Human Testis-Determining Factor SRY Binds to the Major DNA Adduct of Cisplatin and a Putative Target Sequence with Comparable Affinities†

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ABSTRACT: *cis*-Diamminedichloroplatinum(II) (*cis*-DDP or cisplatin) is a widely used anticancer drug that is most effective against tumors of the testis. Although cisplatin is believed to mediate its cytotoxicity through the formation of DNA adducts, the precise biochemical mechanisms underlying its antitumor activity and selectivity for testicular tumors remain elusive. Of significance are the high-mobility group (HMG) domain and other proteins that bind specifically to cisplatin−DNA adducts. The present study focuses on the testis-specific HMG domain protein human SRY (hSRY). The full-length hSRY protein and its HMG domain region alone were expressed in *Escherichia coli* and purified to homogeneity. The affinities and specificities of full-length hSRY and the hSRY−HMG domain for 20 bp DNAs containing a single *cis*-[Pt(NH$_3$)$_2$\{d(GpG)-N7(1),-N7(2)\}] intrastrand cross-link or a putative hSRY target site in the CD3ϵ gene enhancer (AACAAAG) were determined in electrophoretic mobility shift assays. Full-length hSRY bound to the major 1,2-d(GpG) cisplatin adduct with a $K_{d(app)}$ of 120 ± 10 nM and exhibited a 20-fold specificity over unmodified DNA. The HMG domain of hSRY was sufficient for this interaction. The hSRY−HMG domain recognized the 1,2-d(GpG) intrastrand cross-link with higher affinity [$K_{d(app)}$ = 4 ± 0.7 nM] but with lower specificity (5-fold) than the full-length protein. The affinities of full-length hSRY and the hSRY−HMG domain for a single cisplatin−DNA adduct were comparable to those for the putative target sequence AACAAAG. These data suggest that cisplatin−DNA adducts may compete with specific DNA sequences *in vivo* for the binding of human SRY. A possible role for this testis-specific protein in the cytotoxicity and organotropic specificity of cisplatin for testicular tumors is proposed.

cis-Diamminedichloroplatinum(II) (*cis*-DDP or cisplatin;[1] Figure 1A) is one of the most widely used drugs for treatment of human malignancies. It is exceptionally effective against testicular cancer (*1*), affording cure rates in excess of 90% (*2*). The antitumor activity of cisplatin is generally attributed to its covalent interaction with DNA (*3*). Cisplatin−DNA adducts are primarily 1,2-d(GpG) (65%) and 1,2-d(ApG) (25%) intrastrand cross-links and, at a lower frequency, 1,3-d(GpNpG) intrastrand and DNA interstrand cross-links (*4*−*6*). In each case, the platinum is coordinated to the N7 atom of the purine base. DNA adducts are believed to mediate cytotoxicity by inhibiting DNA replication and transcription (*3*) and, ultimately, by activating apoptosis (*7*). However, the detailed biochemical pathway between adduct formation

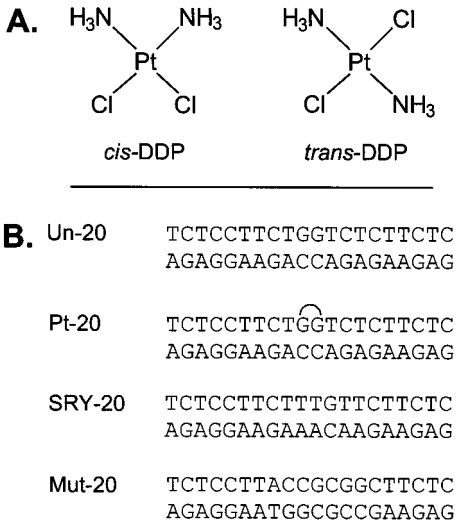

FIGURE 1: Platinum compounds and 20 bp DNA probes used in binding studies. (A) Structures of the therapeutically active compound cisplatin (*cis*-DDP) and the clinically ineffective trans isomer of cisplatin, *trans*-DDP. (B) Sequences of the 20 bp probes.

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[1] Abbreviations: bp, base pair; BSA, bovine serum albumin; *cis*-DDP or cisplatin, *cis*-diamminedichloroplatinum(II); DTT, dithiothreitol; esd, estimated standard deviation; EDTA, ethylenediaminetetraacetic acid; HMG, high-mobility group; HMG1, high-mobility group protein 1; hMSH2, human mismatch repair protein; hUBF, human upstream binding factor; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; IPTG, isopropyl β-D-thiogalactopyranoside; LEF-1, lymphoid enhancer-binding factor 1; PMSF, phenylmethanesulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SRY, testis-determining factor encoded by the sex-determining region on the Y chromosome; SDS, sodium dodecyl sulfate; STE, 10 mM Tris-HCl (pH 8), 1 mM EDTA, and 100 mM NaCl; tsHMG, murine testis-specific HMG domain protein; *trans*-DDP, *trans*-diamminedichloroplatinum-(II).

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and cell death has yet to be elucidated (*8*).

Of possible importance to the cytotoxic mechanism of cisplatin are mammalian proteins possessing a common DNA binding motif, the high-mobility group (HMG) domain, that bind selectively to DNA modified with cisplatin, but not to DNA adducts of the clinically inactive cisplatin isomer, *trans*-diamminedichloroplatinum(II) (*trans*-DDP; Figure 1A) (*9*). An unusual property of the HMG domain is its ability to interact with bent DNA structures such as four-way junctions and to induce bends in linear DNA (*10*−*12*). Structural distortions induced in DNA by the major 1,2-intrastrand cross-links of cisplatin (*13*), including bending and unwinding of the DNA helix (*14*−*16*), are believed to serve as structure-specific recognition signals for these proteins (*17*−*20*). By contrast, the cisplatin 1,3-d(GpNpG) intrastrand cross-links, which unwind the DNA to a greater degree than the 1,2-intrastrand adducts (*16*), fail to attract HMG domain proteins (*18*).

Several models may explain how HMG domain proteins could effect cisplatin cytotoxicity (*19*, *21*−*23*). One model proposes that the binding of HMG domain proteins to cisplatin-DNA adducts may impede removal of the lesions by the nucleotide excision repair machinery. Indeed, studies *in vivo* (*23*, *24*) and *in vitro* (*25*, *26*) have supported the hypothesis that HMG domain proteins can sensitize cells to cisplatin by a repair-shielding mechanism. A second model proposes that cisplatin−DNA adducts may act as molecular decoys in cells, titrating HMG domain proteins from their natural binding sites and thereby disrupting their function. In support of this model (*19*), the binding of the ribosomal RNA transcription factor hUBF to its cognate promoter sequence was competed by a cisplatin−DNA adduct concentration much lower than that found in the DNA of cancer patients (*27*).

Current efforts are directed at trying to understand why cisplatin is more effective in treating testicular tumors than tumors of other tissues. Several HMG domain proteins are specifically expressed in the testis (*28*−*34*) and could potentially contribute to the cisplatin sensitivity of testicular tumors by one or both of the mechanisms described above. The present study focuses on the testis-specific HMG domain protein encoded by the human sex-determining gene on the Y chromosome, *SRY* (*28*). The SRY protein has been detected in the genital ridge of the human embryo (*35*) where it is responsible for testis formation. In addition, *SRY* has been detected at the mRNA level in the adult testis (*28*, *36*); however, its role there is poorly understood. Whereas the biological activity of SRY is believed to involve transcriptional regulation, the downstream target genes for SRY regulation have not yet been identified. Human SRY recognizes certain AT-rich sequences in DNA (*37*−*40*), including the consensus binding sequence A/TAACAAT/A obtained by random site selection (*41*), and activates transcription in the context of certain reporter gene constructs (*40*, *42*). Upon DNA binding, hSRY induces a 60−83° bend in the DNA helix as demonstrated by circular permutation assays (*39*, *43*, *44*) and by a recent NMR structure of a hSRY−HMG domain−DNA complex (*45*). hSRY also recognizes four-way junction DNAs irrespective of their sequence (*43*, *46*) and $(CA)_n$ repeats that can adopt such structures (*47*). The ability of hSRY to interact with bent DNA structures and to bend linear DNA has suggested that the protein may modulate transcription by acting architecturally in the assembly of a nucleoprotein complex (*44*).

