Binding of tsHMG, a Mouse Testis-Specific HMG-Domain Protein, to Cisplatin−DNA Adducts†

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ABSTRACT: The anticancer drug cisplatin is particularly effective against testicular tumors. Although the clinical consequences of cisplatin chemotherapy are well-known, the precise mechanism of action remains elusive. Specific recognition of cisplatin-damaged DNA by a class of proteins containing the high-mobility group (HMG) domain DNA-binding motif could play a role in mediating the cytotoxicity of the drug. This study presents a quantitative investigation of binding of the murine testis-specific high-mobility group protein tsHMG to DNA modified by cisplatin. The binding affinity and specificity of this protein to a site-specific 1,2-d(GpG) cisplatin−DNA intrastrand cross-link in a 20 bp probe were determined. A value for the apparent dissociation constant, $K_{d(app)}$, of 24 ± 5 nM was obtained by gel mobility shift assays. Binding competition assays with the corresponding unmodified 20 bp probe gave a ratio (ρ) of nonspecific to specific $K_{d(app)}$ values of 230. A polypeptide containing tsHMG domain A (residues 1−82) was expressed and purified to homogeneity. This domain alone was sufficient for specific recognition of cisplatin-modified DNA with a $K_{d(app)}$ of 300 ± 50 nM and a ρ of 20, a comparatively high discrimination factor. DNase I interference analysis of the adduct-containing strand revealed that tsHMG binding extends over 14 nucleotides, centered around the platinated bases. The domain A polypeptide protection pattern covers a slightly smaller area of 13 nucleotides. The binding affinity and specificity of tsHMG for cisplatin-modified DNA are exceptional compared to those of other HMG-domain proteins studied previously. The possible relevance of these findings to the mechanism of action of cisplatin is discussed.

DNA bending and unwinding are essential for initiating transcription, replication, and site-specific recombination, processes all mediated by proteins. One class of proteins having the ability to induce specific bending in linear DNA and to interact with distorted DNA structures, such as four-way junctions, are those containing a high-mobility group (HMG)[1] domain (*1*). This domain comprises an 80-amino acid DNA-binding motif. The HMG domain is essentially α-helical in structure with a high proportion of proline, aromatic, and basic amino acid residues. The HMG-domain protein family can be divided into two subgroups (*2*), the first of which includes mammalian HMG1 and similar proteins associated with chromatin in eukaryotes. The second subgroup includes transcriptional regulators, such as SRY and LEF-1, and transcription factors such as hUBF and mtTFA. The HMG-domain proteins SRY and LEF-1 bind to specific DNA sequences by using a hydrophobic residue as an intercalative wedge and unstacking several base pair steps. They locally unwind and bend DNA toward the major groove, widening the minor groove (*3*, *4*).

These structural changes are similar to those observed when the N7 atoms of two adjacent guanine bases in a DNA duplex form bonds to the *cis*-diammineplatinum(II) moiety generated by the loss of chloride ions from the anticancer drug cisplatin. The crystal structure of a double-stranded DNA dodecamer containing the cisplatin 1,2-intrastrand d(GpG) cross-link reveals that platinum coordination unwinds and bends the DNA toward the major groove, destacking the two guanosine nucleotides, and opening and flattening the minor groove (*5*, *6*).

Cisplatin is currently one of the most potent antineoplastic agents, particularly useful for treatment of testicular cancer (*7*) but also widely employed for ovarian, breast, and head and neck tumors. Coordination to the heterocyclic N7 atoms of the DNA purine bases is generally accepted as the primary reaction responsible for the anticancer activity of the drug (*8*). The precise mechanism of action remains elusive, however. Cisplatin 1,2-intrastrand cross-links between two adjacent guanine bases, or between an adenine and a guanine base, account for over 85% of all adducts formed (*9*). Platination blocks replication and transcription (*10*), ultimately inducing cell death. The DNA adducts formed by the clinically inactive geometric isomer *trans*-DDP, however, also inhibit DNA and RNA synthesis (*11*−*13*). Differential processing of *cis*- and *trans*-DDP-modified DNA could be a consequence of their disparate binding by cellular proteins. HMG-domain proteins, in particular, bind specifically to cisplatin-modified DNA, which could explain the effectiveness of this chemotherapeutic agent (*14*).

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[1] Abbreviations: HMG, high-mobility group; bp, base pair; BTP or Bis-tris-propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; *cis*-DDP or cisplatin, *cis*-diamminedichloroplatinum(II); FPLC, fast protein liquid chromatography; IPTG, isopropyl β-D-thiogalactopyranoside; Ixr1, intrastrand cross-link recognition protein; LEF-1, lymphoid enhancer binding factor 1; m-mtTFA, mouse mitochondrial transcription factor A; xl-mtTFA, *Xenopus laevis* mitochondrial transcription factor A; PAGE, polyacrylamide gel electrophoresis; β-ME, β-mercaptoethanol; SDS, sodium dodecyl sulfate; SRY, sex-determining region Y protein; *trans*-DDP, *trans*-diamminedichloroplatinum(II); tris, tris(hydroxymethyl)aminomethane; tsHMG, testis-specific high-mobility group protein.

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Several models have been proposed that link the affinity of cellular proteins for cisplatin−DNA adducts with the pharmacological effects of the drug (*15*). One hypothesis suggests that proteins which are critical for cell viability are diverted from their natural promoters by binding to the cisplatin−DNA cross-links, leading to disruption of function. In support of this model, the rRNA transcription factor hUBF binds to *cis*-DDP−DNA adducts with an affinity similar to that for its natural promoter (*16*). According to another hypothesis, replication and transcription of genes essential for cell survival are disturbed by cisplatin modification because the protein−drug−DNA complex prevents the cellular repair apparatus from removing the damage. This repair shielding model is consistent with results obtained *in vivo* and *in vitro* (*17*−*19*), which indicate that HMG-domain proteins inhibit the nucleotide excision repair of cisplatin 1,2-intrastrand cross-links.

