Cisplatin−DNA Binding Specificity of Calf High-Mobility Group 1 Protein†

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ABSTRACT: We have identified a series of proteins with an affinity for cisplatin-damaged DNA using damaged DNA affinity chromatography. We have purified one of these proteins to homogeneity on the basis of a mobility shift assay detecting binding to cisplatin-damaged DNA. The protein was identified as high-mobility group 1 protein (HMG-1) by N-terminal protein sequence analysis. Analysis of a variety of DNA structures revealed that fully duplex DNAs were the best substrates for HMG-1 binding, while partial duplexes were less avidly bound. The decreased levels of binding are attributed to the length of the duplex region of the DNA substrates. A 3-fold increase in binding was observed when a cisplatin-damaged DNA substrate containing a single break in the phosphodiester backbone was joined by DNA ligase. The strict DNA size dependence of binding was also assessed, and a 10-fold increase in binding was observed when the length of the DNA duplex was increased from 44 to 180 base pairs (bp) at the same level of cisplatin damage. HMG-1 binding also was correlated with the degree of cisplatin−DNA damage, suggesting a higher affinity for DNA containing multiple cisplatin adducts. Nuclease degradation of the cisplatin-damaged DNA demonstrated that at the lowest levels of cisplatin damage all of the substrates contained at least one cisplatin adduct. The potential role of HMG-1 in the repair of cisplatin−DNA adducts is discussed.

cis-Diamminedichloroplatinum(II) (cisplatin)[1] is a chemotherapeutic drug used to treat a variety of cancers and is the treatment of choice for ovarian and testicular cancer. Cisplatin forms covalent DNA adducts, induces cell cycle arrest, and initiates the apoptotic pathway (Chu, 1994). Following clinical treatment with cisplatin, recurrence and resistance often occurs (Perez et al., 1993). The mechanism by which cells become resistant to further cisplatin treatment is not completely understood. Increased efflux, decreased influx, and drug sequestration are all possibilities (Johnson et al., 1993; Eastman, 1991; Chu, 1994). Treatment of cisplatin resistant cells with aphidicolin, a potent inhibitor of DNA polymerases α, δ, and ϵ, restored sensitivity to cisplatin, suggesting repair of cisplatin−DNA adducts is involved in the resistance phenomenon (Masuda et al., 1988). Since this initial observation, increasing support has accumulated for DNA repair playing a role in cellular resistance to high levels of cisplatin (Calsou & Salles, 1993; Calsou et al., 1993; Johnson et al., 1994; Oldenburg et al., 1994).

Previous studies have assessed how purified proteins are able to metabolize cisplatin−DNA adducts. Using purified calf DNA polymerase ϵ, it was demonstrated that the 3′ to 5′ intrinsic exonuclease was unable to degrade DNA substrates containing 3′ terminal 1,2 d(GpG) or single dG cisplatin adducts (Huang et al., 1993a). Substrates containing internal 1,2 d(GpG) or dG monoadducts were efficiently degraded up to the position of the cisplatin adduct. The DNA polymerase ϵ synthetic activity was unable to extend a primer−template substrate if the 1,2 d(GpG) adduct was at the 3′ terminus of the primer. In addition, polymerase ϵ was incapable of bypass synthesis over a cisplatin adduct in the template strand (Huang et al., 1993a). Recently, it was shown that DNA polymerase β is capable of bypass synthesis over a site specific cisplatin adduct (Hoffmann et al., 1995). Helicase E has been proposed to be involved in DNA repair on the basis of its enzymatic properties (Turchi et al., 1992a). It was also recently demonstrated that calf DNA helicase E is capable of displacing cisplatin-damaged DNA primers but is unable to translocate across a cisplatin adduct on the template strand (Huang et al., 1993b). The ability to displace damaged DNA is consistent with this role in DNA repair. The coordinated helicase E/polymerase ϵ displacement/synthesis reaction (Turchi et al., 1992b) was also observed using cisplatin-damaged DNA primers (Huang et al., 1993b).

The identification of proteins with the ability to bind damaged DNA has been approached on many fronts. Mobility shift and Southwestern blotting using cisplatin-damaged DNA probes have been extensively utilized to identify cisplatin−DNA binding proteins (Donahue et al., 1990; McLaughlin et al., 1993; Andrews & Jones, 1991; Bissett et al., 1993; Chao et al., 1991; Clugston et al., 1992). In addition, the genes encoding structure specific recognition proteins SSRP1 (Bruhn et al., 1992) and Ixr1 (Brown et al., 1993) have been cloned. SSRP1 has homology to HMG proteins and was shown to bind cisplatin-damaged DNA (Bruhn et al., 1992). Sequence analysis of the Ixr1 gene revealed two HMG boxes, and deletion of *Ixr1* resulted in a cisplatin resistant phenotype (Brown et al., 1993). Consistent with HMG boxes being involved in mediation of binding to

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); D/N, drug/nucleotide; HMG, high-mobility group; PMSF, phenylmethanesulfonyl fluoride; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; bp, base pair; ct, calf thymus; ss, single-stranded.

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Table 1: DNA Oligonucleotides

DNA	sequence (listed 5′ to 3′)
(−20) 18-mer	GTAAAACGACGGCCAGTG
(−47) 25-mer	CGCCAGGGTTTTCCCAGTCACGACC
44-mer	GCACTGGCCGTCGTTTTACGGTCGTGACTGGGAAAACCCTGGCG
75-mer	TACCCGGGGATCCTCTAGAGTCGACCTGCAGGCATGCAAGCTTTTGTTCCCTTTAGTGAGGGTTAATTCCGAGCT
24-mer	ACGTCGGAATTAACCCTCACTAAA

cisplatin-damaged DNA, human HMG-1 and -2 were identified as cisplatin−DNA binding proteins using a damaged DNA precipitation assay (Hughes et al., 1992). Recombinant rat HMG-1 was shown to specifically bind cisplatin-damaged DNA, while no binding was observed with DNA modified by the clinically inactive *trans*-isomer (Pil & Lippard, 1992). More recently, purified HMG-1 was found to inhibit *in vitro* excision of cisplatin-damaged DNA by human cell free extracts (Huang et al., 1994).

While the specific DNA adducts to which HMG-1 binds have been extensively studied, little information concerning the effect of DNA structure on HMG-1 binding is available. In this paper, we provide a characterization of HMG-1 binding with respect to DNA structure and degree of cisplatin damage. The results are discussed with respect to the repair of cisplatin−DNA adducts.

