HMG Domain Proteins Induce Sharp Bends in Cisplatin-Modified DNA†

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ABSTRACT: Circularly permuted linear DNAs of ~100 bp were constructed containing the major adduct of the anticancer drug cisplatin, a *cis*-[Pt(NH_3)$_2$\{d(GpG)-N7(1), -N7(2)\}] intrastrand cross-link, at a specific site. Gel electrophoresis mobility shift assays with these probes were used to investigate the effects of binding of HMG domain proteins to the platinated DNAs. The site-specifically platinated duplexes were recognized by six different HMG domain proteins—HMG1, mtTFA, Ixr1, and HMG domains from HMG1 (domain B), mSRY, and LEF-1—with comparable binding affinities ($K_d \sim 10^{-6}$ to 10^{-7} M). In the presence of the HMG domain proteins, the platinated DNAs were bent significantly more than in their absence, the values being 86 ± 2°, 87−90 ± 5°, and 68 ± 6°, respectively, for the proteins and 65−74 ± 4°, ~50°, and 72 ± 6°, respectively, for the domains. The variability in bend angles suggests that, although the HMG domain proteins share a common ability to bend platinated DNA, specific contacts between the proteins and the platinated duplex are different. The assay further revealed the bend loci to be centered quite near the platinum adduct. The methodology employed in the present study should be generally applicable for synthesizing other small, circularly permuted, covalently modified DNAs which cannot otherwise be readily obtained.

The high-mobility group (HMG) domain is a DNA-binding motif that was first discovered in abundant non-histone components of chromatin and in several proteins that regulate transcription and cell differentiation (Grosschedl et al., 1994). There are at least two families of HMG domain proteins, one having multiple HMG domains that bind to DNA with little or no sequence specificity and the other comprising a single HMG domain that is responsible for sequence-specific DNA binding. Proteins from both families can recognize irregular or bent DNA structures (Lilley, 1992). HMG1, a member of the multiple HMG domain family, binds specifically to cruciform and cisplatin-modified DNA with up to 100-fold greater affinity than to linear double-stranded DNA (Bianchi et al., 1992; Pil & Lippard, 1992).

In addition to the ability of HMG domain proteins to recognize bent DNA structures, they also induce bends in linear DNA. As revealed in circular permutation assays, DNA bends of 130° and 85° were induced by the sequence-specific HMG domain proteins lymphoid enhancer factor-1 (LEF-1) and sex-determining factor SRY, respectively (Ferrari et al., 1992; Giese et al., 1992). Related studies demonstrated the ability of HMG1 and its individual HMG domains to mediate DNA bending by using T4 DNA ligase-dependent cyclization assays (Onate et al., 1994; Paull et al., 1993; Pil et al., 1993). The human mitochondrial HMG domain protein mtTFA also bends DNA (Fisher et al., 1992), a property which appears to be a general feature of all HMG domain proteins.

Previously, we and others established that HMG domain proteins recognize and bind efficiently and selectively to DNA modified by the antitumor drug *cis*-diamminedichloroplatinum(II), or cisplatin (Brown et al., 1993; Bruhn et al., 1992, 1993; Hughes et al., 1992; Pil & Lippard, 1992; Treiber et al., 1994). In particular, HMG1, yeast intrastrand cross-link recognition (Ixr1) protein, mtTFA, and the 81 amino acid HMG domain B of HMG1 all bind to a double-stranded 92 base pair (bp) DNA fragment containing a single, site-specific *cis*-[Pt(NH_3)$_2$\{d(GpG)-N7(1), -N7(2)\}] intrastrand cross-link with comparable dissociation constants ($K_d \sim 10^{-7}$ M) and moderate selectivity (4−100-fold) over unmodified DNA (C. S. Chow and J. P. Whitehead, unpublished results). The cisplatin d(GpG) adduct itself bends the DNA helix by ~32−34° (Bellon & Lippard, 1990), which is probably the signal for HMG domain recognition. In the present article, we report that six HMG domain proteins investigated induce substantial additional bending in the cisplatin-modified DNA and discuss the potential functional implications of this finding. We also employ a useful method for constructing the set of small circularly permuted DNAs (~100 bp) required for the gel-mobility shift assays used to quantitate the bend angles.

MATERIALS AND METHODS

Materials. Recombinant rat HMG1, HMG domain B (residues K86−K165 of HMG1), and Ixr1 were expressed and purified from *Escherichia coli* (Pil & Lippard, 1992; Chow et al., 1994; J. P. Whitehead and S. J. Lippard, unpublished results). Human mtTFA was supplied by D. Clayton (Stanford University), and LEF-1- and mSRY-HMG domains were supplied by R. Grosschedl (University of California, San Francisco). T4 DNA ligase and restriction enzymes were purchased from New England Biolabs (Beverly, MA).