The HMG domain of hSRY has been shown to be sufficient for both sequence- and structure-specific DNA recognition (*38*, *39*, *43*, *46*). No function, however, has been ascribed to the regions of the protein outside the HMG domain. The present study directly compares the DNA binding properties of the full-length hSRY protein and its HMG domain region alone. Binding to both the major 1,2-d(GpG) adduct of cisplatin and a putative target sequence in the CD3ϵ gene enhancer (AACAAAG) is examined. We demonstrate that full-length hSRY exhibits a higher DNA binding specificity than the hSRY−HMG domain. We also show that the 1,2-d(GpG) cisplatin adduct and the putative target sequence AACAAAG are recognized with comparable affinities.

MATERIALS AND METHODS

Materials. The *Escherichia coli* MC15 cell line containing pDS56/4xHis-hSRY was obtained from D. R. Cohen (Australian National University). D. Page and M. Behlke [Massachusetts Institute of Technology (MIT)] provided the GST/hSRY−HMG fusion expression plasmid. The HeLa S3 cell line was supplied by P. A. Sharp (MIT).

Purification of Full-Length hSRY. The full-length human SRY protein (hSRY) modified with four histidine residues on the amino terminus was expressed and purified from pDS56/4xHis-hSRY *E. coli* MC15. The cells were grown in LB media with 100 μg/mL ampicillin and 25 μg/mL kanamycin at 37 °C and induced at an OD_{600} of 0.8−0.9 with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 2 h, the cells were harvested by centrifugation and lysed in a 6 M guanidine hydrochloride buffer at pH 8.0. The cell lysate was passed over a Ni-NTA agarose (Qiagen) column, and the hSRY protein was eluted in guanidine hydrochloride buffer at pH 5.0, as described (*40*). The protein was dialyzed (4 °C) into buffer A [20 mM Hepes-NaOH (pH 7.9), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol] to renature and then loaded onto an SP-Sepharose column (Pharmacia). The column was eluted with a linear gradient from 0.05 to 1.0 M NaCl in buffer A. Full-length hSRY eluted between 0.55 and 0.60 M NaCl. The peak fractions were dialyzed into storage buffer [20 mM Hepes-NaOH (pH 7.9), 50 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol] and stored in aliquots at −80 °C. The protein was homogeneous by Coomassie blue staining of a SDS−polyacrylamide gel. The identity of hSRY was confirmed by N-terminal amino acid sequencing (MIT Biopolymers Laboratory), which revealed the sequence MHHHHLSVFNSDD. The protein concentration was calculated by using the extinction coefficient (29 000 M^{-1} cm^{-1} at 280 nM) measured by the Edelhoch method (*48*). At dilutions of less than 20 nM, hSRY exhibited a slow diminution in DNA binding activity, probably due to tube/surface adsorption; therefore, the protein was freshly diluted into storage buffer before each binding experiment.

Purification of the hSRY−HMG Domain. The HMG domain of human SRY (residues D58−K136) was expressed as a glutathione *S*-transferase (GST) fusion protein in the protease-deficient *E. coli* strain BL21(DE3). Cells containing the GST/hSRY−HMG plasmid were grown in LB media

with 50 μg/mL ampicillin at 37 °C to an OD_{600} of 1.0. IPTG was added to 1 mM, and the cells were induced for 2 h and then harvested. All of the following procedures were performed at 0−4 °C. The cell pellets were resuspended in buffer A [50 mM Tris-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mg/mL Pefabloc (Boehringer Mannheim), 20 μM aprotinin, 50 μM leupeptin, and 50 μM pepstatin]. Lysis was achieved by sonication with 30 s bursts for a total of 5 min. The lysate was centrifuged to pellet cell debris, and nucleic acids were removed by batch adsorption to DEAE-cellulose at 0.45 M NaCl. The supernatant was diluted to approximately 0.3 M NaCl, added batchwise to glutathione−Sepharose-4B beads (Pharmacia), and mixed for 1 h. Protein-bound beads were collected and washed to remove nonbinding proteins as described (*49*). Cleavage of the GST/hSRY−HMG fusion protein while still bound to the glutathione beads was achieved by digestion with human thrombin (Sigma, 0.5% w/w fusion protein) in 50 mM Tris-HCl (pH 8), 150 mM NaCl, and 2.5 mM $CaCl_2$ for 1 h at room temperature. The hSRY−HMG domain protein was eluted in buffer B [50 mM Hepes-NaOH (pH 7.9), 20 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 5% glycerol]. Fractions were combined and applied to a Mono S HR 5/5 FPLC column (Pharmacia) equilibrated in buffer B without PMSF, and the column was eluted with a 0.020 to 1.0 M NaCl linear gradient. The hSRY−HMG domain eluted between 0.55 and 0.60 M NaCl. The peak fractions were dialyzed into storage buffer [20 mM Hepes-NaOH (pH 7.9), 10 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol] and stored in aliquots at −80 °C. The protein was homogeneous by Coomassie blue staining of a SDS−polyacrylamide gel. N-Terminal amino acid sequencing verified the identity of the hSRY−HMG domain. The protein contained 15 additional amino acid residues (GSPGISGGGGGILDS) at the amino terminus due to the location of the thrombin cleavage site within the GST fusion. The concentration of the hSRY−HMG domain was determined by using the extinction coefficient (21 000 M^{-1} cm^{-1} at 280 nM) (*48*). Amino acid analysis (MIT Biopolymers Laboratory) yielded a similar protein extinction coefficient (26 000 M^{-1} cm^{-1} at 280 nM). The hSRY−HMG domain slowly aggregates; the protein was, therefore, diluted into storage buffer immediately before each binding experiment.

Polyclonal Antisera to hSRY. Full-length hSRY was purified by Ni-NTA affinity chromatography followed by preparative SDS−PAGE and electroelution (*50*), combined with complete Freund's adjuvant, and injected subcutaneously into four New Zealand white rabbits (0.5 mg of protein/rabbit). Booster injections in incomplete Freund's adjuvant were given 1, 2, and 3.5 months later. Serum was collected and stored in aliquots at −20 or −80 °C.

Western Blot Analysis. Full-length hSRY and the hSRY−HMG domain were resolved on 15% SDS−polyacrylamide and 16.5% Tricine-SDS−polyacrylamide gels (*51*), respectively, and electroblotted onto an Immune-Lite nylon membrane (Bio-Rad). The blots were probed with hSRY polyclonal antiserum at dilutions of 1:50000 and 1:5000 for detection of full-length hSRY and the hSRY−HMG domain, respectively. Antibody binding was visualized by using a chemiluminescent detection system (Bio-Rad).

DNA Probes. Sequences of the 20 bp DNA probes are shown in Figure 1B. Oligonucleotides were purchased from Integrated DNA Technologies, Inc. (Coralville, IA), or synthesized on an Applied Biosystems PCR Mate 391 DNA synthesizer and purified by polyacrylamide gel electrophoresis. A 20-base oligonucleotide containing a single, site-specific *cis*-[$Pt(NH_3)_2${d(GpG)-N7(1),-N7(2)}] intrastrand cross-link (referred to as Pt-20-TOP) was prepared as described (*16*) and purified from unreacted DNA on a denaturing 20% polyacrylamide gel. The Pt-20-TOP oligonucleotide was analyzed by platinum atomic absorption spectroscopy on a Varian AA-1475 spectrometer equipped with a GTA-95 graphite furnace and shown to contain a single atom of Pt per strand. The presence of platinum was further demonstrated by treatment with cyanide ion to reverse the Pt adduct. Electrospray mass spectrometry confirmed the molecular mass of the platinated oligonucleotide (calculated molecular mass = 6181 Da, observed molecular mass = 6178 Da).