A major focus of current research is explaining the specific toxicity of *cis*-DDP toward testicular tumors. If HMG-domain proteins play an important role in mediating the anticancer activity of cisplatin, they would have to be localized and/or activated in tumor cells. The human testis-determining factor SRY (*20*), and the human mismatch-repair protein hMSH2 (*13*), both expressed in testicular tissue, bind with modest specificity to cisplatin−DNA adducts. Recently, a cDNA clone for a murine testis-specific HMG protein, tsHMG, was isolated from a testis cDNA library (*21*). The sequence of tsHMG is identical with that of the mitochondrial transcription factor m-mtTFA, except that it lacks the amino-terminal mitochondrial targeting peptide. The 23.3 kDa nuclear protein tsHMG has two HMG domains, domain A (residues 1−82) and domain B (residues 108−187), separated by a β-turn. The protein is found in the nuclei of spermatocytes and elongating spermatids. Although the precise function of tsHMG is currently unknown, by analogy to m-mtTFA, it may act as a sequence-specific transcription factor. A structural role could also be envisioned, consistent with the affinity of the protein for supercoiled DNA and its ability to introduce negative supercoils into a relaxed plasmid substrate in a topoisomerase I-dependent manner (*22*, *23*).

Previously, we reported that tsHMG inhibits repair of cisplatin-damaged DNA in cell extracts (*17*). In this study, we describe in a quantitative manner specific interactions between tsHMG and well-defined *cis*-DDP−DNA adducts. In particular, we demonstrate that both tsHMG and its domain A recognize a cisplatin 1,2-d(GpG) cross-link with very high affinity and specificity, report their DNase I footprints, and describe how these results may relate to the clinical efficacy of the drug.

EXPERIMENTAL PROCEDURES

Purification of tsHMG. A revised protocol was employed for the purification of tsHMG. The vector pET-15b-tsHMG was used to overexpress tsHMG in *Escherichia coli* BL21-(DE3) cells as described (*24*). From this vector, tsHMG was obtained as a fusion protein with a polyhistidine N-terminal tag. Induced cells were harvested after 2.5 h, resuspended in lysis buffer A [50 mM Tris-HCl (pH 7.0), 200 mM NaCl, 10% glycerol, 10 mM β-ME, 0.1% TWEEN, 1 mM EDTA, and 10 mg/mL Pefabloc (Boehringer Mannheim)], and sonicated. The resulting lysate was cleared by centrifugation at 40000*g* and 4 °C for 45 min. The supernatant containing the recombinant tsHMG was loaded onto a Heparin cartridge (Bio-Rad) and eluted with a linear salt gradient from 0 to 1 M NaCl [high-salt buffer, 50 mM Tris-HCl (pH 7.0), 1 mM EDTA, 10% glycerol, 10 mM β-ME, and 1 M NaCl] over 600 min. The tsHMG-containing fractions were identified by SDS−polyacrylamide gel electrophoresis (SDS−PAGE), concentrated, and further purified by His-Bind resin chromatography as described by the manufacturer (Novagen). The fractions containing pure recombinant tsHMG were washed into 20 mM Tris-HCl (pH 7.0), concentrated, and stored at −20 °C. Protein purity was determined by Coomassie-stained SDS−PAGE.

Construction of the Expression Plasmid for tsHMG Domain A. Primers were designed for PCR amplification of tsHMG domain A (residue 1−82) and synthesized on an automated DNA synthesizer (Cruachem model PS250). The 54-nucleotide sequence of the primer for the coding strand 5′-GTACGTCTATGAATTCTTAACCAGGAGCT-GATTATGGGTAGCTATCCAAAGAAA-3′ consists of a clamp, an *Eco*RI restriction enzyme site, a ribosomal binding site, a start codon, and the first 18 nucleotides coding for tsHMG domain A. The 36-nucleotide sequence of the primer for the anticoding strand 5′-GAGGTTCAGTCGACTA-CATTTTCGAATATCTGAATG-3′ contains the last 17 nucleotides coding for tsHMG domain A, a stop codon, a *Hin*dIII restriction enzyme site, and a 10-nucleotide clamp. The primers, 50 pmol each, were used in a 100 μL PCR with 125 ng of pET15b-tsHMG plasmid, 0.2 mM dNTPs, 1× Taq reaction buffer, and 5 units of Taq polymerase. The products were analyzed on a 0.8% agarose gel and purified by using a Qiagen PCR kit. After restriction enzyme digestion and purification of the PCR product and the expression vector pLM1, the PCR product was ligated into the vector by using T4 DNA ligase. In the expression plasmid pLM1, the cloned genes are under control of the tac promoter and the rrnBT1T2 transcription terminator. The resulting vector pLM1-tsHMGdomA was sequenced by using the Sequenase DNA sequencing kit, Version 2.0 (USB), to ensure the presence of the appropriate insert and its correct orientation relative to the tac promoter.

Expression and Purification of Recombinant Mouse tsHMG Domain A. The tsHMG domain A polypeptide was expressed and purified from pLM1-tsHMGdomA/BL21-(DE3). The *E. coli* bacteria were grown in LB medium (*24*) in the presence of 0.14 mM ampicillin (Sigma) at 37 °C. Transcription of the cloned gene was induced at an OD_{600} of 0.8 by addition of IPTG (Boehringer Mannheim) to a final concentration of 1.2 mM. After an induction period of 2.5 h, cells were harvested by centrifugation for 15 min at 5000 rpm (GS53 rotor, Sorvall). The cells were resuspended in 120 mL of lysis buffer A and passed through a French pressure cell twice at 1300 psi. Debris was removed by ultracentrifugation at 40 000 rpm for 45 min (Ti45 rotor, Beckman). The supernatant was loaded onto an S-Sepharose column (Pharmacia). The proteins were eluted at a flow rate of 1 mL/min with a linear gradient from 0.05 to 0.5 mM NaCl in 50 mM Tris-HCl (pH 7.0) and 5 mM β-ME over 600 min and collected in 7.5 mL fractions. The tsHMG