DNA Probes. A blunt-ended 92-bp DNA fragment (92BENDPt) containing a single, site-specific *cis*-[Pt(NH_3)$_2$-\{d(GpG)-N7(1), -N7(2)\}] intrastrand cross-link and the corresponding unmodified control 92-bp DNA fragment (92BENDCt) were prepared as described in the literature (Pil & Lippard, 1992). Oligos A, B, C, and GG20-TOP were

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replaced by d(GAGCCGGCTGGACTAGTCGACCGCCTTAGTACTACAGGCC) for A, d(TAGTACTAAGGCGGTCGACTAGTCCAGCCGGCTC) for B, d(TCGACTAGAAGGCCTAGAGGCCTG) for C, and either d(TCTAGGCCTTCT) (Stu12Ct) or d(TCTAG*G*CCTTCT) (Stu12Pt, where the asterisks denote the *cis*-$\{Pt(NH_3)_2\}^{2+}$ binding site) for GG20-TOP. The oligonucleotides were synthesized on a Cruachem PS250 DNA synthesizer, deprotected by standard methods, and purified on 12% denaturing gels. Stu12Ct and Stu12Pt were prepared and purified as described previously (Naser et al., 1988). A 100-bp bending probe (100SRY) containing the consensus sequence for SRY was also prepared by substituting Stu12Pt and oligo C with d(TAGAGCGCTTTGTTCTCAGT) and d(TCGACTACTGAGAACAAAGCGCTCTAGGCCTG), respectively. A 101-bp DNA fragment containing three A_6 tracts, d(GAGCCGGCTGGACTAGTCGACCGCCTTAGTACTACGTGAAAAAAACGTGAAAAAAACGCGAAAAAAACGATGATATCGCTCCAGCTGTTCACTACCCGGGTACT), and its complement were synthesized, annealed, and purified on 12% non-denaturing polyacrylamide gels. The 92-, 100-, and 101-bp fragments were 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, cold-chased with unlabeled ATP and T4 polynucleotide kinase to ensure complete phosphorylation, and purified by passage through Sephadex G-50.

Cyclization Reaction. Reaction mixtures (50-μL volume) contained the following components: ~50 nM blunt-ended linear DNA fragment (5′-end labeled), 400 units of T4 DNA ligase, 3.0 μg of HMG1 (2.4×10^{-6} M), and reaction buffer (70 mM Tris·HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM dithiothreitol, and 2 mM ATP). The protein and DNA were preincubated on ice for 1 h in reaction buffer before the end-joining reaction was initiated by addition of T4 DNA ligase. Reactions with ligase were terminated after 18 h at 4 °C by extraction with phenol/chloroform/isoamyl alcohol (25:24:1, v/v) followed by ether extraction. To each of the deproteinized DNA samples from the cyclization reactions was added 10 μL of loading buffer (10 mM Tris·HCl, pH 7.0, 30% glycerol, and 0.025% (w/v) each of xylene cyanol and bromophenol blue). The samples were electrophoresed in 1× TBE buffer (90 mM Tris, 90 mM boric acid, and 2.5 mM EDTA, pH 8.3) at 300 V for approximately 1.5 h on 5% non-denaturing polyacrylamide gels (29:1 acrylamide: *N,N′*-methylenebisacrylamide). Following gel electrophoresis of the ligation products, the bands corresponding to the 92-, 100-, and 101-bp DNA circles were located by autoradiography, excised, and electroeluted in 1× TBE. The DNA circles were desalted, concentrated, and used directly for restriction enzyme reactions.

Production of Linear Fragments Containing a Permuted cis-$\{Pt(NH_3)_2\}^{2+}$ *Adduct.* DNA circles were digested with restriction enzymes (*Nae*I, *Hinc*II, *Sca*I, *Stu*I, *Pvu*II, *Eco*RV, or *Sma*I; 10−20 units in a 50-μL final volume) for 2 h at 37 °C, which produced the corresponding linear fragments. In some cases the *Nae*I data point could not be obtained because of inconsistent activity obtained with this enzyme. The resulting fragments were extracted once with phenol/chloroform /isoamyl alcohol (25:24:1 v/v) and separated on 12% non-denaturing polyacrylamide gels. The DNA bands were located by autoradiography, excised, and eluted in 1× TBE. The eluted DNA was desalted, concentrated, and used for gel-mobility shift assays.