The Un-20, Pt-20, SRY-20, and Mut-20 double-stranded DNA probes were prepared by the following procedure. The top and bottom complementary strands were 5′-end labeled with [γ-^{32}P]ATP (6000 Ci/mmol, New England Nuclear) and nonradioactive ATP, respectively, in 70 mM Tris-HCl (pH 7.6) and 10 mM $MgCl_2$ and combined at a molar ratio of 0.9:1.0. NaCl was added to a final concentration of 100 mM, and the solution was heated at 80 °C and cooled slowly to 4 °C to allow the strands to anneal. The 20 bp probes were purified on Sephadex G-25 Quick Spin columns (Boehringer Mannheim), diluted into STE [10 mM Tris-HCl (pH 8), 1 mM EDTA, and 100 mM NaCl], and stored at 4 °C. Competitor DNAs were prepared similarly, but both strands were 5′-end labeled with nonradioactive ATP. Double-stranded DNA concentrations were determined by using the relation 1 A_{260} unit = 50 μg/mL. The concentration of Un-20 DNA determined by this method differed by only 4% from that calculated using the experimentally determined extinction coefficients and a hyprochromicity effect of 20% (*52*).

The 100 bp DNA fragment with a single, centrally located *cis*-[$Pt(NH_3)_2${d(GpG)-N7(1),-N7(2)}] intrastrand cross-link (Pt-100) and the unmodified control DNA (Un-100) were prepared as described (*18*, *53*). A 100 bp DNA probe containing a centrally located AACAAAG sequence (SRY-100) was constructed by replacing Pt-20-TOP and C oligonucleotides with d(CCAGATTCTTTGTTACGTTA) and d(TCGACTTAACGTAACAAAGAATCTGGCTGACG), respectively. A 100 bp probe containing the related central sequence AACAATG (TCR-100) was also prepared by substituting the Pt-20-TOP and C oligonucleotides with d(GGCACCCATTGTTCTCTCCC) and d(TCGACTGGGAGAGAACAATGGGTGCCCTGACG), respectively. Final purification of full-length 100 bp duplex DNAs was by 12% native PAGE. The 100 bp probes were radiolabeled with [γ-^{32}P]ATP and purified by passage over Sephadex G-50 Quick Spin columns. Dilutions were made in STE, and the resulting solutions were stored at 4 °C.

Electrophoretic Mobility Shift Assays. For binding assays, full-length hSRY and the hSRY−HMG domain were freshly diluted into their respective storage buffers immediately before use. Varying concentrations of protein were added to 0.1 nM ^{32}P-labeled DNA probe (5000 cpm) in 10 μL

reaction mixtures containing 8% Ficoll, 10 mM Hepes-NaOH (pH 7.9), 60 mM NaCl, 1 mM EDTA, 0.5 mM DTT, and 100 μg/mL BSA. Reaction mixtures were incubated for 30 min on ice, and then 2 μL of loading dye (30% glycerol, 0.125% bromophenol blue, and 0.125% xylene cyanol) was added. Samples were loaded onto prerun, preequilibrated (4 °C) 10% (29:1 acrylamide:bis) polyacrylamide gels containing 2.5% glycerol and 0.5× TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA). The gels were run for 100−120 min at 350 V and then vacuum dried. The bands representing bound and free DNA probe were quantitated by using a Molecular Dynamics PhosphorImager. Under conditions of excess protein, the apparent dissociation constant [$K_{d(app)}$] was defined as the protein concentration required for half-maximal binding. The $K_{d(app)}$ reported for each protein−DNA interaction is an average from three or four experiments. The error was computed as the average deviation.

Competition Assays. Unlabeled competitor DNAs were titrated into binding reaction mixtures containing 0.1 nM ^{32}P-labeled Pt-20 DNA probe (5000 cpm) and 200 nM hSRY or 5 nM hSRY−HMG domain. The competitive binding of protein to labeled Pt-20 DNA probe and unlabeled competitor DNAs is described by eq 1 (*54*, *55*)

$$\Theta = \frac{1}{2T_t}\left[K_T + \left(\frac{K_T}{K_C}\right)C_t + P_t + T_t - \sqrt{\left[K_T + \left(\frac{K_T}{K_C}\right)C_t + P_t + T_t\right]^2 - 4T_tP_t}\right] \quad (1)$$

where Θ is the fraction of bound DNA probe, P_t, T_t, and C_t are the concentrations of protein, radiolabeled DNA probe, and competitor DNA, respectively, and K_T and K_C represent the apparent dissociation constants for the Pt-20 and competitor DNA probes, respectively. Competition curves were fit for the best value of K_C by nonlinear least-squares analysis. Errors were based on the statistical fit of the data and represent ±1 standard deviation. Relative DNA binding affinities were determined by comparing values of K_C for the competitor DNAs. Relative affinities were also approximated by comparing the concentrations of competitor DNAs required for 50% competition. Experiments with each competitor DNA were performed two or three times.

Excision Repair Inhibition Assays. The sequences, synthesis, and characterization of the oligonucleotides containing the 1,2-d(GpG) and 1,3-d(GpTpG) intrastrand cross-links have been described previously (*16*, *26*). Site-specifically platinated DNA probes (156 bp) with a radiolabel four or five nucleotides 5′ to the site of damage were prepared as described (*25*, *26*). The hSRY−HMG domain was diluted into its storage buffer immediately prior to use. Whole cell-free extracts from HeLa S3 cells were prepared as described (*56*). The repair inhibition experiments were performed as reported previously (*26*) with a few modifications. The hSRY−HMG domain protein was incubated with the site-specific cisplatin−DNA repair probes for 10 min at 30 °C, and then 75 μg of HeLa cell-free extract was added and the reaction mixture incubated at 30 °C for an additional 50 min. The final 25 μL reaction mixture contained 1.4−2.1 μL of protein in storage buffer, in addition to 54 ng of pBR322 plasmid as a nonspecific competitor, 0.2 mg/mL BSA, each dNTP at 20 μM, 28 mM Hepes-NaOH (pH 7.9), 48 mM KCl, 4.5 mM $MgCl_2$, 0.36 mM EDTA, 3.6 mM ATP, 0.5 mM DTT, 40 mM NaCl, 10 mM Tris-HCl, 0.2 mM 2-mercaptoethanol, and 1.9% glycerol. The reaction was stopped by adding 10 μg of proteinase K, making the reaction 0.5% in SDS, and incubating at 55 °C for 20 min. The reaction mixtures were extracted, precipitated, and resolved by 10% PAGE as previously described (*26*). The data were quantitated by PhosphorImager analysis.

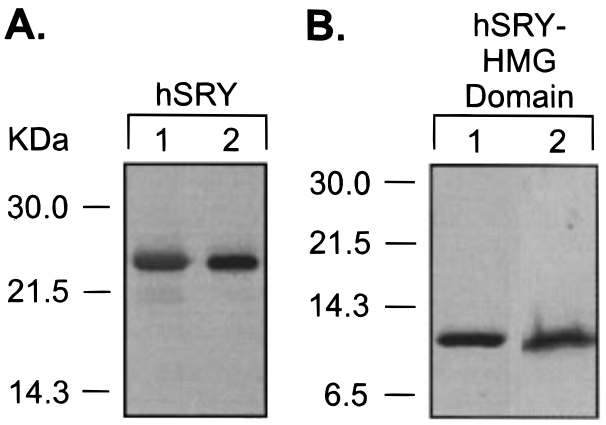

FIGURE 2: Analysis of purified full-length hSRY and the hSRY−HMG domain by Coomassie blue-stained SDS−PAGE and Western blotting. (A) Purified full-length hSRY protein (2 μg, lane 1; 0.4 μg, lane 2) was resolved on a 15% SDS−polyacrylamide gel. (B) Purified hSRY−HMG domain protein (2 μg, lane 1; 1 μg, lane 2) was resolved on a 16.5% Tricine-SDS−polyacrylamide gel. In panels A and B, lane 1 was stained with Coomassie blue and lane 2 was analyzed by Western blotting using hSRY polyclonal antiserum.

