.

**Diamagnetic Restraints.** For initial structure refinements, the deoxyribose sugars in two residues with strong cross-peak intensities in the H8/H6 to H3' region of the 80 ms D$_2$O NOESY spectrum were restrained with standard C$_3$'-endo dihedral angles. Seventeen residues with very little intensity in this region were restrained with standard C$_2$'-endo dihedral angles, and no dihedral angle restraints were applied to three residues with moderate intensity in this region.

NOESY cross-peak volumes were quantitated in Felix (version 95.0, Biosym Technologies). For each isolated and assigned cross-peak, the volume in a standard area was determined. For initial interproton distance restraints, the cross-peak volume in the 200 ms NOESY spectrum of $\text{ds11B}_{\text{red}}$ was converted to a proton-proton distance by using the isolated spin pair approximation (Clore & Gronenborn, 1985). In this approximation, the
thymidine methyl-to-H6 interaction (2.88 Å), the TEMPO geminal methyl-to-
methyl interaction (3.05 Å), and the cytidine H5-to-H6 interaction (2.49 Å) were used to calibrate the methyl-to-proton, methyl-to-methyl, and all other interproton volumes, respectively.

Restrains corresponding to standard hydrogen-bonding distances (Goljer & Bolton, 1994) were applied to those base pairs for which an imino proton was observable, assignable, and exhibited NOE interactions with either the H2 of adenine or the amino protons of cytidine. In those base pairs for which the imino proton was not assignable owing to lack of NOESY cross-peaks, (C1-G22, G6-C17 and C11-G12, Figure 2.1), hydrogen bonding was more loosely restrained (1.8 - 2.4 Å). NOESY cross-peaks between an exchangeable proton in one base and a proton in another base pair, when observed, were converted to distance restraints with a 3.0 - 5.0 Å range. Weak cross-peaks between aromatic protons in consecutive bases in a strand, nH8/H6 to (n+1)H8/H6, excluding the very strong G6 H8 to G7 H8 interaction, if observable in the 300 ms NOESY spectrum in H2O buffer, were also given 3.0 - 5.0 Å distance bounds.

**Paramagnetic Distance Restraints.** In this system an unpaired electron is localized on the nitrogen atom of the nitroxide moiety. The distance from the unpaired electron to a specific proton nucleus can be determined from the paramagnetic contribution to the proton nuclear relaxation rate. The paramagnetic contribution \((T_p^{-1})\) to either the longitudinal \((T_1^{-1})\) or transverse \((T_2^{-1})\) relaxation rate of a magnetic nucleus is proportional to \(1/r^6\), where \(r\) is the electron-proton distance.
The relaxation rate for a proton in \( \text{ds11B}_{\text{para}} \) (\( T_{\text{obs}}^{-1} \)) is the sum of both diamagnetic (\( T_{\text{dia}}^{-1} \)) and paramagnetic (\( T_{\text{para}}^{-1} \)) contributions (eq 1). Since

\[
\frac{1}{T_{\text{obs}}} = \frac{1}{T_{\text{dia}}} + \frac{1}{T_{\text{para}}} \quad (1)
\]

\( T_{\text{dia}}^{-1} \) can be directly determined as the relaxation rate of the proton in \( \text{ds11B}_{\text{dia}} \). \( T_{\text{para}}^{-1} \) can be calculated as the change in relaxation rate in the absence of intermolecular relaxation by using eq 1. For proton nuclei at high magnetic field strengths, where the Larmour frequency of the electron, \( \omega_s \), is much larger than the Larmour frequency of the nucleus, \( \omega_L \), the Solomon-Bloembergen equations (Solomon & Bloembergen, 1956), which relate the paramagnetic contribution of nuclear relaxation rate to electron-proton distance, can be simplified as indicated in eqs 2 and 3 (Kosen, 1989). The correlation time of each electron-proton vector, \( \tau_{\text{c1}} \), is determined from the

\[
\frac{1}{T_{1\text{para}}} = 2.46 \times 10^{-32} \text{cm}^{6} \text{s}^{-2} \left( \frac{3 \tau_{\text{c1}}}{1 + \omega_s^2 \tau_{\text{c1}}^2} \right) \quad (2)
\]

\[
\frac{1}{T_{2\text{para}}} = 1.23 \times 10^{-32} \text{cm}^{6} \text{s}^{-2} \left( \frac{4 \tau_e + \frac{3 \tau_{\text{c1}}}{1 + \omega_s^2 \tau_{\text{c1}}^2}}{1 + \omega_s^2 \tau_{\text{c1}}^2} \right) \quad (3)
\]

ratio \( (T_{1\text{para}}^{-1}/T_{2\text{para}}^{-1}) \) for each resolved proton in a 1D spectrum (eq 4). Correlation times (\( \tau_{\text{c1}} \)) and paramagnetic relaxation rates (\( T_{1\text{para}}^{-1} \) and \( T_{2\text{para}}^{-1} \)) were then used in eqs 2 and 3 to determine electron-proton distances for

\[
\frac{T_{1\text{para}}^{-1}}{T_{2\text{para}}^{-1}} = 2 \left( \frac{3 \tau_{\text{c1}}}{1 + \omega_s^2 \tau_{\text{c1}}^2} \right) \left( \frac{4 \tau_e + \frac{3 \tau_{\text{c1}}}{1 + \omega_s^2 \tau_{\text{c1}}^2}}{1 + \omega_s^2 \tau_{\text{c1}}^2} \right) \quad (4)
\]
protons resolved in the 1D $^1$H NMR spectrum. These distances were employed in the scaling of the paramagnetic distances determined from 2D spectra, as described in the next section.

Additional long range electron-proton distance restraints were estimated from the paramagnetic effect on NOESY cross-peak intensities. Each cross-peak in a NOESY spectrum, which results from the dipolar transfer of magnetization between two magnetically inequivalent nuclei, $i$ and $j$, is affected by the relaxation rates ($T_i^{-1}$ and $T_j^{-1}$) of both nuclei. Although the longitudinal ($T_1$) relaxation times of both $i$ and $j$ are active in the buildup and decay of a NOESY cross-peak, (Bertini & Luchinat, 1996a, La Mar & de Ropp, 1993) the paramagnetic contribution to longitudinal relaxation rate ($T_{1\text{para}}^{-1}$), particularly for moderate to long electron-proton distances (> 10Å), approaches zero at high field strengths (eq 2). The paramagnetic contribution to $T_{2\text{para}}^{-1}$, however, approaches a constant value governed by $4\tau_c$ under these conditions (eq 3). Therefore, for electron-proton distances $\geq 10$ Å at 600 MHz, the paramagnetic contribution to transverse relaxation rate ($T_{2\text{para}}^{-1}$), operative during the frequency selection and acquisition time domains, will dominate proton relaxation in the NOESY spectrum (Figure 2.2).

As previously described (Kosen, 1989), the fractional change in intensity of an NMR signal, assuming $T_2$ relaxation is dominant, also represents the fractional change in $T_2$ (eq 5). Therefore, for a known $T_{2\text{dia}}$ and a given $\tau_{c1}$ value, the change in NOESY cross-peak intensity can be used

$$\frac{I_{\text{dia}} - I_{\text{obs}}}{I_{\text{dia}}} = \frac{T_{2\text{dia}} - T_{2\text{obs}}}{T_{2\text{dia}}}$$

(5)
in eqs 1, 3, and 5 to determine the electron-proton distance. The resulting relationship between change in NOESY cross-peak intensity and electron-proton distance is plotted in Figure 2.3 for a proton with a $T_{2\text{dia}}$ of 0.15 s and $\tau_c$ of 4.5 ns. In the present study, volumes in a standard sized area (2 Hz x 2 Hz) were quantitated for all resolved cross-peaks in selected regions of the 200 ms NOESY spectra of $\text{ds11B}_{\text{dia}}$ and $\text{ds11B}_{\text{para}}$. The paramagnetic cross-peak intensity was scaled until the fractional change in intensity (eq 5) for cross-peaks containing reference protons (A19 H8, A21 H8, G22 H8, T2 CH$_3$ and C16 H6) agreed with the electron-proton distances as determined from 1D experiments. Cross-peaks arising from interactions with A21 H2 were not used in this scaling owing to the weakness of their intensities in the NOESY spectra of $\text{ds11B}_{\text{dia}}$. For structure refinements, the resulting electron-proton restraints were applied to fix the distance from a given proton to the nitroxide nitrogen atom of the 4AT ligand. Typically, 3-5 NOESY volumes were averaged for each assigned proton. Protons having cross-peaks exhibiting $>95\%$ loss in volume (strong class in Figure 2.3, 40 restraints) were assigned an electron-proton distance of $\leq 13\,\text{Å}$. All distances in the medium to weak class (Figure 2.3, 59 restraints) were assigned conservative bounds of $+5\,\text{Å}$ and $-3\,\text{Å}$ from the calculated distance. These asymmetric bounds are to account for the potential underestimation of electron-proton distances due to the interproton distances which give rise to each NOESY cross-peak (Kosen, 1989).

**Structure Refinement.** Restrained molecular dynamics (rMD) simulations were performed in XPLOR (Brünger, 1992) with the paralldg.dna force field modified with parameters for the $\{\text{Pt(NH}_3\}(4\text{AT})\}^{2+}$ moiety and the coordinated guanosine residues as previously described.
(Dunham & Lippard, 1995). The scaling of bonds, angles, dihedral angles and improper angles in this force field was also applied to the added platinum parameters to reflect the requirements of structure refinement with interproton distance restraints (Brünger, 1992). Improper angles that maintain the coplanarity of the individual platinated guanine bases with the Pt-N7 bond, however, were removed (Takahara et al., 1996). Simulations were carried out in vacuum with a distance-dependent dielectric, reduced phosphate charges, and an 11.5 Å cut off for nonbonded interactions.

NMR-derived structure restraints were applied to ds11B in two starting structures, canonical A-form or B-form DNA. Starting structures were generated in QUANTA (version 4.1, Molecular Simulations Inc.), where the energy-minimized \([\text{cis-Pt(NH}_3\text{)(4AT)}]^{2+}\) moiety was docked at a position ~2 Å from the N7 positions of G6 and G7. Conjugate gradient minimization (2000 cycles) was performed on each of these structures prior to 20 ps of restrained molecular dynamics (rMD) in 0.5 fs steps at 300 K. All distance restraints were applied with a square-well potential energy function. Coordinates of the final 2 ps of the dynamics trajectory were averaged, subjected to a maximum of 2000 cycles of conjugate gradient minimization, and submitted for another such rMD cycle. After 3 to 5 rMD cycles, structures resulting from A-form and B-form duplexes were converged (RMSD < 1.2 Å for all non-hydrogen atoms). Converged structures were then subjected to molecular dynamics restrained by the full relaxation matrix approach (Nilges et al., 1991) from 400 K to 100 K in 0.5 fs timesteps and 10 °C temperature steps over 5 ps with an optimal correlation time of 7 ns as determined from a grid search. The weight of the
relaxation matrix and hydrogen-bonding restraints were held constant throughout the refinement.

**Analysis of Refined Structures.** All structures were viewed and compared in QUANTA (version 4.1, Molecular Simulations Inc.). Helical parameters of final structures were determined in CURVES, version 5.1 (Lavery & Sklenar, 1988, Lavery & Sklenar, 1989). Final duplex structures were used to back-calculate regions of the 200 ms D$_2$O NOESY spectrum in the NMR module of QUANTA with a 7 ns correlation time, a leakage rate of 0.9 s$^{-1}$ and a recycle delay of 3.5 s. Back-calculated spectra were then visualized and compared in NMRCOMPASS (version 2.5.1, Molecular Simulations Inc.).

**Results**

**Synthesis, Separation and Identification of Orientational Isomers.** As described previously (Dunham & Lippard, 1995, Hartwig & Lippard, 1992), two orientational isomers result when a mixed amine platinum compound forms a bifunctional d(GpG) adduct on DNA. In the 5' isomer, the substituted amine is proximal to the 5' guanosine, whereas in the 3' isomer (Figure 2.1), the substituted amine is proximal to the 3' guanosine. Modification of d(CTCTCGGTCTC) with 1 yields two products, tsIIA and tsIIB, in a 1:2 ratio which can be separated by HPLC (Figure 2.4). Both of these products contain 1 equivalent of platinum per DNA strand, as determined by atomic absorption spectroscopy, and exhibit three-line EPR signals ($g = 2.006$, $A = 17$ G, Figure 2.5) arising from the nitroxide spin label (Berliner, 1976).

In order to confirm the bifunctional nature of the platinum adducts (Bellon & Lippard, 1990, Eastman, 1986) and to identify the orientational
isomers (Hartwig & Lippard, 1992), both $ts11A_{dia}$ and $ts11B_{dia}$ were subjected to enzymatic digestion analysis according to a published protocol (Eastman, 1986). Digestion of both $ts11A_{dia}$ and $ts11B_{dia}$ resulted in dC and dT monomer components, as well as a single peak eluting later with a different retention time depending on the starting oligonucleotide (Figure 2.6). Following cyanide reversal of these digested samples, coinjections with an authentic standard confirmed that the most slowly eluting peak in the HPLC trace corresponded to d(GGT). Identification of $ts11A_{dia}$ as the 5' isomer and $ts11B_{dia}$ as the 3' isomer was possible by comparison with the HPLC traces of authentic standards prepared from d(GGT) and 1. These assignments were further supported by the observed NOESY connectivities between the 4ATH ligand and base residues 3' to the lesion in $ds11B_{dia}$, shown schematically in Figure 2.1.

**Thermal Stability of Modified Duplexes.** Derivative plots of the melting and cooling curves for $ts11B$ plus 1 equivalent of $bs1l$ revealed a $T_m$ of $\sim30 ^\circ C$ (Figure 2.7). The $\sim29 ^\circ C$ $T_m$ value of $ts11A$ plus 1 equivalent of $bs1l$ was virtually identical. Corresponding studies with the unmodified duplex yielded a $T_m$ $\sim54 ^\circ C$. These results indicate that, although the bifunctional platinum lesion destabilizes the DNA duplex as previously described (Poklar et al., 1996), the orientation of the 4AT ligand does not affect the overall duplex stability.

**NMR Studies.** Nonexchangeable Protons. The nonexchangeable protons in $ds11B_{dia}$ were assigned from COSY and NOESY spectral data by following standard procedures (Wijmenga et al., 1993). Assignments are given in Table 1. Aromatic H8/H6 to sugar H1' connectivities could be traced
without interruption through the unmodified strand d(GAGACCGAGAG) (Figure 2.8A). The corresponding NOESY connectivity along the platinated strand (Figure 2.8B) was more challenging, because of overlap in the aromatic proton region at T2/C3 and T4/C5 base steps and a very weak G6 H1’ to G7 H8 cross-peak. All aromatic-to-aromatic proton connectivities were of very weak intensity except for a strong NOESY cross-peak between the H8 protons of the adjacent platinum-modified guanosines.

The four adenosine H2 protons were readily identified by their long relaxation times in the 1D inversion recovery experiment. Assignments for these resonance were possible from intra-residue H2 to H1’ connectivities observed in the 200 ms D2O NOESY spectrum (Figure 2.8C). In addition, several other weak to moderate H2 cross-peaks were observed, including intrastrand \( nH2 \) to \((n+1)H1’\) cross-peaks and interstrand \( nH2 \) to \((m+1)H1’\) cross-peaks (Figure 2.8C).