Gel-Mobility Shift Assays. Gel-mobility shift assays were carried out under published conditions (Pil & Lippard, 1992). Reaction mixtures containing ~5000 cpm probe, 4% glycerol, 10 mM $MgCl_2$, 50 mM KCl, 10 mM Hepes·NaOH, pH 7.9, 1 mM EDTA, 1 mM spermidine, 0.05% Nonidet P-40, 200 μg/mL BSA, and HMG domain protein in a 10-μL volume were incubated for 30 min on ice prior to loading with 10% glycerol. The concentrations of HMG domain proteins were 3.7, 3.2, 1.2, 1.1, 2.5, and 6.7×10^{-7} M, respectively, for HMG1, Ixr1, mtTFA, HMG domain B, LEF-1-HMG domain, and mSRY-HMG domain. Reaction mixtures with mtTFA and HMG domains also contained 100 ng of chicken erythrocyte DNA as competitor. Reaction mixtures were analyzed by gel electrophoresis on prerun, precooled (4 °C), 0.5× TBE (45 mM Tris, 45 mM boric acid, 1.25 mM EDTA, pH 8.3) 6, 8, or 10% native polyacrylamide gels (29:1 acrylamide: *N,N′*-methylenebisacrylamide, 0.08% ammonium persulfate). The gels were run for 3−4 h at constant voltage (300 V) and temperature (4 °C) in 0.5× TBE. Upon completion of electrophoresis, the gels were vacuum dried onto Whatman 3 mm chromatography paper and exposed to X-ray film (Kodak X-OMAT AR) at −80 °C with an intensifying screen.

RESULTS AND DISCUSSION

Generation of Circularly Permuted DNA Probes. The polyacrylamide gel electrophoresis mobility of small (~20 bp) cisplatin-modified DNA fragments ligated into multimers was useful in detecting systematic variations in the DNA structure such as bending and unwinding (Bellon et al., 1991; Bellon & Lippard, 1990). Unfortunately, this method does not allow the determination of protein-induced bending of linear DNA. The permutation assay developed by Crothers (Wu & Crothers, 1984) is now commonly employed to examine both sequence-directed and protein-induced bending of linear DNAs. The method requires large DNA fragments (200−400 bp) of identical length with a permuted sequence, such that their gel mobility is a function of the bend locus. The gel-mobility shift data are then interpreted to give a bend angle for the particular DNA. Such DNA fragments cannot readily be generated with covalently damaged or cross-linked DNA, however, because of the inability to prepare them by standard cloning techniques.

This problem was solved in the following manner. A 92-bp DNA probe (92BENDPt) was designed that contains a single *cis*-$\{Pt(NH_3)_2\}^{2+}$ 1,2-intrastrand d(GpG) cross-link in the center and seven strategically placed restriction enzyme recognition sites. We then took advantage of the ability of HMG1 to cyclize small DNAs in the presence of T4 DNA ligase (Pil et al., 1993) and generated the corresponding site-specifically platinated 92-bp DNA circle. Cleavage of the circle in seven separate restriction enzyme reactions yielded a set of 92-bp fragments with a circularly permuted sequence (Figure 1). The yield of DNA circles was generally about 50%. For some sequences, however, the efficiency of circle production was lower. In these cases, the linear dimers were cut with restriction enzymes to give the same products, although with lower yields and more side products, as the cleaved circles. The restriction enzyme-generated fragments were incubated with purified HMG domain proteins and analyzed on non-denaturing polyacrylamide gels.

While our work was in progress, Kahn et al. (1994) reported the application of a similar technique for studying

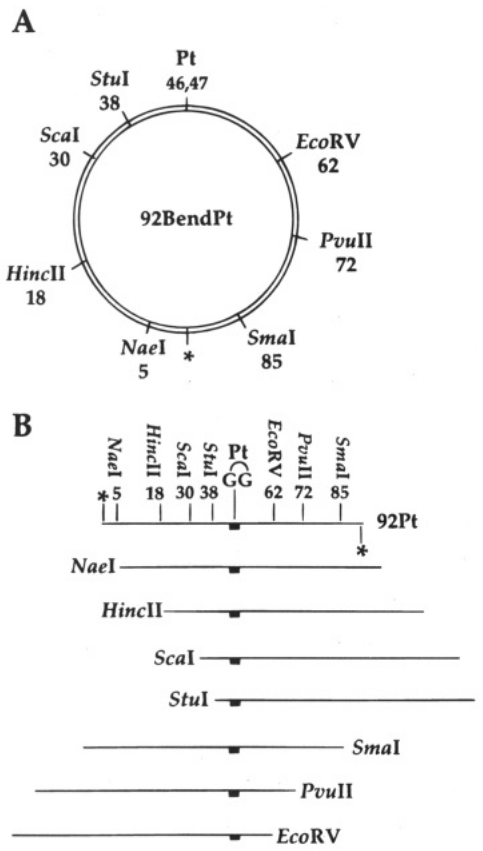

FIGURE 1: Schematic diagram of the 92BENDPt circle and the restriction fragments used in the circular permutation analysis. (A) A 92-bp circle results from ligation of 92BENDPt in the presence of HMG1 and T4 DNA ligase. The cleavage sites for *Eco*RV, *Pvu*II, *Sma*I, *Stu*I, *Sca*I, *Hin*cII, and *Nae*I with the distance from the 5′ end of the platinated strand are indicated. The asterisk marks the location of the radioactive label, and Pt denotes the location of the cisplatin adduct. (B) DNA restriction fragments used for the circular permutation assay with the intrastrand *cis*-[Pt(NH$_3$)$_2${d(GpG)}] cross-link indicated as a solid box. The linear monomer 92BENDPt (92Pt) is shown on top with the radioactive label denoted by an asterisk. Probes are named according to the restriction enzymes used for their preparation.