RESULTS

Purification of Full-Length hSRY and the hSRY−HMG Domain. The full-length human SRY protein (hSRY) modified by the addition of four histidine residues at the amino terminus was purified by nickel chelate affinity chromatography under denaturing conditions followed by renaturation and cation-exchange chromatography. As shown in Figure 2A, the purified protein had an estimated molecular mass of 24 kDa and was homogeneous by Coomassie blue staining of a SDS−polyacrylamide gel (lane 1) and by Western blot analysis using polyclonal antiserum raised against hSRY (lane 2). The HMG domain comprising residues D58−K136 of human SRY (hSRY−HMG domain) was purified under native conditions from a protease-deficient *E. coli* strain. The resulting 11 kDa protein was homogeneous and free of proteolytic degradation products, as determined by SDS−PAGE with Coomassie blue staining and Western blot analysis (Figure 2B).

Full-Length hSRY and the hSRY−HMG Domain Bind to the Major 1,2-d(GpG) Adduct of Cisplatin. Electrophoretic mobility shift assays with globally platinated DNAs (data not shown) demonstrated that full-length hSRY and the hSRY−HMG domain bound specifically to DNA modified with cisplatin, but not to DNA containing adducts of the clinically inactive compound *trans*-DDP (Figure 1A) or to unmodified DNA. The major DNA adducts formed by cisplatin are 1,2-intrastrand cross-links at d(GpG) and d(ApG) sites (*5*). In contrast, *trans*-DDP cannot form these

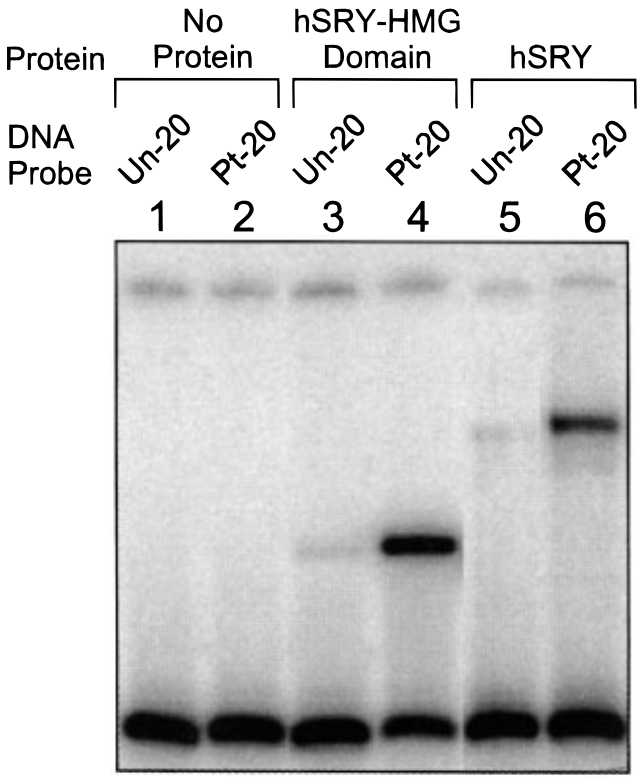

FIGURE 3: Full-length hSRY and the hSRY−HMG domain bind to the major DNA adduct of cisplatin. The radiolabeled 20 bp DNA probes Pt-20 containing a single 1,2-d(GpG) intrastrand cross-link (lanes 2, 4, and 6) and its unmodified control Un-20 (lanes 1, 3, and 5) were incubated with no protein (lanes 1 and 2), 5 nM hSRY−HMG domain (lanes 3 and 4), or 50 nM full-length hSRY (lanes 5 and 6). Lanes 1−4, 5, and 6 are from three separate electrophoretic runs.

adducts owing to geometric constraints (*57*). Thus, the specific binding of human SRY to cisplatin-modified DNA suggests that this protein may recognize the 1,2-intrastrand cross-links. To test this hypothesis, a 20 bp duplex oligonucleotide containing a single, centrally located *cis*-[Pt(NH$_3$)$_2$\{d(GpG)-N7(1),-N7(2)\}] cross-link was prepared. Sequences of the platinated 20 bp probe (Pt-20) and its unmodified control (Un-20) are shown in Figure 1B. In an electrophoretic mobility shift assay, full-length hSRY and the hSRY−HMG domain bound selectively to the Pt-20 probe, but only very weakly to the Un-20 probe (Figure 3). Binding to single-stranded DNA was not observed (data not shown). These results demonstrate that the testis-specific hSRY protein recognizes the major 1,2-d(GpG) adduct of cisplatin and that the HMG domain is sufficient for this interaction.

Human SRY Binds to a Cisplatin Adduct and the Putative Target Sequence AACAAAG with Comparable Affinities. To quantitate the affinity of full-length hSRY for Pt-20 DNA, the protein was titrated into binding reaction mixtures containing a fixed and limiting concentration of radiolabeled probe. A representative experiment is shown in Figure 4A. At hSRY concentrations below maximal binding, a specific hSRY−Pt-20 complex was observed in the gel as a single band. Experiments aimed at defining the stoichiometry of binding suggested that this complex represented a 1:1 interaction (data not shown). At hSRY concentrations above 250 nM, species of lower mobility appeared, probably corresponding to multiple proteins bound to the DNA. Amounts of bound and unbound radiolabeled probe were quantitated, and the fraction of bound Pt-20 DNA was plotted as a function of the log of the hSRY concentration (Figure 4B). The apparent dissociation constant [$K_{d(app)}$] was determined to be 120 ± 10 nM (data summary in Table 1). A similar $K_{d(app)}$ was obtained when only DNA present in the 1:1 highest-mobility complex was quantitated as bound material (data not shown).

In view of the results described above, it was of interest to determine the affinity of full-length hSRY for a DNA target sequence as compared to its affinity for cisplatin-modified DNA. Although a physiological target site has not yet been identified, full-length hSRY binds selectively to the sequence AACAAAG from the CD3ϵ gene enhancer (*37*) and to the related sequence AACAATG (*40*, *41*). These earlier studies, however, did not determine the apparent dissociation constants for hSRY binding. To quantitate the affinity of hSRY for the CD3ϵ gene enhancer sequence, a 20 bp DNA probe (SRY-20) containing AACAAAG was synthesized with the same flanking sequences as Pt-20 (Figure 1B) and used in titration binding experiments with full-length hSRY (Figure 4C). Similar to its interaction with Pt-20 DNA, hSRY formed a specific complex with SRY-20, and at high protein concentrations, multiple hSRY proteins apparently bound to the probe. The binding data yielded a $K_{d(app)}$ of 15 ± 3 nM (Figure 4D). Thus, the results from titration experiments indicate that full-length hSRY has an 8-fold higher affinity for the AACAAAG sequence than for a cisplatin adduct [$K_{d(app)}$ = 120 nM].

To determine whether full-length hSRY and the hSRY−HMG domain differ in their DNA binding properties, the affinity of the hSRY−HMG domain for the Pt-20 and SRY-20 probes was measured under the same conditions used for hSRY binding. Titration of the hSRY−HMG domain into binding reaction mixtures containing a constant amount of the Pt-20 probe produced a specific protein−DNA complex that increased to saturation. Maximal binding was achieved at a protein concentration of 50−100 nM (Figure 5A). At the plateau, the bound Pt-20 DNA fraction was only 0.7, for reasons that are not completely known. The Pt-20-TOP oligonucleotide used to construct the Pt-20 probe was fully platinated as demonstrated by atomic absorption spectroscopy and mass spectrometry. At a protein concentration of 500 nM, however, the total fraction of bound Pt-20 DNA increased to approximately 0.9 due to the appearance of a second band with a lower mobility (data not shown). This species probably represented a second protein bound to the probe, presumably in a nonspecific fashion. The binding data yielded a $K_{d(app)}$ of 4 ± 0.7 nM for the specific interaction between the hSRY−HMG domain and the Pt-20 probe (Figure 5B). Thus, the hSRY−HMG domain exhibited high affinity for the 1,2-d(GpG) cisplatin adduct compared to the full-length hSRY protein [$K_{d(app)}$ = 120 nM]. This difference may in part be a consequence of the small size of the DNA probe. In support of this view, the affinity of hSRY was higher for the 1,2-d(GpG) cross-link contained within a larger 100 bp probe (*vide infra*). Binding experiments with the SRY-20 probe indicated that the hSRY−HMG domain bound to the AACAAAG sequence with a $K_{d(app)}$ of 3 ± 0.4 nM (Figure 5C,D), a value lower than the 20 nM reported previously for this sequence (*44*); note, however, that the flanking base pairs differed in the previous