Stereochemical assignments of the sugar H2’ and H2” protons were determined from NOESY cross-peak intensity in the H1’ to H2’/H2” region of the 80 ms D2O NOESY spectrum. In this region, when mixing times are short and spin diffusion is not dominant, the H1’ cross-peak with H2” is larger than that with H2’ for almost all pseuderotation angles of the deoxyribose ring (Wijmenga et al., 1993).

The \( n(H8/H6) \) to \( n(H3’)\) cross-peak intensity in the 80 ms NOESY spectrum was used as an indicator of deoxyribose sugar pucker (Wijmenga et al., 1993). When the deoxyribose ring adopts predominantly an N-type conformation, the H8/H6 to H3’ distance is ~2.8 to 3.0 Å, whereas a longer distance (~4 to 4.4 Å) results from an S-type sugar pucker. In \( ds11B_{\text{dia}} \), the
most intense cross-peaks in this aromatic to H3′ region are for residues G6 and C11 (Figure 2.9). Moderate intensities were observed for the T2, T4, and T10 residues, but little or no cross-peak intensity was observed in this region for the remaining residues of ds11Bdia.

All nonexchangeable protons of the reduced 4-aminoTEMPO ligand (4ATH) of ds11Bdia could be assigned on the basis of both NOESY and COSY connectivities (Table 1, Figure 2.10). Stereospecific assignments were possible for all 4ATH protons in ds11Bdia since each magnetically inequivalent proton nucleus has a unique chemical shift. This situation contrasts with that for 3′ or 5′ cis-[Pt(NH3)(4ATH){d(GpG)}]+, the dinucleotide model compounds, where chemical shifts for chemically equivalent, but magnetically inequivalent, nuclei were degenerate even at high fields (Dunham & Lippard, 1995). Multiple intraligand connectivities were observed in the NOESY spectrum (Figure 2.10), including strong Ha-CH3d′ and Ha-CH3d cross-peaks as well as weak Ha-CH3e and Ha-CH3e′ cross-peaks indicating a chair conformation of the six-membered TEMPO ring. In addition, strong 1D NOEs were observed between G7 H8 and the CH3d and CH3d′ of the 4AT ligand. These NOEs, in addition to other TEMPO-DNA connectivities (Figure 2.1), were also detected in the 2D NOESY experiment. The stereospecific chemical shifts of the 4ATH protons in ds11Bdia, combined with distinct intra- and inter-residue NOESY connectivities for this ligand, allow one to fix the orientation of 4ATH with respect to the DNA duplex and indicate that the motion of this ligand is minimal on the NMR time scale. Analysis of the relative intensity of the EPR spectral components (M = 0, ± 1) and the linewidth of the M = 0 component in the 25 °C spectrum of 3′ds11para (Figure
2.5) indicated the rotational correlation time of the spin label to be ~ 4.4 ns, in the range (4.2-6.3 ns) expected for an 11 bp duplex (Berliner, 1976, Spaltenstein et al., 1989).

**Exchangeable Protons.** Assignments of the imino (G H1 and T H3) and amino (C NHb and C NHnb) protons in ds11Bdia (Table 1) were based upon observed NOESY connectivities with previously assigned non-exchangeable protons (A H2 and C H5). Although eleven imino protons were recorded in the hydrogen-bonded region of the 1D 1H NMR spectrum (Figure 2.11), only 8 of these exchangeable protons exhibited cross-peaks in the H2O NOESY spectra. The three imino protons for which cross-peaks cannot be observed include those in the two terminal base pairs (G22 H1 and G12 H1) and an internal imino proton near the platinum coordination site (G6 H1). The hydrogen-bonded (NHb) and non-bonded (NHnb) amino protons of the cytosine residues involved in these base pairs (C1, C11 and C17) could be assigned, however. In the 300 ms H2O NOESY spectrum, imino-to-imino connectivities are obtained for base pairs T2-A21 to C5-G18, and again for G7-C16 to T10-A13 (Figure 2.12). Only the lack of the G6 imino resonance interrupts the connection between these two segments.

The strongest peaks in the imino-to-aromatic region of the 300 ms H2O NOESY spectrum are thymidine H3 to adenine H2 and guanosine H1 to cytidine NHb cross-peaks (Figure 2.13). Several other moderate to weak cross-peaks are evident in this region at longer mixing times, the most interesting of which include a weak G18 H1 to C17 NHnb cross-peak and a cross-peak of moderate intensity between G7 H1 and an exchangeable proton at 8.68 ppm (Figure 2.13). Since no other cross-peaks are observed to the resonance at 8.68
ppm, the proton assignment has not been determined. The unidentified resonance is most likely a hydrogen-bonded amino proton of a guanosine or adenosine residue, the orientation of which with reference to G7 and/or the water exchange rate of which has drastically changed due to platinum-induced distortions of the duplex. Observed interproton connectivities involving exchangeable protons in ds11Bdia are schematically depicted in Figure 2.12.

**Structural Implications of Diamagnetic NMR Data.** Although the platinum-modified undecamer forms a stable duplex, the NMR data indicate that the duplex is distorted from canonical B-form DNA. The strong NOE between G6 H8 and G7 H8 is diagnostic of the destacking of the two purine bases due to platinum coordination at the N7 positions (den Hartog et al., 1985). The sugars of two residues (G6 and C11) are predominantly in the N-type (C3-endo) conformation. This conformation, which predominates in A-form duplexes, is not uncommon at 3' terminal residues and was one of the first characteristics to be identified for the 5' purine nucleoside in bifunctional platinated 1,2-intrastrand cross-links (den Hartog et al., 1982, den Hartog et al., 1985, Sherman et al., 1988). Although all expected intra- and inter-residue H6/H8 to H1' cross-peaks were observed for ds11Bdia, some intensities were much weaker than would be expected for a canonical B-DNA duplex, for example, G6 H1' to G7 H8.

The schematic in Figure 2.12 clearly illustrates the lack of exchangeable proton connectivities at the center of the platinum-modified duplex. The absence of cross-peaks to the imino proton of G6, even at 10 °C, indicates that the G6-C17 bp is very accessible to water. Although the G7-C16 and T8-A15
base pairs exhibit the expected intra- and inter-residue exchangeable proton connectivities, many of these cross-peaks are relatively weak, suggesting only moderate stability. In addition, the medium intensities of the $nA H2$ to $(m+1)H1'$ interstrand cross-peaks suggest distortion of the DNA duplex. These cross-peaks have previously been identified as potential indicators of minor groove width deviations and base pair propellar twisting present in bent DNA structures (Goljer & Bolton, 1994).

*The Paramagnetic Effect on NMR Spectra of ds11B.* Because there is minimal anisotropy in the electronic g-factor of the unpaired electron in the nitrooxide spin label (Berliner, 1976), significant proton chemical shift changes between the diamagnetic and paramagnetic forms of ds11B do not occur. As a consequence, simply overlaying the assigned diamagnetic spectrum and the corresponding region of the paramagnetic spectrum allows for proton chemical shifts in ds11B$_{para}$ to be assigned. Because of the relatively slow relaxation of the nitrooxide radical (Berliner, 1976, Bertini & Luchinat, 1996b), effective dipolar coupling with nuclear transitions occurs, resulting in a distance-dependent broadening of proton NMR signals. An example is shown in Figure 2.14 for the aromatic region of the 1D $^1$H NMR spectra of ds11B$_{para}$ and ds11B$_{dia}$. In the paramagnetic duplex, signals from aromatic resonances close to the 4AT ligand relax quickly and cannot be detected, whereas signals from resonances farthest from the unpaired electron are still observable. Quantitation of this distance-dependent relaxation was carried out and the resulting $T_{1para}$ and $T_{2para}$ relaxation times for those protons which are well resolved in the 1D spectrum of ds11B$_{dia}$ and still observed in ds11B$_{para}$ are reported in Table 2. Also given in this table are $\tau_c$ values.
calculated from $T_{1para}$ and $T_{2para}$ by using eq 4, and electron-proton distances calculated according to eqs 2 and 3.

In Figure 2.15, the H1' to H2'/H2'' regions of the 200 ms NOESY spectra of ds11B$_{dia}$ and ds11B$_{para}$ are compared. Qualitatively, one can see that the presence of the unpaired electron simplifies the spectral region. Cross-peaks originating for proton pairs proximal to the unpaired electron are no longer observed. For cross-peaks in this and two other spectral regions containing strongly coupled proton pairs (H8/H6 to H2'/H2''/CH$_3$ and H5 to H6), the change in NOESY intensity due to the unpaired electron was quantitated and converted to an electron-proton distance as described in the Materials and Methods Section. These electron-proton distances are depicted, superimposed on canonical B-form DNA, in Figure 2.16A; restraints (upper bound violations of 3-6 Å) shown in Figure 2.16B cannot be accommodated by this classical structure.

**Structural Implications from the Paramagnetic Data.** As just illustrated (Figure 2.16B), the long range electron-proton distance restraints are not compatible with a canonical B-form DNA structure. The upper bound violations indicate that the 5' end of the duplex must be closer to the platinum lesion in the major groove in order to satisfy the restraints. These paramagnetic NMR data for ds11B thus provide direct experimental evidence that the platinum-modified DNA is significantly bent toward the major groove.

**Structure Refinement with NMR-Derived Restraints.** Structures were generated in two steps. In an initial rMD step (Gronenborn & Clore, 1989) (60 to 80 ps), hydrogen-bonding, dihedral angle, and interproton distance
restraints were used to refine A- and B-DNA starting structures to convergence. A total of 311 short range distance restraints were used in initial structure refinements of \textbf{ds11B}_{\text{dia}}. Of these restraints, 87 were used to define sugar ring conformations (37 H1' to H2'/H2''; 26 H1' to H3'/H4'; 6 H3' to H4'; 18 H3' to H2'/H2''), 108 restricted the $\chi$ angles (38 H8/H6 to H1'; 45 H8/H6 to H2'/H2''; 24 H8/H6 to H3'; 1 H5 to H2'/H2''), 28 addressed base stacking interactions (13 nH8/H6 to (n+1)N8/H6; 1 nH8 to (n+1)H5; 2 nH2 to (n+1)H1; 3 nH1 to (n+1)H2; 6 nCH3 to (n+1)NHb/NHnb; 1 nH1 to (n+1)H3; 1 nNHnb to (n+1)H1), 12 related the two independent strands (3 H2 to H1'; 5 H3 to H1; 4 H1 to NHb), 7 oriented the 4AT ligand with respect to the duplex, and 31 represented various intra- and inter-residue methyl-to-proton interactions.

In the final step, structures from this initial rMD were subjected to molecular dynamics refinement restrained with the full relaxation matrix so as to correct for inaccuracies in interproton distances determined by the two-spin model. For this relaxation matrix refinement of the DNA structure, \(\sim1300\) cross-peak volumes of the above assigned peaks from the five NOESY spectra of \textbf{ds11B}_{\text{dia}} were tabulated, in order of mixing time, for use as interproton restraints in the iterative RELAX function of XPLOR (Brünger, 1992). Two sets of structures were generated. One set was restrained with conventional interproton restraints (denoted "dia"), and the second set with the addition of the long range electron-proton distance restraints (denoted "dia+para"). The RMSDs and R-factors of the final refined structures are listed in Table 3.

In Figure 2.17, final structures from 4 representative refinements are overlaid with blue and red indicating dia and dia+para restrained structures,
respectively. Both sets of final structures deviate significantly from A- and B-
DNA starting structures, although a B-DNA duplex fits the NOESY intensities
much better than an A-DNA duplex (Table 3). Significant bending toward the
major groove is observed in these structures (Figure 2.17), as is significant
widening of the minor groove near the platinum-modification site (Figure
2.18A). The largest differences in the dia and dia+para restrained structures
occur at the ends of the duplex. The 5' end of the duplex, in particular, is bent
more toward the major groove in all dia+para structures (Figure 2.17). This
difference in the location of the DNA ends is reflected in the RMSD of the dia
and dia+para structures, which is 1.5 - 2.0 Å for the heavy atoms of all 11 base
pairs, but only 0.8 - 0.9 Å when just the central 6 base pairs are compared.

The large majority of the backbone torsion angles (Tables 4 and 5) fall
within the range observed for A- and B-DNA structures (Baleja et al., 1990),
with only a few α and γ values, predominantly in the central four base pairs,
lying outside of these limits. Overall, the helical parameters of the dia and
dia+para restrained structures are very similar. The largest differences occur
in the base pair parameters of the T2-A21, C3-G20 and T4-A19 base pairs.
Comparisons of selected base pair and base pair step parameters are available
in Figure 2.19. The distortions caused by the bifunctional platinum lesion in
these structures include a large positive stretch and buckle at the G6-C17 base
pair, and significant opening and stagger at the G7-C16 base pair. Both dia and
dia+para structures also show significant unwinding (< 25° twist), large
positive roll (~50°), and increased shift values at the G6-G7 base pair step.

When the H8/H6 to H1'/H5 region of the 200 ms NOESY spectrum
(Figure 2.20) of ds11Bdia is compared to those back-calculated from dia and
dia+para refined structures, differences between the calculated and observed spectra are subtle. In general, the two sets of predicted spectra agree equally well with the experimental data. Spectral data calculated from both the dia and dia+para structures, however, lack a T10 H6 to C11 H5 cross-peak and, in both, the intensity for the A13 H2 to T10 H1' cross-peak is too high (Figure 2.20). In addition, several cross-peak intensities are too weak in the spectrum predicted from the dia structure, including A13 H8 to G12 H1' and A19 H8 to G18 H1' (Figure 2.20).

Discussion

Characterization and Duplex Stability of a Spin-Labeled Platinated Oligonucleotide. The isomer distribution resulting from modification of tsll with 1 is the same as that observed for modification of calf thymus DNA with cis-[Pt(NH3)(CyNH2)Cl2], the major metabolite of a family of oral Pt(IV) antitumor agents (Barnard et al., 1996, Giandomenico et al., 1995, Kelland et al., 1992). Although platination of the dinucleotide d(GpG) with 1 yields equal amounts of both 5' and 3' orientational isomers (Dunham & Lippard, 1995), modification of the single-stranded 11mer under the same conditions results in a significantly greater amount of the 3' isomer (2:1 ratio of 3' to 5'). The predominance of this isomer, which is also observed in modification of DNA with the cyclohexylamine analog (Hartwig & Lippard, 1992), may be attributed to the presence of a phosphate group 5' to the site of platinum modification. Hydrogen-bonding has been observed between the 5' ammine ligand in cisplatin and a phosphate on DNA (Sherman et al., 1988, Takahara
et al., 1996), and this interaction may be sterically hampered by a bulky substituent on the 5' amine (Hartwig & Lippard, 1992).

Although the 3' isomer is preferred upon modification of the 11mer sequence with 1, the melting profiles of the 3' and 5' isomeric modified duplexes are equivalent. Formation of the bifunctional lesion in either orientation decreases the melting temperature by \( \sim 24 \, ^\circ\text{C} \), comparable to values measured for short DNA duplexes modified with cisplatin at a single d(GpG) site (Van Hemelryck et al., 1984). Clearly the distortion caused by the bifunctional adduct provides a more significant energy perturbation to the duplex than any steric interactions with the substituents on the modified amine ligand.

