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Distinct DNA-binding properties of the high mobility group domain of murine and human SRY sex-determining factors

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ABSTRACT **The mammalian sex-determining gene SRY (sex-determining region on Y chromosome) encodes a member of the high mobility group (HMG) family of regulatory proteins. The HMG domain of the SRY protein represents a DNA binding motif that displays rather unusually weak evolutionary conservation of amino acids between human and mouse sequences. Together with the previous finding that the human (h) SRY gene is unable to induce a male phenotype in genetically female transgenic mice, these observations raise questions concerning the DNA binding properties of SRY proteins. Here, we present data that indicate that the DNA binding and bending properties of the HMG domains of murine (m) SRY and hSRY differ from each other. In comparison, mSRY shows more-extensive major-groove contacts with DNA and a higher specificity of sequence recognition than hSRY. Moreover, the extent of protein-induced DNA bending differs for the HMG domains of hSRY and mSRY. These differences in DNA binding by hSRY and mSRY may, in part, account for the functional differences observed with these gene products.**

Male sex determination in mammals is controlled by genes located on the Y chromosome. The identification and isolation of a gene, termed sex-determining region on Y chromosome (SRY), provided some insight into the testis-determination pathway. The importance of the SRY genes for mammalian testis differentiation has been demonstrated by two sets of experiments. (*i*) Analysis of naturally occurring mutations in humans, causing sex reversal, localized several of these mutations to the high mobility group (HMG) domain of the human (h) SRY gene (1–3). (*ii*) Gene transfer of cloned genomic DNA carrying the murine (m) SRY gene into the mouse germ line indicated that this DNA fragment was capable of inducing testis differentiation in genetically female mouse embryos (4). However, gene transfer of the hSRY gene was unable to induce the development of the male phenotype in female mouse embryos.

SRY proteins contain an HMG domain that is related to the DNA binding motifs of several other biochemically defined regulators of transcription and genetically defined regulators of cell specification (5–13). The HMG domains of hSRY and mSRY were shown to interact with the specific nucleotide sequence 5′-CTTTGTT, which was identified as a binding site for the HMG-domain proteins, TCF-1 and IRE-ABP (8, 14, 15). The family of HMG-domain proteins is somewhat unusual because its members recognize DNA with substantially different DNA binding specificity. Proteins with multiple HMG domains have typically no or low sequence specificity of DNA binding. For example, HMG-1 protein has no detectable sequence specificity (16) and the RNA polymerase I transcription factor UBF has weak sequence specificity (5, 17, 18). Promoter recognition by UBF, however, can be augmented by interaction with another protein complex, termed SL-1 (19). In contrast, proteins with a single HMG domain, including LEF-1 (6, 7), TCF-1 (8), and the human sex-determining factor SRY (14, 15), were found to interact with DNA in a sequence-specific manner. DNA binding by these proteins was found to be characterized by two unusual features. (*i*) HMG-domain proteins contact DNA primarily through the minor groove of the double helix (20–22). (*ii*) DNA binding by LEF-1 and SRY was shown to be accompanied by very sharp bends in the DNA helix (21, 23).

Comparison of the amino acid sequences of the HMG domains associated with the h- and mSRY proteins indicated a 70% identity, which represents a significantly lower evolutionary conservation than the typically >90% sequence identity observed with mammalian homologs of other DNA binding domains (24–27). Thus, the inability of the hSRY gene to induce sex reversal in female mouse embryos could, in principle, be explained by amino acid changes that have resulted in different DNA binding properties of the h- and mSRY HMG domains. Alternatively, the species specificity of SRY function could be accounted for by interactions of h- and mSRY with different and/or possibly species-specific regulatory proteins. Here, we compared DNA binding by h- and mSRY and found differences in the specificity of sequence recognition and DNA contacts. These differences may contribute to the species-specific function of this regulatory protein.

MATERIALS AND METHODS

Plasmid Construction and Expression of h- and mSRY HMG-Domain Peptides. Two overlapping oligonucleotides, together containing the coding region for the hSRY HMG-domain peptide [amino acids 5–92 (25)], were annealed, extended with DNA polymerase, and ligated into plasmid pGEX-3X (Pharmacia). The peptide contained two additional amino acid residues at the N terminus (GI) and three additional amino acid residues at the C terminus (NSS), due to the position of the translational stop codon in pGEX-3X. The mSRY HMG-domain peptide was as described (21). The glutathionine *S*-transferase–SRY HMG fusion proteins were expressed and purified as described (20).