domain A-containing fractions were identified by an SDS−PAGE 10−20% tris-glycine Mini-Gel (Bio-Rad) and eluted between 85 and 190 mM NaCl. Fractions were pooled and diluted 1:2 with water prior to loading onto an Econo-Pac heparin cartridge (Bio-Rad). The same gradient was applied, the protein being eluted between 130 and 163 mM NaCl. The protein-containing fractions were concentrated in Millipore Ultrafree 5K filter units (MWCO 5000) and loaded onto an FPLC size exclusion column (Superdex 75 Pharmacia). The tsHMG domain A polypeptide was eluted with PBS (phosphate-buffered saline) over 450 min at a flow rate of 0.5 mL/min and washed into 10 mM BTP (Sigma). Purity was determined by mass spectrometry. Electrospray mass spectra were obtained on a Hewlett-Packard 5989B mass spectrometer operated in the positive ion mode. The instrument was tuned on a peptide mixture and on myoglobin just prior to analysis. Samples were dissolved in a mixture of 80:20 water/2-propanol with 2% propionic acid or 50:50 water/methanol with 0.05−1% acetic acid and then flow injected into a stream of the same solvent delivered at 1−20 μL/min with a syringe pump. Typically, 10 scans were averaged and the resulting spectra were deconvoluted with the appropriate routine in the HP Chemstation software. Protein concentrations were determined from A_{280} by using the extinction coefficient (ϵ = 22 500 M^{-1} cm^{-1}). The extinction coefficient was obtained by measuring the A_{280} of a solution containing a known amount of protein determined by amino acid analysis.

DNA Probes. The globally cisplatin-modified 123 bp probe and the corresponding unmodified probe were prepared as described in the literature (*25*). The cisplatin-modified (20-Pt) and unmodified (20-Ct) 20 bp probes were obtained as previously discussed (*26*). The 92 bp probes, containing a single site-specific 1,2-*cis*-[Pt(NH_3)$_2${d(GpG)-N7(G1),-N7-(G2)}] cross-link in the center (92-Pt) and the corresponding unmodified oligonucleotide (92-Ct), were prepared according to a published protocol [92BENDPt and 92BENDCt (*27*)]. DNA probes for the footprinting assays were obtained from the blunt-ended 92 bp oligonucleotides. The top strand was labeled by digesting 92-Pt and 92-Ct, respectively, with the restriction enzyme *Ava*I for 1 h at 37 °C, which removes the last five nucleotides on the 3′ side, and a subsequent fill-in reaction with [α-^{32}P]dCTP and unlabeled dGTP by the Klenow fragment of DNA polymerase, resulting in the two 87 bp probes, 87-Pt and 87-Ct. The Klenow fragment in the reaction mixtures was removed by phenol extraction (*24*). The unincorporated label was removed by passing the sample over a Sephadex G25 spin column (Boehringer Mannheim). The labeled footprinting probes were ethanol precipitated twice before carrying out Maxam−Gilbert sequencing and binding reactions. The Maxam−Gilbert sequencing reactions specific for the nucleotides guanine and cytosine plus thymine were performed for each labeled strand of the 87 bp duplex oligonucleotides according to a published procedure (*24*). DNA concentrations were determined by measuring the A_{260} value and assuming $1OD_{260}$ = 50 μg/mL (*24*).

Southwestern Blot Assays. Samples were separated on a 12% SDS−PAGE gel and electroblotted overnight at 4 °C and 29 mA onto 0.45 μm nitrocellulose membranes (S&S NC, Schleicher and Schuell). The resulting blots were processed as previously described in the literature (*28*). The assay for platinum-specific binding was carried out with a 123 bp probe by following a published procedure (*25*).

Gel Mobility Shift Assays. All binding reactions for gel mobility shift assays were carried out in 1× binding buffer [10 mM HEPES (pH 7.2), 4% glycerol, 10 mM $MgCl_2$, 50 mM KCl, 1 mm EDTA, 1 mM spermidine, and 0.05% NP40] in a total volume of 10−15 μL. Binding reaction mixtures with the 92 bp probe also contained 2.5 μg of salmon sperm competitor DNA. The reaction mixtures were incubated for 15 min on ice prior to addition of 2 μL of loading dye (40% sucrose and 0.01% xylene cyanol) and subsequent analysis by gel electrophoresis on prerun, precooled (4 °C), 0.5× TBE [45 mM Tris, 45 mM boric acid, and 1 mM EDTA (pH 8.0)], 5 or 7.5% native polyacrylamide gels [29:1 acrylamide/*N*,*N*′-methylenebis(acrylamide)]. The gels were run for 1−2 h at 300 V and 4 °C and then vacuum-dried onto Whatman 3 MM chromatography paper and exposed to X-ray film (Kodak X-OMAT AR). For quantitative measurements of bound and free oligonucleotide, performed by phosphorimager analysis (Molecular Dynamics PhosphorImager), only the fraction of free or complexed DNA entering the gel was taken into account.

Apparent Dissociation Constants. Gel mobility shift assays to determine the apparent dissociation constants were carried out with a 20 bp probe d(TCTCCTTCTG*G*TCTCTTCTC)·d(GAGAAGAGACCAGAAGGAGA), where the asterisks denote the cisplatin-modified purine sites. Reaction mixtures contained a fixed concentration of the [γ-^{32}P]dATP-labeled 20 bp probe (7.5−30 nM). Varying amounts of tsHMG or tsHMG domain A were titrated into the binding reaction mixtures. Bands corresponding to bound and unbound probe were quantitated, and the data were fit by nonlinear least-squares analysis (KaleidaGraph for Macintosh, Version 3.0). Apparent dissociation constants from direct titration of the DNA probe with tsHMG were estimated from fits to the Langmuir isotherm [eq 1 (*29*)]

$$\theta = \frac{P}{P + K_d} \quad (1)$$

where θ is the fraction of radiolabeled DNA bound, P is the total protein concentration, and K_d is the apparent dissociation constant $K_{d(app)}$, assuming a protein:DNA binding ratio of 1:1. Apparent dissociation constants were determined in two or three independent experiments. The reported errors were based on a statistical fit of the data and represent ±1 standard deviation.