**Comparisons with Structural Studies of Cisplatin-Modified Oligonucleotides.** Several structural elements have previously been identified in DNA modified with cisplatin at the N7 positions of adjacent purine residues (Yang & Wang, 1996). Structural studies of single- and double-stranded DNAs have indicated that cisplatin-modification of adjacent guanosine residues induces a change in the sugar conformation of the 5' coordinated nucleoside, from C2'- to C3'-endo. Recent NMR solution studies have also revealed disruption of hydrogen-bonding and/or increased water accessibility at the 5' coordinated G-C base pair (de los Santos & Patel, Gelasco & Lippard, 1997, Herman et al., 1990, Yang et al., 1995), with detection and assignment of the 5' G imino proton proving to be very difficult, even at low temperatures. These elements are shared by the undecamer duplex modified with a spin-labeled asymmetric platinum compound investigated here. In the crystal structure of d(CCTCTG*G*TCTCC)-(GGAGACCAGGG) (Takahara
et al., 1996), unconventional hydrogen-bonding is observed at the base pair to the 3' side of the platinum coordination site. The adenine amino group is no longer within hydrogen bonding distance of its Watson-Crick partner, but instead interacts with the O6 group of guanine in the preceding base pair. Although direct evidence for such a feature in the NMR solution structure is unavailable, the imino proton in the corresponding T-A pair in ds11Bdia (T8 H3) is very broad, and the unidentified cross-peak to G7 H1 in the preceding base pair may indeed arise from such an unconventional hydrogen-bonding scheme.

Data from early NMR studies (den Hartog et al., 1985) indicated that the unmodified decamer duplex, d(TCTCGGTCTC)-(GAGACCGAGA), adopts a right-handed, B-form helical structure in solution. The unmodified 11 bp sequence used in the present study, which is identical in sequence and has an added C-G base pair on the 5' end, is similarly expected to have B-form character such that any observed differences from B-DNA would derive from platinum-modification. NOESY cross-peak intensities between both exchangeable and nonexchangeable proton pairs in ds11Bdia deviated by the greatest extent from B-form DNA near the platinum-modification site, just as the major changes in chemical shifts reported upon modification of the corresponding decamer with cisplatin were restricted to the central four base pairs (den Hartog et al., 1985).

The final structures of 3'ds11, refined with dia and dia+para restraints, show both similarities and differences to the previously reported cisplatin-modified dodecamer crystal structure (Takahara et al., 1996) and the octamer NMR structure (Yang et al., 1995). The minor grooves of these duplexes
(Figure 2.18) widen near the platinum lesion, and the magnitude of widening is comparable for the dia, dia+para, and dodecamer structures (maximum \(-10-11\ \text{Å}\)) but significantly less in the octamer duplex (\(-7-8\ \text{Å}\)). The widened minor groove, particularly near the platinum coordination site, has been implicated in the recognition of such DNA duplexes by cellular proteins (Takahara et al., 1996). Since \(-10-11\ \text{Å}\) minor groove widths are observed in the dia and dia+para structures reported here, as well as in the dodecamer crystal structure, this structural feature can be confidently attributed to formation of the platinum 1,2-intrastrand cross-link rather than restricted by crystal packing forces in the solid state.

The dia+para NMR and the dodecamer crystal structure are compared in Figure 2.21. Since the sequences of these two modified duplexes are not identical, the overlay at the top of this figure represents the best superposition of heteroatoms in the platinum coordination sphere, the coordinated guanine bases, and the sugar-phosphate backbone. The overall topologies of the dia+para NMR and dodecamer structures are remarkably similar, with an RMSD of 1.98 Å for the heteroatoms described above. Comparisons of the dodecamer crystal structure to the dia NMR or octamer NMR structures result in corresponding RMSDs of 2.26 Å and 2.78 Å, respectively. Visually, the dodecamer and dia+para NMR structures display comparable directions and magnitudes of curvature. The dodecamer duplex, which is predominantly an A-form helix, exhibits a wide central hole when viewed directly down its 5' end (Takahara et al., 1996). This property is characteristic of A-DNA and is also clearly evident when the dia+para NMR structure is viewed similarly (Figure 2.21, center). This feature arises even though only
two of the 22 sugar residues in this duplex have A-type (C3'-endo) sugar conformations. The global helix axis, as determined in the program CURVES (Lavery & Sklénar, 1988, Lavery & Sklénar, 1989), passes through the center of this hole at the 5' end of the dodecamer crystal structure but through the base pairs in the corresponding portion of the dia+para NMR structure. Because the helical axes of these two very similar duplexes are so differently defined in CURVES (Figure 2.22), the bend angle as determined by the program for the dodecamer is ~40°, whereas a much larger value results for the undecamer structure (~80°). Bend angle analysis described previously relies on the determination of local helix axes (Takahara et al., 1996). As with the global helix axes, these local axes are defined very differently for the dodecamer X-ray and the dia+para NMR structures, again resulting in large differences in bend angle values calculated for the two duplexes. Despite this difficulty in rigorously calculating bend angles for comparison of these duplex structures, the overall shape of the structures is very similar, with substantial bending of the duplex toward the major groove (Figure 2.21, top). For comparison, gel mobility experiments which measure macroscopic duplex properties afford bend angles of 32-34° for such adducts (Bellon & Lippard, 1990).

The similarities between the dia+para NMR and dodecamer X-ray duplex topologies do not extend to the roll angle (ρ) between the platinated guanine bases. Although a positive roll is observed in all four structures (Table 3), the magnitudes are significantly larger for the dia and dia+para structures reported here. The roll angles observed in the dodecamer crystal and octamer NMR structures are accompanied by greater displacement of the
platinum atom from the plane of the 5′ guanine base (Table 3). This displacement may be related to the identity of the DNA flanking base pair. In both the dodecamer and octamer structures the internal sequence is d(TpGpG)-d(CpCpA), whereas the sequence in the present study is d(CpGpG)-d(CpCpG). It is possible that the 5′ CG step, which has been reported to have a large wedge angle (Hartmann & Lavery, 1996), can modulate the roll of the platinated d(GpG) site. Another possible source for the roll angle and differences is the nature of the platinum compound. The presence of a bulky substituent on the amine ligand in the major groove may affect the relative orientations of the modified guanine bases, even though the nitroxide group is positioned toward the 3′ side of the lesion.

Effects of Paramagnetic Distance Restraints on Structure Refinement. It has been suggested that NMR data for duplex DNA modified with cisplatin at a d(GpG) site are best explained by a localized distortion of the duplex, such as a kink, rather than a more gradual change in structure (den Hartog et al., 1985, Herman et al., 1990, Yang et al., 1995). Conventional NMR NOESY data reflect mainly nearest neighbor interactions (< 5 Å), however, and are limited in their ability to characterize such disturbances in duplex structure. Although many small localized changes in a predominantly linear helix may cause a significant distortion in the global helix topology, the individual changes may be well within the error of interproton distance or a dihedral angle determinations. The long range electron-proton distance restraints described in the present paramagnetic NMR investigation address this deficiency, allowing a more accurate determination of global duplex structure in solution.
The application of nitroxide spin labels for biological macromolecular structure determination has a rich history (Berliner, 1976, Berliner, 1979), but most of this work focused on proteins and protein-ligand interactions. The need for the additional structure restraints afforded by nitroxide spin labels, however, was soon superseded by technical advances in multidimensional and multinuclear NMR methods. These advances have allowed for the determination of high resolution protein structures in solution in the absence of long range paramagnetic restraints (Wüthrich, 1995), since protein folding often brings non-adjacent residues inside the 5 Å NOESY window.

The persistent lack of non-nearest neighbor restraints in duplex oligonucleotides, which do not form folded globular protein-like structures, has made it difficult to obtain well-determined structures in a similar manner. To our knowledge, this is the first study in which data from a covalently attached nitroxide spin label has been used to refine the global structure of any duplex oligonucleotide in solution.

Introduction of the 4AT ligand to afford long range distance restraints, first applied to refine the solution structure of a platinum-modified single-stranded dinucleotide monophosphate (Dunham & Lippard, 1995), has contributed to our understanding of the present platinum-modified duplex undecamer in several ways. In the first place, visual inspection of the long range distances restraints mapped onto a canonical B-form undecamer duplex clearly illustrate that the DNA, particularly at the 5' end of the duplex, must bend substantially toward to major groove in order to accommodate the data (Figure 2.16B). Such a conclusion would have been difficult, if not
impossible, to make from visual inspection of a series of short-range interproton distances.

Secondly, the addition of the paramagnetic restraints to refine the duplex structure did not affect the agreement of the final structure with the conventional diamagnetic data. Instead, dia or dia+para restrained refinements of this duplex yielded structures which comparably converged from A-form and B-form starting structures and which agreed equally well with the NOESY intensity build up data (Table 3). In addition, only subtle differences in NOESY data back-calculated from either the dia or dia+para structures were observed (Figure 2.20). This evidence, together with the very similar helical parameters of the duplexes resulting from these two sets of refinements (Figure 2.21), indicates that the same local structure is achieved from both the dia and dia+para restraints.

Finally, although all long range distance restraints are satisfied in the dia+para refined structures, approximately 25-30 long range distance restraints are violated in each dia structure, the upper-bound sum of which is greater than 45-50 Å. Agreement with all long range distance restraints, a result of dia+para refinement, produces duplex structures with an overall shape comparable to that of the cisplatin-modified dodecamer structure determined by X-ray methods. These results suggest that the duplex conformation observed in the dodecamer crystal structure persists in solution. Differences in local base pair parameters, such as the C3'-endo sugar pucker of the A-DNA segment of the dodecamer, can be attributed to the helix end/groove packing interactions in the solid state. The changes in refined undecamer duplex structure resulting from the inclusion of the long range distance restraints
may reflect gradual changes in duplex morphology which are poorly addressed by conventional NMR observables.

Conclusion

The addition of a large number of conservative long range electron-proton distance restraints has resulted in a solution structure of a nitroxide spin-labeled platinum-modified DNA duplex which agrees with interproton restraints in the diamagnetic analog but could not be deduced from these interproton restraints alone. Although the nitroxide spin label was introduced in the form of a modified platinum moiety for this study, others have developed methods to attach nitroxide spin labels directly to DNA residues, either through covalent base (Kryak & Bobst, 1990, Spaltenstein et al., 1989) or phosphate modifications (Nagahara et al., 1992). By employing such methods, long range distance restraints might be determined for other non-canonical DNA structures, such as those formed by A-tracts (dAn where n ≥ 3), the overall shapes of which, including magnitude and direction of bending, have been difficult to characterize by conventional diamagnetic NMR methods (Goljer & Bolton, 1994, Hartmann & Lavery, 1996, Harvey et al., 1995). The use of spin labels in DNA, particularly for duplexes modified with cisplatin and related drugs, may also contribute significantly to the characterization of ternary complexes formed between such DNA molecules and other macromolecules. NMR-derived long range distance restraints may reveal structural components that dictate cellular protein recognition of these platinum-modified DNAs, for example, and could help to determine the link between DNA structure and cytotoxic function of platinum antitumor agents.
References


de los Santos, C., & Patel, D. J. Personal communication.


Fichtinger-Schepman, A. M. J., van der Veer, J. L., den Hartog, J. H. J.,
Giandomenico, C. M., Abrams, M. J., Murrer, B. A., Vollano, J. F.,
Chem. 34, 1015-1021.
Goljer, I., & Bolton, P. H. (1994) in Two-Dimensional NMR Spectroscopy:
Applications for Chemists and Biochemists (Croasmun, W. R., & Carlson, R.
Harvey, S. C., Dlakic, M., Griffith, J., Harrington, R., Park, K., Sprous, D., &
Herman, F., Kozelka, J., Stoven, V., Guittet, E., Girault, J.-P., Huynh-Dinh, T.,
133.
Kelland, L. R., Murrer, B. A., Abel, G., Giandomenico, C. M., Mistry, P., &
Table 1. Proton chemical shifts (ppm) and assignments in 3'ds1dia.

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TEMPO<sup>c</sup>  Ha  Hb  Hb'  Hc  Hc'  CH₃<sup>d</sup>  CH₃<sup>d'</sup>  CH₃<sup>e</sup>  CH₃<sup>e'</sup>  
2.93  2.35  2.38  1.62  1.65  1.16  1.13  1.25  1.28

<sup>a</sup>Chemical shifts were measured at 23 °C and internally referenced to d₄-TSP at 0.0 ppm. <sup>b</sup>The abbreviations NHb and NHnb refer to the hydrogen-bonded and non-hydrogen-bonded amino protons in cytosine. <sup>c</sup>Letter designations of protons as shown in Figure 2.1. <sup>d</sup>Not assigned.
Table 2.2. Paramagnetic $^1$H relaxation times, unique $^1$H-$^1$H correlation times, and electron-proton distances determined for six resolved protons in 3'ds11\textsubscript{dia}.\textsuperscript{a}

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<tr>
<th>Proton</th>
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<th>$T_{2\text{para}}$ (s)</th>
<th>$10^9\tau_{c1}$ (s)</th>
<th>Electron-$^1$H distance (Å)</th>
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<td>A19 H8</td>
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<td>3.5</td>
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<td>15.4 ± 0.9</td>
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\textsuperscript{a}Errors are reported as ± 2 standard deviations.
Table 3. RMSDs$^a$, R-factors$^b$ and selected structural parameters for refined duplexes compared to canonical DNA forms and other high resolution structures.