Electrophoretic Mobility Shift Assay and Methylation Interference Analysis. DNA binding reactions and analysis of the protein–DNA complexes were essentially as described (6). For methylation interference analysis, double-stranded TCR-11 oligonucleotide (Fig. 1) was labeled either at the 5′ end of the coding or noncoding strand and methylated with dimethyl sulfate (28). DNA binding reactions were performed using 50 fmol of the single end-labeled probe, 500 ng of sonicated salmon sperm DNA, and 20 ng of recombinant m- or hSRY HMG-domain peptide.

Circular Permutation Analysis and Determination of the Bending Angle. Circularly permuted DNA fragments containing the SRY binding site were generated as described (21). Bending angles were estimated by taking the ratio of the

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Abbreviations: HMG, high mobility group; h, human; m, murine.

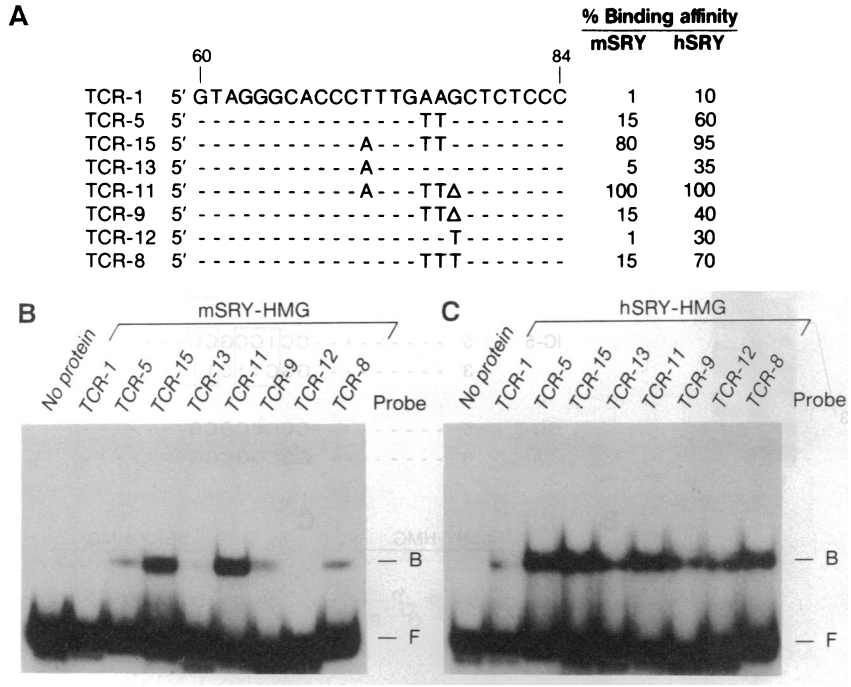

FIG. 1. HMG domains of m- and hSRY display differences in the specificity of DNA binding. (*A*) Structure of various oligonucleotides containing putative SRY binding sites. The nucleotide sequence of the coding (top sequence) strands is shown with dashes representing identical nucleotides. The open triangle indicates a nucleotide deletion. Relative binding affinities of purified HMG-domain peptides of m- and hSRY to the various oligonucleotides are indicated on the right with the maximal binding affinity of each peptide set to 100%. The values represent the average of four experiments. (*B* and *C*) Electrophoretic mobility shift assay of purified m- and hSRY HMG-domain peptide with various double-stranded DNA probes. Each peptide at 5 ng was incubated with 5'-end-labeled DNA probes in the presence of 5 ng of salmon sperm DNA. The complexes were separated by electrophoresis through a native polyacrylamide gel and visualized by autoradiography. The positions of the bound DNA probe (B) and the unbound DNA probe (F) are indicated.

mobilities of the fastest- and slowest-migrating complexes in the mobility shift assay (29). The bending center was determined by plotting the relative mobilities of the complexes as a function of the distances from the center of the TCR-11 or TCR-15 binding site to the ends of the DNA probes (29).