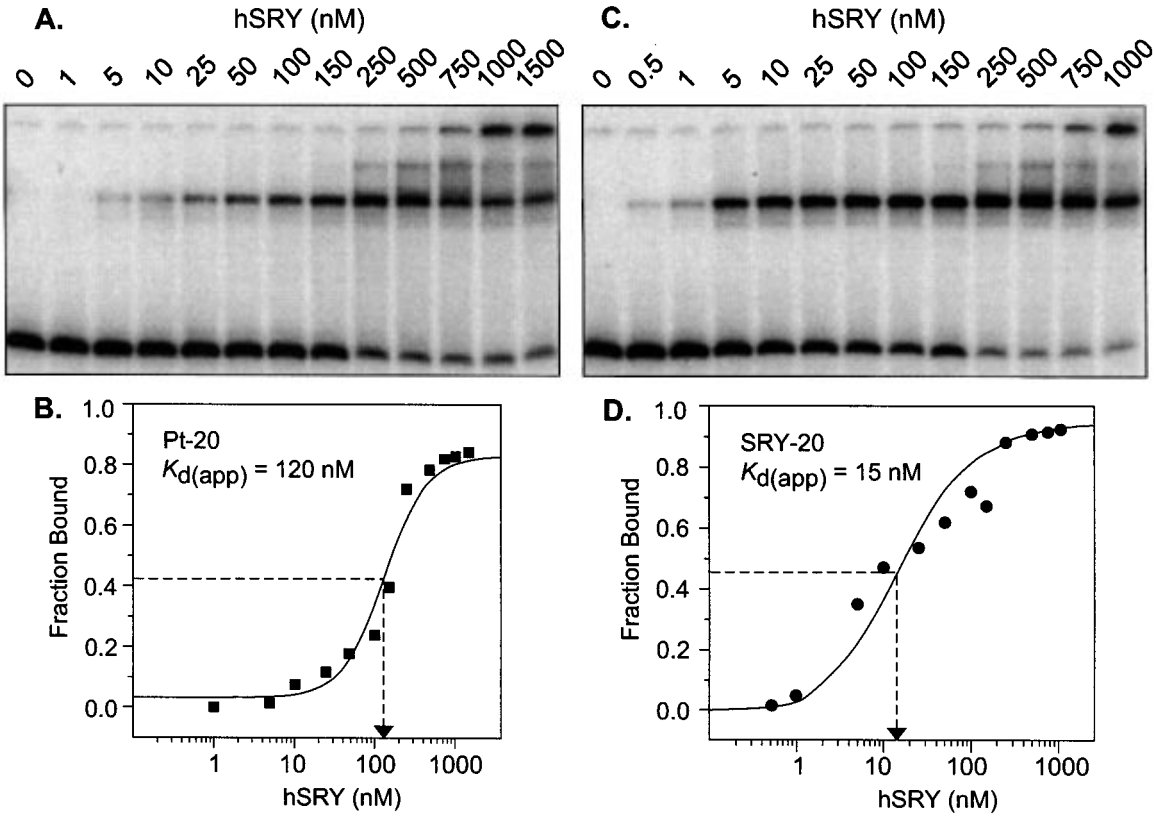

FIGURE 4: Interactions of full-length hSRY with Pt-20 and SRY-20 probes. (A) Full-length hSRY (1−1500 nM) was titrated into binding reaction mixtures containing 0.1 nM (5000 cpm) ^{32}P-labeled Pt-20 probe. Lane 1 contains Pt-20 probe in the absence of protein. (B) The fraction of bound Pt-20 DNA in each lane from A was plotted as a function of the log of the hSRY concentration. The protein concentration giving half-maximal binding [$K_{d(app)}$] is indicated by the broken line. (C) Full-length hSRY (0.5−1000 nM) was titrated into binding reaction mixtures containing 0.1 nM (5000 cpm) ^{32}P-labeled SRY-20 probe. (D) Plot of the data from panel C.

Table 1: DNA Binding Affinities and Specificities of hSRY and the hSRY−HMG Domain

DNA probes	hSRY		hSRY−HMG domain	
	$K_{d(app)}$	specificity[b]	$K_{d(app)}$	specificity[b]
Pt-20	120,[a] 115 nM[b]	20-fold,[c] 2.3-fold[d]	4 nM[a]	5-fold,[c] 1.5-fold[d]
SRY-20	15,[a] 50 nM[b]	60-fold,[e] 40-fold[f]	3 nM[a]	20-fold,[e] 10-fold[f]
Pt-100	nd[h]	nd[h]	4 nM[a]	5-fold[g]
SRY-100	nd[h]	nd[h]	3 nM[a]	nd[h]

[a] Determined by titration. [b] Determined by competition assays. [c] Preference for Pt-20 over Un-20. [d] Preference for SRY-20 over Pt-20. [e] Preference for SRY-20 over Mut-20. [f] Preference for SRY-20 over Un-20. [g] Preference for Pt-100 over Un-100. [h] Not determined.

work. Our results (Table 1) demonstrate that the hSRY−HMG domain interacts with the SRY-20 probe with a slightly higher affinity than that of full-length hSRY [$K_{d(app)}$ = 15 nM]. Additionally, the hSRY−HMG domain recognizes SRY-20 and Pt-20 [$K_{d(app)}$ = 4 nM] with almost equal affinities. Taken together, our binding data from titration experiments with full-length hSRY and the hSRY−HMG domain indicate that human SRY binds to a cisplatin adduct and the target sequence AACAAAG with comparable affinities. This view is further supported by competition binding experiments (*vide infra*).

Competition Studies Demonstrate Higher DNA Binding Specificity for Full-Length hSRY Compared to Its HMG Domain Alone. To determine the DNA binding specificity of full-length hSRY, competition experiments were performed with specific probes Pt-20 and SRY-20 and negative control probes Un-20 and Mut-20; as shown in Figure 1B, Un-20 is the analogous unmodified control for Pt-20, and Mut-20 has AACAAAG in SRY-20 replaced with CCGCGGT, a sequence shown previously to be a poor binding site for hSRY (*37*). Unlabeled competitor DNAs were titrated into binding reaction mixtures containing constant amounts of full-length hSRY and ^{32}P-labeled Pt-20 probe. A plot of a representative set of experiments is shown in Figure 6. These data were fit to a competitive binding equation (eq 1) (*54*, *55*) to determine the apparent dissociation constants for competitor DNAs. The equation yielded apparent dissociation constants of 115 ± 20 and 50 ± 10 nM for the Pt-20 and SRY-20 DNAs, respectively (Table 1). These results are in reasonable agreement with titration experiments (Figure 4) and demonstrate that full-length hSRY recognizes a cisplatin adduct and the sequence AACAAAG with comparable affinities. In contrast to the specific probes Pt-20 and SRY-20, the Un-20 and Mut-20 DNAs competed poorly for hSRY binding with apparent dissociation constants of 2000 ± 350 and 3000 ± 500 nM, respectively. The binding specificity, as defined by the preference of full-length hSRY for Pt-20 over Un-20, was 20-fold, whereas the preference for a target sequence (SRY-20) over a mutated sequence (Mut-20) was 60-fold (Table 1). Similar results were obtained from two other independent sets of experiments.