EXPERIMENTAL PROCEDURES

Materials. Unlabeled nucleotides were from Pharmacia (Piscataway, NJ), and radiolabeled nucleotides were from New England Nuclear (Boston, MA). Sequenase version 2.0 was from United States Biochemical (Cleveland, OH). Mung bean nuclease was from Gibco/BRL (Gaithersburg, MD). All other reagents were purchased from standard suppliers. Oligonucleotides were synthesized on a Molecular Biosystems 390 DNA synthesizer and purified by 15% polyacrylamide/7 M urea preparative DNA sequencing gel electrophoresis (Sambrook et al., 1989). The DNA oligonucleotides used in this study are presented in Table 1. The 44 bp fully duplex DNA substrate was prepared by annealing 10 pmol of the 44-mer with 20 pmol of the 25-mer DNA oligonucleotide, heating to 65 °C for 10 min, and cooling to room temperature over the next hour in buffer containing 50 mM Tris-HCl (pH 7.5), 1 mM DTT, 50 mM NaCl, and 10 mM $MgCl_2$. The DNA was extended and labeled with 100 μM dATP, dCTP, dTTP, and [α-^{32}P]dGTP (3000 Ci/mmol, 100 μCi) using Sequenase. Extension reactions were performed for 30 min and then chased with 100 μM unlabeled dGTP to assure the 25-mer was fully extended. The specific activity of each DNA was determined by spotting an aliquot on DE-81 filter paper and washing three times in 0.5 M sodium phosphate buffer at pH 7.5 and radioactivity determined by liquid scintillation counting. Unincorporated nucleotides were removed by Sephadex G-50 spin column chromatography (Penefesky, 1977). The 130 bp and 180 bp fully duplex DNA substrates were prepared by polymerase chain reaction (PCR) amplification of the plasmid pBS+ (Stratagene, La Jolla, CA) as previously described (Turchi et al., 1994). The PCR products were gel purified by electrophoresis through 3% NuSeive GTG low-melting point agarose (FMC Bio products). The excised fragments were melted at 65 °C for 5 min and purified on Qiagen columns as described by the manufacturer (Qiagen, Chatsworth, CA). The purified DNA was quantified using the Hoechst 33258 fluorescent dye assay and a TKO minifluorometer according to the manufacturers' protocol (Hoefer, CA).

DNA Platination. The ^{32}P-labeled DNA substrates were incubated with varying concentrations of cisplatin in buffer containing 10 mM $NaHPO_4$ (pH 7.5) and 3 mM NaCl for 16 h at 37 °C in the dark. The DNA was purified from unreacted cisplatin by G-50 spin column chromatography (Penefsky, 1977). The radioactivity recovered was determined by liquid scintillation counting an aliquot of the eluate, and on the basis of the specific activity obtained in the original labeling reactions, the femtomoles of DNA recovered was calculated.

Mobility Shift Assay. Mobility shift assays were performed in 20 mM HEPES (pH 7.0), 1 mM DTT, 0.001% NP-40, 50 mM NaCl, 50 μg/mL bovine serum albumin, and 50 fmol of [^{32}P]DNA. Reactions were allowed to reach equilibrium by incubation on ice for 30 min. Reaction products were separated by native polyacrylamide gel electrophoresis (PAGE) at 150 V for 2 h. The gels were dried, and the products were visualized by autoradiography and quantified by phosphorimager analysis using ImageQuant software in volume integration mode (Molecular Dynamics, CA).

Purification of Calf DRP-2/HMG-1. A calf thymus S-100 homogenate was prepared from 400 g of fetal thymus tissue and fractionated on a Biorex-70 column and DEAE−Sepharose column as previously described (Siegal et al., 1992; Turchi & Bambara, 1993). Calf DRP-2/HMG-1 activity was monitored by mobility shift analysis. DEAE fractions containing maximum activity were pooled and dialyzed versus buffer A [50 mM Tris-HCl (pH 7.8), 20% glycerol, 1 mM DTT, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM PMSF] supplemented with 100 mM NaCl. The DEAE pool was loaded onto a 10 mL Whatman P-11 column equilibrated in the same buffer. Protein was eluted with a linear gradient from 100 to 600 mM NaCl in buffer A. Peak fractions were pooled and diluted to a conductivity of buffer A + 100 mM NaCl and loaded onto a 5 mL heparin−Sepharose column equilibrated in the same buffer. A linear gradient from 100 to 750 mM NaCl was used to elute proteins. Peak fractions were pooled and diluted in buffer B (50 mM KPO_4, 20% glycerol, 1 mM DTT, 1 mM EDTA, and 1 mM PMSF) to a conductivity equal to buffer B + 100 mM KCl. This fraction was applied to a 5 mL S-Sepharose column equilibrated in the same buffer. Protein was eluted with a linear gradient from 100 to 500 mM KCl in buffer B. Peak fractions were assayed and stored at −70 °C. The purified DRP-2/HMG-1 was desalted and further purified in batches on a 1 × 28 cm AcA34 gel filtration column equilibrated in buffer A + 100 mM NaCl. Twenty-five micrograms were applied, and 0.5 mL fractions were collected following the void volume. Ten microliters were employed in a mobility shift assay, and peak fractions were pooled and stored at −70 °C. Fifty microliters of each fraction were analyzed by 12% sodium dodecyl sulfate−

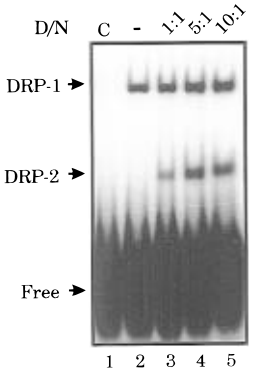

FIGURE 1: Mobility shift identification of DRP-1 and DRP-2. The ^{32}P-labeled 44 bp fully duplex DNA was constructed and treated with cisplatin at the D/N ratio stated in the figure, as described in Experimental Procedures. Lane 1 is a control reaction performed in the absence of added protein. The DNA substrates were purified and mobility shift reactions performed using 30 ng of protein, as described in Experimental Procedures. Reaction products were separated by 8% native PAGE and visualized by autoradiography. The free DNA and two DNA−protein complexes are denoted by the arrows and labeled accordingly.

polyacrylamide gel electrophoresis, performed according to Lammelli (1970). Gels were fixed and stained with silver according to Morrisey (1981). The final AcA-34 pool of DRP-2/HMG-1 was used for all experiments employing calf DRP-2/HMG-1.