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<th>Duplex</th>
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<th>R-factor</th>
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$^a$Root-mean-squared deviation of all non-hydrogen atoms in six final structures. $^b$Sixth-root residuals calculated as described by James et al. (1990) and Brünger (1992). $^c$Mean of R-factors calculated for six final structures. $^d$Average roll ($\rho$) angle between the two modified guanine bases as calculated by CURVES (Lavery et al., 1988). $^e$Takahara et al., 1996. $^f$Yang et al., 1995.
Table 2.4. Backbone parameters (°) for diamagnetic refinement of 3'ds11.\textsuperscript{a}

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<th>ε</th>
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\textsuperscript{a}Backbone parameters calculated by CURVES are reported for an average of six structures.
Table 2.5. Backbone parameters (°) for diamagnetic and paramagnetic refinement of 3' ds11.a

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*aBackbone parameters calculated by CURVES are reported for an average of six structures.
**Figure 2.1.** Schematic representation of the 3' orientational isomer of tsll modified with 1. The oxidized and reduced forms of the 4-aminoTEMPO ligand are drawn, and observed TEMPO to DNA interproton NOEs are indicated by arrows.
Figure 2.2. Plot of the paramagnetic contribution to proton relaxation rate versus electron-proton distance at 600 MHz for a proton with a 4.5 ns correlation time.
Figure 2.3. The change in NOESY cross-peak intensity plotted versus the electron-proton distance for a proton with a 0.15 s $T_{2\text{dia}}$ and a 4.5 ns correlation time.
Figure 2.4. HPLC separation of products resulting from ts11 reaction with 1. Unreacted starting material and orientational isomer identities are indicated.
Figure 2.5. EPR spectra at 25 °C of
(A.) $\text{cis-}[\text{Pt(NH}_3\text{)}(4\text{-aminoTEMPO}((\text{H}_2\text{O})_2)]^{2+}$
and (B.) ds11B$_{para}$.
Figure 2.6. Reversed phase HPLC separation of enzymatic digestion products of (A) ts11A\textsubscript{dia} and (B) ts11B\textsubscript{dia}. Peak a coelutes with 5\textit{cis}-[Pt(NH\textsubscript{3})(4ATH)(d(GpGpT)))] and peak b coelutes with 3\textit{cis}-[Pt(NH\textsubscript{3})(4ATH)(d(GpGpT))].
Figure 2.7. Plot of the first derivative of UV-vis melting and cooling profiles for 3'\text{ds}11_{\text{dia}}.
Figure 2.8A  H8/H6 to H1'./H5 region of the 200 ms NOESY spectrum of 3'ds11dia. The nH8/H6-nH1' and nH1'-(n+1)H8/H6 connectivities are shown for bs11.
Figure 2.8B. H8/H6 to H1'/H5 region of the 200 ms NOESY spectrum of 3'ds11_dia. The $n$H8/H6-$n$H1' and $n$H1'-(n+1)H8/H6 connectivities are shown for 3'ts11_dia.
Figure 2.8C. H8/H6 to H1'/H5 region of the 200 ms NOESY spectrum of 3'ds11\textsubscript{dia}.
Labeled cross-peaks are assigned as: a, A15 H2 to H15 H1'; b, A15 H2 to C9 H1';
c, A15 H2 to C16 H1'; d, A21 H2 to H21 H1'; e, A21 H2 to C3 H1';
f, A21 H2 to G22 H1'; g, A13 H2 to A13 H1'; h, A19 H2 to A19 H1'.
Figure 2.9. Stack plot of the $nH8/H6$ to $nH3'$ region of the 80 ms NOESY spectrum of 3'8ds11_dia. Peaks with moderate to strong intensities are labeled.
Figure 2.10. The aliphatic region of the 80 ms NOESY spectrum of 3'ds11_dia with observed 4ATH proton connectivities indicated. The 4ATH methyl protons (Figure 2.1 for numbering) and thymine methyl protons are labeled on the 1D projection.
Figure 2.11. The 591 MHz 1D imino proton spectrum of 3' ds11_dia at 15 °C. The peaks marked with an asterisk were not assigned.
Figure 2.12. Schematic representation of inter residue connectivities observed for exchangeable protons in the 300 ms H$_2$O NOESY spectrum of 3' ds11$_{dia}$: Solid and dashed lines indicate strong and medium/weak intensities, respectively.
Figure 2.13. Imino to aromatic/amino region of the 300 ms H$_2$O NOESY spectrum of 3'ds11$_{dia}$. Labeled peaks are: a, T10 H3 to A13 H2; b, T2 H3 to A21 H2; c, T4 H3 to C5 H6; d, T4 H3 to A19 H2; e, T4 H3 to C3 NH$_b$; f, T4 H3 to C5 NH$_b$; g, T8 H3 to A15 H2; h, G7 H1 to A15 H2; i, G7 H1 to A15 H2; j, G7 H1 to C16 NH$_b$; k, G7 H1 to unassigned exchangeable proton; l, G14 H1 to C9 NH$_b$; m, G14 H1 to A13 H2; n, G14 H1 to A15 H2; o, G14 H1 to C9 NH$_b$ and G20 H1 to C3 NH$_b$; p, G20 H1 to C3 NH$_{nb}$; q, G20 H1 to A19 H2; r, G18 H1 to C17 NH$_{nb}$; s, G18 H1 to C5 NH$_{nb}$; t, G18 H1 to C5 H6; u, G18 H1 to A19 H2; v, G18 H1 to C5 NH$_b$. 

D1 (ppm)  

7.2  

7.8  

8.4  

D2 (ppm)  

13.6  

12.8
Figure 2.14. Overlay of the aromatic region of the 1D $^1$H NMR spectra of 3'ds11\textsubscript{para} (top) and 3'ds11\textsubscript{dia} (bottom). Proton chemical shift assignments are indicated.
Figure 2.15. The H1’ to H2’/H2” region of the 200 ms NOESY spectra of (A) 3’ds11_di and (B) 3’ds11_par. Assignments in the H1’ dimension are indicated.
Figure 2.16. $3'\text{ds11}_{\text{dia}}$ represented in a canonical B-type helix form, with superposition of (A) all long range distance restraints and (B) the subset of long range distance restraints with upper bound violations. The termini of the platinum-modified strand are indicated.
Figure 2.17. Overlay of dia (blue) and dia+para (red) restrained duplex structures resulting from relaxation matrix refinement. The best fit of the 6 internal base pairs was used to overlay the structures.
Figure 2.18. Continuous measurements of minor groove widths for (A) dia (open circles) and dia+para (closed circles) restrained structures, and for (B) the cisplatin-modified dodecamer crystal structure (open circles) and the cisplatin-modified octamer NMR structure (closed circles) as calculated in CURVES, version 5.1. Standard values along the minor grooves of canonical A- and B-DNA are indicated.
Figure 2.19A. Plots of helical base pair parameters for dia (open circles) and dia+para (closed squares) refined structures. Each point represents the mean value for six structures and is plotted with error bars indicating the standard error of these values.
Figure 2.19B. Plots of helical base pair step parameters for dia (open circles) and dia+para (closed squares) refined structures. Each point represents the mean value for six structures and is plotted with error bars indicating the standard error of these values.
Figure 2.20. Experimental and predicted H8/H6 to H5/H1' regions of the 200 ms NOESY spectrum of 3'ds11_{dia}. Notable cross-peaks are assigned as: 

- a, T10 H8 to C11 H5;
- b, T4 H1' to T4 H6/C5 H6;
- c, A13 H2 to T10 H1';
- d, A19 H2 to G20 H1';
- e, G18 H8 to C17 H5;
- f, A15 H2 to T8 H1'.

Solid and dashed boxes indicate high and low cross-peak intensities, respectively, compared to the observed data.
Figure 2.21. At the top: Stereoviews of the cisplatin-modified dodecamer crystal structure (yellow) and the dia+para restrained NMR structure (red). In the center: The same duplexes are oriented such that the 5' axis is directed toward the viewer. At the bottom: The central 4 bp of each duplex are shown from the major groove. In all views of both duplexes, the platinum coordination sphere is grey.
Figure 2.22. Stereoview of the global helical axes as determined by CURVES for the dia+para restrained structure (light) and the dodecamer crystal structure (dark).
Chapter III

HMG-Domain Protein Interactions with Cisplatin-Modified DNA
Introduction

Protein recognition of specific DNA sequences and structures triggers many cellular functions. The activity of the anticancer drug cisplatin has been attributed to its ability to bind to and modify the structure of DNA, forming adducts that are specifically recognized by cellular proteins (McA’Nulty & Lippard, 1995). The processing of cisplatin-DNA adducts by the cell involves their recognition by proteins including those required for replication, transcription, repair and apoptosis (Pil & Lippard, 1997). Accordingly, considerable attention has been paid to identifying and characterizing human proteins which bind with high affinity and specificity to the major cisplatin intrastrand cross-links. The present study investigates several proteins in the HMG-1/-2 family which recognize cisplatin-modified DNA (Chow et al., 1995, Farid et al., 1996, Locker et al., 1995) and shield the major 1,2-intrastrand d(GpG) and d(ApG) adducts from excision repair (Zamble et al., 1996).

Although only limited structural information is available for HMG-domain interactions with cisplatin-modified DNA (Berners-Price et al., 1997, Chow et al., 1994, Kane & Lippard, 1996, Locker et al., 1995), several high resolution solution studies of isolated HMG domains (Broadhurst et al., 1995, Hardman et al., 1995, Jones et al., 1994, Read et al., 1993, Weir et al., 1993) and of HMG domains bound to unmodified duplex DNA (Hardman et al., 1995, Love et al., 1995, Werner et al., 1995) have been carried out. These investigations reveal that, despite mild (~30%) sequence conservation, the L-shaped fold of the alpha-helical domain is highly conserved, even across protein classes and in the presence of DNA. The concave surface of the HMG domain, including residues in the extended N-terminal region, in helices I and
II, and at the C-terminus (Figure 3.17), contacts the oligonucleotide. A single binding mode in the DNA minor groove occurs for the sequence-specific HMG domains (Love et al., 1995, Werner et al., 1995), but multiple binding modes may be present in solution for structure-specific HMG domains binding to duplex DNA (Hardman et al., 1995).

Initial experiments in this study utilize spectroscopic and electrophoretic methods to investigate HMG-domain protein recognition as a function of several variables. These variables include the platinum ligands and their orientation, the length of the modified DNA duplex, and the ribose content of the oligonucleotide. In previous studies of HMG-domain protein binding to cisplatin-modified DNA, the platinated DNA probe was either globally modified and thus had a variety of adducts or was site-specifically modified and contained a single 1,2-d(GpG), 1,2-d(ApG) or 1,3-d(GpTpG) cross-link embedded in a region of DNA of constant sequence context. Because footprinting studies reveal that the interaction of HMG-domain proteins with cisplatin-modified DNA extends over more than a turn of duplex (Locker et al., 1995, McA’Nulty et al., 1996, Treiber et al., 1994), we were interested to determine whether the DNA sequence flanking the platinum lesion would influence the affinity and specificity of the resulting complex. The presence of a site-specific platinum lesion precludes the use of current combinatorial methodologies to optimize the flanking DNA sequence. A series of individual 15-bp oligonucleotides d(CCTTCN_1G*G*N_2TCTTC)-(GAA-GAN_3CCN_4GAGAGG), where asterisks denote N7-modification of guanosine with cisplatin, N_1 and N_2 = dA, dC or T and N_3 and N_4 are their Watson-Crick complements, was therefore prepared to study the effects of DNA
sequence on HMG-domain binding to cisplatin-modified DNA. Although sixteen DNA sequences are possible by varying the sites flanking the central d(GpG) core, only nine were investigated. The seven sequences not examined contain three or more consecutive guanine residues, which renders difficult the isolation of pure N1G*G*N2 products. The information obtained here should be useful both for the design of potential anticancer drug candidates in the platinum family and for guiding experiments to determine the structures of the complexes by NMR or X-ray diffraction methods.

Materials

Cisplatin was a gift from Johnson Matthey AESAR/Alfa Co. All DNA synthesis reagents were purchased from Cruachem, Inc. The pT7HMG1bA plasmid was kindly supplied by M. E. Bianchi. Purified HMG1 and tsHMGdomA proteins were provided by Q. He and U.-M. Ohndorf, and tS12G*G*, tS16TG*G*AC, and 159 base pair probes were provided by C. S. Chow, U.-M. Ohndorf and D. B. Zamble, respectively.

Sample Preparation

Oligonucleotide Probes. Tables 3.1 to 3.3 list the oligonucleotides used in this study together with their abbreviations. Oligonucleotides were synthesized in micromole quantities on a Cruachem PS250 DNA synthesizer by employing conventional solid support phosphoramidite chemistry. Fully protected ribonucleotide-containing sequences, r(gaagaaccagagagg) and d(GAAGA)r(accagagagg), were purified by using reversed phase HPLC (C18 radial pak cartridge, Waters, nonlinear 0 to 100% methanol gradient in 200
mM NaOAc buffer, pH 7.2) prior to deprotection. These oligonucleotides were further purified on a 20% denaturing polyacrylamide gel (7.5 M urea, 19:1 acrylamide:bisacrylamide, 90 mM TRIS-borate, 1 mM EDTA, pH 8.3, 300V, ~10 h) and desalted prior to use.

Deoxyribonucleotides were synthesized with the final trityl group cleaved, purified by using anion exchange HPLC (Dionex NucleoPac PA-100 9 x 250 mm column, 200 to 400 mM NaCl gradient in 25 mM NH₄OAc pH 6.0, 10% acetonitrile), and desalted on a G25 Sephadex (Pharmacia) column prior to use. All oligonucleotides containing a single d(GpG) target site for platinum modification were allowed to react with 1.0 to 1.25 equiv of cis-[Pt(NH₃)₂(H₂O)₂]²⁺ (Takahara et al., 1996) or cis-[Pt(NH₃)(4AT)X₂]²⁺ where X = DMF/NO₃⁻ (Dunham & Lippard, 1995) in 10 mM sodium phosphate buffer, pH 6.0, for several hours at 37°C or room temperature. Oligonucleotide reactions with [Pt(NH₃)₂(H₂O)₂]²⁺ were monitored by anion exchange HPLC (Dionex NucleoPac PA-100 4 x 250 mm column, 50 to 400 mM NaCl gradient in 25 mM NH₄OAc pH 6.0, 10% acetonitrile) and the major product was isolated in each case. The reaction of ts20GG with cis-[Pt(NH₃)(4AT)X₂]²⁺ was monitored by reversed phase HPLC (C₄, VYDAC, 4.6 x 250 mm column with linear gradients of acetonitrile in 0.1 M NH₄OAc, pH 6.0) and the two major products, designated ts20A and ts20B, were separated on a preparatory scale column (C₄, VYDAC, 22 x 250 mm).

The modified oligonucleotides were determined by atomic absorption spectroscopy (Varian 1475 graphite furnace spectrometer operating in peak height mode at 265.9 nm) to have one platinum atom per strand. Formation of the desired cisplatin 1,2-intrastrand adducts was confirmed by enzymatic
digestion as described previously (Figures 3.1 - 3.4). Oligonucleotide strand concentrations were determined from $A_{260}$ values and theoretically estimated extinction coefficients (Borer, 1975).

**HMG-Domain Proteins.** HMG1domB, K86-K165 of rat HMG1 (Ferrari et al., 1994), was expressed in *E. coli* (BL21-DE3) from a previously reported plasmid (Chow et al., 1995). The domain was isolated and purified as described (Chow et al., 1995) with the addition of a final FPLC size-exclusion purification step (high load Superdex 75, Pharmacia, 1 ml/min, 11.8 mM PBS, pH 7.4). HMG1domA, M1-F89 of rat HMG1, was expressed in the same bacterial strain from the pT7HMG1bA plasmid (Falciola et al., 1994). Cells were grown and harvested according to published procedures (Falciola et al., 1994) and the protein was purified by using the same protocol as for HMG1domB.

Protein concentrations were determined by optical absorption at 278 nm using extinction coefficients of 14,000 M$^{-1}$cm$^{-1}$ and 12,160 M$^{-1}$cm$^{-1}$ for HMG1domA and HMG1domB, respectively. The extinction coefficient for each protein was determined from the combined measurements of optical absorbance at 278 nm and protein concentrations from amino acid analyses (MIT Biopolymers Lab). The isoelectric point (pI) for each protein sequence was determined by using the Pepsort algorithm available in the GCG program (Version 7.0, Genetics Computer Group, Madison, WI). Protein sequence alignments were performed in the AMPS program (Version D2.0, G. Barton, Oxford).
Spectroscopic Studies

Electron Paramagnetic Resonance Spectroscopy

**Experimental.** EPR spectra were recorded on an X band (9.8 GHz) Bruker ESP300 spectrometer at 295 K and externally referenced to TEMPOL, $g = 2.006$. For each sample, a total of 24 to 128 scans were acquired over a 100 G sweep width centered at 3470 G with a modulation amplitude of 1.0 G, a modulation frequency of 12.5 KHz, and 20 mW of microwave power. All spectral data were exported in ASCII format and analyzed in KaleidaGraph (version 3.0, Abelbeck software). A 100 μL sample in a 1.5 mm capillary tube was inserted into a 3.0 mm quartz tube for measurements. Oligonucleotides (8 to 32 μM) were prepared in 100 mM sodium phosphate buffer, pH 6.2 and protein samples were exchanged into 100 mM KCl, 10 mM sodium phosphate buffer, pH 6.6 prior to addition.