The binding specificity of the hSRY−HMG domain was also investigated by competition assays. Unlabeled competitor DNAs were incubated with the hSRY−HMG domain and ^{32}P-labeled Pt-20 probe in binding reaction mixtures. A plot of one set of experiments is shown in Figure 7. Similar data

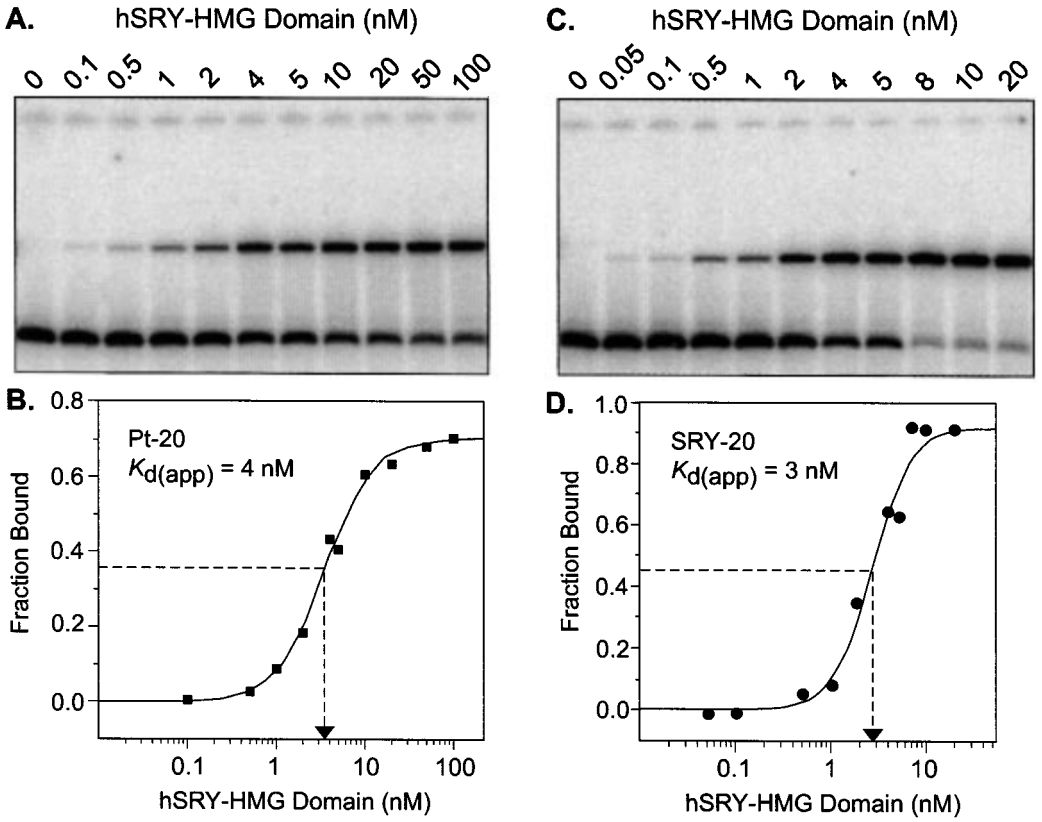

FIGURE 5: Interactions of the hSRY−HMG domain with Pt-20 and SRY-20 probes. (A) Titration of the hSRY−HMG domain (0.1−100 nM) into binding reaction mixtures containing 0.1 nM ^{32}P-labeled Pt-20 probe. (B) Plot of the data from panel A. The protein concentration giving half-maximal binding [$K_{d(app)}$] is indicated by the broken line. (C) Titration of the hSRY−HMG domain (0.05−20 nM) into binding reaction mixtures containing 0.1 nM ^{32}P-labeled SRY-20 probe. (D) Plot of the data from panel C.

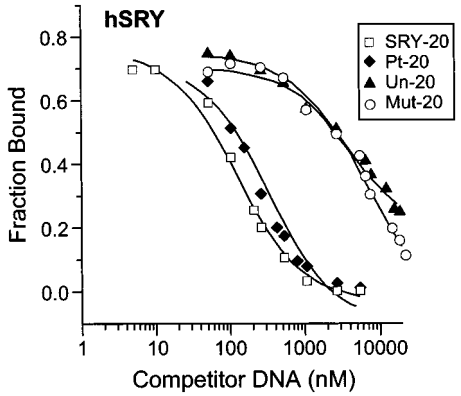

FIGURE 6: Specificity of DNA binding by full-length hSRY determined by competitive binding experiments. Unlabeled competitor DNAs Pt-20, Un-20, SRY-20, and Mut-20 were titrated into binding reaction mixtures containing 200 nM hSRY and 0.1 nM (5000 cpm) ^{32}P-labeled Pt-20 probe. Data from one set of experiments are plotted as the fraction of bound radiolabeled Pt-20 probe versus the concentration of unlabeled competitor DNA: (□) SRY-20, (◆) Pt-20, (▲) Un-20, and (○) Mut-20.

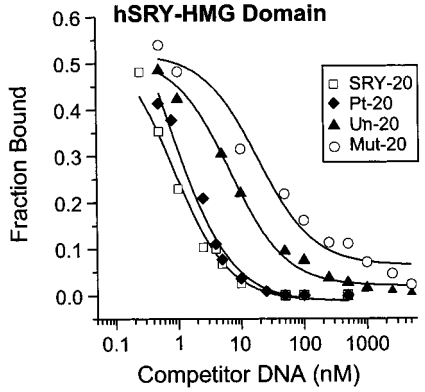

FIGURE 7: Specificity of DNA binding by the hSRY−HMG domain. Unlabeled competitor DNAs Pt-20, Un-20, SRY-20, and Mut-20 were titrated into binding reaction mixtures containing 5 nM hSRY−HMG domain and 0.1 nM (5000 cpm) radiolabeled Pt-20 probe. A graphical representation of one set of experiments is shown: (□) SRY-20, (◆) Pt-20, (▲) Un-20, and (○) Mut-20.

resulted from a second set of experiments. Due to a poor fit of the data to the competitive binding equation, apparent dissociation constants could not be determined with confidence. Therefore, relative DNA binding affinities were approximated by comparing the concentrations of competitor DNAs required to reduce Pt-20 binding by 50%. This method indicated that the hSRY−HMG domain recognized the Pt-20 and SRY-20 DNAs with very similar affinities, in agreement with titration experiments (Figure 5). The hSRY−HMG domain, however, exhibited an overall lower DNA binding specificity than full-length hSRY (summarized in Table 1). In particular, the hSRY−HMG domain displayed only a 5-fold preference for Pt-20 over Un-20 DNA. Additionally, the selectivity of the hSRY−HMG domain for SRY-20 over Mut-20 was 20-fold. To confirm relative binding affinities obtained by competition assays, the binding of full-length hSRY and the hSRY−HMG domain to the Un-20 and Mut-20 control probes was also examined in titration experiments. Very sharp binding transitions and multiple proteins binding to multiple sites on the probes (data not shown), however, precluded our ability to perform thermodynamic analysis with confidence.

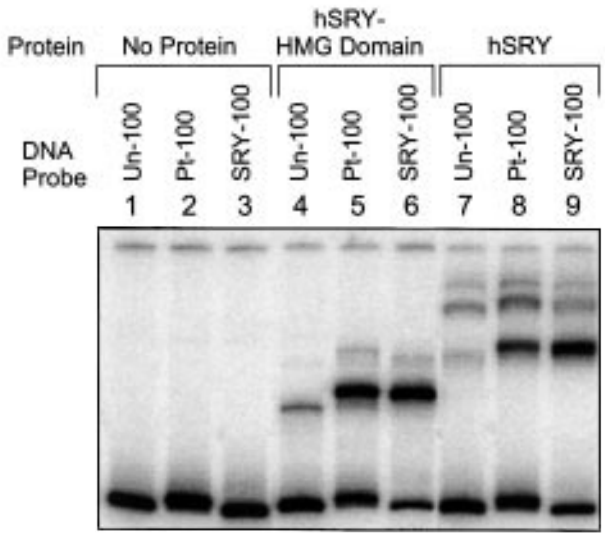

FIGURE 8: Binding of full-length hSRY and the hSRY−HMG domain to 100 bp DNA probes. The radiolabeled 100 bp DNA probes Pt-100 containing a single 1,2-d(GpG) intrastrand cross-link (lanes 2, 5, and 8), its unmodified control Un-100 (lanes 1, 4, and 7), and SRY-100 containing the sequence AACAAAG at its center (lanes 3, 6, and 9) were incubated with no protein (lanes 1−3), 5 nM hSRY−HMG domain (lanes 4−6), or 20 nM full-length hSRY (lanes 7−9).