RESULTS

Identification of Cisplatin−DNA Binding Proteins. We have initiated the study of proteins involved in the metabolism of cisplatin−DNA adducts. HeLa cell free extracts were initially fractionated on a cisplatin−DNA cellulose column. Cell free extracts were prepared from 2 L of HeLa cells in mid to late log phase and were applied to the cisplatin−DNA cellulose column in buffer A containing 100 mM NaCl. The column was washed with the same buffer and bound protein eluted with buffer A supplemented with 750 mM NaCl. The eluate was diluted to a conductivity equal to 100 mM NaCl and applied to a 20 mL phosphocellulose column. The phosphocellulose column was washed and bound protein eluted as described above. The eluted proteins were assayed for cisplatin−DNA binding using a mobility shift assay employing a ^{32}P-labeled 44 bp fully duplex DNA substrate which was constructed as described in Experimental Procedures. The 44 bp DNA substrate has potential sites for 1,2 d(GpG), 1,2 d(ApG), and 1,3 d(GpXpG) intrastrand adducts, dG monoadducts, and interstrand adducts. We chose this substrate so as not to exclude binding proteins which may exhibit specificity for a certain type of cisplatin adduct. The results of a typical mobility shift experiment are shown in Figure 1. In the absence of added protein, the free DNA substrate is observed migrating at the bottom of the gel (lane 1). A single protein−DNA complex migrating slower in the gel, termed DRP-1, was observed when the undamaged 44 bp DNA was employed (lane 2). Two distinct protein−DNA complexes were observed when the 44 bp DNA was damaged with cisplatin, the original DRP-1 shift, and a faster migrating protein−DNA complex termed DRP-2 (lanes 3−5). These results are consistent with other reports demonstrating two specific shifts using human cell extracts (Donahue et al., 1990; Andrews & Jones, 1991; Bissett et al., 1993; Chao et al., 1991). The minor increase in the level of DRP-1 binding observed upon damage with cisplatin (lanes 3−5) was variable in our assays. The faster-migrating shifted band termed DRP-2 was only observed in reactions involving the cisplatin-damaged DNA (Figure 1, lanes 3−5). The protein responsible for this shift, therefore, requires cisplatin for mediation of binding. The degree of cisplatin adduct formation on the DNA was assessed by nuclease degradation experiments and demonstrated that at a 1/1 D/N ratio greater than 90% of the DNA substrates contained at least one platinum adduct (data not shown). A low level of interstrand cross-links was also observed upon platination at a 1/1 D/N ratio. At D/N ratios of 5/1 and 10/1, the DNA substrates contained multiple adducts and a greater proportion of interstrand cross-links. The increased binding of DRP-2 is the result of either a higher affinity for DNA substrates containing multiple cisplatin adducts or a greater affinity for a specific adduct formed only at the higher D/N ratios. Initial attempts to purify DRP-2 from HeLa cells resulted in yields too low to definitively identify the protein responsible for the mobility shift. Therefore, using the mobility shift assay, we purified DRP-2 from calf thymus, as described in Experimental Procedures.

Comparison of Human and Calf DRP-2. To confirm that we had purified the same activity from human and calf, we performed a mobility shift with each separate protein and then equally mixed (Figure 2A). Preliminary experiments were performed to determine the protein concentration of each preparation necessary to obtain approximately equal binding activity (data not shown). Titration of human DRP-2 (lanes 2−6) yielded a single shifted band, identical in migration to that of calf DRP-2 (lanes 7−11). Lanes 12−16 show the results obtained when a mixture of calf and human DRP-2 was employed in a mobility shift. Again, a single shifted band was observed, demonstrating that calf and human DRP-2 have an identical mobility upon native polyacrylamide gel electrophoresis. As mentioned above, the amount of protein employed in the assays was varied to obtain a similar level of DNA binding for both DRP-2 preparations. The HeLa preparation of DRP-2 has a higher specific activity, and therefore, less protein was required to obtain similar levels of DNA binding. The maximum binding for each preparation was between 4 and 5 fmol.

To further demonstrate that in fact the calf and human DRP-2 proteins are similar, we assessed the cisplatin requirement for DNA binding. The 44 bp fully duplex [^{32}P]-DNA was treated with varying levels of cisplatin and analyzed in a mobility shift assay with both calf and human DRP-2. The results shown in Figure 2B demonstrate a nearly identical cisplatin dose response for the human and calf DRP-2. The protein level was 40 and 126 ng per reaction for the HeLa and calf DRP-2 preparations, respectively. A higher level of protein was employed to aid in detection of binding at the lower D/N ratios. Increased binding was observed at D/N ratios through 10/1. Quantification of the data was performed, and femtomoles bound was plotted versus D/N ratio. Linear regression analyses revealed identical slopes for both calf and HeLa DRP-2 with regression coefficients of 0.98 (data not shown). These results demonstrate that the calf and human DRP-2 display similar binding characteristics with respect to cisplatin damage dependence and, combined with identical migration in DNA complexes, suggest that the calf and human proteins are analogous. Interestingly, we observed increased binding of DRP-2 to

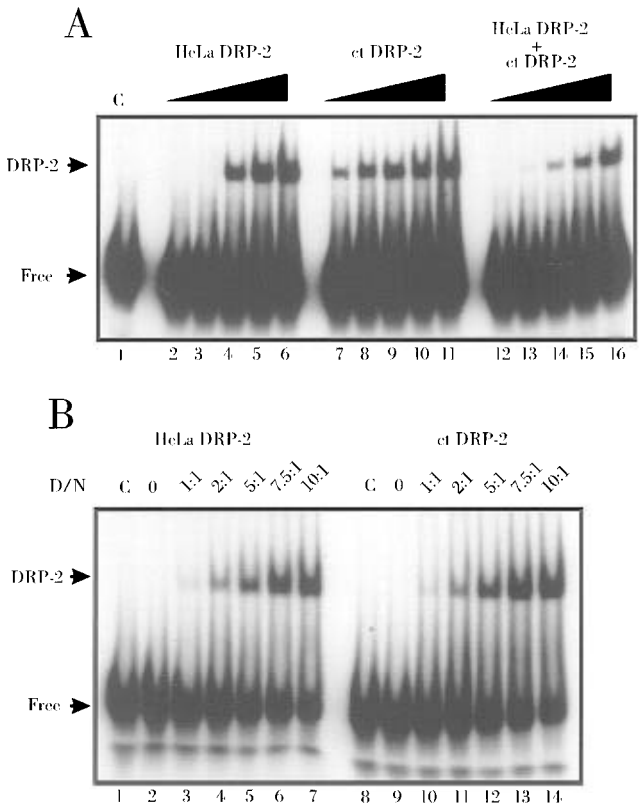

FIGURE 2: Calf and human DRP-2 both display identical migration and have identical cisplatin−DNA binding activity. (A) Increasing concentrations of human DRP-2 (lanes 2−6, 1, 2.5, 5, 10, and 25 ng), calf DRP-2 (lanes 7−11, 4, 8, 16, 40, and 80 ng), or an equal mixture of the two (lanes 12−16, 2, 5, 10, 20, and 50 ng) were analyzed in a mobility shift assay using the cisplatin-damaged 44 bp [^{32}P]DNA substrate. Reaction products were separated and visualized as described in Experimental Procedures. (B) HeLa DRP-2, 40 ng (lanes 2−7), and calf DRP-2, 126 ng (lanes 9−14), were incubated with the ^{32}P-labeled fully duplex 44 bp DNA substrate damaged by incubation with varying levels of cisplatin. The D/N ratio used in each reaction is shown in the figure. Lanes 1 and 8 are control reactions performed without added proteins. Reaction products were separated by 12% nondenaturing PAGE and visualized by autoradiography.