**Results and Discussion.** The nitroxide spin label gives rise to a three-line EPR spectrum due to strong coupling of a single unpaired electron ($g = 2.006$) with the $^{14}$N nucleus ($I = 1, A = 17$ G). For samples in which the nitroxide is tumbling very rapidly in solution ($\tau_r < 10^{-10}$ s), an isotropic spectrum is observed with approximately equal heights for the three spectral components. The EPR spectrum of the spin-labeled platinum compound, $\text{[Pt(NH}_3)(4\text{AT})(\text{H}_2\text{O})_2]^2+\text{,}$ is shown in Figure 3.5 (top) and illustrates the signal resulting from such a rapidly tumbling nitroxide. Line shape analysis (Bobst, 1979) of this spectrum affords an estimated rotational correlation time ($\tau_r$) of 52 ps for the nitroxide spin label in the free platinum compound.
Coordination of this platinum compound to ts20GG significantly changes the line shape of the nitroxide EPR spectrum (Figure 3.5, middle). Although electronic distribution, solvent polarity, and molecular motion can all affect the EPR line shape of the nitroxide (Krugh, 1976), the main contributor to this observed change is the decreased tumbling rate of the spin label ($\tau_r \sim 1.7$ ns for ts20A and 1.6 ns for ts20B) due to covalent attachment to the oligonucleotide. Further signal shape distortions are observed in the EPR spectra upon annealing of ts20A or ts20B with a slight excess of the complementary oligonucleotide (Figure 3.5, bottom). Although similar line shape analysis for the duplex signals estimates $\tau_r$ values $\geq 5$ ns, the assumptions for this simple calculation are not valid for tumbling in this regime. Qualitative differences in the spectra, however, verify that $\tau_r$ increases with the size of the molecule to which the nitroxide is bound.

In Figure 3.6, the changes in the EPR spectra of ds20A and ds20B are illustrated upon addition of 0.5 and 1.0 mol equiv of HMG1domB. This 9.2 kD protein specifically recognizes the cisplatin-modified duplex, ds20G*G*, (Chow et al., 1995) with a dissociation constant of $\sim 0.5 \mu$M. The significant increase in the shoulder of the low field line of the EPR spectrum in both duplexes indicates a longer $\tau_r$ for the nitroxide spin label upon addition of protein. In comparison with other studies of protein interactions with spin-labeled oligonucleotides (Bobst, 1979, Keyes et al., 1996), these spectral changes are consistent with HMG1domB binding to these duplexes. The observation of significant spectral changes after addition of only 0.5 equiv of protein is consistent with a dissociation constant in the micromolar or submicromolar range for this interaction.
Fluorescence Spectroscopy

Experimental. The intrinsic fluorescence of HMG1domB protein was monitored both in the presence and in the absence of duplex oligonucleotides on a Hitachi F-3010 fluorescence spectrometer. Samples were excited at 280 nm with an excitation bandpass of 10 nm. Fluorescence emission was monitored from 300 to 400 nm with an emission bandpass of 5 nm at a scan rate of 30 nm/min. Sample temperature was maintained at 20 °C with a circulating water bath.

Oligonucleotides were annealed according to the following general procedure. Equal amounts of complementary strands were combined in 100 mM sodium phosphate buffer, pH 7.2, and heated at 65 °C for 15 min. Samples were cooled to room temperature over several hours and incubated at 4 °C for 10-15 h before use. Ion exchange HPLC of annealed duplexes indicated < 10 % single-stranded material was present.

A background scan of the initial solution (0 or 0.32 μM duplex in 10 mM MES buffer, pH 6.5) was recorded and subtracted from all subsequent spectra in each titration experiment. To 750 μL of initial solution in a 2 mm x 2 mm fluorescence cell, 1 μL aliquots from a 35 μM protein stock were added. After each addition, samples were thoroughly mixed by repeated pipetting and equilibrated for 1 min. Fluorescence spectra were then recorded and the total fluorescence was integrated over the entire emission range.

Results and Discussion. The quenching of intrinsic protein fluorescence associated with DNA-binding can be analyzed to determine the affinity and stoichiometry of protein-DNA interactions (Kneale, 1994).
HMG1domB exhibits intrinsic fluorescence owing to a single tryptophan and several tyrosine residues. Initial experiments with HMG1domB showed that little fluorescence quenching occurred in the presence of either ds20G*G* or ds15TG*G*T in buffers containing ≥ 250 mM NaCl (data not shown). Moderate fluorescence quenching was observed, however, by the same duplexes in buffers with NaCl concentrations ≤ 100 mM.

Figure 3.7 shows a theoretical plot of intrinsic protein fluorescence (Fl) versus protein concentration, [P], both in the presence and absence of L, a ligand which binds to and quenches the fluorescence of the protein. When such a ligand, an example being DNA, is present in the initial solution, Fl does not increase linearly with [P]. Instead, quenching of protein fluorescence occurs until all of the binding sites are saturated, at which point protein intrinsic fluorescence increases linearly with the same slope as in the absence of quencher (Kohlstaedt & Cole, 1994a, Kohlstaedt & Cole, 1994b). A linear fit through the post saturation data gives an x-intercept, [P]e, which represents the protein concentration at which all binding sites are saturated. The ratio of [P]e to [S], therefore, is the stoichiometry of the protein-substrate interaction.

Plots of fluorescence measured as a function of HMG1domB concentration are shown in Figure 3.8. The control data in both plots illustrate that, in the absence of a quenching agent, the intrinsic fluorescence of HMG1domB increases linearly with protein concentration over the range used in this study. Fluorescence data for HMG1domB in the presence of 20 base pair oligonucleotides (Figure 3.8A) illustrates that both ds20G*G* and ds20GG quench the fluorescence of HMG1domB. The break point for these titration data, however, occurs at a much lower protein concentration (~1
μM) for ds20G*G* than for ds20GG. For HMG1domB in the presence of 0.32 μM of the cisplatin-modified probe, a linear fit of the post saturation data using the same slope as the control curve afforded an x-intercept of 0.42 μM protein. This intercept corresponds to a protein:DNA ratio of 1.3:1.0 for the observed interaction with ds20G*G*. For the unmodified probe, however, the later saturation allowed for an estimate of ≥ 3.0:1.0 stoichiometry.

The analogous experiments in the presence of 15-bp oligonucleotides, shown in Figure 3.8B, contrasted those with the longer duplex in that both the cisplatin-modified and unmodified 15-bp duplexes resulted in very similar titration curves. Analysis of these data afforded protein to DNA ratios of ~ 1.4:1.0 for HMG1domB interaction with either ds15TGTT or ds15TG*G*T. It is possible that the greater surface area of the 20-bp duplex (Figure 3.9) allows for more than one protein-binding event, whereas the 15-bp probes are sufficiently short to limit such additional binding.

For both the 15- and 20-bp probes, subtle differences are observed in the presaturation region of the curves for the platinated and control duplexes (Figure 3.8). For either length oligonucleotide, the quenching of HMG1domB fluorescence is greater with the cisplatin-modified duplex. Assuming the fluorescence extinction for the HMG1domB complex to be the same for the platinated and unplatinated forms, these presaturation data are consistent with a stronger interaction between HMG1domB and the cisplatin-modified duplexes.
NMR Spectroscopy

**Experimental.** Uniformly enriched $^{15}$N-HMG1domB was obtained as described in the protein sample preparation section except that expression was performed in cells grown on minimal media with 99.4% $^{15}$N-ammonium chloride as their sole nitrogen source. The purified protein sample was exchanged into buffer I (10 mM MgCl$_2$, 50 mM NaCl, 10 mM sodium phosphate buffer, pH 6.6) by centrifiltration at 4 °C (centricon, MWCO 3000, Amicon). $^{15}$N-1H HSQC spectra of a 4 mM protein sample in 90%H$_2$O/10%D$_2$O buffer I were recorded at 591 MHz on a home built spectrometer (Francis Bitter Magnet Laboratory) at 20 °C with 128 experiments and a 5000 Hz sweep width in the $t_1$ dimension, and 1024 data points and a 10000 Hz sweep width in the $t_2$ dimension. Titrations from 0.25 to 1.25 mol equiv of ds15TG*G*T (21 mM stock in buffer II) were added to the protein solution, mixed and equilibrated for ~1 hr, followed by data collection.

**Results and Discussion.** In order to obtain more detailed structural information for the interaction between an HMG-domain protein and a cisplatin-modified DNA, the formation of an HMG1domB-ds15TG*G*T complex was attempted at NMR concentrations. The $^{15}$N-1H HSQC spectrum of the protein, as well as the 1D 1H spectrum of the imino region, was monitored as the duplex concentration was increased. The HSQC spectrum of the protein alone (Figure 3.10A) and after 0.25 equiv of ds15TG*G*T (Figure 3.10B) are shown. Spectra following subsequent additions were identical to that observed in Figure 3.10B. With increasing duplex concentration, increasing amounts of a white-grey precipitate formed and the protein backbone $^{15}$N$\alpha$-1H$\alpha$ cross peaks in the HSQC spectrum lost dispersion as well
as signal to noise. Although the white grey precipitate did redissolve when the sodium chloride concentration was increased to 510 mM, the spectra did not improve (Figure 3.10C). These results indicate that, in this experiment, the duplex was not forming a discrete, characterizable complex with the protein, perhaps due to protein denaturation or oligomerization of the protein-DNA complex. Further optimization of the interaction between an isolated HMG-domain and a cisplatin-modified duplex may be required before the structure of the desired protein-DNA complex can be determined by NMR methods.

**Gel Electrophoretic Studies**

**Experimental**

Single-stranded oligonucleotide probes (10 to 20 pmol) were 5'-end labeled with T4 polynucleotide kinase (20 units, NEB) and υ-32P-ATP (50 μCi, NEN), purified on G25 Quick Spin columns (Boehringer Mannheim), and ethanol precipitated. Radiolabeled probe concentrations were determined as previously described (Batey & Williamson, 1996). Radiolabeled duplexes were formed by annealing 10^6 cpm of one strand with 1.5 to 10 equiv of its complement in buffer III (10 mM HEPES, pH 7.5, 50 mM LiCl, 10 mM MgCl2) at 45º C for 15 min followed by a 10 h incubation at 4º C. Unlabeled duplexes for competition experiments were annealed by combining equimolar amounts of the two complementary strands at concentrations of 250 μM to 1 mM in buffer III at 45º C for 15 min followed by 10 h incubation at 4º C.
Oligonucleotide duplexes (0.4 to 5.0 nM, 5000 cpm) were titrated with protein in 10 μL sample volumes in buffer IV (10 mM HEPES, pH 7.5, 10 mM MgCl₂, 50 mM LiCl, 100 mM NaCl, 1 mM spermidine, 0.2 mg/mL BSA, 0.05% Nonidet P40). For all gel mobility shift experiments, samples were incubated on ice for 1 h and made 7% in sucrose and 0.017% in xylene cyanol prior to loading on running, precooled (4° C), prerun (300 V, 1-2 h) native polyacrylamide gels (29:1 acrylamide:bisacrylamide, 3.3% cross-linking, 22.5 or 45 mM TRIS-borate, 1 mM EDTA, pH 8.3). Gels were electrophoresed for 1-2 h before drying under vacuum (80° C, 1 h) and exposure to film (10-40 h). Quantitative measurements of bound and free oligonucleotide were performed by phosphorimager analysis following 2-12 h exposures (Molecular Dynamics PhosphorImager). Apparent dissociation constants, K_d, were estimated from non-linear least squares fits of binding data to the Langmuir isotherm (eq 1) (Lohman & Mascotti, 1992), where θ is the fraction of bound oligonucleotide probe and P is the total protein concentration.

\[ \theta = \frac{P}{P + K_d} \]  

(1)

In order to determine the stoichiometry of binding, duplex oligonucleotides (100 nM, 5000 cpm) were titrated with 0 to 3 equiv of HMG1domA protein in buffer IV. The stoichiometry of binding was assessed from plots of θ versus protein equivalents. The point of intersection of two lines, one fit to presaturation and the other to postsaturation data, was taken as an indication of the stoichiometry of the protein-DNA complex.

For competition assays, unlabeled competitor oligonucleotide probe (0-200 μM) was titrated against radiolabeled AG*G*A duplex (0.45 nM, 5000 cpm)
in buffer IV) complexed with HMG1domA. By following a previously described protocol (Long & Crothers, 1995), both oligonucleotides were mixed prior to addition of the protein at a concentration which afforded 50-70% binding of the labeled probe in the absence of competitor. Apparent relative dissociation constants, $K_{rel}$, were determined from non-linear least squares fits of the competition data to eq 2 (Long & Crothers, 1995) where $K_{rel}$ is the ratio of $K_t$, the apparent dissociation constant of the labeled probe, to $K_c$, the apparent dissociation constant of the competitor, and $P_t$, $T_t$, and $C_t$ are the total concentrations of protein, radiolabeled probe, and competitor probe, respectively.

\[
\theta = \frac{1}{2T_t} \left\{ \frac{K_t + K_{rel}C_t + P_t + T_t}{\sqrt{\left[ K_t + K_{rel}C_t + P_t + T_t \right]^2 - 4T_tP_t}} \right\}
\] (2)

Results and Discussion

**Protein Recognition of the Orientational Isomers Formed by an Asymmetric Platinum Compound with a Bulky Amine Ligand.** Gel mobility shift assays of ds20GG, ds20G*G*, ds20A and ds20B in the presence of HMG1domB are shown in Figure 3.11. As previously reported (Chow et al., 1995), the cisplatin-modified duplex (ds20G*G*) forms a specific complex with HMG1domB (lane 3) under conditions for which no protein interaction is observed with the unmodified duplex (ds20GG, lane 1). Two duplexes, previously studied by EPR methods (see preceding section), were formed from the products of ts20 modification with the asymmetric spin-labeled platinum compound $\text{cis-}[\text{Pt(NH}_3\text{)(4AT)X_2}]^{2+}$ (Dunham & Lippard, 1995) and represent the 5’ and 3’ orientational isomers at the d(GpG) site. The final lanes in this
gel represent the analogous binding titrations of HMG1domB with these two duplexes, ds20A and ds20B. Although the orientation of the 4AT ligand in each isomer has not been definitively identified, the absence of dG after enzymatic digestions indicated that both are the desired bifunctional d(GpG) adducts. As observed in lanes 5 and 7 (Figure 3.11), a discrete complex is formed between HMG1domB and each of these duplex orientational isomers.

