A Single HMG Domain in High-Mobility Group 1 Protein Binds to DNAs as Small as 20 Base Pairs Containing the Major Cisplatin Adduct†

Christine S. Chow, Carmen M. Barnes, and Stephen J. Lippard*

Department of Chemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: Proteins containing a relatively new DNA-binding motif known as the high-mobility group (HMG) domain bind specifically to DNA modified by the anticancer drug cisplatin, but not to unmodified DNA (McA'Nulty & Lippard, 1995). Southwestern-blot analyses of the binding of proteolytic fragments of HMG1 to a 123-bp globally platinated DNA demonstrate that the HMG domains A and B of HMG1 are responsible for its specific interactions with cisplatin-modified DNA. An 81 amino acid recombinant protein representing a single HMG motif, HMG1 domain B, binds with an affinity ($K_d = 10^{-7}$ M) equal to that of HMG1 itself to 92- and 100-bp DNAs containing the major adduct of cisplatin, a *cis*-[$Pt(NH_3)_2$-{d(GpG)-N7(1), -N7(2)}] intrastrand cross-link, at a specific site. The isolated HMG domain B binds with comparable affinity to cisplatin-modified DNAs having as few as 20 bp. The related human mitochondrial HMG domain protein mtTFA also recognizes the 123-bp globally platinated DNA, providing further evidence that HMG domains are responsible for modulating binding of this class of proteins to cisplatin-modified DNA. This work provides direct biochemical evidence in support of conclusions drawn previously from analyses of sequence conservation (Bruhn et al., 1992) that HMG domains are the key elements in protein binding to cisplatin-modified DNA.

A new DNA-binding motif known as the HMG[1] domain has recently been identified (Grosschedl et al., 1994; Lilley, 1992). This domain is based on a repeating 80 amino acid sequence that comprises approximately two-thirds of the high-mobility group 1 (HMG1) protein. The HMG domain has been discovered in a number of proteins, including hUBF, the rRNA gene promoter upstream-binding factor (Jantzen et al., 1990); mtTFA, the mitochondrial DNA-binding factor (Parisi & Clayton, 1991); TCF-1, the T-cell transcription factor 1 (van de Wetering et al., 1991); LEF-1, the lymphoid enhancer-binding factor (Travis et al., 1991); and SRY, the testis-determining factor (Sinclair et al., 1990). In parallel work, an 81-kDa HMG domain mammalian protein called SSRP1, the structure-specific recognition protein 1, was discovered which binds DNA structural elements such as bent or unwound conformations that occur upon covalent binding of the antitumor drug *cis*-diamminedichloroplatinum(II) (*cis*-DDP, or cisplatin) (Bruhn et al., 1992; Toney et al., 1989). More recently, a *Drosophila* homologue of the human SSRP1 (Bruhn et al., 1993) and a yeast SSRP, the intrastrand cross-link recognition (Ixr1) protein (Brown et al., 1993), were cloned and sequenced; both of these proteins contain at least one HMG domain and bind to cisplatin-modified DNA.

cis-DDP is a clinically important anticancer drug used in the treatment of several human malignancies (Loehrer & Einhorn, 1984). There is strong evidence that DNA is the principal cellular target for *cis*-DDP (Roberts & Thomson, 1979), which has significant consequences for DNA replication and transcription. Conversely, *trans*-DDP, the geometric isomer of *cis*-DDP, is ineffective as a chemotherapeutic agent and does not form DNA adducts that are recognized by HMG domain proteins, even though this compound can block replication at doses equitoxic to those of *cis*-DDP (Bruhn et al., 1990). Thus, the selective interactions of SSRP1 and related HMG domain proteins with *cis*-DDP−DNA adducts may contribute to its biological activity. The exact role of HMG domain proteins in mediating the toxicity of *cis*-DDP is unknown, although several models have been proposed (Brown et al., 1993; Donahue et al., 1990; Huang et al., 1995; Treiber et al., 1994).

Work with recombinant rat HMG1 expressed in *Escherichia coli* has revealed that this HMG domain protein binds strongly ($K_d = 10^{-7}$ M) and selectively (nonspecific $K_d = 10^{-5}$ M) to covalent *cis*-DDP intrastrand d(GpG) and d(ApG) adducts, further implicating the importance of the HMG domain in binding to platinated DNA as a substrate (Pil & Lippard, 1992). Complementary studies have shown that both HMG1 and HMG2 purified from human cell extracts have the ability to bind cisplatin-modified DNA (Hughes et al., 1992). The *cis*-DDP 1,2-d(GpG) and -d(ApG) intrastrand cross-links bend the DNA helix by approximately 34° in the direction of the major groove (Bellon & Lippard, 1990) and unwind it by 13° (Bellon et al., 1991). HMG1 also binds preferentially to AT-rich sequences (Brown & Anderson, 1986), Z-DNA or B−Z junctions (Waga et al., 1988), DNA cruciform structures (Bianchi et al., 1989), and tandemly repeated poly(CA)·poly(TG) DNA sequences (Gaillard &

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* To whom correspondence should be addressed.

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[1] Abbreviations: HMG, high-mobility group; *cis*-DDP or cisplatin, *cis*-diamminedichloroplatinum(II); *trans*-DDP, *trans*-diamminedichloroplatinum(II); bp, base pair; *E. coli, Escherichia coli*; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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Strauss, 1994). Although no clear consensus sequence among these DNAs is apparent from comparisons, they are all unwound and/or bent, suggesting that such distortions are important for the preferential recognition and binding of HMG1. In addition, several groups have reported that HMG1 itself can mediate DNA bending in a T4 DNA ligase-catalyzed ring closure assay using small DNA fragments, less than 150 bp (Onate et al., 1994; Paull et al., 1993; Pil et al., 1993).

Recently we reported that HMG domain proteins induce sharp bends in cisplatin-modified DNA (Chow et al., 1994). In the present, complementary study, we demonstrate that a single HMG domain is sufficient for binding the *cis*-DDP 1,2-d(GpG) intrastrand cross-link. In addition, we have employed deletion clones of rat HMG1, limited proteolytic digestions of HMG1, Southwestern blotting, and gel mobility shift assays to localize regions of the protein that are important for modulating its specific binding to cisplatin-modified DNAs ranging in size from 123 down to 20 bp. We also examined the binding of another related HMG domain protein, mtTFA, to DNA modified by cisplatin. These experiments provide direct evidence that the HMG domain is responsible for the specificity of binding to DNA containing the major *cis*-[$Pt(NH_3)_2${d(GpG)-N7(1), -N7(2)}] intrastrand cross-link. They also define small platinated DNA and HMG domain protein components capable of forming specific complexes for eventual characterization by NMR and X-ray structural methods.

MATERIALS AND METHODS

Materials. The rat HMG1 clone pT7-RNHMG1 was obtained from M. E. Bianchi (Dipartimento di Genetica e Microbiologia, Universitá di Pavia). D. A. Clayton (Department of Developmental Biology, Stanford University School of Medicine) provided human mtTFA. The expression vector pLM1 and the BL21(DE3) strain were a gift from G. L. Verdine and L. Chen (Department of Chemistry, Harvard University). Oligonucleotides, primer K86 (5′-TAGGGCGAATTCTTAACCAGGGAGCTGATTATGAAAAAGAAGTTCAAGGACCCCAATGCC-3′), and primer K165 (5′-TCTAGGCAAAGCTTTTATTTTGCTCTGTAGGCAGCAATATC-3′) were synthesized by the Biopolymers Laboratory (MIT) and purified by gel electrophoresis. Oligonucleotides for the 20-, 92-, and 100-bp probes were synthesized on a Cruachem PS250 DNA synthesizer by the phosphoramidite method. The deprotected oligonucleotides were purified by polyacrylamide gel electrophoresis (12%, 8 M urea) and stored in 10 mM Tris·HCl, pH 7.0, at −20 °C. T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (Beverly, MA).