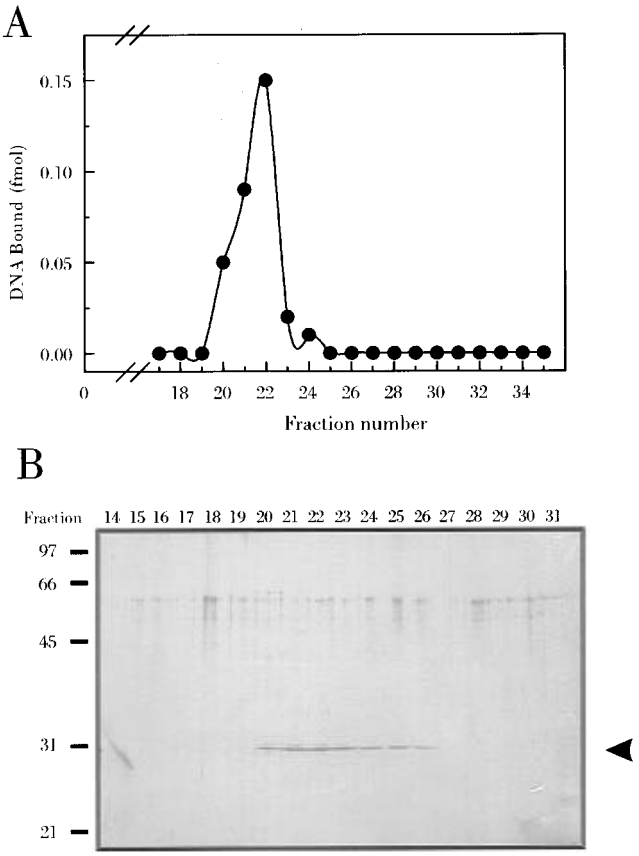

FIGURE 3: AcA34 gel filtration of calf DRP-2. Twenty-five micrograms of the final pool of calf DRP-2 was fractionated on an AcA34 column as described in Experimental Procedures. (A) An aliquot of each column fraction, 10 μL, was assayed for cisplatin−DNA binding by mobility shift assay using the duplex [^{32}P]44-mer platinated at a D/N ratio of 10/1. The amount of DNA bound was quantified by phosphorimager analysis using volume integration mode and ImageQuant software. (B) Fifty microliters from each fraction was analyzed by 12% SDS−PAGE, as described in Experimental Procedures. The position of molecular weight markers is shown on the left, and the arrowhead denotes the protein that comigrates with DRP-2 activity.

the more highly damaged DNA substrates, suggesting an increased affinity for substrates containing multiple cisplatin−DNA adducts.

Characterization of DRP-2 Protein. To identify the protein responsible for DNA binding, the final pool of calf DRP-2 was analyzed by AcA34 gel filtration chromatography. The results shown in Figure 3A reveal a peak of DNA binding activity, as determined by mobility shift analysis, in fractions 20−22. A protein of 30 kDa was found to coelute with calf ctDRP-2 binding activity (Figure 3B). Bands observed on the gel between 50 and 60 kDa are artifacts of the silver-staining procedure and are observed in all lanes independent of added protein. The elution position of DRP-2 corresponds to a high-molecular mass complex, similar to HeLa DRP-2 (data not shown), suggesting a multimeric complex of 30 kDa subunits. The elution of the 30 kDa protein does not correlate exactly with binding activity at the trailing edge of the peak of binding activity. This is likely the result of difficulty in quantification of the low level of DNA binding observed. AcA-34 gel filtration analyses of less pure fractions of calf DRP-2 reveal the 30 kDa protein eluted at the same position and comigrates with cisplatin−DNA binding activity (data not shown).

Identification of DRP-2 as HMG-1. The subunit molecular mass of DRP-2 and the ability to bind cisplatin-damaged DNA suggested that it may be a high-mobility group protein. DRP-2 was separated by 15% SDS−PAGE, transferred to a PVDF membrane, and stained with 0.1% Coomassie blue. Following destaining, the band at 30 kDa was excised, dried, and subjected to automated N-terminal sequencing. The results revealed complete identity to the published sequence for HMG-1 for the first 18 amino acids (Kaplan & Duncan, 1988), after which the signal decreased below detectable limits. These results provide conclusive evidence that DRP-2 is homologous to HMG-1.

Specificity of DRP-2/HMG-1 Binding to Partial Duplex DNA Substrates. To further characterize the specificity of DRP-2/HMG-1 DNA binding, we have assessed the DNA substrate specificity using a variety of DNA structures containing cisplatin damage. We have demonstrated that DRP-2/HMG-1 will not bind to single-stranded (ss) DNA treated with cisplatin (data not shown). Figure 4 shows the results of HMG-1 binding to partial duplex DNA substrates containing either 5′ or 3′ single strand tails. The ss44-mer oligonucleotide was 5′ end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. The first partial duplex substrate was constructed by annealing the 25-mer primer to the 5′ [^{32}P]-

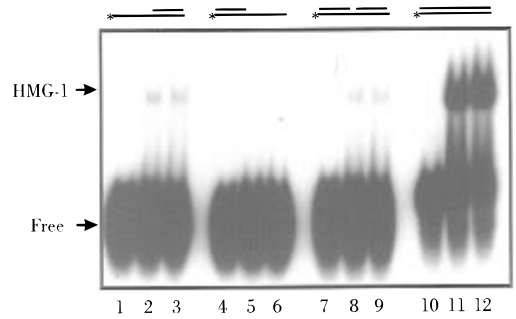

FIGURE 4: DRP-2/HMG-1 binding to cisplatin-damaged partial duplex DNA substrates. DNA substrates were prepared by labeling the 5′ terminus of the single-stranded 44-mer with [γ-^{32}P]ATP and T4 polynucleotide kinase. The partial duplex substrates were prepared as described in the text and are depicted schematically above the figure. The asterisk denotes the 5′ ^{32}P label on the 44-mer template. Lanes 1−3 show a 25-mer duplex with a 19-nucleotide 5′ single strand tail. Lanes 4−6 show an 18-mer duplex with a 25-base 3′, and 1-base 5′ single strand tail. In lanes 7−9, both primers were annealed, generating a nick between the two primers and a 1-base 5′ tail. In lanes 10−12, the 25-mer was annealed and extended with deoxynucleotides and Sequenase to generate a fully duplex DNA. Each DNA substrate was gel purified by 12% nondenaturing polyacrylamide gel electrophoresis, treated with cisplatin at a 10/1 D/N ratio, and analyzed for DRP-2/HMG-1 binding using 50 fmol of DNA per reaction, as described in Experimental Procedures. The mixtures in lanes 1, 4, 7, and 10 were incubated in the absence of protein; the mixtures in lanes 2, 5, 8, and 11 contained 12.5 ng of DRP-2/HMG-1, and the mixtures in lanes 3, 6, 9, and 12 contained 50 ng of DRP-2/HMG-1. Products were separated by 12% nondenaturing PAGE and visualized by autoradiography.