Competition Assays. Relative dissociation constants were determined in a competition assay. The tsHMG protein (30 nM) or tsHMG domain A (400 nM) was incubated for 10 min on ice with varying concentrations of unlabeled 20-Pt or 20-Ct in a total volume of 13 μL. After addition of [γ-^{32}P]-dATP-labeled 20-Pt (7.5 nM), the reaction mixtures were incubated for an additional 15 min to establish a concentration at which 10−80% of the labeled DNA was bound. Reaction mixtures were resolved by electrophoresis as described for the gel mobility shift assays. The bands corresponding to bound and unbound probe were quantitated by using phosphorimager analysis. The competitive binding of a protein to the labeled DNA probe and the unlabeled competitor DNA can be described by eq 2 (*30*)

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

where K_{rel} is the ratio of K_t, the apparent dissociation constant of the labeled probe, to K_C, the apparent dissociation constant of the competitor, and P_t, T_t, and C_t are the concentrations of protein, radiolabeled probe, and competitor probe, respectively. Competition data were fit to eq 2, and errors were based on the statistical fit of the data and represent ±1 standard deviation. Each data point was determined in three or four independent experiments.

DNase I Footprint. DNase I (Worthington, DPFF grade) was dissolved at 10 mg/mL in 50 mM Tris-HCl (pH 7.0), stored in 20 μL aliquots at −80 °C, and thawed on ice immediately before use. Reaction mixtures were prepared as follows. A 100 000 cpm aliquot of [α-^{32}P]dCTP-labeled cisplatin-modified 87 bp probe, 2 μg of salmon sperm competitor DNA, and different concentrations of tsHMG or tsHMG domain A polypeptide were incubated on a 20 μL scale in binding buffer [5×, 100 mM HEPES (pH 7.2), 500 mM KCl, 35 mM $MgCl_2$, 7.5 mM $CaCl_2$, 0.5 mM EDTA, and 0.5 mg/mL BSA] and kept on ice for 15 min. Binding reactions with the full length tsHMG protein were resolved on a 5% band shift gel. The bands corresponding to the specific complex were excised, crushed, and immersed in 1× binding buffer containing 3 μg/μL DNase I for 3 min. The digestion was stopped by addition of 5 mM EGTA (pH 7.3) and 50 μg/mL chicken erythrocyte DNA (stop solution). After the solution was filtered to remove the polyacrylamide gel pieces, the DNA was ethanol precipitated twice. The binding reaction mixtures containing tsHMG domain A were digested by addition of 2 μL of DNase I to a final concentration of 3 or 1 μg/μL, at room temperature for 1 min. The digestions were terminated by addition of 100 μL of stop solution. The DNA was precipitated with cold ethanol. The resulting DNA pellets were dried and redissolved in 8 μL of loading buffer (90% formamide, 10 mM EDTA, 0.05% bromophenol blue, and 0.05% xylene cyanol) and electrophoresed in 1× TBE buffer on 8% denaturing polyacrylamide gels. The gels were dried and subjected to autoradiography at −80 °C in the presence of an intensifying screen.

RESULTS

Purification of tsHMG and Detection of Specific Binding to Cisplatin-Modified DNA. Coomassie blue staining of an SDS−PAGE gel confirmed that the 29 kDa protein was >90% pure after purification as described in Experimental Procedures (Figure 1A). For the Southwestern assay, the purified tsHMG protein was resolved on a 12% SDS−PAGE and subsequently electroblotted onto nitrocellulose. When the blot was probed with a ^{32}P-labeled cisplatin-modified 123 bp oligonucleotide, a band was observed corresponding to an apparent molecular mass of approximately 29 kDa (Figure 1B). This result is consistent with the predicted molecular mass of tsHMG fused to the N-terminal polyhistidine tag and thrombin cleavage site. A small amount of higher-molecular mass proteins able to hybridize with cisplatin-modified DNA was also observed. When an identical blot was probed with an unmodified 123 bp DNA duplex, no bands appeared (data not shown). This initial result encouraged a more thorough investigation of tsHMG binding to cisplatin-modified DNA.

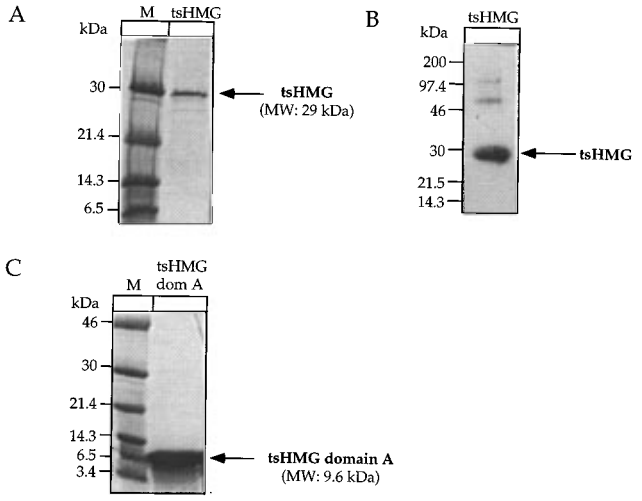

FIGURE 1: Analysis of purified full length tsHMG and tsHMG domain A by Coomassie blue-stained SDS−PAGE and Southwestern blotting. Molecular mass markers (M) are indicated. (A) Resolution of full length tsHMG on a 15% SDS−PAGE. (B) Southwestern analysis of tsHMG, probed with a globally cisplatin-modified 123 bp DNA. (C) Resolution of tsHMG domain A on a 4−20% SDS−PAGE tris-glycine gel.

Expression and Purification of tsHMG Domain A. The full length tsHMG contains two HMG domains. Domain A was chosen for investigating the ability of a single HMG domain to confer specific recognition of a cisplatin−DNA cross-link to the protein. As shown in Figure 1C, the purification protocol described for the domain A polypeptide yielded homogeneous protein. The molecular mass for this protein was determined to be 9493.5 Da by electrospray ionization mass spectrometry (calculated mass of 9494.0 Da). Amino acid analysis and N-terminal sequencing confirmed that the desired protein was obtained without an additional methionine start codon.