HMG1 protein recognition was previously reported for a 123-bp fragment modified with Pt(dansen)Cl₂, an asymmetric fluorescent cisplatin analog (Hartwig et al., 1992). Because this 123-bp probe was globally platinated, the protein-recognition contributions of different adducts and the orientation of these adducts could not be assessed. The experiments described in the present study employing a site-specifically platinum-modified 20-bp DNA duplex provided the first evidence that both orientational isomers formed upon modification of a d(GpG) site with an asymmetric platinum compound impart HMG-domain protein recognition for a DNA duplex. Although detailed quantitation of these interactions was not obtained, HMG1domB affinity is comparable for both orientational isomers ds20A and ds20B (lanes 6 and 9), and for the same duplex modified with cisplatin (ds20G*G*, lane 3). More recent studies with an asymmetric platinum complex of L-lysine have yielded similar results with a site-specifically modified 15-bp oligonucleotide (Sandman & Lippard, 1997). The size or orientation of these exogenous platinum ligands do not affect HMG domain recognition of bifunctional DNA adducts, supporting the conclusion that the protein recognizes structural distortions in the DNA duplex resulting from platinum coordination, rather than directly contacting the platinum moiety.
**HMG-Domain Protein Recognition as a Function of Duplex Length.** In an effort to identify a lower limit for the length of a cisplatin-modified duplex required for specific HMG-domain protein recognition, a series of oligonucleotides were investigated. Titrations of ds12G*G*, ds15TG*G*T and ds20G*G* with HMG1domB are shown in Figure 3.12. A band of shifted mobility, indicating the formation of a protein-DNA complex, is observed for both the 15-bp (lane 6) and 20-bp (lane 9) duplexes.

Although the cisplatin-modified 12 bp sequence is the exact portion of a longer oligonucleotide previously shown to be recognized specifically by both HMG1domB and HMG1 (Chow et al., 1994), it does not form a specific complex with HMG1domB under the conditions examined here (lane 3). These results suggest that a minimum of ~15 base pairs is required for HMG domain recognition of a cisplatin-modified DNA duplex. This minimal length is consistent with the 14- to 15-bp footprint found for nuclease digestion of several HMG-domain proteins bound to cisplatin-modified DNA (Locker et al., 1995, McA‘Nulty et al., 1996, Ohndorf et al., 1997, Treiber et al., 1994). These footprinting experiments also indicate that the platinum adduct occupies the central portion of the protected region, which extends ~6 base pairs to both the 3’ and the 5’ side of the platinum adduct.

**HMG-Domain Proteins and DNA-RNA Heteroduplexes.** The bifunctional platinum d(GpG) lesion can induce A-type helix characteristics in duplex DNA (Takahara et al., 1995, see Chapter 2 of this work). These characteristics, including a wide and shallow minor groove, are a likely target for minor-groove binding HMG-domain proteins. Since duplex oligonucleotides containing ribose sugars have been shown to exhibit A-type
helix characteristics in solution (Fedoroff et al., 1993, Horton & Finzel, 1996, Salazar et al., 1993a, Salazar et al., 1993b, Salazar et al., 1994), the possibility that cisplatin-modified DNA-RNA hybrids could have enhanced affinity for such proteins was investigated. None of the three control duplexes, DNA duplex TGGT, DNA-RNA hybrid hTGGT, or DNA-RNA chimeric hybrid cTGGT (Table 3.3), was recognized specifically by either isolated domain of HMG1 (Figures 3.13A and B). The incorporation of a site-specific, cisplatin-modified d(GpG) cross-link imparted HMG-domain binding only for an all DNA duplex (ds15TG*G*T, Figures 3.14A and 3.14B, lanes 1-3). The incorporation of ribose sugars abolished protein binding similarly for both HMG1domA (Figure 3.14A) and HMG1domB (Figure 3.14B). To exclude the possibility that the absence of protein-binding was a result of hTG*G*T or cTG*G*T duplex instability, native gels were run under more highly resolving conditions (data not shown). The ds15TG*G*T, hTG*G*T and cTG*G*T duplexes exhibited decreased gel mobility compared to the labeled DNA single strand alone, consistent with the formation of double-stranded oligonucleotides under these conditions.

The lack of HMG-domain protein binding to a DNA-RNA hybrid (hTGGT) and to the chimeric hybrid (cTGGT) indicated that a propensity to form a more A-type helix conformation does not necessarily impart protein-oligonucleotide affinity. In addition, the substitution of ribose for deoxyribose sugars in the corresponding cisplatin-modified duplexes appears to offset any platinum-dependent structural distortion recognized by HMG1domA and HMG1domB. Although the 2'-hydroxyl groups of the ribose sugars have been implicated as important for protein recognition of DNA-RNA hybrids
(Horton & Finzel, 1996), their presence along the minor groove of the double helix could well interfere with specific protein-oligonucleotide contacts essential for HMG-domain binding. If cisplatin-modification of DNA-RNA hybrid or chimeric structures were important in the antitumor mechanism of the drug, then the present study suggests that HMG-domain proteins will not participate in such a mechanism.

**Recognition of ds15AG*G*A by Several HMG-Domain Proteins.** A 15-bp oligonucleotide, ds15AGGA, both with and without a single cis-[Pt(NH$_3$)$_2$(d(GpG))] modification was investigated for binding to several structure-specific HMG-domain proteins. Gel mobility shift assays with full length HMG1, the first HMG domain in the testis-specific HMG protein (tsHMGdomA), and the two isolated domain of HMG1 (HMG1domA and HMG1domB) are shown in Figure 3.15. HMG1 protein, consisting of both A and B domains and an acidic tail region, did not bind to the unmodified probe (Figure 3.15A, lanes 2-4) and exhibited only minimal interaction with the cisplatin-modified probe (Figure 3.15B, lanes 2-4). The three isolated HMG domains from proteins in the structure-specific class all exhibited minimal binding to the unmodified probe (Figure 3.15A, lanes 5-16). Smearing of the unmodified probe occurs at increasing HMG1domA concentrations (lanes 10-12) and represents nonspecific (platinum-independent) interactions which are absent for tsHMGdomA (lanes 6-8) and HMG1domB (lanes 14-16). As indicated by the presence of a shifted band which increases with protein concentration (Figure 3.15B), tsHMGdomA, HMG1domA and HMG1domB all recognize ds15AG*G*A. At the highest tsHMGdomA concentration (Figure 3.15B, lane 8), the emergence of a second shifted band may indicate that
oligomers of the protein also have the ability to bind to this DNA duplex. Since only a single shifted band forms following incubation of ds15AG*G*A with either HMG1domA or HMG1domB, detailed titration studies were carried out. The titration of ds15AG*G*A with HMG1domB is shown at the top of Figure 3.16. Nonlinear fits of the titration data for both HMG1domA and HMG1domB to eq 1 (Figure 3.16, bottom) afforded $K_d$ values reported in Table 3.4.

The lack of HMG1 binding to the cisplatin-modified 15-bp duplex is not unexpected since Pil (Pil, 1993) previously observed that HMG1 recognition of a site-specifically cisplatin-modified probe required $> 60$ bp. In addition to oligonucleotide size, electrostatic considerations may influence the interaction between ds15AG*G*A and HMG1. Owing to the presence of the acidic tail, the full length HMG1 protein has a much lower pI and net negative charge at pH 7.5 compared to the isolated HMG domains (Table 3.5). In previous studies, the negative charge density of the acidic tail abolished binding of an HMG1domB-acidic tail fragment to cisplatin-modified DNA (Chow et al., 1995).

All three isolated HMG domains specifically recognize this DNA duplex when a bifunctional cisplatin 1,2-intrastrand cross-link is present. Differences are noted, however, in the affinities of each of these proteins for ds15AG*G*A. As shown in Figure 3.15B, the affinities for ds15AG*G*A increase in the order tsHMGdomA < HMG1domB < HMG1domA. Although they have similar affinities for four-way junction DNA (Bianchi et al., 1992), HMG1domA and HMG1domB interact differently with the same cisplatin-modified duplex. In fact, the $K_d$ values determined for the interactions of
HMG1domA or HMG1domB with ds15AG*G*A differ by approximately 2 orders of magnitude (Table 3.4).

Factors such as protein size, net charge and amino acid composition can contribute to the differences in binding observed for isolated HMG-domain proteins. The gel mobilities of the tsHMGdomA-ds15AG*G*A complex and the HMG1domB-ds15AG*G*A complex are significantly greater (greater mobility = less distance shifted) than that observed for the HMG1domA-ds15AG*G*A complex (Figure 3.15B, compare lanes 7 and 15 to lane 11). Since gel mobility is a function of size and charge, the lower mobility of the HMG1domA-ds15AG*G*A complex may result from the larger size and higher net positive charge of HMG1domA as compared to the other domains (Table 3.5).

In particular, the length of the basic C-terminal tail and the amino acid composition within the DNA-binding regions of these HMG domains may affect their interactions with the platinum-modified DNA duplex. The inclusion of a short, basic C-terminal region in HMG1domA could account for its greater affinity for ds15AG*G*A when compared to the other domains (Figure 3.17). Although this basic region did not affect the affinity of HMG1domA for globally platinated DNA (Farid et al., 1996), a similar but longer region in the LEF-1 domain spans the major groove of the consensus DNA target, establishing multiple contacts and increasing the affinity for and the bend angle of a consensus DNA sequence (Lnenicek-Allen et al., 1996, Love et al., 1995). Moreover, the basic region following the HMG domain in HMG-D, the Drosophila analog of HMG1, increases the protein affinity for four-way junction and linear DNA (Payet & Travers, 1997). The string of basic
residues following HMG1domB in the full HMG1 sequence (Bianchi et al., 1992) and tsHMGdomA in the full tsHMG sequence (Boissonneault & Lau, 1993) was not included in the present constructs, and their affect on binding or bending of cisplatin-modified DNA has not been reported.

**Stoichiometry and Nonspecific Interactions.** The expression in eq 1 allows for the quantitation of a simple bimolecular binding interaction with 1:1 stoichiometry. Verification of such a stoichiometry between an isolated HMG domain and a short cisplatin-modified duplex was therefore attempted by using gel electrophoretic methods. The ds15AG*G*A probe was titrated with stoichiometric amounts of HMG1domA (Figure 3.18, top). A plot of θ versus equivalents of added protein (Figures 3.18, bottom) was linear at both pre- and post-saturation limits. The intersection of the best fit lines to the experimental data indicated that 1.0 ± 0.2 equiv of HMG1domA was required for saturation of binding to ds15AG*G*A.

Because a discrete, shifted band did not appear in gel mobility shift studies of unplatinated duplexes or single-stranded oligonucleotides with isolated HMG-domain proteins, nonspecific interactions formed by these probes could not be directly quantitated. Instead, platinum- and duplex-independent binding of HMG-domain proteins to oligonucleotide probes was investigated by using competition assays. As indicated in Figure 3.19 (top), addition of unlabeled ds15AG*G*A duplex competes away the binding of HMG1domA to labeled ds15AG*G*A. A plot quantitating these competition data is presented in the bottom panel of Figure 3.19, together with competition data from analogous experiments in which ds15AGGA, ts15AGGA or ts15AG*G*A were used as competitors. The dissociation
constants for the ds15AG*G*A and ds15AGGA competitors, as determined from the best fits of these data to eq 2, are given in Table 3.4. Because the single-stranded oligonucleotides were such poor competitors, results for these oligonucleotides could not be accurately fit to eq 2.

Binding to single-stranded DNA by HMG1 protein has been reported (Kohlstaedt & Cole, 1994a) and, under certain salt and pH conditions, can be greater than interaction of HMG1 with linear double-stranded DNA (Butler et al., 1985, Kohlstaedt & Cole, 1994a). Recognition of non-canonical DNA structures such as cruciform DNA by HMG1 or its isolated domains, however, is more effective than recognition of linear double-stranded or single-stranded DNA (Bianchi et al., 1992). The competition studies with ts15AGGA and ts15AG*G*A reported here agree with the latter results and indicate that the interaction of these single-stranded oligonucleotides with HMG1domA is more than 3 orders of magnitude weaker than the structure-specific recognition of the cisplatin-modified ds15AG*G*A duplex.

The stoichiometry and competition results demonstrate that a single, bifunctional platinum lesion can increase the affinity of an HMG-domain protein for a 15-bp DNA duplex by almost three orders of magnitude (Table 3.4). In accord with previous studies of isolated HMG-domain interactions with cisplatin-modified DNA (Berners-Price et al., 1997, Chow et al., 1995, Farid et al., 1996, Kane & Lippard, 1996), HMG1domA forms a specific complex with ds15AG*G*A but not with the corresponding unmodified duplex or single-stranded oligonucleotides. Under the conditions reported here, the interaction between HMG1domA and ds15AG*G*A is very strong ($K_d = 1.6 \pm 0.2$ nM), has 1:1 stoichiometry (Figure 3.18), and decreases by a factor of ~1000
(\(K_d = 1.6 \pm 0.3 \, \mu M\)) when the bifunctional platinum lesion is not present (Figure 3.19). A 1000-fold specificity factor is unprecedented for the interaction between an isolated HMG domain and a site-specifically cisplatin-modified duplex and is greater than the specificity of full length HMG1 binding to site-specifically cisplatin-modified DNA (Pil & Lippard, 1992).

**DNA Sequence Context Modulates HMG Domain Recognition of Cisplatin-Modified 15-bp DNA Duplexes.** Initial NMR titrations of HMG1domB with ds15TG*G*T, described in the previous section, indicated that optimization of protein-DNA complex formation, either by variation of the DNA sequence or the protein composition, would be required for high resolution structural studies. In an attempt to deduce an optimal protein-DNA complex for such studies, the effect of the DNA sequence context on HMG-domain protein recognition of a cisplatin 1,2-intrastrand cross-link was investigated. A series of nine cisplatin-modified 15-bp DNA duplexes (ds15N1G*G*N2, Table 3.2), which differ in base pair composition directly adjacent to the platinum lesion, was screened for binding by HMG1domA and HMG1domB. Figure 3.20 (top) shows the gel mobility shift assay of each oligonucleotide in this series with HMG1domA at a constant protein concentration. Qualitative comparisons of \(\theta\) for each oligonucleotide, averaged from three independent experiments, with 5 nM HMG1domA or 300 nM HMG1domB are presented at the bottom of Figure 3.20. Titration experiments were carried out for the strongest and weakest binding oligonucleotides for both proteins (data not shown), and \(K_d\) values (Table 3.4) were determined from nonlinear fits of these data to eq 1. Similar titration experiments between HMG1domA and ds16TG*G*AC were also performed.
and afforded the $K_d$ value reported in Table 3.4. To be sure that differences in protein-binding to this series of 15-bp oligonucleotides did not result from intra- to interstrand platinum adduct rearrangements (Yang & al., 1996), following all binding studies, the radiolabeled duplexes were resolved on a 20% denaturing gel (data not shown). No evidence for intrastrand cross-link formation was observed for any sequence under any preparation conditions.