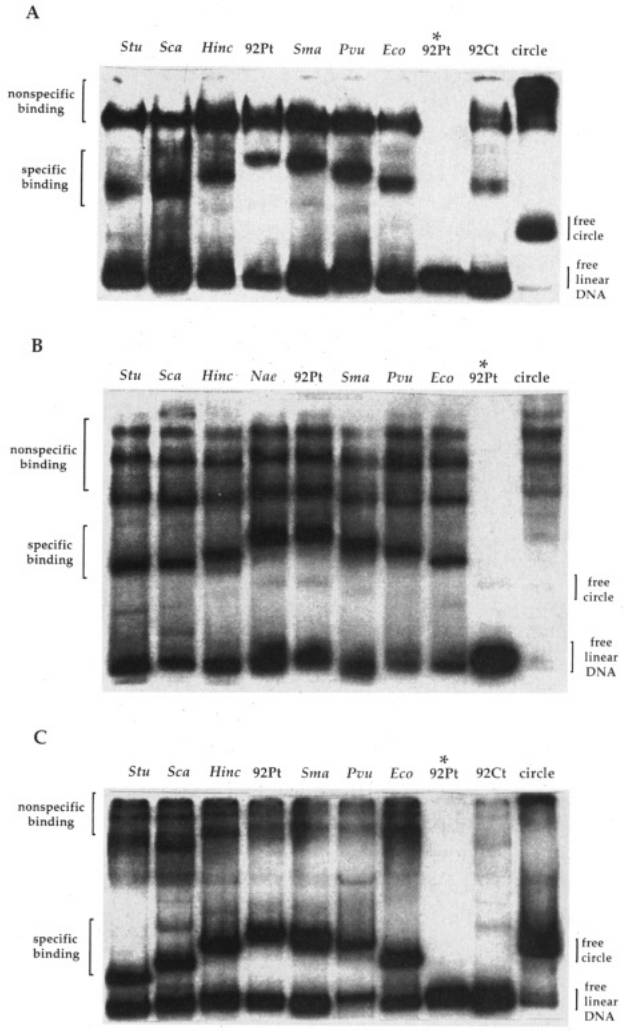

FIGURE 2: Electrophoretic mobility shift assays of HMG domain proteins binding to circularly permuted cisplatin-modified DNA probes. (A) Electrophoretic analysis in 10% non-denaturing polyacrylamide of products obtained by incubating 92-bp DNA probes with HMG1. In all lanes except 92Pt*, which contains no protein, the protein concentration was 3.7×10^{-7} M. Probes are defined in Figure 1. 92Pt refers to the linear monomer 92BENDPt, and circle refers to the 92BENDPt circular monomer. Lanes marked 92Ct contain unmodified 92-bp DNA (92BENDCt). (B) Analysis of the same samples as in (A) on a 6% non-denaturing polyacrylamide gel. (C) Repeat of (A), but substituting 3.2×10^{-7} M Ixr1 for HMG1.

nonclonable internal DNA loop sites in a circular permutation assay. The method is thus generally applicable and should be especially useful for generating DNA covalently modified with reagents where it is difficult to make circularly permuted, site-specific adducts by more conventional techniques. In our experiments, we did not employ DNAs having 5′-overhanging ends, which would have facilitated the labeling and ligation procedures. We wanted to avoid DNA end effects in band shift assays on the uncyclized probes, which were used to determine K_d values (vide infra). The use of HMG1 to enhance the cyclization of small, blunt-ended DNAs allowed us to synthesize the desired circularly permuted substrates from the same probes.

HMG1 Induces Sharp Bends in Cisplatin-Modified DNA. Figure 2A shows the results of HMG1 binding to the set of circularly permuted platinated probes in a gel-mobility shift assay (10% polyacrylamide). The formation of specific protein−DNA complexes required the presence of HMG domain protein as well as the cisplatin adduct, although nonspecific binding was also evident. We use the terms specific and nonspecific in this context to refer to *platinum-dependent* and *platinum-independent* binding, respectively. Specific complexes with the cisplatin adduct in the center of the 92-bp DNA (fragment 92Pt) migrated more slowly than complexes with the platinum binding site near the ends (fragments *Stu*I and *Eco*RV). There were also slight differences in the mobilities of the free platinated DNA probes, but these were insufficient for extraction of the bend angle induced by platination.