Gel Mobility Shift Assays of tsHMG and tsHMG Domain A with Cisplatin-Modified DNA. To investigate further the ability of tsHMG to bind specifically to cisplatin-modified DNA, a gel mobility shift assay was performed. The protein was titrated with increasing amounts of tsHMG in binding reaction mixtures which contained either 92-Pt (Figure 2A) or 92-Ct (Figure 2B) in the presence of a 500-fold excess of salmon sperm competitor DNA. With increasing concentrations of tsHMG, more 92-Pt DNA was recruited to a slower migrating form due to formation of a specific tsHMG−DNA complex. Half-maximal binding occurs at a concentration of approximately 0.4 μM tsHMG. In the gel mobility shift assay performed with the corresponding unmodified probe 92-Ct, a band accounting for a distinct complex grows at higher protein concentrations, resulting in half-maximal binding at approximately 6 μM. At tsHMG concentrations of >1.4 μM, an increasing amount of 92-Ct is prevented from entering the gel, possibly due to formation of higher-molecular mass protein−DNA aggregates. This phenomenon is also observed with the 92-Pt probe, although to a lesser extent. Formation of these high-molecular mass complexes and the need for salmon sperm DNA as a competitor in the binding reactions did not allow for an accurate determination of the dissociation constant with this probe. Gel mobility shift assays carried out in the absence

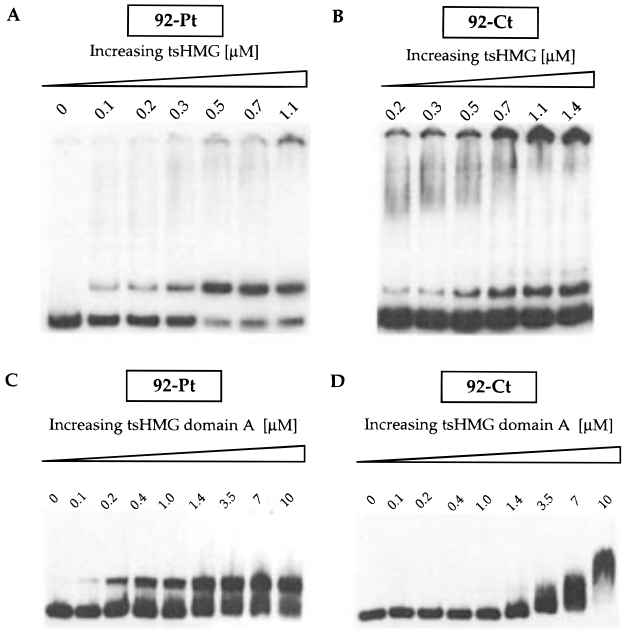

FIGURE 2: Binding of full length tsHMG and tsHMG domain A to 92 bp DNA probes. 92-Pt contains a single 1,2-d(GpG) intrastrand cross-link, and 92-Ct is the unmodified control. Increasing amounts of tsHMG were titrated into binding reaction mixtures containing 2.5 μg of salmon sperm competitor DNA and (A) 5 ng of labeled 92-Pt or (B) 5 ng of labeled 92-Ct. The tsHMG domain A polypeptide was titrated into binding reaction mixtures containing 2.5 μg of salmon sperm competitor DNA and (C) 5 ng of labeled 92-Pt or (D) 5 ng of labeled 92-Ct.

of competitor salmon sperm DNA resulted in a band shift pattern similar to that observed with 20 bp probes (vide infra), with three distinct bands of retarded mobility (data not shown). The significantly higher amount of nonspecific binding and the formation of protein−DNA aggregates at much lower tsHMG concentrations precluded quantitative analysis.

The affinity of tsHMG domain A for *cis*-DDP−DNA adducts was also investigated. This polypeptide displays DNA binding behavior similar to that of the full length tsHMG. In the gel mobility shift assays with the 92-Pt DNA, only a single retarded band is observed, accounting for formation of a specific complex (Figure 2C). Titration of the 92-Ct with increasing amounts of protein resulted in a smeared band of continuously decreasing mobility, attributed to nonspecific binding (Figure 2D).

DNase I Footprint. The structures of the complex between tsHMG or tsHMG domain A and cisplatin-modified DNA were probed by nuclease digestion with DNase I. The 87 bp probes were obtained from the 92 bp probes used in the gel mobility shift assays by restriction enzyme digestion and a subsequent fill-in reaction with radiolabeled [α-^{32}P]dCTP. Figure 3 shows the DNase I digestion pattern in which 87-Ct, as well as the modified analog, 87-Pt containing one cisplatin 1,2-d(GpG) intrastrand cross-link, was bound by tsHMG and tsHMG domain A and subsequently treated with the enzyme. Maxam−Gilbert sequencing reactions were run in parallel (data not shown). The digestion pattern of the complexed, platinated probe 87-Pt shows that the cleavage frequency varies along the sequence, reflecting the structural distortions induced by the platinum lesion. The region protected from DNase I digestion by tsHMG in the complex extends over 14 bp, 6 bp on either side of the cisplatin adduct. Domain A covers a slightly smaller area of 13 bp, 6 bp toward the 5′ end and 5 bp on the 3′ side. When the large diameter of DNase I is taken into account (*31*), however, the length of DNA protected by complex formation with tsHMG or tsHMG domain A is probably closer to 10 or 9 bp, respectively. Compared to that of the full length protein, the cleavage pattern of the tsHMG domain A complex displays an additional cutting site 1 bp downstream from the platinum lesion. No difference in cutting frequency was observed in the presence or absence of protein for the unmodified 87-Ct probe.

Determination of the Apparent Dissociation Constants $K_{d(app)}$. A 20 bp probe containing a single centered 1,2-*cis*-[Pt(NH_3)$_2${d(GpG)-N7(G1),-N7(G2)}] intrastrand cross-link (20-Pt) was employed for quantitative studies, to minimize nonspecific protein−DNA interactions. Binding titrations of tsHMG and tsHMG domain A were carried out under identical conditions. The fraction bound, θ, was determined by gel mobility shift assays and subsequent phosphorimager analysis. A plot of the binding data is presented in Figure 4. The θ versus protein concentration data were fit by nonlinear least squares to eq 1. The gel mobility shift performed with tsHMG displays three distinct bands of retarded mobility accounting for formation of three different complexes (data not shown). For quantitation of the fraction bound (θ), all three shifted bands were taken into account, affording a $K_{d(app)}$ value of 24 ± 5 nM. Because the aggregation state of the protein has not been determined, this $K_{d(app)}$ value assumes that tsHMG binds as a monomer. The formation of several shifted bands may be a consequence of protein multimerization, which would alter the $K_{d(app)}$ values but not the ratio of specific to nonspecific binding constants.

Titration of 20-Pt with tsHMG domain A resulted in formation of one single band of lower mobility. No specific shift is observed with the unmodified probe 20-Ct (data not shown). Quantitation of the fraction bound and a fit of the resulting binding isotherm produced a $K_{d(app)}$ value of 300 ± 54 nM (Figure 4), approximately 13-fold higher than the value determined for the full length protein.