Construction of pHB1. The plasmid pHB1 containing the HMG domain expression cassette was constructed by PCR amplification of the desired DNA sequence in pT7-RN-HMG1 (Bianchi, 1991) coding for amino acids 86−165 of HMG1. Primer 1 (K86), which contains an *Eco*RI site, the ribosome-binding site, and the start codon, and primer 2 (K165), which contains a *Hin*dIII site and the stop codon, were employed. The expression cassette was subcloned into the *Eco*RI and *Hin*dIII cut expression vector pLM1, a phagemid in which the cloned genes are under the control of the *tac* promoter and the $rrnBT_1T_2$ transcription terminator (MacFerrin et al., 1993). The insertion of the PCR fragment was confirmed by restriction mapping and DNA sequencing.

Expression and Purification of the HMG Domain Polypeptide. The HMG domain polypeptide (residues 86−165) was expressed and purified from pHB1/*E. coli* BL21(DE3). Cells were grown in LB medium (10 g of bactotryptone, 5 g of yeast extract, and 10 g of NaCl per liter) with ampicillin (75 μg/mL) at 37 °C and were induced at OD_{600} = 0.8 by the addition of isopropyl β-D-thiogalactopyranoside (IPTG, Boehringer Mannheim) to a final concentration of 1 mM. After 2 h, the cells were harvested by centrifugation, resuspended in 1/40 vol of cold lysis buffer (50 mM Tris, 5 mM β-mercaptoethanol, 20 mM NaCl, 10 mM EGTA, 10 mM EDTA, and 1 mM PMSF, pH 7.2), and lysed by passing through a French press at 1500 psi. Sodium chloride was added to the lysate to a final concentration of 0.5 M. Debris was removed from the lysate by centrifuging in a Beckman 45Ti rotor at 40 000 rpm for 30 min. The supernatant was made 2.6 M in ammonium sulfate, cleared by centrifugation in the 45Ti rotor, concentrated and desalted by using an Amicon concentrator (diaflo ultrafilter YM2, MW cutoff 1000), washed with column buffer A (50 mM Tris, 5 mM β-mercaptoethanol, 50 mM NaCl, pH 7.3), and loaded onto an S Sepharose column (Pharmacia). The column was washed with buffer A until A_{280} returned to baseline, and the proteins were eluted with a linear gradient from 0.05 to 0.5 M NaCl. The fractions containing HMG domain polypeptide eluted between 0.20 and 0.25 M NaCl and were identified by 18% SDS−PAGE and Coomassie Blue R-250 staining. The fractions containing HMG domain polypeptide were pooled, diluted with an equal volume of buffer A, and loaded onto a heparin Econo-Pac column (Bio-Rad). The proteins were eluted with a linear gradient from 0.05 to 0.5 M NaCl. Again, the HMG domain fractions eluted between 0.20 and 0.25 M NaCl and were identified by 18% SDS−PAGE. The fractions were combined, concentrated, and stored in buffer A at −20 °C. The protein concentration was determined by a Bio-Rad protein assay using bovine serum albumin (BSA) as a standard or by using the extinction coefficient (ε = 12 500 M^{-1} cm^{-1} at 280 nM). The yield was 5−10 mg of purified HMG protein per liter of culture.

Expression and Purification of ^{35}S-Labeled HMG1. Recombinant rat HMG1 was expressed and purified as described (Pil & Lippard, 1992). ^{35}S-Labeled HMG1 was expressed and purified from pT7-RNHMG1/*E. coli* BL21-(DE3)pLysE. Cells were grown in M9 medium (6 g of Na_2HPO_4, 3 g of KH_2PO_4, 1 g of NH_4Cl, and 0.5 g of NaCl per liter) with 1 mM $MgSO_4$, 0.2% glucose, and 100 μg/mL ampicillin at 37 °C for 6 h (OD_{600} = 0.8). The cells were harvested by centrifugation and resuspended in fresh M9 medium with 1 mM $MgSO_4$, 0.2% glucose, 100 μg/mL ampicillin, and 0.005% 18 L-amino acid mixture (w/v) (minus L-methionine or L-cysteine). Following incubation of the resuspended cells at 37 °C for 30 min, protein synthesis was induced by addition of IPTG to a final concentration of 1 mM and incubation of the cells at 37 °C for an additional 30 min. Rifampicin (Sigma; stock solution was 20 mg/mL in methanol) was added to a final concentration of 200 μg/mL, and after 1 h at 37 °C, the newly synthesized proteins were labeled by the addition of L-[^{35}S]methionine and L-[^{35}S]-cysteine (Dupont/NEN, 4 mCi to 400 mL of cells in M9 media, 3.3 nM). After 45 min at 37 °C, the cells were harvested by centrifugation and resuspended in 1/30 vol of cold lysis buffer (50 mM Tris, 5 mM β-mercaptoethanol, 20 mM NaCl, 10 mM EGTA, 10 mM EDTA, 10 mM $MgCl_2$,

1 mg/mL DNAse I, 1 mg/mL lysozyme, and 1 mM PMSF, pH 7.3). The cells were lysed by exposure to repeated freeze (−80 °C)−thaw (4 °C) cycles. Debris was removed from the cells by centrifugation, and the protein was isolated and purified from the lysate as described previously (Pil & Lippard, 1992). The ^{35}S-labeled HMG1 concentration was determined by using an extinction coefficient at 280 nm of 20 500 M^{-1} cm^{-1}, and the amount of radioactivity was determined by using a Beckman Model LS6500 scintillation counter.

DNA Probes. The DNA molecules used in these experiments had four different sequences and were prepared in the following manner. The 123-bp fragment was obtained by digesting a commercially available 123-bp ladder (Gibco-BRL) with *Ava*I restriction enzyme and purifying the resulting 123-bp DNA with 4-bp 5′ overhangs (Andrews & Jones, 1991). The 123-bp fragment was platinated at a drug-to-nucleotide ratio (r_b) of 0.023; 3′-end labeled by a fill-in reaction with the Klenow fragment of DNA polymerase I, [α-^{32}P]dCTP, and an equimolar mixture of dATP, dGTP, and dTTP; and purified by passing through a Sephadex G-50 Quick Spin column (Boehringer Mannheim). A blunt-ended 100-bp DNA fragment (100Pt) containing a single, site-specific *cis*-[Pt(NH_3)$_2$\{d(GpG)-N7(1), -N7(2)\}] intrastrand cross-link and the corresponding unmodified 100-bp DNA control fragment (100Ct) were prepared as described in the literature (Pil & Lippard, 1992), 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, and purified by passing through Sephadex G-50 Quick Spin columns (Boehringer Mannheim). Two 92-bp fragments (92Pt and 92Ct), also with blunt ends and containing either a single *cis*-[Pt(NH_3)$_2$\{d(GpG)-N7(1), -N7(2)\}] intrastrand cross-link or an unmodified d(GpG) site, were prepared according to the literature (Pil & Lippard, 1992), but the dodecamers d(TCTAGGCCTTCT) (Stu12) and *cis*-[Pt(NH_3)$_2$\{d(TCTAGGCCTTCT)\}] (Stu12Pt) were substituted for GG20-TOP and *cis*-Pt-GG20-TOP, respectively. Oligo C was replaced by d(TCGACTAGAAGGCCTAGACTGACG) (oligo C-24). Oligos Stu12, Stu12Pt, GG20-TOP, and *cis*-Pt-GG20-TOP were prepared and purified as described previously (Bellon & Lippard, 1990; Naser et al., 1988). The 92-bp fragments were 5′-end labeled and purified in the same way as the 100-bp fragments. A 20-bp double-stranded fragment was produced by annealing *cis*-Pt-GG20-TOP to its complement (GG20-BOTTOM), which was 3′-end labeled with terminal transferase and [α-^{32}P]ddATP, in Tris·HCl and 50 mM NaCl, pH 7.0. The 20-bp fragment was also modified at its 5′-ends with fluorescein (bottom) and rhodamine (top) for use in another set of experiments (C. S. Chow, C. M. Barnes, and S. J. Lippard, unpublished). These modifications did not affect the K_d values (data not shown).