To assess the effect of DNA sequence context in an extended oligonucleotide, gel mobility shift assays were also carried out between HMG-domain proteins and two site-specifically cisplatin-modified 15-bp sequences embedded in a 159-bp DNA duplex. The probes were constructed as previously described (Zamble et al., 1996) with the incorporation of $\text{ts15N}_1\text{G}^*\text{G}^*\text{N}_2$ ($\text{N}_1 = \text{N}_2 = \text{dA or dC}$) and the corresponding $\text{bs27N}_3\text{CCN}_4$ ($\text{N}_3 = \text{N}_4 = \text{T or dG}$) internal fragments (see Table 3.1). In Figure 3.21A, no interaction of either HMG1domA or HMG1domB is observed with the unmodified ds159AGGA probe (lanes 2-5) in the presence of 200 ng of chicken erythrocyte competitor DNA. Under the same conditions, complex formation was near the detection limit for either protein with the cisplatin-modified ds159CG$^*\text{G}^*\text{C}$ (lanes 7-10). HMG1domA, however, recognized ds159AG$^*\text{G}^*\text{A}$ with a dissociation constant < 50 nM (estimated from half-maximal binding, lanes 12 and 13), while this same probe was only weakly bound by HMG1domB (lanes 14 and 15). Full-length HMG1 protein binding to these long probes was also investigated (Figure 3.21B). Although a small amount of nonspecific recognition of ds159AGGA occurred at the highest HMG1 concentrations (lane 5), moderate binding with ds159CG$^*\text{G}^*\text{C}$ (lanes 9 and 10)
and significantly stronger binding to ds159AG*G*A (lanes 14 and 15) was observed.

**HMG1domA Binding to ds15N1G*G*N2.** The interaction of HMG1domA with a site-specifically cisplatin-modified 15-bp DNA duplex is modulated by the nature of the base pairs flanking the platinum lesion. The apparent dissociation constants measured for HMG1domA binding to this series of cisplatin-modified duplexes range over 2 orders of magnitude from 1.6 ± 0.2 nM to 517 ± 60 nM (Table 3.4). A striking trend was observed for the DNA sequence preference of HMG1domA binding to dsN1G*G*N2 oligonucleotides. The dominant base pair preference was apparent 3' to the platinum lesion where, regardless of N1, affinity decreased as N2 = dA > T > dC (Figure 3.20). This sequence preference persisted in the site-specifically cisplatin-modified 159-bp probes (Figure 3.21A).

The base pair located 3' to the platinum lesion has unique hydrogen-bonding and minor groove accessibility as shown in the solid state structure of a cisplatin-modified dodecamer (Takahara et al., 1996). In addition, footprinting studies revealed that the position immediately 3' to the platinum lesion, irrespective of the nature of the base, is one of only two positions in cisplatin-modified DNA which is hypersensitive to cleavage by the minor groove binding protein, DNase I (Locker et al., 1995, Schwartz & Leng, 1994, Visse et al., 1991).

There are two likely explanations for the N2-N3 base pair preference of HMG1domA binding to cisplatin-modified DNA. The first is that, in the absence of an HMG domain, the platinated DNA duplexes in solution will be differentially flexible and/or bendable depending on the nature of the N2-N3
base pair (A-T, T-A, or C-G). Preliminary thermal denaturation data (Pilch et al., 1997) indicate that, although the overall cisplatin-induced thermal destabilizations of ds15TG*G*T ($\Delta \Delta G_{25^\circ C} = 5.3$ kcal/mol) and ds15CG*G*C ($\Delta \Delta G_{25^\circ C} = 4.1$ kcal/mol) are similar, the enthalpic ($\Delta \Delta H_{TGGT} > \Delta \Delta H_{CGGC}$) and entropic ($\Delta \Delta S_{TGGT} > \Delta \Delta S_{CGGC}$) contributions are very different. These findings suggest that there may be significant differences in these cisplatin-modified duplexes in the absence of HMG domain proteins. Clearly, additional comparisons of duplex stabilities and high resolution structures of site-specifically cisplatin-modified duplexes in which the nature of the 3' base pair is varied, are required to assess the extent of such contributions.

The second possibility is that the observed DNA sequence preferences arise from base-specific protein-DNA contacts. The high resolution protein-DNA structures available for hSRY (Werner et al., 1995) and LEF-1 (Love et al., 1995) HMG domains complexed with their consensus DNA binding sequences $d(GCACAAAC).d(GTTTGTGC)$ and $d(GAGCTTCAAAGGGTG).d(C-ACCCCTTTGAAGCTC)$, provide some clues about key protein-DNA contacts which may be responsible for the observed $N_2$ preferences of HMG1domA interaction with ds15N$_1$G*G*N$_2$. In the SRY-DNA and LEF-1-DNA complexes, a hydrophobic residue at position 17 (Figure 3.17, HMG1domA numbering), an isoleucine in SRY and a methionine in LEF-1, intercalates between two adjacent adenosine bases. These nucleotides, denoted by bold face in the above sequences, form the major bend locus of the duplex. The roll at the point of intercalation, 19° in hSRY-DNA and 52° in LEF1-DNA, is comparable to that induced by cisplatin-modification at the G*G* site in a DNA duplex (Takahara et al., 1996, Takahara et al., 1997). An A-T base pair
occurs in the SRY and LEF-1 DNAs just 3' to the intercalated bases and corresponds to the A-T base pair which is preferred by HMG1domA in the cisplatin-modified duplex. A hydrogen bond is formed between the keto oxygen atom at position 2 of the thymine base in this pair (T₁₀ in SRY-DNA; T₂¹ in LEF-1-DNA) and the protein residue at position 42 (HMG1domA numbering scheme, Figure 3.17) in both the SRY and LEF-1 domains. This protein residue is highly conserved as either serine or asparagine in all sequence-specific HMG domains. Although position 42 tends to be occupied by alanine, valine or lysine in the structure-specific HMG domains, HMG1domA has a serine in this position. Like S₉₄ of SRY and N₃₃₀ of LEF-1, where numbering refers to the full length proteins, S₄₂ in HMG1domA may form a hydrogen bond to O₂ of this thymine base in ds₁₁₅N₁G*G*N₂, accounting for the preference for dA at the N₂ position.

Studies were therefore carried out with a short, cisplatin-modified duplex in which the base pair two steps removed from the 3' side of the platinum lesion was modified to agree with the target sequence in the hSRY-DNA complex. This duplex, ds₁₁₆TG*G*AC (Table 3.1), contains a C-G base pair at this position. The guanine base in the corresponding C-G base pair of the hSRY-DNA complex (Werner et al., 1995) forms a specific contact with S₃₃ of hSRY (residue at position 39 in Figure 3.17, HMG1domA numbering). Since HMG1domA also has a serine residue at this position, the contribution of S₃₉ to the interaction with ds₁₁₆TG*G*AC was investigated. Gel titration studies indicated a $K_d$ value of $7.2 \pm 1.4 \text{ nM}$ for this interaction. This binding is comparable to that of HMG1domA with ds₁₁₅TG*G*A (Table 3.4). Assuming that no other specific protein-DNA contacts are disrupted, the
result indicates that the putative S39 contact does not significantly improve the protein-DNA interaction.

**HMG1domB Binding to ds15N1G*G*N2.** As observed with HMG1domA, the base pairs flanking the platinum lesion also modulate HMG1domB binding to a 15-bp cisplatin-modified duplex (Figure 3.20). The apparent dissociation constants measured for these interactions span more than one order of magnitude from 48 ± 9 nM to 1.3 ± 0.2 μM (Table 3.4), with subtle but observable trends in base pair preferences. Unlike HMG1domA, HMG1domB affinity decreased with $N_1 = dA > T = dC$, and with $N_2 = T > dA ≥ dC$ (Figure 3.20, bottom).

The most notable difference between HMG1domA and HMG1domB preferences is at the base pair flanking the 3' side of the platination site (N2-N3). Although both proteins prefer $N_2 = T > dC$ (Figure 3.20), HMG1domA has a strong preference for $N_2 = dA$ at this position, whereas HMG1domB does not. The previous argument highlighting the potential importance of the protein residue at position 42 (Figure 3.17, HMG1domA numbering), in addition to supporting the $N_2 = dA$ preference of HMG1domA, also provides a potential explanation for the lack of such a preference with HMG1domB. HMG1domB, like many other structure-specific HMG domains, has an alanine residue at position 42, which is unlikely to form base-specific contacts at this apparently crucial base pair.

Although the base pair preferences are not the same for HMG1domA and HMG1domB binding to ds15N1G*G*N2, the best oligonucleotide probes for either domain have A/T base pairs flanking the platinum lesion (AG*G*A for HMG1domA and AG*G*T for HMG1domB). The fewer
number of hydrogen bonds in an A/T base pair should increase the flexibility of the DNA near the platinum lesion, facilitating additional DNA bending observed upon protein binding (Chow et al., 1994). Increased bendability around the platinum lesion, however, does not explain all observed preferences, since HMG1domA prefers AG*G*A over TG*G*T and HMG1domB prefers AG*G*T over TG*G*A (Figure 3.20, bottom). Preferences for DNA bases surrounding the platinum lesion in a cisplatin-modified duplex were previously proposed to facilitate HMG-domain binding (Takahara et al., 1996). Analysis of the solid-state structure of d(CCTCTG*G*TCTCC).GGAGACCAGAGG suggested that, upon further bending, potential hydrogen-bonding interactions within the distorted duplex would be stabilized. In particular, it was suggested that duplex stability would be enhanced for N1 = T > dA = dC, which does not agree with the present experimental findings. It is clear that factors other than duplex flexibility and intraduplex hydrogen bonding are important in recognition of these cisplatin-modified DNAs by HMG domains.

**HMG1 Binding to ds159N1G*G*N2.** The above results suggest that, if the DNA binding observed for isolated HMG1domA and HMG1domB is representative of their activity within the full length HMG1 protein, then these domains may not contribute equally to HMG1 recognition of cisplatin-modified DNA. In fact, one might predict that HMG1domA, having a higher affinity for the cisplatin intrastrand cross-links in these 15-bp probes, should dominate the interaction between HMG1 and cisplatin-modified DNA. Because the 15-bp duplex oligonucleotides are not of sufficient length to address the binding of the full length HMG1 protein, site-specifically modified
15mer sequences were each incorporated into a 159-bp probe. For these studies, two sequences were selected which afford strong (AG*G*A) and weak (CG*G*C) recognition for both HMG1domA and HMG1domB (Figure 3.20). Although the DNA binding of HMG1 (Kd ~1.0-1.5 μM) to ds159AG*G*A is attenuated compared to that of HMG1domA (Kd < 50 nM), the same sequence-context dependence of cisplatin 1,2-intrastrand cross-link recognition is observed for both proteins (ds159AG*G*A >> ds159CG*G*C). Due to minimal binding (Figure 3.21A, lanes 9-10 and 14-15), little difference was observed for HMG1domB with the same probes. This result is consistent with HMG1domA dominating protein recognition of such platinum adducts by full length HMG1. Of possibly greater importance, however, is the verification that these sequence-context preferences of HMG domain binding are observed for the full length HMG1 protein, and are not limited to the chosen constructs of the isolated HMG domains.

**DNA Sequence Context Effects on Nonspecific HMG Domain Interactions.** Since the base pairs surrounding a d(GpG) site can modulate HMG-domain protein recognition of a cisplatin-modified duplex, the effect of these flanking sequence variations on nonspecific interactions was also investigated. Competition experiments (Figure 3.19) revealed that, for ds15CG*G*C (one of the weakest binding probes to HMG1domA), removal of the bifunctional platinum lesion resulted in only 2- to 3-fold loss in affinity (Kd = 1.6 ± 0.2 μM, Table 3.4). This apparent dissociation constant, which represents the nonspecific binding of HMG1domA to ds15CGGC, is the same as that observed for the interaction with ds15AGGA (1.6 ± 0.3 μM, Table 3.4) and suggests that the platinum-independent binding of HMG1domA to
duplex DNA is not affected by the DNA target sequence. In addition, competition experiments with ts15TGGT (data not shown) and the HMG1domA-ds15AG*G*A complex are identical to those with ts15AGGA (Figure 3.19, bottom). Although these single-stranded oligonucleotides are extremely poor competitors and their dissociation constants with the isolated HMG domain protein can only be estimated ($K_d > 1.3 \mu M$), it is clear that DNA sequence context does not affect this nonspecific interaction.

These data support the proposal that structure-specific HMG domains may have multiple binding modes to duplex DNA (Berners-Price et al., 1997, Teo et al., 1995). One mode, representing platinum-independent binding, is insensitive to DNA sequence context, in agreement with previous studies of structure-specific HMG-domain proteins with unmodified duplex or four-way junction DNA (Read et al., 1995). A second binding mode, which is specific for a cisplatin 1,2-intrastrand $d(GpG)$ lesion in duplex DNA, is very sensitive to DNA sequence context surrounding the platination site (this work).

The observed HMG-domain preferences for DNA sequence context at a cisplatin intrastrand cross-link should be considered in proposed mechanisms of protein-mediated cisplatin antitumor activity. Until now, possible mechanisms that link HMG-domain proteins to the biological activity of cisplatin, reviewed in McA’Nulty & Lippard (1995), have tacitly assumed that all cisplatin 1,2-intrastrand DNA cross-links bind with similar affinity to a given HMG-domain protein. Since the DNA sequence context surrounding the cisplatin 1,2-intrastrand cross-link can modulate HMG-domain protein affinity by more than 2 orders of magnitude, a subset of such platinum lesions
within optimum DNA sequence contexts could be more effectively shielded from repair and/or affect the natural functions of essential HMG-domain proteins. In this manner, the lethality of each platinum lesion on the genome may be dictated by the surrounding DNA sequence context as well as the particular bases to which platinum is coordinated. It is likely that other proteins, such as components of excision repair, mismatch repair, and the apoptotic apparatus, will recognize cisplatin 1,2-intrastrand cross-links differentially according to the DNA sequence context surrounding the platinum lesion.

Conclusions
In the absence of high resolution structural information, the spectroscopic and electrophoretic studies described herein provide details of the HMG-domain protein interaction with the major cisplatin adduct of duplex DNA. Gel studies revealed that a 15-bp duplex containing a centrally located cisplatin d(GpG) adduct was sufficient for specific recognition by the isolated HMG domains from two cellular proteins. Both EPR titrations and band shift assays identified complex formation between HMG1domB and either orientational isomer of a 20-bp duplex site-specifically modified with the asymmetric, paramagnetic platinum compound, cis-[Pt(NH₃)(4AT)Cl₂]. Fluorescence studies with HMG1domB and gel studies with HMG1domA determined ~1:1 stoichiometry for these protein interactions with short, cisplatin-modified DNA duplexes. Loss of HMG-domain recognition upon ribose sugar substitution in ds15TG*G*T indicated that a predominance for an A-type helix is not an exclusive requirement for this specific interaction. A
strong \((K_d = 1.6 \pm 0.2 \text{ nM})\), highly specific (1000-fold), duplex dependent interaction was identified for HMG1domA interaction with ds15AG*G*A, representing the largest specificity for HMG-domain binding to cisplatin-modified DNA reported to date.