Relative Binding Affinities. To assess the specificity of protein binding to platinated DNA, competition experiments were carried out in which the relative binding affinities of tsHMG and tsHMG domain A for 20-Pt versus the unmodified probe 20-Ct were measured. A representative set of experiments is presented in Figure 5. A fit of the data for tsHMG yields a $K_{d(app)}$ of 30 ± 5 nM for the specific competitor 20-Pt, in very good agreement with the binding titration experiments. The $K_{d(app)}$ value for the nonspecific competitor is 6.9 ± 3.5 μM. The specificity index, defined as the ratio (ρ) of nonspecific to specific dissociation constants, is 230 for tsHMG (Table 1).

Compared to the full length protein, tsHMG domain A bound platinated DNA with much lower specificity; $K_{d(app)}$(20-Pt) = 0.59 ± 0.34 μM, and $K_{d(app)}$(20-Ct) = 11 ± 5 μM (Figure 6). These results afford a ρ value of approximately 20 for tsHMG domain A.

DISCUSSION

Binding Affinity and Specificity. In this investigation, we demonstrated that the nuclear HMG-domain protein tsHMG binds with high affinity and specificity to the major

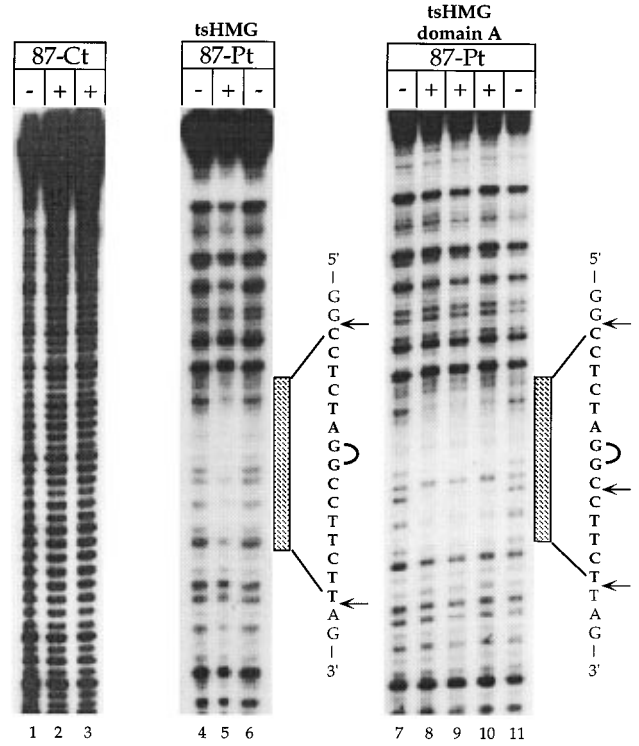

FIGURE 3: The 87 bp DNA fragment containing a single cisplatin-d(GpG) 1,2-intrastrand cross-link is protected from DNase I cleavage in the presence of tsHMG and tsHMG domain A. The sequence of the protected region is shown on the right. Arrows indicate the cleavage sites. The DNA sequence was determined by Maxam−Gilbert sequencing reactions run in parallel (data not shown). The analogous unmodified 87-Ct DNA probe gives the same DNase I cleavage pattern both in the presence and in the absence of tsHMG or tsHMG domain A. Digestion of the unmodified 87-Ct probe (lane 1) without protein, (lane 2) with 1.6 μM tsHMG, and (lane 3) with 6.2 μM tsHMG domain A. Digestion of 87-Pt (lanes 4, 6, 7, and 11) with no protein added, (lane 5) with 1.6 μM tsHMG, and (lanes 8−10) with 6.2 μM tsHMG domain A.

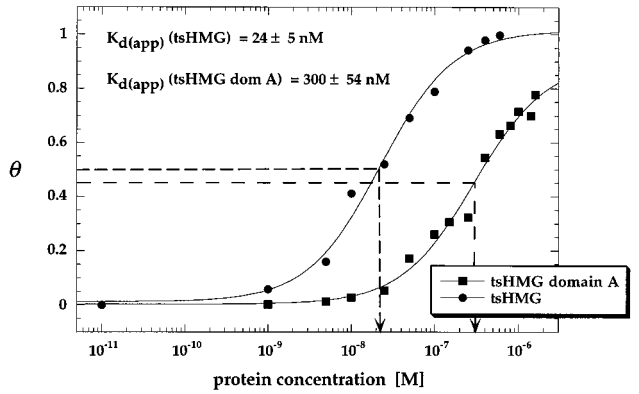

FIGURE 4: Results for full length tsHMG (solid circles) and tsHMG domain A (solid squares) binding to 20 bp DNA probes. Data are plotted as the fraction of radiolabeled probe bound (θ) versus the concentration of protein. Solid lines are the best least-squares fit to eq 2. The protein concentration giving half-maximal binding, $K_{d(app)}$, is indicated by the broken line.

cisplatin−DNA adduct. The value of 30 ± 5 nM for the apparent dissociation constant $K_{d(app)}$ for full length protein binding to a 20 bp probe containing a single cisplatin 1,2-d(GpG) adduct corresponds to an approximately 230-fold preference for platinated DNA over unplatinated DNA. This specificity of tsHMG for platinated DNA is much higher than that observed for other HMG-domain proteins, including