Southwestern Blotting. Protein samples were resolved by SDS−PAGE on 12%, 15%, or 18% separating gels [29:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)], and electroblotted onto 0.45-μm nitrocellulose membranes (S&S NC, Schleicher and Schuell). The resulting blots were processed as described in the literature (Singh et al., 1988). To assay for DNA binding, nitrocellulose filter-bound proteins were incubated in binding buffer (30 mM Hepes·NaOH, pH 7.5, 10 mM $MgCl_2$, and 0.25% nonfat dry milk), using 20 mL per 20 × 20 cm filter with either cisplatin-modified or unplatinated ^{32}P-labeled 123-bp fragment or 100-bp probe (5×10^5 cpm). Chicken erythrocyte DNA was added as a competitor for nonspecific DNA-binding proteins at 5 μg/mL. The incubations were run for 3 h at ambient temperature with gentle agitation. Unbound DNA was removed by washing the filter twice for 10 min at 4 °C with 50 mL of binding buffer lacking $MgCl_2$. Protein−DNA complexes were detected by autoradiography (Kodak X-OMAT AR) with the use of an intensifying screen at −80 °C.

Limited Proteolytic Cleavage. Protease from *Staphylococcus aureus* strain V8 and clostripain from *Clostridium histolyticum* were purchased from Sigma in lyophilized form and were each resuspended to a final concentration of 500 ng/μL in 10 mM Tris, pH 7.8. Clostripain was activated for 1 h prior to use with 7.5 mM DTT. Full-length HMG1 (5 μg in 10 μL of buffer; 100 mM Tris, 50 mM EDTA, and 20 mM β-mercaptoethanol, pH 7.8) was allowed to equilibrate for 15 min at ambient temperature. Protease (1 μL) was added, and incubation was continued for 2 min. Protease digestion was stopped by the addition of 10 μL of sample buffer (125 mM Tris, 20% glycerol, 0.4% SDS, and 0.2 M DTT, pH 8.8) and boiling for 5 min. The protein fragments were resolved on 15% SDS−polyacrylamide gels [29:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)] and co-electrophoresed with molecular mass standards (Bio-Rad broad range). Calibration curves of the distance migrated versus the log molecular mass were constructed in order to obtain the calculated molecular mass of the protein fragments on the basis of their migration in the gel. Southwestern blots, as described above, were employed to test the ability of the protein fragments to bind to platinated DNA, but the denaturation and renaturation steps were omitted.

N-Terminal Sequencing of Protease V8 and Clostripain Cleavage Products. Following enzymatic cleavage of HMG1 (25 μg) with protease V8 (1 μg) or clostripain (1 μg) as described above, the products were resolved on 15% SDS−polyacrylamide gels [37.5:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)] and electroblotted in Tris−glycine buffer onto a 0.2-μm PVDF protein sequencing membrane (Bio-Rad). The membrane was washed with deionized water and stained with Coomassie Blue R-250. The membrane was destained with 50% methanol in water, washed with deionized water, dried, and submitted to the Biopolymers Lab (MIT) for N-terminal sequencing.

Gel Mobility Shift Assays. Gel mobility shift assays were carried out and K_d values calculated according to published procedures (Pil & Lippard, 1992). Reaction mixtures containing 5000 cpm probe, 4% glycerol, 10 mM $MgCl_2$, 50 mM KCl, 10 mM Hepes·NaOH, pH 7.9, 1 mM EDTA, 1 mM spermidine, 0.05% Nonidet P-40, 200 μg/mL BSA, and varying concentrations of HMG domain protein or full-length HMG1 in a 10-μL volume were incubated for 15 min on ice prior to loading with 10% glycerol. The HMG domain B reactions were also run in the presence of salt (0.5 M NaCl) or hexaamminecobalt(III) (0.5 to 5 mM). Reaction mixtures were analyzed by gel electrophoresis on prerun, precooled (4 °C), 0.5× TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3), 10% native polyacrylamide gels (29:1 acrylamide/*N*,*N*′-methylenebis(acrylamide), and 0.08% ammonium persulfate]. The gels were run at 300 V for 3 h at constant voltage and temperature in 0.5× TBE. Following completion of electrophoresis, the gels were vacuum dried onto Whatman 3 MM chromatography paper and exposed to X-ray film (Kodak X-OMAT AR) at −80 °C with an intensifying screen.

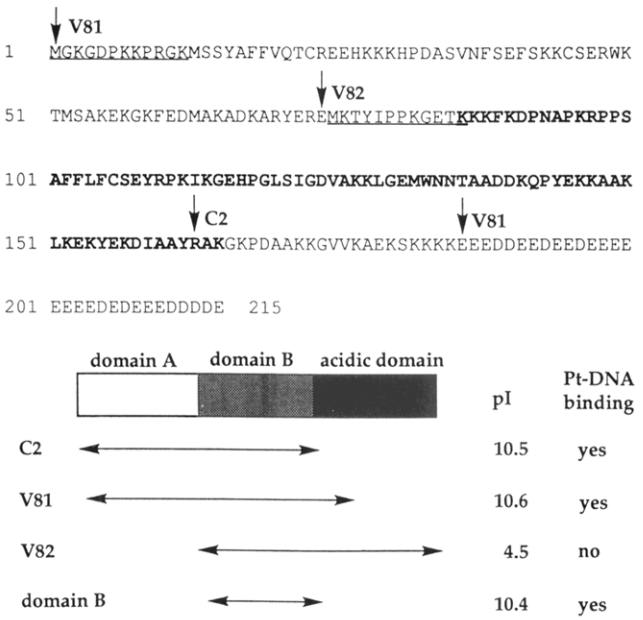

FIGURE 1: Summary of protease V8 and clostripain cleavage results for rHMG1. The full-length sequence of rat HMG1 is shown with proteolytic cleavage sites indicated by arrows. The underlined portions indicate the N-terminal sequences determined for the proteolytic fragments. Clostripain cuts at R163, and protease V8 promotes cleavage at residues M1, E186, and E74. The sequence of expressed HMG domain B is shown in boldface. DNA-binding properties and calculated isoelectric points of the HMG1 proteolytic fragments are indicated schematically below the sequence.