Interactions of Full-Length hSRY and the hSRY−HMG Domain with 100 bp DNA Probes. To test whether the affinity and specificity exhibited by full-length hSRY and the hSRY−HMG domain were affected by the length of the DNA, the following 100 bp DNA probes were constructed: Pt-100 containing a single, centrally located 1,2-d(GpG) cisplatin cross-link, Un-100, the analogous unmodified control for Pt-100, and SRY-100 containing a centrally located AACAAAG sequence. Figure 8 shows the binding of full-length hSRY and the hSRY−HMG domain to these DNAs at protein concentrations of 20 and 5 nM, respectively. Titration experiments (data not shown) indicated that full-length hSRY bound to the Pt-100 probe with an approximately 3-fold higher affinity than it did to Pt-20. Similarly, hSRY appeared to recognize the SRY-100 probe with a 2-fold-increased affinity compared to SRY-20, suggesting that the longer DNA probe may be better able to accommodate the full-length protein. The binding of multiple proteins to these probes, however, complicated our ability to determine specific apparent dissociation constants.

In contrast with full-length hSRY, the hSRY−HMG domain interacted with the Pt-100 and SRY-100 probes to form predominantly a single complex (Figure 8). In saturation binding experiments, half-maximal binding occurred at hSRY−HMG domain concentrations of 4 ± 1 and 3 ± 0.7 nM for the Pt-100 and SRY-100 probes, respectively. These apparent dissociation constants were identical to those obtained for the 20 bp probes (Table 1). Competition assays indicated that the hSRY−HMG domain displayed a 5-fold selectivity for Pt-100 over Un-100 DNA, a preference similar to that for the 20 bp DNAs (Table 1).

The sequence AACAATG has been reported to be a better target site for full-length hSRY (*41*) and the hSRY−HMG domain (*39*, *44*) than AACAAAG. In agreement with the earlier report, full-length hSRY appeared to bind to a 100 bp DNA probe (TCR-100) containing a centrally located AACAATG sequence with a slightly higher affinity than it did to SRY-100. The hSRY−HMG domain, however, bound to the TCR-100 [$K_{d(app)} = 3 \pm 0.4$ nM] and SRY-100 [$K_{d(app)} = 3 \pm 0.7$ nM] DNAs with equal affinities.

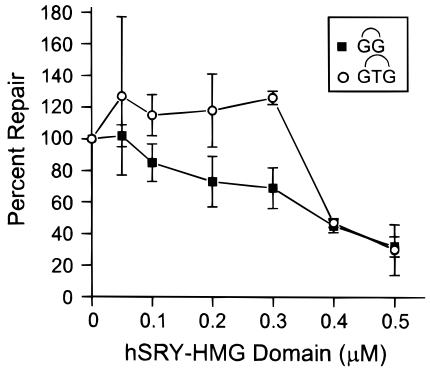

FIGURE 9: Effect of the hSRY−HMG domain on excision repair of the 1,2-d(GpG) and 1,3-d(GpTpG) cisplatin intrastrand cross-links *in vitro*. The 1,2-d(GpG) (■) and the 1,3-d(GpTpG) (○) cross-linked 156 bp substrates were incubated with the hSRY−HMG domain (0.05−0.5 μM) for 10 min at 30 °C before addition of the HeLa cell-free extract and further incubation for 50 min. The level of repair in the absence of the hSRY−HMG domain is designated 100%. Data points are averages of two or three experiments. Error bars represent ± 1 esd.

The hSRY−HMG Domain Specifically Inhibits Repair of the 1,2-d(GpG) Cisplatin Adduct in Vitro. We have proposed that cisplatin adduct-binding proteins may enhance drug cytotoxicity by shielding cisplatin−DNA lesions from the cellular repair machinery (*22*−*24*). Indeed, several HMG domain proteins inhibit repair of the 1,2-d(GpG) and 1,2-d(ApG) intrastrand cross-links, but not the 1,3-d(GpTpG) intrastrand adducts (*25*, *26*). In these studies, an *in vitro* excision repair assay was employed in which incubation of site-specifically platinated DNA substrates with HeLa cell-free extract released the adducts in 27−29 nucleotide fragments. To investigate the ability of human SRY to influence the repair of cisplatin−DNA adducts, excision repair assays were carried out in which 156 bp DNA substrates containing site-specific 1,2-d(GpG) or 1,3-d(GpTpG) intrastrand cross-links were preincubated with the hSRY−HMG domain before addition of the HeLa cell-free extract. As shown in Figure 9, repair of the 1,2-d(GpG) adduct was inhibited with increasing concentrations of the hSRY−HMG domain (0.1−0.5 μM). In contrast, repair of the 1,3-d(GpTpG) adduct was modestly stimulated at protein concentrations up to 0.3 μM, perhaps by protein-induced bending of the DNA substrate (*39*, *43*−*45*), and then repair was inhibited at higher protein concentrations. These repair inhibition assays were performed several times, and consistently, repair of the 1,2-d(GpG) adduct was blocked by the hSRY−HMG domain at concentrations of 0.1−0.3 μM whereas repair of the 1,3-d(GpTpG) adduct was not inhibited at these protein concentrations. Thus, it appears that a specific mode of binding by the hSRY−HMG domain selectively hindered repair of the 1,2-d(GpG) adduct. Excision repair assays were also performed with the full-length hSRY protein, demonstrating that repair of the 1,2-d(GpG) adduct was specifically inhibited at a concentration of hSRY similar to that of the hSRY−HMG domain (data not shown).

DISCUSSION

We have demonstrated that human SRY recognizes the major DNA adduct of cisplatin with high affinity and specificity. Full-length hSRY binds to a 20 bp probe containing a single 1,2-d(GpG) cisplatin intrastrand cross-link (Pt-20) with a $K_{d(app)}$ of 115−120 nM and displays a 20-fold preference for Pt-20 over Un-20, the unmodified control probe (Table 1). The HMG domain of hSRY is sufficient for cisplatin−DNA recognition. The hSRY−HMG domain binds to the 1,2-d(GpG) intrastrand cross-link with a $K_{d(app)}$ of 4 nM and exhibits a 5-fold specificity over unmodified DNA; for comparison, the specificity of another testis-specific protein, tsHMG, was more than 1 order of magnitude higher (U. M. Ohndorf, J. P. Whitehead, N. L. Raju, and S. J. Lippard, submitted for publication). The binding of full-length hSRY and the hSRY−HMG domain to a target site in the CD3ϵ gene enhancer (AACAAAG) was also examined under the same conditions used for cisplatin adduct binding. Full-length hSRY binds to a 20 bp probe containing this sequence (SRY-20) with a $K_{d(app)}$ of 15−50 nM, whereas the hSRY−HMG domain binds to the same probe with a $K_{d(app)}$ of 3 nM. Taken together, our results (Table 1) demonstrate that the affinity of human SRY for the 1,2-d(GpG) cisplatin adduct is comparable to that for a putative target sequence. A similar analysis demonstrated that the four-way junction *z* is also recognized by the hSRY−HMG domain with an affinity similar to that of the sequence AACAAAG (*44*).

Recent structural studies lend insight to the molecular basis for recognition of two distinct targets by human SRY: intrinsically bent DNA structures (cisplatin adducts/four-way junctions) and linear DNA sequences. Gel electrophoresis studies have demonstrated that the 1,2-d(GpG) cisplatin intrastrand adduct unwinds the DNA helix 13° (*16*) and induces a bend of 34−40° in the direction of the major groove (*14*, *15*). A recent X-ray crystal structure of the 1,2-d(GpG) cross-link in duplex DNA (*13*) has further revealed a hybrid DNA structure of A and B forms with an expanded minor groove. Coordination of platinum to its guanine ligands destacks the bases, and a considerable strain is generated by displacement of the platinum atom from the guanine ring planes. It has been proposed that HMG domain protein binding, which increases duplex bending to 70−90° (*20*), might relieve this strain (*13*). Four-way junction DNA shares several structural features with the 1,2-d(GpG) cisplatin adduct, including sharp bend angles, unstacked bases, and a widened minor groove at the crossover point of the junction (*58*−*60*).