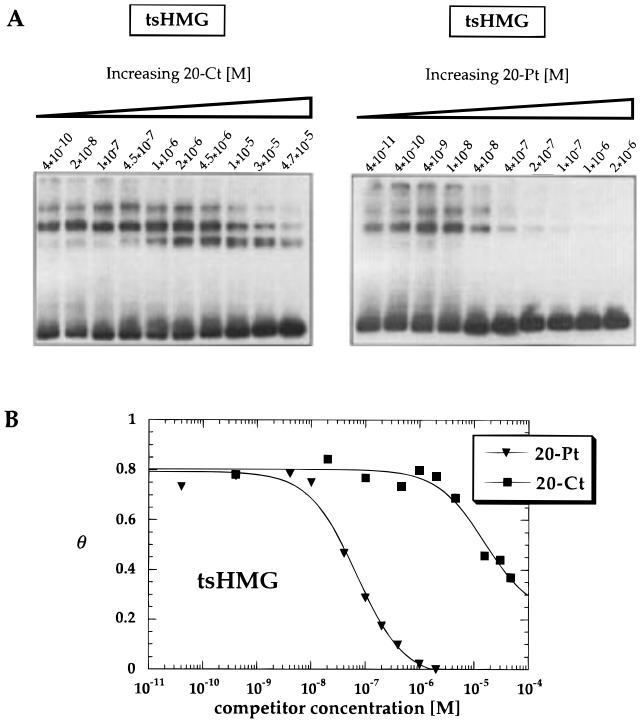

FIGURE 5: Specificity of DNA binding by full length tsHMG determined by competitive binding experiments. (A) Gel mobility shift assays of binding reactions where unlabeled competitor DNAs 20-Pt or 20-Ct were titrated into binding reaction mixtures containing 30 nM tsHMG and 7.5 nM (4000 cpm) ^{32}P-labeled 20-Pt probe. (B) Data from one set of experiments are plotted as the fraction of bound radiolabeled 20-Pt probe versus the concentration of unlabeled competitor DNA 20-Pt (solid triangles) and 20-Ct (solid squares).

Table 1: Binding Affinities and Specificities of m-tsHMG and the Isolated HMG Domain A

protein	$K_{d(app)}$ for *cis*-DDP-modified DNA (nM)	$K_{d(app)}$ for unmodified DNA (μM)	ρ value for *cis*-DDP-modified DNA
tsHMG	24 ± 5[a] 30 ± 5[b]	6.9 ± 3.5[b]	∼230
tsHMG domain A	300 ± 54[a] 590 ± 340[b]	11 ± 5[b]	∼20

[a] Determined by titration. [b] Determined by competition assay.

Ixr1 (*19*) and hUBF (*16*). Only the binding affinity and specificity of HMG1 for the 1,2-d(GpG) adduct are comparable (*25*). The dissociation constants for these other proteins were determined with much longer DNA probes and different adduct-flanking sequences, however, which renders comparisons less meaningful. The apparent dissociation constants for the human testis determining factor SRY (*20*), a sequence-specific HMG-domain protein, were determined by using the same probes as described here. SRY has a specificity ratio, ρ, of approximately 20 for the platinated 20-Pt probe (*20*), 1 order of magnitude lower than that determined for tsHMG. The results for several of these studies are summarized in Table 1.

The tsHMG protein contains two DNA-binding domains separated by a 25-amino acid basic linker and a basic tail. The two HMG domains differ widely in their isoelectric points (p*I*). At neutral pH, tsHMG domain A (residues 1−82) is positively charged (pI = 10.4), whereas tsHMG domain B (residues 108−187) is uncharged (pI = 6.9). The

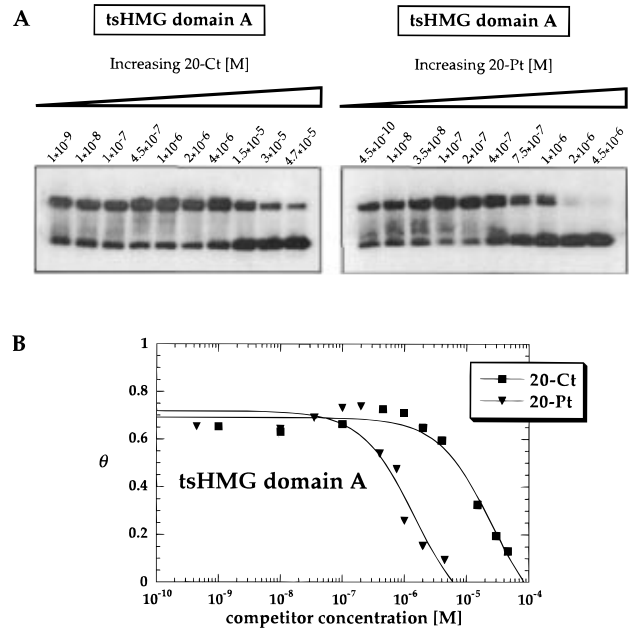

FIGURE 6: Specificity of DNA binding of tsHMG domain A as determined by competitive binding experiments. (A) Gel mobility shift assays of binding reactions, where unlabeled competitor DNAs 20-Pt or 20-Ct were titrated into binding reaction mixtures containing 350 nM tsHMG domain A and 7.5 nM (4000 cpm) ^{32}P-labeled 20-Pt probe. (B) Data from one set of experiments are plotted as the fraction of bound radiolabeled 20-Pt probe versus the concentration of unlabeled competitor DNA 20-Pt (solid triangles) and 20-Ct (solid squares).

sequence homology between tsHMG domain A and rat HMG1 domain A, which binds specifically to cisplatin-modified DNA (*32*), is ~33%. In an attempt to assess the contribution of a single HMG domain in the binding of tsHMG to cisplatin-damaged DNA, a construct containing tsHMG domain A was cloned, overexpressed, and purified. Band shift analysis with the 92-Pt probe shows that domain A is a sufficient functional unit for binding to the unplatinated probe as well as the platinated DNA. The apparent dissociation constant of 300 ± 54 nM determined with the cisplatin-modified 20 bp probe is 1 order of magnitude higher than that observed for tsHMG, which translates into a free energy of binding difference (ΔG) of 5.8 kJ/mol at 4 °C. Although the domain A fragment exhibits 10-fold less specificity for 20-Pt compared to that of the full length protein, it still has a relatively high ρ value of 20. The decreased stability of the protein−Pt−DNA complex observed with the isolated domain A is consistent with a previous qualitative investigation of the interaction between a single domain of HMG1 and cisplatin-damaged DNA (*32*). This complex dissociated in ≤300 mM NaCl, whereas the complex with full length HMG1 proteins was stable at concentrations of >0.5 M NaCl. These data, in addition to those described here, suggest that the presence of multiple HMG domains potentiate the affinity of the proteins for cisplatin-modified DNA. Moreover, regions adjacent to the HMG domains, such as the basic linker and tail in tsHMG, could be important not only for conferring sequence specificity of the protein for its natural target sequence but also for its ability to recognize the cisplatin structural modification.