RESULTS

Expression and Purification of the HMG Domain Proteins. HMG1 is made up of three domains: the basic N-terminal domain (HMG domain A), a homologous, basic central domain (HMG domain B), and a highly acidic C-terminal domain. In order to test the ability of a single HMG domain to bind specifically to cisplatin 1,2-intrastrand cross-links, an expression vector was constructed by using the ECPCR method of MacFerrin et al. (1990) to overproduce a specific protein fragment corresponding to the HMG domain B. This domain, rather than domain A, was chosen because of its greater homology to SSRP1 (Bruhn et al., 1992) and the fact that its solution structure has been determined by NMR spectroscopy (Read et al., 1993; Weir et al., 1993). The HMG domain polypeptide, corresponding to residues K86 to K165 of rat HMG1, was expressed in milligram quantities in pHB1/*E. coli* BL21(DE3) upon induction with IPTG and conveniently purified by passing through a cation-exchange column followed by heparin chromatography. The sequence of the expressed HMG domain is depicted in boldface type in Figure 1. The polypeptide is 81 amino acids in length and extends just beyond the region of sequence conservation defined as HMG domain B. As shown in Figure 2, the expressed protein was homogeneous and had an apparent molecular mass of 8900 Da, as determined by Coomassie staining of a 15% SDS−polyacrylamide gel and comparison with molecular mass standards. The N-terminal sequence, MKKKF, with the additional methionine encoded by the start codon, was confirmed.

^{35}S-Labeled HMG1 was expressed and purified from pT7-RNHMG1/*E. coli* BL21(DE3)pLysE by using [^{35}S]methionine and [^{35}S]cysteine, as described in Materials and Methods. As shown in Figure 3A (lane 1), the resulting protein was homogeneous and had an apparent molecular mass of 31 kDa, as determined by Coomassie staining of a 12% SDS−polyacrylamide gel or by autoradiography and comparison with molecular mass standards.

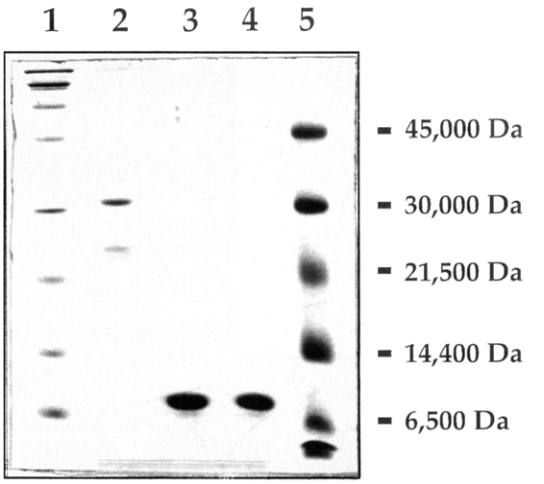

FIGURE 2: Isolation of HMG domain B of rHMG1. HMG domain B polypeptide of HMG1 produced in *E. coli* BL21(DE3) cells and purified on S Sepharose appears on a Coomassie-stained 15% SDS−polyacrylamide gel in lanes 3 and 4 with a molecular mass of 8.9 kDa. Full-length HMG1 in lane 2 has an apparent molecular mass of 31 kDa (a degradation product is also apparent at ~25 kDa). The molecular mass markers are shown in lanes 1 and 5, the values of which are given to the right of the gel.

Southwestern Blot Assays. The existence of proteins in mammalian cellular extracts that are capable of binding to *cis*-DDP−DNA adducts with high affinity was established previously by Southwestern blot assays (Chao et al., 1991; Donahue et al., 1990; Toney et al., 1989). This method initially revealed the existence of two classes of proteins that recognize *cis*-DDP-damaged DNA having molecular mass values of approximately 28 and 80−100 kDa. The gene encoding the 81-kDa SSRP1, which was cloned and sequenced, revealed the existence of an HMG domain (Bruhn et al., 1992; Toney et al., 1989). In a Southwestern blot of purified rat HMG1, the protein was probed with *cis*-DDP-modified 123-bp DNA, but not with unmodified DNA (Pil & Lippard, 1992). In addition, the HMG domain proteins Ixr1 (Brown et al., 1993) and hUBF (Treiber et al., 1994) were also shown by Southwestern analysis to bind selectively to cisplatin-modified DNA. Therefore, the Southwestern blot is useful for studying interactions of HMG domain proteins with *cis*-DDP-damaged DNA.

Figure 4 shows a comparison in binding of HMG-domain proteins to *cis*-DDP-modified DNA. HMG1 (lane 1), the HMG domain polypeptide (lane 2), and mtTFA (lane 3) were resolved by 18% SDS−PAGE and electroblotted onto nitrocellulose. When probed with unmodified ^{32}P-labeled DNA, no binding to any of the proteins was apparent (data not shown). When probed with ^{32}P-labeled 123-bp DNA globally modified with cisplatin, binding to full-length HMG1, HMG domain B, and mtTFA was observed. In addition, mtTFA appeared as both a monomer and a dimer in the assay, both of which were recognized by cisplatin-modified DNA. These results demonstrate that a single HMG domain is sufficient for recognition of cisplatin-modified DNA and that various HMG domain proteins similarly can bind specifically to these substrates.

Limited Proteolytic Cleavage. Carballo et al. (1983) demonstrated that, when HMG1 from calf thymus was

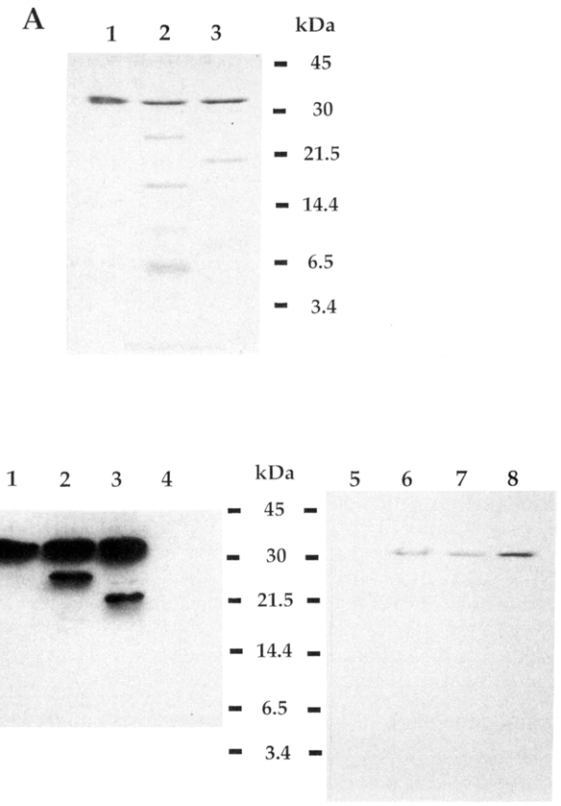

FIGURE 3: Resolution of (A) full-length ^{35}S-labeled HMG1 and HMG1 proteolytic fragments by 15% SDS−PAGE and (B) Southwestern-blot analysis of HMG−domain proteins binding to cisplatin-modified DNA. (A) Lane 1: full-length HMG1. Lane 2: protease V8 cleavage of HMG1. Lane 3: clostripain cleavage of HMG1. In all enzyme reactions, 500 ng of protease was allowed to react with 5 μg of unlabeled HMG1 plus 360 ng of ^{35}S-labeled HMG1 for 2 min. (B) HMG domain proteins were resolved in duplicate by 15% SDS−PAGE and electroblotted onto nitrocellulose that was divided for use in blots with cisplatin-damaged 123-bp DNA ($r_b = 0.023$) (lanes 1−4) or unmodified DNA (lanes 5−8). Lanes 1 and 8: full-length HMG1. Lanes 2 and 7: protease V8 cleavage of HMG1. Lanes 3 and 6: clostripain cleavage of HMG1. Lanes 4 and 5: HMG domain B.