44-mer template. The resulting DNA has a 25 bp duplex region and a 19-base 5′ ssDNA tail. This substrate was gel purified, platinated, and assayed for DRP-2/HMG-1 binding, and only modest binding was observed (lanes 1−3). The second substrate (lanes 4−6) had an 18-mer primer annealed at a position one base 3′ of the 5′ terminus of the template. This generated an 18 bp duplex with a 25-base 3′ ssDNA tail and a 1-base 5′ overhang on the opposite termini. Following gel purification and platination, no binding was detected (lanes 4−6). The next substrate had both the 25-mer and 18-mer primers annealed to the 44-mer template and generated a single 5′ base overhang on the template strand and a nick between the two primers. Modest binding was observed with this substrate (lanes 7−9), on the order of that observed with the 25-mer alone annealed to the 44-mer. As a control, the 25-mer was annealed to the 44-mer and extended with Sequenase and dNTPs to generate a fully duplex DNA. Again, following gel purification and platination, DRP-2/HMG-1 binding was assessed, and this substrate was avidly bound (lanes 10−12). Quantification of binding revealed that 50 ng of DRP-2/HMG-1 bound 12.7 fmol of the fully duplex DNA, 0.80 fmol of the substrate with a nick, and 0.85 fmol of the 3′ tailed substrate.

DRP-2/HMG-1 Binding to Nicked and Ligated DNA Substrates. The results obtained in Figure 3 demonstrate that a fully duplex cisplatin-damaged DNA is bound most avidly by DRP-2/HMG-1. The decreased level of binding observed with the substrate containing the two primers annealed could be the result of the nick between the primers or the single base 3′ recessed terminus. The ss44-mer oligonucleotide was again 5′ ^{32}P labeled, and both the 18-mer and 25-mer primers were annealed. Half of the reaction was then ligated with T4 DNA ligase and ATP. The

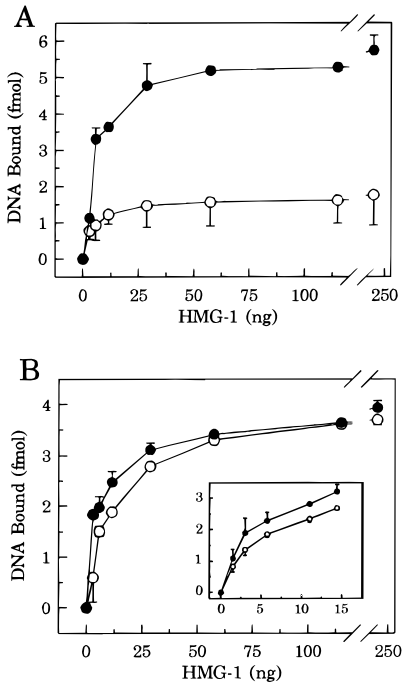

FIGURE 5: DRP-2/HMG-1 binding to nicked and ligated cisplatin-damaged DNA substrates. (A) The ss 44-mer oligonucleotide was 5′ labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and then the 25-mer and 18-mer were annealed, generating a nick between the two primers. Half of the reaction was ligated with T4 DNA ligase (filled circles) and half untreated, leaving the nick between the two primers (open circles). The two substrates were gel purified by 12% nondenaturing PAGE and platinated at a D/N ratio of 10/1, as described in Experimental Procedures. Mobility shift assays were performed with 50 fmol of DNA substrate and varying levels of DRP-2/HMG-1. The amount of DNA bound was quantified by phosphorimager analysis. (B) The 18-mer was annealed to the 44-mer and labeled by extension with [α-^{32}P]dCTP and Sequenase. The 25-mer was then annealed, and again, half of the reaction was ligated with T4 DNA ligase (filled circles) and half untreated, leaving a nick between the primers (open circles). The inset shows lower levels of DRP-2/HMG-1 binding to the same substrates. Gel purification, platination, and mobility shift analyses were performed as described above. The data presented are the average and standard deviation of two individual experiments.

substrates were assessed for integrity by electrophoresis through denaturing DNA sequencing gel, and 95% of the substrates treated with DNA ligase were joined (data not shown). Each DNA, nicked and ligated, was gel purified and platinated and binding assessed by titration of DRP-2/HMG-1 from 1.5 to 250 ng in the reactions. Quantification of the mobility shift assay is shown in Figure 5A and demonstrates a greater than 3-fold increase in binding to the ligated substrate at saturation. This data suggest that a break in the phosphodiester backbone of the DNA results in decreased affinity of DRP-2/HMG-1. Interestingly, when the converse experiment was performed, extending the 3′ recessed terminus to generate a full duplex DNA, different results were obtained (Figure 5B). The 18-mer was annealed and extended with [α-^{32}P]dCTP to label the DNA substrate and generate a blunt end. The 25-mer was then annealed, and again, half of the reaction was ligated and each DNA platinated. Titration of DRP-2/HMG-1 revealed identical binding to the nicked and ligated substrates at high DRP-2/HMG-1 levels. The level of binding observed at saturation is intermediate to that observed for the nicked and ligated substrates containing the 1-base 3′ recessed terminus (Figure 5A) but more closely to that of the ligated DNA. The inset

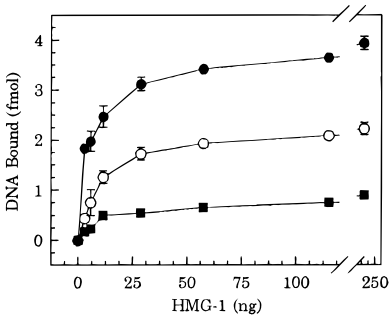

FIGURE 6: Single strand regions do not inhibit DRP-2/HMG-1 binding to cisplatin-damaged DNA. A partial duplex containing a 5′ single-stranded tail was constructed by annealing the 25-mer to the 44-mer and labeling by extension with Sequenase, [α-^{32}P]dATP, dTTP, and dGTP. Following gel purification and platination, DRP-2/HMG-1 binding was assessed and quantified (open circles). Digestion of the 5′ single strand region was performed with mung bean nuclease to generate a fully duplex 31-mer. This DNA was gel purified and platinated at a D/N ratio of 10/1 and assessed for DRP-2/HMG-1 binding (filled squares). The 44 bp fully duplex DNA is included for reference (filled circles) and was constructed as described in the legend to Figure 5A.

to Figure 5B shows data obtained by titrating down the level of DRP-2/HMG-1. At low levels of DRP-2/HMG-1, there is a slight preference for binding the ligated substrate, consistent with Figure 5A, however, not to the same extent. Apparently, the combination of the nick and the 1-base 3′ recessed terminus results in a significant decrease in the level of binding at high DRP-2/HMG-1 levels. Interestingly, we also observed an increased level of DRP-2/HMG-1 binding to platinated DNA containing 5′ phosphorylated termini (data not shown). The results taken together suggest that small perturbations in the structure of the DNA substrate can have dramatic effects on DNA binding. The mechanism by which these small differences mediate binding is currently under investigation.