A bent DNA structure is also induced by human SRY when it binds to specific sequences in linear DNA. A recent NMR solution structure of a complex between the hSRY−HMG domain and a specific DNA site (GCACAAAC) in the Mullerian inhibitory substance gene promoter revealed the severely distorted nature of the bound DNA (*45*). In the complex, the DNA structure is an A/B-type hybrid with a widened minor groove and the DNA is bent 70−80° toward the major groove owing to the partial intercalation of an isoleucine residue between A·T base pairs. The striking resemblance between this distorted DNA conformation induced by the hSRY−HMG domain and the intrinsically bent 1,2-d(GpG) adduct of cisplatin (*13*) suggests a rationale for structure-specific recognition by human SRY. In particular, the 1,2-d(GpG) adduct may attract hSRY by closely mimicking a stabilized DNA structure that occurs in the hSRY−target DNA complex. An isoleucine intercalation also occurs when the hSRY−HMG domain binds to four-way junction DNA, suggesting that the protein uses a single binding surface to interact with both specific sequences and structures (*46*). The competitive binding of both full-length hSRY and the hSRY−HMG domain to the sequence AACAAAG and the 1,2-d(GpG) cisplatin adduct (Figures 6 and 7) further supports this view.

To date, no function has been attributed to the regions of human SRY outside the HMG domain region. A comparison of SRY amino acid sequences from several mammalian species has revealed that the HMG domain is the only conserved region of the protein and that the sequences outside this region diverge completely (*61*, *62*). A goal of our study was to compare directly the DNA binding properties of full-length hSRY and the hSRY−HMG domain *in vitro*. Our results from titration experiments (Table 1) indicate that full-length hSRY binds to a cisplatin adduct and the sequence AACAAAG with slightly lower affinity than its HMG domain alone. These data suggest that the non-HMG domain regions of hSRY may have a negative effect on DNA binding affinity, perhaps through a localized region of negative charge. At pH 7.9 in the binding buffer, full-length hSRY and the hSRY−HMG domain have calculated net charges of +12 and +13, respectively. The results from competition studies (Table 1) demonstrate that full-length hSRY displays a higher DNA binding specificity than the hSRY−HMG domain. The solution structure of a hSRY−HMG domain-target DNA complex indicates that DNA binding occurs on the concave surface formed by helices 1 and 3, and further DNA contacts are made by side chains from the N- and C-terminal regions (*45*). One possible explanation for the increased DNA binding specificity exhibited by full-length hSRY is the generation of favorable DNA contacts by the regions of the protein just outside of the HMG domain. In the solution structure, Arg-75, Pro-76, Arg-77, and Arg-78 at the far C-terminal end of the hSRY−HMG domain are disordered in solution (*45*). The residues of hSRY adjacent to this C-terminal region may help to anchor it to the DNA, resulting in additional favorable interactions. Consistent with this view, a highly basic C-terminal region next to the HMG domain of LEF-1 extends the DNA binding surface by making extensive contacts with the sugar−phosphate backbone (*63*). Full-length LEF-1 and the LEF-1−HMG domain containing this basic C-terminal region exhibit very similar DNA binding properties *in vitro* (*64*−*66*). Residues adjacent to the N-terminal region of the hSRY−HMG domain might also contact DNA in the full-length protein. In a recent study (*67*), photochemistry was used to cross-link a 15 bp oligonucleotide containing a single, 1,2-d(GpG) cisplatin adduct to a single residue (Lys-6) in HMG1 domain B. Since the platinum is bound to the N7 guanine atoms in the major groove, the formation of a platinum−protein cross-link suggests that the N terminus of domain B contacts the major groove of the DNA. A lysine residue (Lys-53, numbering according to ref *61*) located just outside of the HMG domain of hSRY might interact with DNA in a similar manner. Parallel footprinting studies employing full-length hSRY and the hSRY−HMG domain need to be performed in order to

identify any additional DNA contacts made by regions outside the HMG domain.

Clinical experience has indicated that testicular germ cell tumors are exquisitely sensitive to cisplatin-based chemotherapy (*1*, *68*, *69*). Recent evidence suggests that this hypersensitivity may be related to a reduced capacity to repair cisplatin-induced DNA damage since several testicular germ cell tumor lines are deficient in their ability to remove DNA adducts of cisplatin (*70*−*74*). One model proposed to explain how the HMG domain protein may mediate cisplatin cytotoxicity suggests that the binding of these proteins to cisplatin−DNA lesions may impede removal of the adducts by DNA repair enzymes (*21*, *22*). *In vivo* studies in yeast have indicated that HMG domain proteins can sensitize cells to cisplatin by a repair-shielding mechanism (*23*, *24*). Human SRY and other testis-specific HMG domain proteins may contribute to the specificity of cisplatin for testicular tumors by a similar mechanism. In support of this view, human SRY has been detected at the mRNA level in testicular tumor tissue (*75*) and at the protein level in a testicular embryonal carcinoma cell line (*35*), which is deficient in cisplatin adduct removal (*73*). Furthermore, we have shown that human SRY selectively inhibits repair of the 1,2-d(GpG) adduct in an *in vitro* excision repair assay (Figure 9 and data not shown). Several other HMG domain proteins are also most highly expressed in the adult testis (*29*−*34*). In particular, the mouse testis-specific HMG domain protein tsHMG selectively binds to the 1,2-d(GpG) adduct and inhibits its repair *in vitro* (*26*). Recent evidence also indicates that the human mismatch repair protein hMSH2, which does not contain an HMG domain, binds to cisplatin−DNA adducts (*76*, *77*) and is overexpressed in testicular tissue (*76*). Taken together, these results suggest that, if the aforementioned or other as yet unidentified testis-specific proteins are expressed in testicular tumors and are able to inhibit repair of cisplatin−DNA adducts, these proteins may contribute to the limited ability of testicular tumor cells to remove cisplatin−DNA adducts.

One complication with the above model is that HMG1, which is very abundant in all cells (*78*), may compete with the testis-specific HMG domain proteins for occupancy of cisplatin−DNA adducts in testicular tumor cells. Higher concentrations of HMG1 (1−2 μM) (*25*), however, are required to achieve the same level of repair inhibition of the 1,2-d(GpG) adduct in *in vitro* excision repair assays, compared to the testis-specific proteins hSRY (0.1−0.3 μM) (Figure 9) and tsHMG (0.025−0.1 μM) (*26*). Thus, the concentration needed to achieve repair inhibition may differ among individual HMG domain proteins. Further information about repair inhibition, cellular concentration, and affinity for cisplatin−DNA compared to that for target DNA is needed in order to evaluate critically a role for the testis-expressed HMG domain proteins in sensitizing testicular tumor cells to cisplatin.

Finally, the ability of hSRY to bend DNA and to bind to prebent structures like cisplatin−DNA suggests that DNA bending may be central to the biological function of hSRY in sex determination. In a recent study, mutant HMG domain proteins from XY human females with complete gonadal dysgenesis were found to differ in their DNA bending and binding activities. In particular, one mutant protein bound to the sequence AACAAAG with almost normal affinity but produced a considerably smaller DNA bend angle (*44*). The apparent requirement for a precise geometry in the hSRY−DNA complex suggests that hSRY may bend the DNA in order to facilitate interactions between other DNA binding proteins in the assembly of a higher-order nucleoprotein complex (*44*), as proposed for LEF-1 (*11*, *79*). Such protein−protein contacts may be essential for the regulation of gene transcription by hSRY. The determination of a hSRY−cisplatin−DNA adduct structure might further our understanding of the architecture of this transcriptional complex in addition to providing insight into how HMG domain proteins may modulate the activity of an important anticancer drug.

ACKNOWLEDGMENT

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