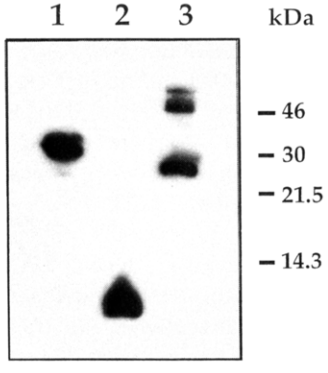

FIGURE 4: Southwestern blot analysis of HMG domain proteins binding to cisplatin-modified DNA. HMG proteins (1 μg each) were resolved by 18% SDS−PAGE and electroblotted onto nitrocellulose for use in blots with cisplatin-damaged 123-bp DNA ($r_b = 0.023$). Lane 1: HMG1. Lane 2: HMG domain B. Lane 3: mtTFA (monomer and dimer). The positions of the molecular mass markers are indicated at the right.

degraded with protease V8, specific protein fragments were produced. In their experiments, short periods of digestion with the enzyme produced two specific peptides that were progressively degraded over time. The results indicated that HMG1 contains preferential cleavage sites for the enzyme, which presumably arise because of the structure of the folded protein. Folding of the protein into its stable secondary or tertiary structures would appear to render some of the possible cleavage sites inaccessible to the enzyme. In the present study, we have examined clostripain- and protease V8-promoted cleavage of ^{35}S-labeled recombinant rat HMG1. The ^{35}S-labeled fragments were resolved on 15% SDS−polyacrylamide gels (Figure 3A) and tested for their ability to recognize specific cisplatin−DNA adducts by transfer to nitrocellulose membranes and probing with ^{32}P-labeled *cis*-DDP-modified DNA in a Southwestern assay (Figure 3B).

In reactions with clostripain (arginine specific, nine possible sites), two minor cleavage products, C1 and C3, and one major product, C2, were produced and separated on a 15% SDS−polyacrylamide gel (Figure 3A, lane 3). The fragments C1, C2, and C3 corresponding to molecular masses of 29, 21, and 9 kDa, respectively, were all visible because of the ^{35}S-labeling. Cleavage products not containing a cysteine or methionine residue would not appear on this gel. In a parallel SDS−polyacrylamide gel, the HMG1 fragments were separated, transferred to a nitrocellulose membrane, and probed with 123-bp DNA, globally modified with cisplatin, in a Southwestern blot assay. This assay revealed fragments capable of binding to cisplatin-modified DNA, including any present but not visible in Figure 3A, owing to the lack of a ^{35}S label. When probed with unmodified ^{32}P-labeled DNA, no binding to full-length protein or the proteolytic fragments was apparent (Figure 3B, lanes 5−8), although a small amount of radioactivity from the ^{35}S label was evident. Only the intermediate-sized fragment (C2) was capable of binding to the globally platinated DNA (Figure 3B, lane 3). This result indicated that only one of the protein fragments contained the domain that was necessary for binding to cisplatin-modified DNA. It should be noted, however, that the smaller protein fragments did not transfer to the nitrocellulose as efficiently as the larger fragments. Since we were limited in protein, there was an insufficient quantity of the small fragments to get conclusive results on a Southwestern blot. We were thus unable to determine conclusively whether the 9-kDa fragment contained the element required for cisplatin−DNA recognition. There were additional problems in the transfer of the smaller fragments to nitrocellulose. In order to get efficient transfer of the small quantities of the larger fragments available, we had to modify the conditions (shorter transfer times at higher voltages) from those used in Figure 4. This change led to loss of the smaller fragments, as is evident from the control lane (lane 4), which originally contained purified HMG domain B.

In order to determine the sequences of the fragments that are involved in binding to platinated DNA, full-length HMG1 was digested with protease, and the fragments were resolved by 15% SDS−PAGE and electroblotted onto a PVDF membrane for N-terminal sequencing. The N-terminal sequence of C2, MGKGDPKKPR, and the observed molecular mass of the fragment suggested that cleavage occurred at R163 (Figure 1). The sequencing revealed that the C2 fragment contained both HMG domains, but not the acidic C-terminal domain. Clostripain promoted cleavage at the C-terminal edge of, but not within, HMG domain B. The cleavage data support the fact that HMG motifs are folded, stable domains with internal arginine residues that are protected from cleavage (Carballo et al., 1983; Cary et al., 1983; Read et al., 1993; Weir et al., 1993). In addition, this result is consistent with previously described work carried

Table 1: Properties of HMG1 Protease Fragments

fragment	N-terminal sequence	mol mass obsd (kDa)	sequence content	p*I*	Pt−DNA binding	acidic domain
C2	MGKGDPKKPR	21	1−163	10.5	yes	−
V81	GKGDPKKPRGK	23	2−186	10.6	yes	partial
V82	MKTYIPPKGET	17	75−215	4.5	no	+
domain B	MKKKF	9	85−163	10.4	yes	−

out using HMG2, which has two HMG domains capable of binding to DNA globally modified with cisplatin (Lawrence et al., 1993).

Cleavage with protease V8, which cuts at aspartic and glutamic acid residues (56 possible sites), produced three major fragments, V81, V82, and V84, and one minor fragment, V83, having molecular masses of 23, 17, 6.5, and 10 kDa, respectively (Figure 3A, lane 2). Only one (V81) of the three major fragments seen in Figure 3A was capable of binding to cisplatin-modified 123-bp DNA in a Southwestern blot assay (Figure 3B, lane 2). Fragment V81 had the N-terminal sequence GKGDPKKPRGK, which suggested that cleavage occurred at residues M1 and E186 to produce a 23-kDa fragment. Like fragment C2, V81 contained both HMG domains, but not the acidic tail, and recognized cisplatin-modified DNA. Fragment V82 had the N-terminal sequence MKTYIPPKGET and a sequence content of M75−E215. Again, the cleavage between E74 and M75 occurred between the HMG A and B domains, but not at the other possible D and E sites, further supporting the conclusion that the HMG domain must be a distinct structural element. The digestion product V82 did not bind to cisplatin-modified DNA, but it did contain a single HMG domain. The calculated p*I* for the fragment is 4.5, however, which suggests that the highly negative character of this fragment may prohibit its binding to DNA.

The results of protease V8 and clostripain cleavage are summarized in a schematic diagram in Figure 1 and in Table 1. We conclude from these proteolytic digestion experiments and the Southwestern analysis shown in Figure 4 that a combination of A and B HMG domains or the isolated domain B is sufficient for binding to cisplatin-modified DNA. In a related study with HMG2 (Lawrence et al., 1993), it was concluded that two HMG domains could recognize cisplatin-modified DNA, but that individual domains A and B exhibited only weak binding. Perhaps differences between the amino acid sequences of HMG1 and HMG2 affect cisplatin−DNA recognition. Alternatively, the individual domains that were isolated from HMG2 were not folded properly and therefore failed to bind tightly to the *cis*-DDP-modified DNA. For example, NMR studies (Weir et al., 1993) demonstrated that a "minimal"-sized B-domain is required for proper folding. Further work is required to resolve this apparent inconsistency, although our results with the expressed material clearly indicate that a single domain is sufficient for recognition of cisplatin-modified DNA.