Results obtained in Figure 4 also suggest that the 5′ single strand tail may affect binding. To determine the effect of the 5′ tail on direct binding, we employed mung bean nuclease to degrade the single strand 5′ tail and generate a fully duplex DNA of 31 bases. In this experiment, the 25-mer was annealed and labeled by extension with dGTP, dTTP, and [α-^{32}P]dATP until the first dCMP in the template strand is reached. One-half of the resulting 31 bp duplex containing a 13-base 5′ single strand tail was digested with mung bean nuclease to degrade the ssDNA. This resulted in the generation of a 31 bp fully duplex DNA. The 31 bp fully duplex digested DNA and undigested control DNA containing the 5′ tail were gel purified and treated with cisplatin, and DRP-2/HMG-1 binding was assessed by mobility shift analysis. This allowed direct comparison of the two substrates, each identical in sequence and degree of platination but differing by the presence of a 5′ 13-base single strand region. The results shown in Figure 6 demonstrate that the fully duplex 31-mer is inefficiently bound by DRP-2/HMG-1 where the tailed substrate shows a 3-fold increase in binding. Quantification of binding to the 44 bp fully duplex DNA substrate is shown on the same graph and demonstrates that binding correlates with DNA length as shown by the increased level of binding of the 44 bp DNA compared to that of the 31 bp DNA. Comparison of the 31 bp duplex with and without the single strand tail revealed enhanced binding of DRP-2/HMG-1 to the substrate containing the ss tail. The 31 bp region contains three individual sites for 1,2 d(GpG) adducts, while the 44 bp DNA has five potential 1,2 d(GpG) adduct sites. The differences in binding are attributed to the increased number of adducts. This is supported by the increased level of binding with the 31 bp duplex with the 5′ ss tail substrate where adducts on the single strand portion may enhance binding to the duplex region. Both HMG boxes are bound in the 44-mer duplex DNA which then results in a greater level of binding, while the 31 bp DNA may not be long enough to allow binding to each HMG box.

DNA Size Dependence of DRP-2/HMG-1 Binding to Cisplatin-Damaged DNA. The results in Figure 6 suggest there may be a size effect on DRP-2/HMG-1 binding. Also, despite the increased number of potential 1,2 d(GpG) adducts on the 44 bp DNA, we were unable to observe a second complex or supershift that would result from two DRP-2/HMG-1 proteins binding to a single substrate even at the highest concentrations of DRP-2/HMG-1 employed. Chow et al. (1995) demonstrated that a truncated HMG-1 construct containing just box A could bind to duplex DNA substrates 20 bases in length with a single 1,2 d(GpG) adduct. Footprint experiments of HMG-1 on cisplatin-damaged DNA demonstrated protection of a 24-base region (Locker et al., 1995), suggesting that the DNA substrates used thus far may be too short to support multiple binding events. We constructed three additional fully duplex DNA substrates of 75, 130, and 180 bp and treated each with cisplatin at the same D/N ratio to achieve the same degree of damage per substrate. The results shown in Figure 7 demonstrate each of the 75-mer, 130-mer, and 180-mer duplex DNA substrates supported binding, and supershifts were observed with each substrate (Figure 7A,B). Titration of DRP-2/HMG-1 revealed substantially increased binding to the longer DNAs. The amount of DRP-2/HMG-1 required for binding saturation of the 75, 130, and 180 bp DNA substrates, approximately 6 ng, was considerably less than that observed for the 44 bp DNA, 25 ng. In addition, the DNA bound at saturating DRP-2/HMG-1 was greater for the 75 and 130 bp DNA substrates, nearly 100% or 50 fmol, while the 5−6 fmol of the 44-mer was bound at saturating DRP-2/HMG-1. These results demonstrate a positive correlation between DNA length and binding affinity, consistent with the results presented in Figure 6.

DRP-2/HMG-1 Binding as a Function of Degree of DNA Damage. HMG-1 has been shown to bind to cisplatin 1,2 d(GpG) and 1,2 d(ApG) adducts but not to single dG or transplatin adducts (Pil & Lippard, 1992). The results presented in Figures 1 and 2B demonstrate a positive correlation of the level of DRP-2/HMG-1 binding to the extent of cisplatin−DNA damage. We expanded the study of assessing binding to a longer substrate, 75 bp, varying the levels of cisplatin damage over a 100-fold range. A fully duplex internally labeled 75 bp DNA was employed and incubated with cisplatin at D/N ratios of 0.01/1, 0.1/1, and 1/1. The platinated DNA was purified from unreacted cisplatin by spin column chromatography and the degree of platination assessed by nuclease digestion. We employed T7 DNA polymerase which contains an active 3′ to 5′ exonuclease activity. The results from the digestion are shown in Figure 8. Each of the substrates was incubated in the absence (lanes 1−4) or presence (lanes 5−8) of T7 DNA