In addition, this work provides the first evidence that variations in DNA and protein composition significantly affect the HMG-domain protein interaction with cisplatin-modified DNA in vitro. The information obtained here should be particularly useful for guiding experiments to determine the structures of HMG-domain protein complexes with cisplatin-modified duplex DNAs. The large range in protein binding constants resulting from modest changes in DNA sequence suggest that base variations farther from the platinum lesion, perhaps even those outside the 15-bp protein-binding region, may also affect HMG-domain protein binding (Benight et al., 1995). Sequence-dependent recognition of cisplatin-DNA adducts may also apply to cellular proteins outside of the HMG-1/-2 family, such as damage recognition proteins hMSH2 (Mello et al., 1996) and XPAC (Jones & Wood, 1993). In addition, alterations in flanking sequence may affect the binding of proteins to DNA damaged by agents other than cisplatin. Finally, these studies imply that, in vivo, the DNA sequence surrounding a platinum lesion in the genome, in addition to the nature of the platinum lesion, may be very important for recognition by cellular proteins. If cellular protein recognition and subsequent biological processing of cisplatin-modified DNA is active in the antitumor mechanism of this drug, then one may envision a generation of platinum drugs which optimally recruit cellular proteins by targeting not only GG sites but their specific sequence context as well.
References


Table 3.1. Deoxyribonucleotide Sequences and Abbreviations for the 12-, 16-, 20-, and 159-bp DNA duplexes\textsuperscript{a}

<table>
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<tr>
<th>Abbreviation</th>
<th>Duplex Oligonucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ds12GG</td>
<td>5'-TCTAGGCCTTCT-3'</td>
</tr>
<tr>
<td></td>
<td>3'-AGATCCGGAAGA-5'</td>
</tr>
<tr>
<td>ds16TGGAC</td>
<td>5'-CCTCTCTGGAGCTTCC-3'</td>
</tr>
<tr>
<td></td>
<td>3'-GGAGAGACCTGGAAGG-5'</td>
</tr>
<tr>
<td>ds20GG</td>
<td>5'-TCTCCTCTGGTCTTCTC-3'</td>
</tr>
<tr>
<td></td>
<td>3'-AGAGGAAGACCAGAGAAGAG-5'</td>
</tr>
<tr>
<td>ds159AGGA\textsuperscript{b}</td>
<td>5'-CCTCTCAGGATCTTC-3'</td>
</tr>
<tr>
<td></td>
<td>3'CTTAAGGGAGAGTCTAGAAGACTCCG-5'</td>
</tr>
<tr>
<td>ds159CGGC\textsuperscript{b}</td>
<td>5'-CCTCTCCGGCTCTTC-3'</td>
</tr>
<tr>
<td></td>
<td>3'CTTAAGGGAGAGGCCGAGAGACTCCG-5'</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Bold face type indicates the site of bifunctional platinum modification. \textsuperscript{b}This sequence corresponds to the internal fragment used in ligations as described by Zamble et al. (1996).
Table 3.2. Deoxyribonucleotide Sequences and Abbreviations for the Platinum-Modified Strands of 15-bp DNA duplexesa

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Duplex Oligonucleotide Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts15AGGA</td>
<td>5'-CCTCTCAGGATCTTC-3'</td>
</tr>
<tr>
<td>ts15AGGT</td>
<td>5'-CCTCTCAGGTTCTTC-3'</td>
</tr>
<tr>
<td>ts15AGGC</td>
<td>5'-CCTCTCAGGCTCTTC-3'</td>
</tr>
<tr>
<td>ts15TGGA</td>
<td>5'-CCTCTCTGGATCTTC-3'</td>
</tr>
<tr>
<td>ts15TGGT</td>
<td>5'-CCTCTCTGGTTCTTC-3'</td>
</tr>
<tr>
<td>ts15TGGC</td>
<td>5'-CCTCTCTGGCTCTTC-3'</td>
</tr>
<tr>
<td>ts15CGGA</td>
<td>5'-CCTCTCCGGATCTTC-3'</td>
</tr>
<tr>
<td>ts15CGGT</td>
<td>5'-CCTCTCCGGTTCTTC-3'</td>
</tr>
<tr>
<td>ts15CGGC</td>
<td>5'-CCTCTCCGGCTCTTC-3'</td>
</tr>
</tbody>
</table>

aBold face indicates basepair variations.
Table 3.3. Ribose-containing Oligonucleotide Sequences and Abbreviations$^a$

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>hTGGT</td>
<td>5′-CCTCTCTGTCTTC-3′</td>
</tr>
<tr>
<td></td>
<td>3′-ggagaccaagaag-5′</td>
</tr>
<tr>
<td>cTGGT</td>
<td>5′-CCTCCTCTGTCTTC-3′</td>
</tr>
<tr>
<td></td>
<td>3′-GGAGAgaccaagaag-5′</td>
</tr>
</tbody>
</table>

$^a$Upper case and lower case lettering denotes deoxyribonucleotides and ribonucleotides, respectively, whereas bold face indicates the site of bifunctional platinum modification.
Table 3.4. $K_{d(app)}$ Values Determined for HMG-domain Protein Interactions with Oligonucleotides$^a$

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>HMG1domA</th>
<th>HMG1domB</th>
</tr>
</thead>
<tbody>
<tr>
<td>ds15AG<em>G</em>A</td>
<td>$1.6 \pm 0.2 \text{ nM}$</td>
<td>$134 \pm 18 \text{ nM}$</td>
</tr>
<tr>
<td></td>
<td>$6.8 \pm 0.8 \text{ nM}^b$</td>
<td></td>
</tr>
<tr>
<td>ds15AG<em>G</em>T</td>
<td></td>
<td>$48 \pm 9 \text{ nM}$</td>
</tr>
<tr>
<td>ds15TG<em>G</em>A</td>
<td>$2.9 \pm 0.5 \text{ nM}$</td>
<td></td>
</tr>
<tr>
<td>ds16TG<em>G</em>AC</td>
<td>$7.2 \pm 1.4 \text{ nM}$</td>
<td></td>
</tr>
<tr>
<td>ds15TG<em>G</em>C</td>
<td>$127 \pm 17 \text{ nM}$</td>
<td></td>
</tr>
<tr>
<td>ds15CG<em>G</em>A</td>
<td>$8 \pm 3 \text{ nM}$</td>
<td>$1.1 \pm 0.2 \mu\text{M}$</td>
</tr>
<tr>
<td>ds15CG<em>G</em>C</td>
<td>$517 \pm 60 \text{ nM}$</td>
<td>$1.3 \pm 0.2 \mu\text{M}$</td>
</tr>
<tr>
<td>ds15AGGA</td>
<td>$1.6 \pm 0.3 \mu\text{M}^b$</td>
<td></td>
</tr>
<tr>
<td>ds15CGGC</td>
<td>$1.6 \pm 0.2 \mu\text{M}^b$</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Measured by direct titration unless otherwise specified. $^b$Measured from competition experiments.
Table 3.5. Selected Characteristics of HMG-Domain Proteins

<table>
<thead>
<tr>
<th>Protein</th>
<th>pI\textsuperscript{a}</th>
<th>Net Charge\textsuperscript{b}</th>
<th>Mass (kD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HMG1</td>
<td>5.5</td>
<td>-5</td>
<td>25</td>
</tr>
<tr>
<td>HMG1\textsubscript{domA}</td>
<td>10.6</td>
<td>+12</td>
<td>10.4</td>
</tr>
<tr>
<td>HMG1\textsubscript{domB}</td>
<td>10.5</td>
<td>+9</td>
<td>9.2</td>
</tr>
<tr>
<td>tsHMG\textsubscript{domA}</td>
<td>10.4</td>
<td>+6</td>
<td>9.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Calculated with the GCG Sequence Analysis Software Package, Version 7.0.

\textsuperscript{b}Charge calculated at pH 7.5.
Figure 3.1. Reversed phase HPLC traces of enzymatic digestions of cisplatin-modified and unmodified DNAs (Table 3.2). Peak identities were determined by coinjections with original standards, where dC, dG, dA and T are the mononucleosides, dA* is a side-product from the digestion of dA with excess P1 nuclease, and Pt(dGpG) is [Pt(NH$_3$)$_2$(d(GpG))]$^+$. 
Figure 3.2 Reversed phase HPLC traces of enzymatic digestions of cisplatin-modified and unmodified DNAs (Table 3.2). Peak identities were determined by coinjections with original standards, where dC, dG, dA and T are the mononucleosides, dA* is a side-product from the digestion of dA with excess P1 nuclease, and Pt(dGpG) is $[\text{Pt(NH}_3\text{)}_2\text{d(GpG)}]^+$. 
Figure 3.3. Reversed phase HPLC traces of enzymatic digestions of cisplatin-modified and unmodified DNAs (Table 3.2). Peak identities were determined by coinjections with original standards, where dC, dG, dA and T are the mononucleosides, dA* is a side-product from the digestion of dA with excess P1 nuclease, and Pt(dGpG) is [Pt(NH$_3$)$_2$(d(GpG))]$^+$. 
**Figure 3.4.** Reversed phase HPLC traces of enzymatic digestions of cisplatin-modified and unmodified DNAs (Table 3.1). Peak identities were determined by coinjections with original standards, where dC, dG, and T are the mononucleosides, and Pt(dGpG) is cis-[Pt(NH$_3$)$_2$(dGpG)]$^+$. 
Figure 3.5. EPR spectra of cis-[Pt(NH$_3$)$_3$(4AT)(H$_2$O)$_2$]$^{2+}$, the two orientational isomers formed upon modification of ts20, and the two duplex oligonucleotides formed after annealing with bs20.
Figure 3.6. EPR spectra of ds20A and ds20B isomers upon addition of HMG1domB.
Figure 3.7. Theoretical plot of protein fluorescence upon addition of protein ($P$) to ligand ($L$).
Figure 3.8. Fluorescence quenching of HMGdomB in the presence of cisplatin-modified (A) 20-bp and (B) 15-bp oligonucleotides.
Figure 3.9. Relative sizes of short DNA duplexes (shown as B-form helices) and HMG1domB (Weir et al., 1993).
Figure 3.10. Change in $^{15}$N-$^1$H HSQC of HMGdomB upon addition of ds15TG*G*T. (A) HMGdomB, 150 mM NaCl. (B) HMGdomB + 0.25 equiv of ds15TG*G*T, 150 mM NaCl. (C) HMGdomB + 1.0 equiv of ds15TG*G*T, 500 mM NaCl.
Figure 3.11. Gel mobility shift assays of 20-bp oligonucleotides (10 μM) with HMGdomB in 100 mM sodium phosphate, pH 7.3 at 4°C: Lane 1, ds20GG with 40 μM HMGdomB; Lanes 2-3, ds20G*G*; Lanes 4-5, ds20A; Lanes 6-7, ds20B. Platinum modified duplexes contain 0 and 16 μM HMG1domB in each pair of lanes.
Figure 3.12. Gel mobility shift assays of 60nM ds12G*G* (lanes 1-3), 60nM ds15TG*G*T (lanes 4-6) and 60 nM ds20G*G* (lanes 7-9). Protein concentrations are 0 nM (lanes 1, 4, and 7), 60 nM (lanes 2, 5, and 8) and 600 nM (lanes 3, 6, and 9).
Figure 3.13. Gel mobility shift assays of 5 nM ds15TGTT (lanes 1-3), hTGTT (lanes 4-6) and cTGTT (lanes 7-9) with (A) HMG1domA and (B) HMG1domB. Protein concentrations are 0 nM (Lanes 1,4 and 7), 250 nM (lanes 2, 5 and 8), and 500 nM (lanes 3, 6 and 9).
Figure 3.14. Gel mobility shift assays of 5 nM ds15TG*G*T (lanes 1-3), hTG*G*T (lanes 4-6) and cTG*G*T (lanes 7-9) with (A) HMG1domA and (B) HMG1domB. Protein concentrations are 0 nM (Lanes 1, 4 and 7), 250 nM (lanes 2, 5 and 8), and 500 nM (lanes 3, 6 and 9).
Figure 3.15. Gel mobility shift assays of (A) ds15AGGA (0.45 nM) and (B) ds15AG*A*G*A (0.45 nM) with HMG1 (lanes 1-4), tsHMGdomA (lanes 5-8), HMG1domA (lanes 9-12) and HMG1domB (lanes 13-16). Protein concentrations in each quartet increase as follows: 0 nM, 10 nM, 100 nM, and 1000 nM.
Figure 3.16. Gel mobility shift assay analysis of the titration of ds15AG*G*A (0.45 nM) with HMGdomB (0.1 nM to 178 μM) (top). Plot of the fraction of bound DNA vs. [HMGdomA] (closed circles) or [HMGdomB] (open circles) with the superimposed fits to eq 1 (bottom).
Figure 3.17. Sequence alignment and secondary structure of the HMG domains from several proteins. Numbers at the start of each amino acid sequence represent the residue numbering in the full length proteins.
Figure 3.18. Gel mobility shift assay of 100 nM dsAG*G*A with increasing amounts of HMG1domA (top). Fit of the binding data to determine the stoichiometry of the interaction (bottom). The intersection of the two lines indicates that saturation of binding of the duplex occurs as 1.0 ± 0.2 equivalents of protein.
Figure 3.19. Gel mobility shift assay analysis of the self-competition of ds15AG*G*A-HMG1domA complex with unlabeled competitor ds15AG*G*A (top). Plot of the fraction of bound DNA vs. competitor concentration for ds15AG*G*A (triangles), ds15AGGA (open circles), ds15CGGC (closed circles), ts15AGGA (open squares), and ts15AG*G*A (closed squares) competitors with the ds15AG*G*A-HMG1domA complex (bottom). Fits of the data to eq 3, where possible, are superimposed.
Figure 3.20. Gel mobility shift assay of each DNA sequence, ds15N₁G*G*N₂ (Table 3.2) with 5.0 nM HMG1domA (top). Lane 1 contains ds15AG*G*A without protein. Bar graph illustrating the fraction of bound DNA (θ) for each sequence (bottom) at 5.0 nM HMG1domA (black bars) and 300 nM HMG1domB (gray bars). Values are the average of three independent experiments and error bars represent ± 1 standard deviation.
Figure 3.21. Gel mobility shift assays of:
(A) ds159AGGA (lanes 1-5), ds159CG*G*C (lanes 6-10), and ds159AG*G*A (lanes 11-15) with HMG1domA (50 nM, lanes 2, 7 and 12; 100 nM, lanes 3, 8 and 13) and HMG1domB (50 nM, lanes 4, 9 and 14; 100 nM, lanes 5, 10 and 15).

(B) ds159AGGA (lanes 1-5), ds159CG*G*C (lanes 6-10), and ds159AG*G*A (lanes 11-15) with HMG1 (0, 0.5, 1.0, 1.5, and 2.0 μM).
BIOGRAPHY

The author was born Shari Ann Uldrich on June 2, 1970 in Toms River, NJ. After 12 years of catholic education she graduated from Monsignor Donovan High School in 1988. She then attended Drew University, where she participated in research in the laboratories of Drs. George de Stevens and Mary-Ann Pearsall. After graduating summa cum laude with honors in Chemistry in 1992, a passion for bioinorganic chemistry led her to the laboratory of Dr. Stephen J. Lippard. While pursuing her graduate work at MIT, she met and married Stephen Dunham. Following graduation, she will return to her home state as a Visiting Assistant Professor of Chemistry at Drew University.