The extent of cisplatin-modified DNA bending by specific HMG1 binding was estimated by comparing the ratio of mobilities of the slowest and fastest migrating species in the polyacrylamide gels with published values for A tract DNA standards (Thompson & Landy, 1988). In this manner, the bend angle of cisplatin-modified DNA induced by HMG1 was calculated to be 86 ± 2°, an average over three independent determinations. This value is significantly greater than the 32−34° bend induced by the *cis*-{Pt(NH$_3$)$_2$}$^{2+}$ 1,2-intrastrand d(ApG) and d(GpG) cross-links alone (Bellon & Lippard, 1990). The locus of HMG1-induced bending in the DNA duplex was determined by plotting the relative mobilities of the complexes as a function

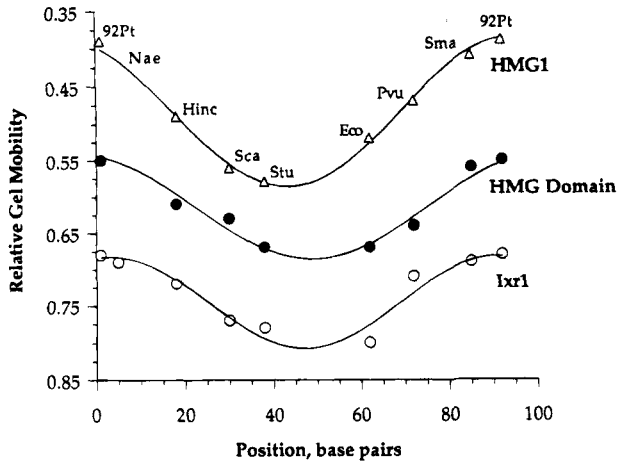

FIGURE 3: Determination of bend centers for cisplatin-modified DNA in the presence of HMG domain proteins. Relative mobilities of protein−DNA complexes in Figure 2 are plotted as a function of the position of the restriction enzyme cleavage site from the 5′ end of the platinated strand. Bend centers were estimated by fitting the data to cosine curves and extrapolating the maximum value to a position on the DNA fragment. HMG1 data averaged from Figure 2A and other experiments not shown are depicted as triangles, and the HMG domain B data are shown as solid circles. Ixr1 data from Figure 2C and other experiments not shown, some of which included the *Nae*I data point, are depicted as open circles.

of the distance from the 5′ end of the platinated strand to the restriction enzyme sites (Figure 1) (Wu & Crothers, 1984). The data were fit by least squares to a cosine function (Kerppola & Curran, 1991), and the maximum revealed that the bend center occurred at, or near, the platinum binding site (44 ± 2 bp, Figure 3). The empirical equation $\mu_M/\mu_E = \cos(\alpha/2)$, where μ_M and μ_E are the relative mobilities of DNA bent in the middle and at the end, respectively, can also be used to obtain the bend angle (α). This equation gave α values which were within 5% of those obtained by using A tract standards, irrespective of whether μ_M and μ_E were measured directly from the gels or derived from the cosine curves.

Previous work suggested that the severe bending of DNA caused by HMG1 is largely a result of nonspecific binding (Paull et al., 1993; Pil et al., 1993), because a variety of DNA sequences of varying lengths can be cyclized in the presence of protein and T4 DNA ligase whether or not bound platinum is present. As indicated in Figure 2A, this nonspecific binding leads to slow moving bands in the electrophoresis gels, whereas complexes of intermediate and variable mobility arise from specific binding of the protein to the cisplatin adduct. The relative mobilities of bands corresponding to nonspecific binding were independent of the presence or position of the platinum adduct. A very weak band of intermediate mobility in the 92Ct lane is ascribed to nonspecific HMG1 binding to the unplatinated probe, since its mobility was not altered in control experiments with circularly permuted DNAs and with higher protein concentrations (data not shown).

In order to examine the nonspecific binding component alone, an excess of HMG1 was added to a set of circularly permuted DNAs prepared with no platinum at the d(GpG) site (92BENDCt samples). Complexes with the control sequence in the center of the 92-bp DNA had the same mobility as those with the site located near the ends (data not shown). The bands that we assign to nonspecific binding in the platinated DNA−protein gels (Figure 2A) had mobilities identical to those of these control fragments, confirming the assignment. The unusually slow mobility of these bands suggests that they might be multimers of DNA−protein complexes and is also consistent with severely bent DNA structures resembling those of circles.