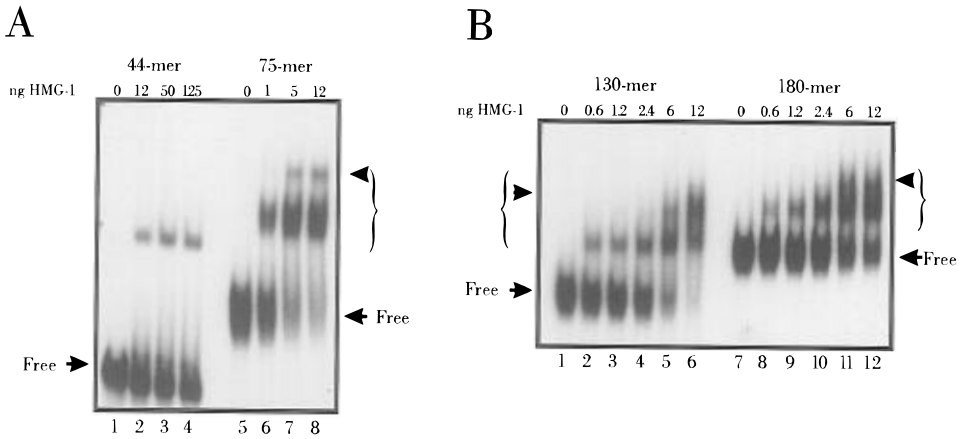

FIGURE 7: Cisplatin-damaged DNA length dependence of DRP-2/HMG-1 binding. The 44 bp fully duplex DNA was prepared as described in Experimental Procedures. The 75 bp DNA substrate was prepared by annealing the 24-mer to the 75-mer and labeling by extension with Sequenase and [α-^{32}P]dGTP, dATP, dTTP, and dCTP. The 130 and 180 bp fully duplex DNA substrates were generated by PCR amplification of the pBS+ plasmid, as previously described (Turchi et al., 1994), and labeled 5′ with [γ-^{32}P]ATP and T4 polynucleotide kinase. Each DNA was gel purified and platinated at a 10/1 D/N ratio. (A) The 44-mer and 75-mer substrates were incubated with the level of DRP-2/HMG-1 denoted in the figure and products separated by 8% nondenaturing PAGE and visualized by autoradiography. (B) The 130 and 180 bp substrates were incubated with the level of DRP-2/HMG-1 denoted in the figure and products separated by 6% nondenaturing PAGE and visualized by autoradiography. The arrows indicate the mobility of the free DNA, the brackets indicate the migration of DRP-2/HMG-1−DNA complexes, and the arrowheads indicate the supershifts. Note the amount of DRP-2/HMG-1 used in panel A, lanes 1−4, is 10-fold greater than that used in lanes 5−8 and panel B.

polymerase. The unplatinated 75 bp DNA is completely degraded by T7 polymerase to products smaller than 10 bases (lane 5). Platination at a D/N ratio of 0.01/1 results in the slower migration of the 75 bp (lane 2), and T7 polymerase generates a clear banding pattern indicative of cisplatin damage and nuclease inhibition at the sites of damage. The positions of the adducts correspond to d(GpG) sequences on the labeled DNA strand. In addition, we did observe a substantial amount of low-molecular mass products, below the 10-base marker. The DNA substrate was prepared by annealing a 24-mer to the 3′ terminus of the 75-mer single-stranded oligonucleotide and labeling by extension of the 24-mer. The extension was performed with [α-^{32}P]dGTP and unlabeled dCTP, dATP, and dTTP, followed by chasing with an excess of unlabeled dGTP. The low-molecular mass products are generated by digestion of the DNA, releasing a single [α-^{32}P]dGMP that is 3′ to the first 1,2 d(GpG) adduct on a given substrate. The adducts block the exonuclease activity and allow determination of the sites of adduct formation and the percentage of substrates containing adducts. Quantification of the results demonstrated 60% of the label was in the low-molecular mass fraction for the DNA damaged at a D/N ratio of 0.01/1. Taking into account that only one strand was labeled and the ^{32}P label was internal, conservatively, greater than 75% of the DNA substrates have at least one cisplatin−DNA adduct. Platination at a D/N of 0.1/1 resulted in an even slower migration (lane 3) and an intensification of the banding pattern (lane 6), indicating a higher proportion of the DNA substrates were damaged with cisplatin, greater than 95%, and the adducts are found at essentially the same sites. We also observed a significant level of interstrand adducts which migrate at a position corresponding to greater than 140 bases. These are denoted by the brace on the right side of Figure 8. Platination at a level of 1/1 resulted in the generation of interstrand adducts on greater than 95% of the DNA substrates (lane 4) and minimal degradation by T7 DNA polymerase (lane 8). Titration of DRP-2/HMG-1 in reactions with each of these DNA substrates is presented in Figure 9 and demonstrated saturation of binding at 8−10 fmol for the 0.01/1 and 35 fmol bound for the 0.1/1 and 1/1 D/N ratios. The low level of binding observed in the 0.01/1 DNA is likely the result of a decreased affinity for less damaged DNA. The increased binding to the more highly damaged DNA is likely the result of an increased affinity for DNA containing multiple adducts. The results obtained from nuclease degradation experiments (Figure 8) argue against increased binding being the result of an increased number of substrates being damaged. The lack of nuclease digestion, the high degree of interstrand adducts in substrates platinated at a 1/1 D/N ratio, and the high level of DRP-2/HMG-1 binding observed clearly demonstrate DRP-2/HMG-1 can form a stable complex with DNA extensively damaged and containing cisplatin interstrand cross-links. In addition, these results are completely consistent with the data presented in Figures 1 and 2B demonstrating increased binding to DNA substrates containing a greater degree of cisplatin damage.

DISCUSSION

Cisplatin has been the subject of intense study since its anticancer activity was first demonstrated. One area of this research has concentrated on the interaction of cisplatin with cellular components and how chemotherapeutic efficacy is imparted. It is generally accepted that the intracellular target of cisplatin is DNA, and blocking DNA replication induces a G_2 cell cycle block which activates the apoptotic pathway (Chu, 1994). Crude cell extracts and mobility shift assays have been employed by a number of labs to identify proteins capable of binding cisplatin-damaged DNA.

In this report, we have combined the mobility shift assay using a high-specific activity DNA probe and initial fractionation of cell extracts on a cisplatin-damaged DNA affinity column to identify and purify proteins which can bind to cisplatin-damaged DNA. The first protein purified, DRP-2, has been unequivocally identified as HMG-1. This result was not unexpected as HMG-1 has been demonstrated to bind cisplatin-damaged DNA in a damaged DNA cellulose precipitation assay (Hughes et al., 1992) and in mobility shift

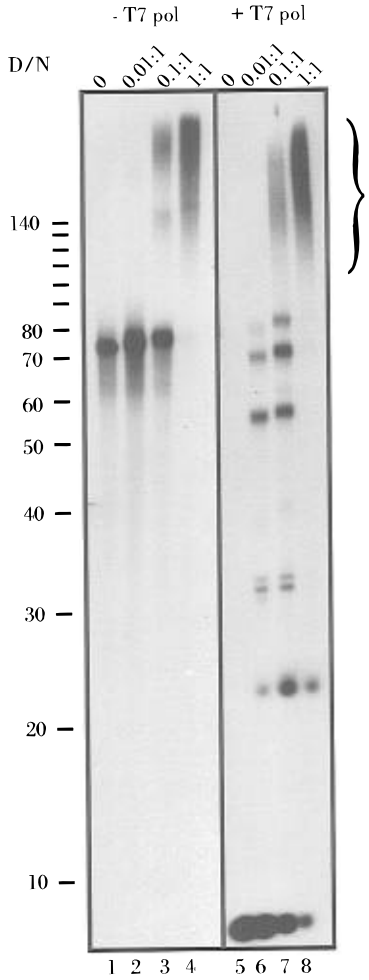

FIGURE 8: Structure of 75 bp DNA platinated at various D/N ratios. The 75 bp fully duplex DNA was prepared as described in the legend to Figure 7. Platination was performed at D/N ratios of 0.01/1, 0.1/1, and 1/1, as described in Experimental Procedures. The DNA substrates were incubated in the absence (lanes 1−4) or the presence (lanes 5−8) of 0.8 unit of T7 DNA polymerase for 15 min at 37 °C. Products were analyzed by 10% polyacrylamide/7 M urea DNA sequencing gels and visualized by autoradiography. The position of a 10-base ladder is shown on the left side of the figure. The D/N ratios are shown at the top, and the migration of DNAs with interstrand cross-links is denoted by the brace.