DNA Probes. In an effort to study the binding of the HMG domain polypeptide to a specific cisplatin adduct, a 100-bp probe containing a single *cis*-[Pt(NH_3)$_2${d(GpG)-N7(1), -N7(2)}] intrastrand cross-link was prepared as described in the literature (Pil & Lippard, 1992). The oligonucleotides that were used for the construction of the 100-bp probe are shown in Figure 5. In addition, a 92-bp probe containing a single *cis*-{Pt(NH_3)$_2${d(GpG)-N7(1), -N7(2)}] adduct was prepared according to the same procedure (Figure 5B). The 92-bp

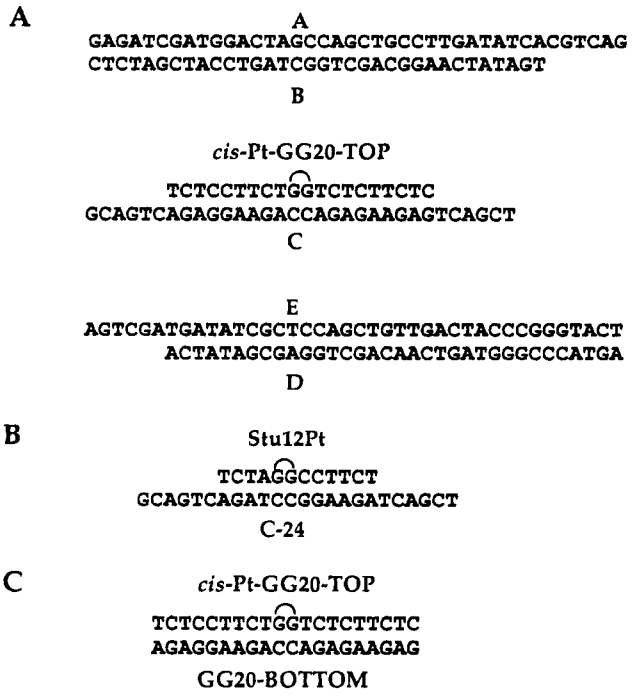

FIGURE 5: DNA probes with single intrastrand *cis*-[Pt(NH_3)$_2$-{d(GpG)-N7(1), -N7(2)}] adducts. Panel A depicts the six oligonucleotides (A, B, C, D, E, and *cis*-Pt-GG20-TOP) used in the ligation reaction to form the 100-bp probe. Panel B shows two oligonucleotides (Stu12Pt and C-24) that are substituted for GG20-TOP and C to produce the 92-bp probe. The sites of cisplatin modification on *cis*-Pt-GG20-TOP and Stu12Pt are indicated by semicircles. The 20-bp *cis*-Pt-GG20 probe is shown in panel C.

probe differed from the 100-bp probe in that the middle 20 bp of the 100-mer (5′-TCTCCTTCTGGTCTCTTCTC-3′) were substituted in the 92-mer with a 12-mer (5′-TCTAG-GCCTTCT-3′). This change was made in order to examine the influence of nucleotide sequence context on binding of DNA to HMG domain proteins. In order to study the size dependence, we synthesized the complement to Pt-GG20 to make a double-stranded 20-mer with a single *cis*-DDP−d(GpG) cross-link (Figure 5C).

Interactions of the HMG Domain Polypeptide with cis-DDP-Modified DNA. We employed the 81 amino acid HMG domain B polypeptide in gel mobility shift assays in order to determine whether it is a platinum−DNA binding portion of HMG1. Dissociation constants were calculated according to the literature (Pil & Lippard, 1992). In an experiment with 100- and 92-bp DNA (Figure 6), protein-DNA complexes were apparent at low protein concentrations (7×10^{-8} M). These experiments were performed under conditions in which binding to *cis*-DDP-modified DNA by HMG1 was apparent; the HMG domain concentrations ranged from 10^{-6} to 10^{-9} M. The isolated, purified HMG domain B was capable of binding strongly (Figure 6B; $K_d = 4 \times 10^{-7}$ M) to the 92-bp DNA probe that contained a single, site-specific *cis*-DDP adduct and exhibited binding with a 2.5-fold lower affinity ($K_d = 1 \times 10^{-6}$ M) to the unmodified 92-bp DNA.

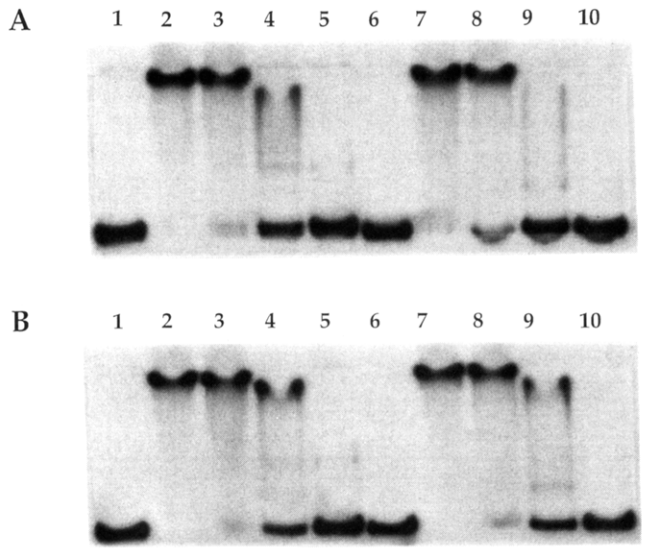

FIGURE 6: Binding of HMG domain B to *cis*-DDP-modified 100- or 92-bp DNA. (A) Lanes 1−5 contain cisplatin-modified 100-bp DNA, and lanes 6−10 contain unmodified 100-bp DNA. Lanes 1 and 6 do not contain protein. Lanes 2−5 and 7−10 contain 600, 60, 6, and 0.6 ng of HMG domain polypeptide, respectively. (B) Lanes are as described for (A), but with 92-bp DNA substituted for 100-bp DNA.

The dissociation constant for HMG1 and *cis*-DDP-modified 100-bp DNA was reported to be 3.7×10^{-7} M with a 100-fold lower affinity for unmodified DNA (Pil & Lippard, 1992). Similar binding by the HMG domain B to the 100-bp cisplatin-modified DNA was observed (Figure 6A; $K_d = 3 \times 10^{-7}$ M), but with only a 3−4-fold lower affinity for the unmodified DNA. The difference in specific versus nonspecific binding, although small, is quite reproducible and clearly observable on these gels. For example, comparison of lanes 3 and 8 or lanes 4 and 9 in Figure 6A reveals more shifted material in the first member of each pair, which contains the platinated DNA probes. The differences are even more subtle, but again real, in Figure 6B (compare, for example, lanes 3 and 8), owing to the lower affinity difference for the modified versus the unmodified probes.

The higher nonspecific affinity of the HMG domain polypeptide when compared to HMG1 is probably due to the high net positive charge of the protein under the binding conditions used in these investigations. At pH 7.9 the HMG domain B has a net charge of +8.5 (pI = 10.45), whereas HMG1 has a net charge of −6.2 at identical conditions (pI = 5.5). Favorable electrostatic interactions with the DNA backbone may enhance the nonspecific binding of the HMG domain polypeptide to DNA. The similar specific binding of the HMG domain and full-length HMG1 to both 92- and 100-bp DNAs (K_d values $\sim 10^{-7}$ M) supports the conclusions of earlier work (Bruhn et al., 1992) from analysis of sequence conservation that the HMG domain itself is the key element in binding to cisplatin-modified DNA. In the presence of cationic species such as $[Co(NH_3)_6]^{3+}$, the nonspecific binding of HMG domain B is reduced (data not shown), whereas the nonspecific binding of HMG1 is enhanced. Similarly, the addition of 0.5 M NaCl reduces the electrostatic binding of HMG domain B to DNA, but enhances the specific binding to cisplatin-modified DNA. These results suggest a role for the acidic domain of HMG1 in regulating nonspecific DNA binding by the protein and are consistent with published work (Sheflin et al., 1993; Stros et al., 1994a,b).