Differences in protein−DNA complex formation were observed in gel retardation assays of tsHMG and domain A with 20-Pt. The full length protein formed three shifted bands, whereas only one such band formed for domain A. Three complexes were detected previously with the mitochondrial homolog m-mtTFA upon binding to its natural recognition site (*33*). Together, these results suggest that the full length proteins contain specific domains for protein multimerization, which could have functional significance. In support of this notion, xl-mtTFA binds as a tetramer to its recognition sequence, possibly activating bidirectional transcription (*34*).

Structural Considerations. Parallel footprinting analysis of complexes between full length tsHMG or domain A and a cisplatin-modified 87 bp probe provides insight into their structures. The minor groove footprinting reagent DNase I contacts phosphate groups of both strands (*35*). The ability of the enzyme to cut DNA is therefore dependent on the accessibility of the minor groove, suggesting that both DNA rigidity and groove width are important. The digestion patterns of free DNA and its complexes with tsHMG or tsHMG domain A are similar, displaying no detectable footprint. This result is interesting because a tsHMG−87-Ct complex was isolated as a retarded band in the gel mobility shift assay. Perhaps the low affinity of tsHMG for the unmodified probe allows the complex to dissociate during the footprinting experiment. Alternatively, tsHMG could be bound in a nonspecific manner along the length of the DNA, rendering a discrete footprint unobservable in this procedure. The structural distortions introduced by the cisplatin 1,2-d(GpG) intrastrand cross-link are reflected in the digestion pattern of 87-Pt, as in previous reports (*36*). In the digestion pattern of the tsHMG complex, the protein protects an area of 10 bp on the adduct-containing strand, corrected for the intrinsic size of the nuclease, centered at the platinum lesion. Two significant differences are revealed in a comparison between this footprint and that for the tsHMG domain A complex. Domain A covers an area of similar size 5′ to the 1,2-d(GpG) cross-link, but a smaller region is protected on the 3′ side, extending over only 3 bp, compared to 4 bp for the full length protein. This difference suggests that there are fewer specific contacts made by the truncated domain A polypeptide, possibly contributing to its weaker binding interaction and lower ρ value. In addition, the nuclease cleavage pattern for domain A revealed the appearance of an unprotected d(CpC) phosphate bond 3′ to the cisplatin 1,2-d(GpG) cross-link, which retained its sensitivity to DNase I cleavage. The absence of this cleavage site in the protection pattern of the full length tsHMG complex may arise either from better shielding by the protein or from a more significantly distorted structure. Possibly, other components of the tsHMG protein, e.g. HMG domain B, the basic linker, or the tail, extend toward the 3′ end upon binding to the DNA, thereby protecting the d(CpC) cleavage site.

For all known complexes of HMG-domain proteins with cisplatin−DNA adducts, including Ixr1 (*19*), hUBF (*16*), and HMG1 domains A and B (*37*), the footprint sizes are comparable to those obtained here. The protected area always covers 13−14 bp of the adduct-containing strand, even though the molecular masses of the proteins differ by almost 1 order of magnitude, ranging from 94 kDa for hUBF to 9.6 kDa for tsHMG domain A. In contrast, footprint sizes for the putative natural recognition sequences are much larger, spanning areas of ~40 bp for hUBF (*38*) and ~30 bp for h-mtTFA (*39*). These results suggest that not all of the DNA contacts made with the natural binding site are involved in recognition of the cisplatin 1,2-d(GpG)-intra-

strand cross-link. Sequence-specific contacts could be essential for tsHMG binding to its recognition sequence, but not required for binding to cisplatin-modified DNA. Deformation of the helix, which imposes the greatest energetic barrier to the formation of protein−DNA complexes, would be facilitated by the prebent nature of the cisplatin-modified DNA substrate. Moreover, the additional bending from 40 to 85−90°, which occurs upon protein binding to cisplatin-modified DNA, would allow the platinum atom to move into the planes of the guanine bases, providing stability to the complex (*6*).

Functional Implications. Several functions have been suggested for tsHMG (*22*, *23*). By analogy to m-mtTFA, the nuclear protein tsHMG might act as a transcriptional activator, since it contains the same C-terminal domain as the mitochondrial isoform (*40*). Alternatively, tsHMG could play a structural role consistent with its unusually high affinity for nonspecific DNA sequences (*22*). The presence of tsHMG in elongating spermatids (*21*, *23*) suggests that it may compact nuclear DNA during spermatogenesis, when highly basic proteins are replaced with histones.

We now consider how tsHMG might mediate the specific toxicity of cisplatin in testicular cancer. Localization of tsHMG to the testes and its extremely high affinity and specificity for cisplatin-damaged DNA make it an interesting candidate for performing such a role. The tsHMG protein occurs in the nuclei of spermatocytes of all seminiferous tubules of testis (*23*). There is evidence suggesting that the pachytene spermatocytes are progenitors of germ cell tumors (*41*), although the pathway of malignant transformation of early meiotic germ cells is still under intense study. In agreement with the shielding hypothesis (*15*), tsHMG inhibits *in vitro* excision repair of the cisplatin 1,2-intrastrand cross-links but not the minor, 1,3-intrastrand cross-links in HeLa cell free extracts (*17*) at remarkably lower concentrations than other HMG domain proteins (*17*, *20*). Such differences in excision repair shielding efficiency are consistent with the superior binding affinity and specificity of tsHMG to cisplatin-modified DNA.

The high degree of sequence homology between tsHMG and m-mtTFA suggests that the binding properties of m-mtTFA to cisplatin-damaged DNA are similar to those of tsHMG. Elevated levels of platinum occur on the mitochondrial genome in human malignant melanoma cells treated with cisplatin (*42*), a finding attributed to the lack of a mitochondrial DNA repair system (*43*). Thus, the platinum lesions could titrate mtTFA away from its natural binding site, thereby disrupting its function as a transcription factor.

In summary, the discovery of mouse tsHMG as a nuclear protein specifically expressed in testis tissue and binding with high relative affinity to Pt(II)−DNA lesions is an encouraging development in unraveling the mechanism of cisplatin-mediated cytotoxicity. Whether there is a human analog, and determining if it plays a role in the therapeutic effect of the drug, remain to be investigated.

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