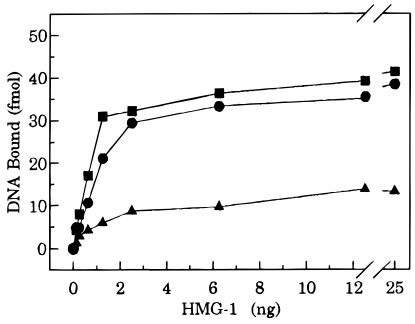

FIGURE 9: DRP-2/HMG-1 binding to cisplatin interstrand cross-linked DNA. The 75 bp fully duplex DNA, 50 fmol, platinated at 0.01/1 (triangles), 0.1/1 (squares), and 1/1 (circles) was tested for DRP-2/HMG-1 binding. Determination of the amount of DNA bound was performed as described in Experimental Procedures.

assays (Pil & Lippard, 1992). We have extended these studies further by determining the DNA substrate specificity of DRP-2/HMG-1. Our results clearly demonstrate fully duplex DNA substrates containing multiple platinum adducts are bound by DRP-2/HMG-1 with the degree of binding correlating to DNA length and degree of cisplatin damage. Interestingly, we were unable to detect binding to any substrates damaged with transplatin at a variety of D/N ratios (data not shown). If the structural DNA distortion induced by the adducts provided for binding, we might expect that transplatin damage would induce binding. Other reports have demonstrated that single site specific transplatin adducts do not support binding (Pil & Lippard, 1992). Our results demonstrate that increasing cisplatin D/N ratios result in increased binding of DRP-2/HMG-1. These data suggest that either DNAs with multiple adducts are more efficiently bound compared to DNAs with fewer adducts or a specific platinum adduct that mediates binding is only formed at higher D/N ratios. The latter is unlikely in light of our results demonstrating that the position of adducts does not change over the range we have tested on the 75 bp (Figure 8) and on the 44 bp DNA substrate (data not shown). Previous work demonstrated that 1,2 d(GpG) adducts are the major adduct formed and preferentially bound by HMG-1 (Pil & Lippard, 1992). However, the 44 bp DNA substrate contains a d(GpG) sequence near the 3′ terminus of the primer strand. When the strand containing the d(GpG) is 5′ labeled with ^{32}P, nuclease digestion experiments demonstrate 100% of the substrates contain an adduct at d(GpG) positions (data not shown). These data suggest that additional adduct formation results in increased binding. Denaturing PAGE analysis of the damaged DNA substrates revealed a small portion of interstrand cross-links that could not account for the increase in binding observed, as only 5% interstrand cross-links were observed on the 44 bp DNA (data not shown). Despite the increased affinity for highly damaged DNA substrates, no supershifts were observed on the 44 bp DNA substrate, suggesting a single DRP-2/HMG-1 bound in each complex. The increased ability to bind DNA substrates containing multiple adducts may be the result of the interaction of both HMG domain A and B with two separate cisplatin adducts. In light of recent results demonstrating binding of a fragment of HMG-1 containing only domain B (Chow et al., 1995), it is possible that an increase in affinity would be observed if two individual sites were available for binding. Lawrence et al. (1993) demonstrated two individual domains of HMG-2 were capable of low-level binding to cisplatin-damaged DNA, while both domains together resulted in increased binding. These results corroborate our data using full length DRP-2/HMG-1 and demonstrating increased binding to DNA containing multiple adducts. The mechanism by which an increased level of cisplatin damage on individual DNA substrates affects binding of the individual HMG domains remains to be addressed. This line of experimentation is currently underway.

Evidence for multiple binding events was obtained when larger DNA substrates were employed. Supershifts were obtained with DNA substrates as short as 75 bp in length. Interestingly, we never observed binding saturation using the 31 and 44 bp DNA substrates. No supershift was apparent with either of these substrates, despite increasing the level of DRP-2/HMG-1 in the reaction mixture 10-fold above that required to saturate the 75, 130, and 180 bp DNA substrates. In addition, we were able to saturate binding to the 75, 130, and 180 bp DNA substrates, and in each of these, we observed supershifts with DRP-2/HMG-1. An important

observation is that greater than 95% of the 75-mer DNA platinated at a 1/1 D/N ratio contains interstrand cross-links, and this DNA is efficiently bound by DRP-2/HMG-1 and resulted in supershifts. Interstrand adducts can result in the formation of a branched DNA, which have been demonstrated to bind HMG-1 in the absence of cisplatin damage (Bianchi et al., 1989). These adducts serve to anchor two HMG-1 molecules to a single DNA. Interestingly, transplatin can form interstrand cross-links, yet we were unable to detect DRP-2/HMG-1 binding to DNA substrates containing multiple transplatin adducts. Interstrand cross-links are effectively repaired in eukaryotic cells (Jones et al., 1991; Zhen et al., 1992) and are not thought to be responsible for the chemotherapeutic efficacy of cisplatin.

Recent reports have demonstrated that HMG-1 can inhibit incision of cisplatin-damaged DNA substrates catalyzed by whole cell extracts *in vitro* (Huang et al., 1994). The role this abundant nuclear protein plays in the metabolism of cisplatin−DNA adduct remains unclear. The ability to bind damaged DNA is likely a fortuitous event and likely unrelated to its still undefined cellular function. HMG-1 is thought to be involved in almost all aspects of DNA metabolism, and one model to explain the chemotherapeutic efficacy of cisplatin is that HMG-1 inhibits repair of cisplatin−DNA adducts by obviating binding of DNA repair proteins involved in recognition of DNA damage (Chu, 1994; Huang et al., 1994). Consistent with this hypothesis, increased DNA repair has been implicated as a mechanism for acquired resistance to cisplatin (Masuda et al., 1988; Johnson et al., 1994). This implies resistant cells may have subverted the action of HMG-1 and may provide some insight into the role HMG-1 plays in DNA metabolism, an area we are currently investigating.

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