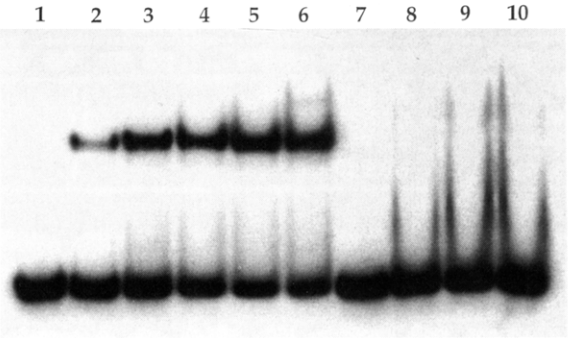

FIGURE 7: Binding of HMG domain B to *cis*-DDP-modified 20-bp DNA. Lanes 1−6 contain cisplatin-modified 20-bp DNA. Lanes 7−10 contain unmodified 20-bp DNA. Lanes 1 and 7: no protein. Lanes 2−6: 5, 10, 30, 50, and 80 ng of HMG domain B, respectively. Lanes 8−10: 50, 80, and 120 ng of HMG domain B, respectively. All reactions were carried out in the presence of 0.5 M NaCl.

Assays in which a globally platinated DNA probe is used to investigate protein binding are sometimes better able to discriminate specific versus nonspecific binding because they contain a variety of adducts. In the Southwestern blot experiment, for example, we employed a 123-bp DNA fragment containing an average of 5.6 cisplatin adducts, and these probes were better able to discriminate HMG domain binding to platinated versus unplatinated DNAs. The effects of nucleotide sequence content on the binding of site-specifically cisplatin-modified DNA to HMG domain proteins were therefore also examined. The 92-bp probe is related to the 100-bp probe (Pil & Lippard, 1992), but modified in the center by a sequence corresponding to the recognition sequence of *StuI*. The dissociation constant for HMG domain B and platinated 100-bp DNA is on the same order as that for the platinated 92-bp probe (10^{-7} M). These results reveal that little sequence specificity is involved in binding of the HMG domain to cisplatin-modified DNA.

The size dependence for efficient binding of cisplatin-modified DNA for HMG1 has previously been reported to be >60 bp (Pil, 1993). In contrast, the HMG domain B was able to bind with equal affinity to DNAs as small as 20 bp in length that contain a single cisplatin−d(GpG) cross-link. Figure 7 shows the result of HMG domain B binding to a double-stranded cisplatin-modified 20-mer. The K_d for specific binding is 5×10^{-7} M with a greater than 4-fold lower nonspecific binding to unmodified 20-mer ($K_d > 2 \times 10^{-6}$ M). HMG domain B did not exhibit any binding to single-stranded 20-bp control DNAs (data not shown). Other results in our laboratory reveal that HMG domain B can bind to DNAs as small as 15 bp with K_d values on the order of 10^{-6} M (S. A. Uldrich and S. J. Lippard, unpublished work).

DISCUSSION

The present investigation demonstrates that a single DNA-binding motif known as the HMG domain is sufficient for the recognition of cisplatin-modified DNA by eukaryotic proteins. A number of proteins with one or more HMG domains such as HMG1 (Pil & Lippard, 1992), HMG2 (Hughes et al., 1992), human SSRP1 (Bruhn et al., 1992), yeast SSRP, Ixr1 (Brown et al., 1993), *Drosophila* SSRP (Bruhn et al., 1993), hUBF (Treiber et al., 1994), mtTFA

(this study), and LEF-1 (Chow et al., 1994) recognize DNA modified with the antitumor drug cisplatin. Here we demonstrated that HMG domain B of HMG1 is sufficient for *cis*-DDP−DNA binding. The average sequence identity between the HMG domains of these proteins is only ~25%. In addition, no clear consensus sequence arises from comparisons between the HMG family members, with the exception of two invariant residues and several semiconserved aromatic and charged residues. All of these proteins share, however, the ability to recognize a 34° bend directed toward the major groove of the DNA helix by *cis*-DDP. These results are also consistent with *structure-specific,* rather than sequence-specific, binding of the DNA because several different sequences containing a single *cis*-DDP−d(GpG) cross-link are recognized by the HMG domain. Bianchi and co-workers (Bianchi et al., 1992; Ferrari et al., 1992) found similar results with the isolated HMG domains A and B binding to four-way junctions, which are also bent DNA structures. The degree of specificity could well depend on sequence context of the adduct, however, a subject that we are currently addressing in ongoing work.

The three-dimensional solution structure of HMG1 domain B was recently determined (Read et al., 1993; Weir et al., 1993). The domain is made up of three α helices that form an L shape; an 80° angle between the two arms of the L is defined by a cluster of conserved aromatic residues. Mutational analysis (Falciola et al., 1994) shows that one of these highly conserved aromatic residues (W49) has no effect on the binding of HMG domain A to a four-way junction, but instead affects stability and folding of the domain. Conversely, a semiconserved arginine residue (R10) is important for interactions with the four-way junction and exhibits a 5- to 10-fold decrease in binding when changed to a glycine residue. This basic residue resides on the concave surface of the HMG domain and may be important for specific DNA contacts. It has not yet been determined, however, which of the amino acids in domain B are important for recognition of cisplatin-modified DNA and whether they are the same residues that are responsible for four-way junction recognition.

The HMG domain B also facilitates the DNA bending activities of HMG1 (Onate et al., 1994; Paull et al., 1993; Pil et al., 1993). The HMG1-induced circularization of short DNA fragments does not require cisplatin modification. In fact, the platinum adduct has no effect on the DNA cyclization, which is caused by nonspecific binding of HMG1 or its B domain. Distortion of the DNA induced by cisplatin may resemble the naturally occurring binding site of HMG1, however, and the bendability in DNA afforded by the drug may additionally facilitate conversion of the double helix into its preferred binding configuration. Thus, a modest amount of specific binding to the cisplatin-modified DNA is observed, and the adduct serves to form a structural conformation that is favored by the HMG domain. The small mitochondrial protein mtTFA is an HMG domain protein which also has both nonspecific and specific DNA-binding properties (Fisher et al., 1992). This protein has the ability to wrap and bend random DNA, but it also binds specifically to a promoter sequence and causes a localized, sequence-directed bend. The properties of mtTFA are quite analogous to those of HMG1, and therefore it is not surprising that mtTFA also recognizes cisplatin-modified DNA. In addition, mtTFA is a mitochondrial accessory protein that is required for regulating transcription events involving promoter recognition by mammalian mitochondrial RNA polymerase. Similarly, HMG1 was recently shown to have multiple functions as both a transcriptional activator and a repressor (Ge & Roeder, 1994; Onate et al., 1994).