Figure 2B shows the results for the same samples run on a lower percentage polyacrylamide non-denaturing gel. Specific binding was still observed on this 6% gel, but the mixtures of nonspecific protein−DNA complexes were resolved into separate bands. Nonspecific binding produced a series of protein−DNA complexes having mobilities almost identical to those of the set of shifted circular DNAs bound to HMG1. This result suggests that the nonspecific complexes have structures resembling those of the circles. When the data were analyzed by plotting the relative mobilities for the specific complexes as a function of the distance of the restriction enzyme sites from the DNA ends, the same 86° bend angle and 44-bp bend locus were determined as previously found for the higher percentage polyacrylamide gels. Although we do not have a quantitative measure of the protein:DNA ratios for either the specific or the nonspecific complexes, it is possible that multiple protein molecules are bound in both cases.

A-Tract Bending and SRY/LEF-1-Induced Bending at an HMG Box Consensus Sequence. The DNA probes in the present study were smaller than those used to generate the A tract bending standard curves (Thompson & Landy, 1988). We therefore performed a control to test the validity of using smaller DNAs (~100 bp) in the permutation assay. A 101-bp A tract DNA standard was generated which contained three A_6 sequences spaced at 10-bp intervals and six different restriction enzyme sites. On the basis of previous work, this 101-bp DNA should have a bend angle ranging from 51° to 63°, assuming a contribution of 17−21° per A_6 tract (Koo & Crothers, 1988; Koo et al., 1990). The 101-bp DNA was cyclized in the presence of HMG1 and T4 DNA ligase and cut with restriction enzymes in six separate reactions to yield a set of circularly permuted A tract DNAs. The mobilities of these DNAs were measured on non-denaturing polyacrylamide gels and compared to the reported values for A tract standards (Thompson & Landy, 1988). In this manner, the bend angle of the 101-bp DNA induced by the three A tracts was calculated to be 56° with the bend locus at 55 bp (located at the 3′ end of the second A_6 tract). These results are consistent with previous work done with larger DNAs (Koo et al., 1990).

In a second control experiment, we examined the DNA bending induced by mSRY- and LEF-1-HMG domains at an HMG box consensus sequence (Figure 4). Both mSRY and LEF-1 are single-HMG-domain proteins which bind to the consensus sequence 5′-(A/T)(A/T)CAAAG-3′ and produce sharp bends (85° and 130°, respectively) in the DNA centered around this site (Ferrari et al., 1992; Giese et al., 1992, 1994). We prepared a 100-bp DNA with the consensus sequence 5′-AACAAAG-3′ in the center, cyclized it in the presence of HMG1 and T4 DNA ligase, cut it with a set of restriction enzymes, and compared the mobilities of its fragments in the presence of the mSRY- and LEF-1-HMG domains (Giese et al., 1992). The bend angles ranged from 80° for mSRY to 102−125° for LEF-1. Our calculated bend angles may differ from the literature values because we chose a different consensus sequence. Giese et al. (1994) reported

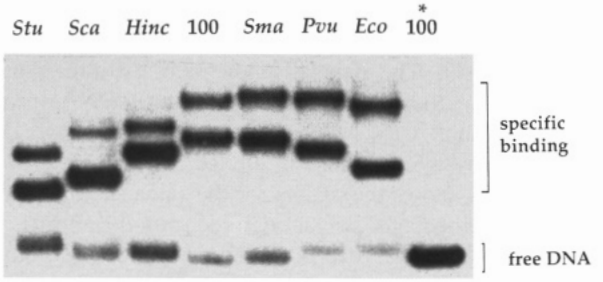

FIGURE 4: Electrophoretic mobility shift assay of HMG domain from LEF-1 binding to circularly permuted 100SRY DNA probe (recognition sequence AACAAAG) on a 10% non-denaturing polyacrylamide gel. In all lanes except 100*, which contains no protein, the LEF-1-HMG domain concentration was 2.5×10^{-7} M. Probes are defined in Figure 1. Lanes 100 and 100* refer to 100 SRY DNA linear probe.

recently that variations in the consensus sequence can alter the DNA bend angles induced by both mSRY and hSRY. In our study, the LEF-1-HMG domain interacted with 100-bp DNA probes to form two complexes rather than single protein−DNA complexes, as shown in Figure 4. The less shifted complexes had a calculated DNA bend angle of 102° and a bend locus of 49 bp. The more shifted DNA contained a bend that was slightly bigger (125°), and the bend locus was shifted away from the center by 3 bp (to 46 bp). Perhaps the LEF-1-HMG domain is bound as a dimer or multimer in the more highly shifted bands. Although the differences in bend angles and loci are small, possibly within experimental error, measurement of different samples on the same gel under identical conditions indicates they are probably real. Overall, the results presented here are consistent with the literature and indicate that the small DNA duplexes used in our assay will not affect the validity of the final results.

HMG Domain Proteins (Ixr1, mtTFA) Also Induce Bends in Cisplatin-Modified DNA. Two other HMG domain proteins were tested for their ability to induce bending of cisplatin-modified DNA. As indicated in Table 1, they too have DNA-bending activity that is specific for cisplatin-modified DNA. Figure 2C shows results for Ixr1 binding to the set of circularly permuted DNAs. Binding of Ixr1 or mtTFA to a probe with the cisplatin adduct near the center (92Pt) formed complexes that migrated at rates slower than those of complexes generated with a DNA probe containing the platinum site near the end of the molecule (*Stu*I). Plotting the relative mobility of the Ixr1−DNA complexes and extrapolating the data to the maximum revealed the bend center to be at the cisplatin binding site (Figure 3). Similar results were obtained with mtTFA. The protein-induced DNA bend angles were calculated to be 68 ± 6° for Ixr1 and 87−90 ± 5° for human mtTFA.

Binding of Ixr1 produced a series of specific protein−DNA complexes with varying mobilities as well as non-specific complexes that all had slower, but identical, mobilities. Unlike HMG1, however, Ixr1 exhibited a much lower affinity for circular DNA (<100-fold) than for cisplatin-modified DNA. Ixr1 also induced a smaller bend angle than HMG1 in cisplatin-modified DNA. These results demonstrate that there are distinct differences among members of this class of proteins in their ability to recognize prebent DNA and to induce DNA bending. The multi-HMG-domain protein mtTFA produced several specific protein-DNA complexes with bend angles ranging from 87° to 95° (± 5°), with the faster migrating species corresponding to complexes with smaller bend angles, in addition to some nonspecific protein−DNA species. Human mtTFA also recognized circular DNA with greater than 100-fold specificity over cisplatin-modified DNA. These results are consistent with the facts that mtTFA, like HMG1, can bend or wrap DNA in a nonspecific manner (Fisher et al., 1992) and also bends cisplatin-modified DNA to the same extent as HMG1.

HMG Domains of HMG1 (Domain B), mSRY, and LEF-1 Exhibit Variable Bending Activities in the Presence of Cisplatin-Modified DNA. We also tested the ability of single HMG domains to induce additional bending in cisplatin-modified DNA; the results of these tests are included in Table 1. Previous qualitative work revealed that the 81 amino acid HMG domain B of HMG1 has DNA bending capabilities similar to those of full-length HMG1 (Onate et al., 1994; Paull et al., 1993; Pil et al., 1993). This activity is relatively nonspecific and was examined with DNA fragments as small as ~80 bp in cyclization assays. We used the 92-bp circularly permuted DNA fragments containing a single cisplatin adduct to quantitate the bend angle induced by specific binding of the HMG domain to cisplatin-modified DNA (data plotted in Figure 3). The induced bending was calculated to be 65 and 74° (± 4°), values comparable to that of full-length HMG domain proteins. Similarly, the bend locus was at the platinum binding site, and nonspecific protein−DNA complexes were observed as much more slowly migrating species on the gel. The multiple bands appearing in the mobility shift assay revealed that the HMG domain has two specific binding modes which induce bend angles in the DNA, with the faster migrating species having larger bend angle, 74°. As with HMG1, the isolated HMG domain recognized prebent, circular DNA with high affinity.

The smaller bend angles induced by HMG domain B compared to full-length HMG1 suggest that some differences occur in specific binding by these proteins. HMG domain B is sufficient for DNA cyclization, but this activity is probably a consequence of nonspecific binding (Onate et al., 1994; Paull et al., 1993; Pil et al., 1993). The HMG domain B also binds to cisplatin-modified 92-bp DNA with lower specificity (4-fold difference in nonspecific versus specific binding) (C. S. Chow, unpublished results) than HMG1 (100-fold difference) (Pil & Lippard, 1992). Thus, the ability of the HMG domains to induce sharp bends in cisplatin-modified DNA may be related to their ability to bind in a specific manner. In the case of HMG1, individual contacts made by both HMG domains A and B may be important for further bending of the DNA.

The 92-bp DNAs were bent by 72° and ~50° in the presence of the LEF-1- and murine (m) SRY-HMG domains, respectively. LEF-1-HMG domain and HMG1 domain B induced similar bend angles in cisplatin-modified DNA, but the bend locus for LEF-1 was shifted by 6 bp away from the platinum binding site. The dissociation constants for these two proteins and cisplatin-modified 92-bp DNA were comparable (~10^{-7} M). In contrast, the mSRY-HMG domain demonstrated a weaker affinity ($K_d \sim 10^{-6}$ M) for the cisplatin-modified DNA and induced a much smaller bend angle. We could not obtain a bend locus for mSRY because of the poor quality of the data. The isolated HMG domains exhibited only a 4−10-fold difference in specific versus nonspecific binding (C. S. Chow, unpublished results).