The roles of the HMG domains in mediating the true functions of HMG1 and the antitumor properties of cisplatin are not yet fully understood. Several models for the latter activity have been proposed, including the shielding of the toxic platinum−DNA adducts from repair (Brown et al., 1993; Huang et al., 1994) and DNA "hijacking", in which HMG proteins are titrated away from their normal binding sites by the cisplatin adducts (Treiber et al., 1994). The DNA-binding (this work) and -bending (Chow et al., 1994) properties of the HMG motif, as well as the interplay between its specific and nonspecific interactions with DNA, are likely to be significant in regulating its activity. HMG domain proteins appear to have important roles in DNA replication, transcription, repair, recombination, and packaging by a looping mechanism that involves an assembly of multiprotein complexes. Thus the antitumor mechanism of cisplatin may involve recognition of its major DNA adducts by the abundant HMG proteins which leads to alterations in DNA conformation and subsequent involvement of other proteins or factors.

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REFERENCES

Andrews, P. A., & Jones, J. A. (1991) *Cancer Commun. 3*, 1−10.
Bellon, S. F., & Lippard, S. J. (1990) *Biophys. Chem. 35*, 179−188.
Bellon, S. F., Coleman, J. H., & Lippard, S. J. (1991) *Biochemistry 30*, 8026−8035.
Bianchi, M. E. (1991) *Gene 104*, 271−275.
Bianchi, M. E., Beltrame, M., & Paonessa, G. (1989) *Science 243*, 1056−1059.
Bianchi, M. E., Falciola, L., Ferrari, S., & Lilley, D. M. J. (1992) *EMBO J. 11*, 1055−1063.
Brown, J. W., & Anderson, J. A. (1986) *J. Biol. Chem. 261*, 1349−1354.
Brown, S. J., Kellett, P. J., & Lippard, S. J. (1993) *Science 261*, 603−605.
Bruhn, S. L., Toney, J. H., & Lippard, S. J. (1990) *Prog. Inorg. Chem. 38*, 477−516.
Bruhn, S. L., Pil, P. M., Essigmann, J. M., Housman, D. E., & Lippard, S. J. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2307−2311.
Bruhn, S. L., Housman, D. E., & Lippard, S. J. (1993) *Nucleic Acids Res. 21*, 1643−1646.
Carballo, M., Puigdomenech, P., & Palau, J. (1983) *EMBO J. 2*, 1759−1764.
Cary, P. D., Turner, C. H., Mayes, E., & Crane-Robinson, C. (1983) *Eur. J. Biochem. 131*, 367−374.
Chao, C. C.-K., Huang, S.-L., Lee, L.-Y., & Lin-Chao, S. (1991) *Biochem. J. 277*, 875−878.
Chow, C. S., Whitehead, J. P., & Lippard, S. J. (1994) *Biochemistry 33*, 15124−15130.

Donahue, B. A., Augot, M., Bellon, S. F., Treiber, D. K., Toney, J. H., Lippard, S. J., & Essigmann, J. M. (1990) *Biochemistry 29*, 5872−5880.

Falciola, L., Murchie, A. I. H., Lilley, D. M. J., & Bianchi, M. E. (1994) *Nucleic Acids Res. 22*, 285−292.

Ferrari, S., Harley, V. R., Pontiggia, A., Goodfellow, P. N., Lovell-Badge, R., & Bianchi, M. E. (1992) *EMBO J. 11*, 4497−4506.

Fisher, R. P., Lisowsky, T., Parisi, M. A., & Clayton, D. A. (1992) *J. Biol. Chem. 267*, 3358−3367.

Gaillard, C., & Strauss, F. (1994) *Science 264*, 433−436.

Ge, H., & Roeder, R. G. (1994) *J. Biol. Chem. 269*, 17136−17140.

Grosschedl, R., Giese, K., & Pagel, J. (1994) *Trends Genet. 10*, 94−100.

Huang, J.-C., Zamble, D. B., Reardon, J. T., Lippard, S. J., & Sancar, A. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 10394−10398.

Hughes, E. N., Engelsberg, B. N., & Billings, P. C. (1992) *J. Biol. Chem. 267*, 13520−13527.

Jantzen, H.-M., Admon, A., Bell, S. P., & Tjian, R. (1990) *Nature 344*, 830−836.

Lawrence, D. C., Engelsberg, B. N., Farid, R. S., Hughes, E. N., & Billings, P. C. (1993) *J. Biol. Chem. 268*, 23940−23945.

Lilley, D. M. J. (1992) *Nature 357*, 282−283.

Loehrer, P. J., & Einhorn, L. H. (1984) *Ann. Intern. Med. 100*, 704−713.

MacFerrin, K. D., Chen, L., Terranova, M. P., Schreiber, S. L., & Verdine, G. L. (1993) *Methods Enzymol. 217*, 79−102.

McA'Nulty, M. M., & Lippard, S. J. (1995) in *Nucleic Acids & Molecular Biology* (Eckstein, F., & Lilley, D. M. J., Eds.) Springer, Berlin (in press).

Naser, L. J., Pinto, A. L., Lippard, S. J., & Essigmann, J. M. (1988) *Biochemistry 27*, 4357−4367.

Onate, S. A., Prendergast, P., Wagner, J. P., Nissen, M., Reeves, R., Pettijohn, D. E., & Edwards, D. P. (1994) *Mol. Cell. Biol. 14*, 3376−3391.

Parisi, M. A., & Clayton, D. A. (1991) *Science 252*, 965−969.

Paull, T. T., Haykinson, M. J., & Johnson, R. C. (1993) *Genes Dev. 7*, 1521−1534.

Pil, P. M. (1993) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge.

Pil, P. M., & Lippard, S. J. (1992) *Science 256*, 234−237.

Pil, P. M., Chow, C. S., & Lippard, S. J. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 9465−9469.

Read, C. M., Cary, P. D., Crane-Robinson, C., Driscoll, P. C., & Norman, D. G. (1993) *Nucleic Acids Res. 21*, 3427−3436.

Roberts, J. J., & Thomson, A. (1979) *Prog. Nucleic Acids Res. Mol. Biol. 22*, 71−133.

Sheflin, L. G., Fucile, N. W., & Spaulding, S. W. (1993) *Biochemistry 32*, 3238−3248.

Sinclair, A. H., Berta, P., Palmer, M. S., Hawkins, J. R., Griffiths, B. L., Smith, M. J., Foster, J. W., Frischauf, A.-M., Lovell-Badge, R., & Goodfellow, P. N. (1990) *Nature 346*, 240−244.

Singh, H., LeBowitz, J. H., Baldwin, A. J., Jr. & Sharp, P. A. (1988) *Cell 52*, 415−423.

Stros, M., Reich, J., & Kolibalova, A. (1994a) *FEBS Lett. 344*, 201−206.

Stros, M., Stokrova, J., & Thomas, J. O. (1994b) *Nucleic Acids Res. 22*, 1044−1051.

Toney, J. H., Donahue, B. A., Kellett, P. J., Bruhn, S. L., Essigmann, J. M., & Lippard, S. J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 8328−8332.

Travis, A., Amsterdam, A., Belanger, C., & Grosschedl, R. (1991) *Genes Dev. 5*, 880−894.

Treiber, D. K., Zhai, X., Jantzen, H.-M., & Essigmann, J. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 5672−5676.

van de Wetering, M., Oosterwegel, M., Dooijes, D., & Clevers, H. (1991) *EMBO J. 10*, 123−132.

Waga, S., Mizuno, S., & Yoshida, M. (1988) *Biochem. Biophys. Res. Commun. 153*, 334−339.

Weir, H. M., Kraulis, P. J., Hill, C. S., Raine, A. R. C., Laue, E. D., & Thomas, J. O. (1993) *EMBO J. 12*, 1311−1319.

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