Table 1: Protein-Induced Bend Angles of Cisplatin-Modified 92-bp DNA[a]

protein	HMG domains	specificity class	bend angle (deg)	binding affinity (specific) (M)	bend locus (bp)
HMG1	2	structure	86 ± 2	$\sim10^{-7}$	44 ± 2
Ixr1	2	unknown	68 ± 6	$\sim10^{-7}$	46 ± 2
HMG domain B	1	structure	74 ± 4, 65 ± 4[b]	$\sim10^{-7}$	46, 44 ± 2[b]
mtTFA	2	structure	87 ± 5, 90 ± 5[b]	$\sim10^{-7}$	46, 46 ± 2[b]
LEF-1 domain	1	sequence	72 ± 6	$\sim10^{-7}$	40 ± 2
mSRY domain	1	sequence	~50	$\sim10^{-6}$	[c]
A-tract DNA			56	[d]	55
mSRY domain/AACAAAG	1	sequence	80	[d]	49
LEF-1 domain/AACAAAG	1	sequence	102, 125[b]	$>10^{7}$	49, 46[b]

[a] A-tract DNA (101 bp) and 100-bp AACAAAG DNA with LEF-1- or mSRY-HMG domains are shown for comparison. [b] For experiments with two specific protein−DNA complexes, results for the lower shifted band are listed first and those for the higher shifted band are listed second. [c] Insufficient data. [d] Not applicable.

In the case of mSRY, the weaker specific binding affected our ability to obtain accurate bending data. Nonetheless, the results reveal differences in the ability of the individual HMG domains to induce DNA bending. Although these three HMG domains share the ability to recognize prebent, cisplatin-modified DNA, they induce additional DNA bending to varying degrees. The changes in bend loci also indicate that these proteins bind differently to cisplatin-modified DNA.

Recognition of Cisplatin-Modified DNA Is Shared by HMG Domain Proteins That Are Functionally Unrelated: Implications for the Mechanism of Action. Many members of the HMG class of DNA-binding proteins have the ability to recognize altered structural forms of DNA, in particular DNA modified by the antitumor agent cisplatin (Brown et al., 1993; Bruhn et al., 1992, 1993; Hughes et al., 1992; Pil & Lippard, 1992; Treiber et al., 1994). Some of these proteins have the additional property of recognizing DNA in a sequence-specific manner, for example, LEF-1 and SRY ($(A/T)_2C(A/T)_3G$) or SSRP/T160 (V(D)J recombination signal), whereas others exhibit little or no sequence selectivity (hUBF, HMG1, HMG2, mtTFA) (Grosschedl et al., 1994). HMG domain proteins have the ability to recognize prebent DNA, but also bend linear DNA by both sequence-specific (LEF-1, SRY) and nonspecific (HMG1, HMG2, mtTFA) interactions. In the present study, we demonstrate that cisplatin-modified DNA is recognized and bent by HMG1 and related HMG domain proteins and quantitate the degree of such bending induced by the structure-specific binding mode.

The HMG domain proteins HMG1, Ixr1, and mtTFA, as well as the isolated HMG domains from HMG1, mSRY, and LEF-1, cause a 50−90° bend in the DNA that is centered in the vicinity of the cisplatin adduct. Although all members of this class induce additional bending of cisplatin-modified DNA, the variability in bend angle and locus indicates that individual proteins make different specific contacts with the DNA. This result reflects the lack of sequence homology, usually only ~25%, among HMG domains in different proteins (Laudet et al., 1993). Even HMG domain proteins within the same family, for example, human and mouse SRY, exhibit variations in sequence specificity and bending activities (Giese et al., 1994; Grosschedl et al., 1994). Thus, in general, HMG domain proteins have similar abilities to bend and recognize prebent DNA, but amino acid sequence variability helps to differentiate them on a functional level.

Recent work has revealed that HMG domain proteins selectively inhibit repair of DNA containing a cisplatin 1,2-intrastrand d(GpG) cross-link by the human excision repair system in mammalian cell extracts (Huang et al., 1994). These results lend credence to a model in which shielding of the major cisplatin−DNA adducts by HMG domains blocks their repair by the excinuclease system, thus sensitizing cells to the drug (Brown et al., 1993). In parallel work in vitro, the same site-specific cisplatin adduct was able to compete the ribosomal RNA transcription factor hUBF away from its natural binding site on DNA (Treiber et al., 1994). The variable bending of platinated DNA by HMG domain proteins discovered in the present investigation could influence both of these shielding and "hijacking" activities. It would be interesting to examine tumor or cisplatin-resistant cell lines to determine whether shielding or hijacking by HMG domain proteins occurs and correlates with their bend angles as determined in the present investigation. Detailed structural work with different HMG domains and short cisplatin-modified DNA fragments will eventually provide information about specific protein−platinated DNA contacts and provide the insight for understanding the DNA-binding/bending properties of this class of proteins at a molecular level. This information could help unravel the function of the HMG domain proteins in relation to the antitumor mechanism of cisplatin.

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