RESULTS

Previous studies identified the nucleotide sequence 5'-CTTTGTT as a recognition site for the HMG domains of h- and mSRY (14, 15). Recent experiments, however, indicated that the mSRY HMG domain recognized the related nucleotide sequence 5'-CATTGTT with a significantly higher affinity (21). Therefore, as a first step to compare the DNA binding properties of the h- and mSRY HMG-domain peptides, we generated oligonucleotides containing variants of these binding sites (Fig. 1*A*). Purified h- and mSRY HMG-domain peptides were incubated with the various labeled probes and examined for sequence-specific DNA binding in an electrophoretic mobility shift assay. As anticipated from our previous data (21), the mSRY HMG-domain peptide interacted efficiently with the oligonucleotides TCR-11 and TCR-15 (Fig. 1*B*). In contrast, the oligonucleotides TCR-5, TCR-9, TCR-8, and TCR-13 were bound by the mSRY HMG domain at a level 5–15% of that of TCR-11 and virtually no binding was observed with the DNA probes TCR-1 and TCR-12, suggesting a significant specificity of DNA binding by mSRY.

In parallel experiments, we examined binding of the hSRY HMG-domain peptide to the same set of DNA probes (Fig. 1*C*). Although maximal binding was detected with the oligonucleotides TCR-11 and TCR-15, the level of binding of most of the other DNA probes was only 2- to 4-fold lower than that of the TCR-11 probe. The TCR-1 oligonucleotide was the only DNA probe examined that was bound by the hSRY HMG peptide at a level 10 times lower than that of the TCR-11 oligonucleotide. These differences in the relative DNA binding specificity of the m- and hSRY HMG-domain peptides was also confirmed by examining the sensitivity of the peptide–DNA complexes to competition with excess unlabeled DNA. Incubation of both HMG domains with labeled TCR-11 DNA probe and addition of excess unlabeled TCR-11, TCR-1, and TCR-8 oligonucleotides indicated that mSRY–DNA and hSRY–DNA complexes were sensitive to competition with the TCR-11 oligonucleotide, but not with the TCR-1 oligonucleotide. In contrast, only the hSRY–TCR-11 complex, but not the mSRY–TCR-11 complex, was sensitive to competition with excess of TCR-8 oligonucleotide (data not shown). We quantitated the binding affinity of mSRY and hSRY HMG-domain peptides for their recognition site in the TCR-11 oligonucleotide by saturation binding experiments. These experiments indicated that the equilibrium dissociation constant (K_d) for this interaction is 3 nM for mSRY and 50 mM for hSRY (data not shown). Thus, these data suggest that the HMG domains of m- and hSRY recognize the nucleotide sequence 5'-CATTGAA with different affinity and specificity.

A possible explanation for the modest sequence specificity of the hSRY HMG domain was provided by previous methylation interference experiments and by replacements of A·T base pairs with I·C base pairs (22). These experiments indicated that the hSRY HMG domain interacts with DNA primarily through the minor groove. To examine whether the high specificity of DNA binding by the mSRY HMG domain is reflected by a different mode of DNA recognition, we performed methylation interference analysis and A·T → I·C replacement experiments. DNA binding of the mSRY HMG domain was inhibited by methylation of adenosines A73 and A75 of the noncoding strand (Fig. 2*A*, lanes 1–4) and A71 of the coding strand (lanes 5–8). Binding was also inhibited by methylation of the G74 of the coding strand. The methylation interference pattern of mSRY differs from that previously reported for the HMG domain of hSRY (22). In particular, a methylated guanosine in the center of the SRY binding site, G74, interfered with DNA binding by mSRY but not by hSRY. Because the previous data with the hSRY peptide were obtained using a different and suboptimal binding site (TCR-5, Fig. 1), we performed a methylation interference experiment using the higher-affinity binding site TCR-11. Similar to mSRY, DNA binding of the hSRY HMG-domain peptide was inhibited by methylation of A71 of the coding strand (Fig. 2*B*). However, no interference with binding of the hSRY HMG-domain peptide was observed by methylation of G74 (Fig. 2*B*, lanes 1–3). The summary of the methylation interference data in a projection diagram of the DNA double helix suggests an equivalent contribution of

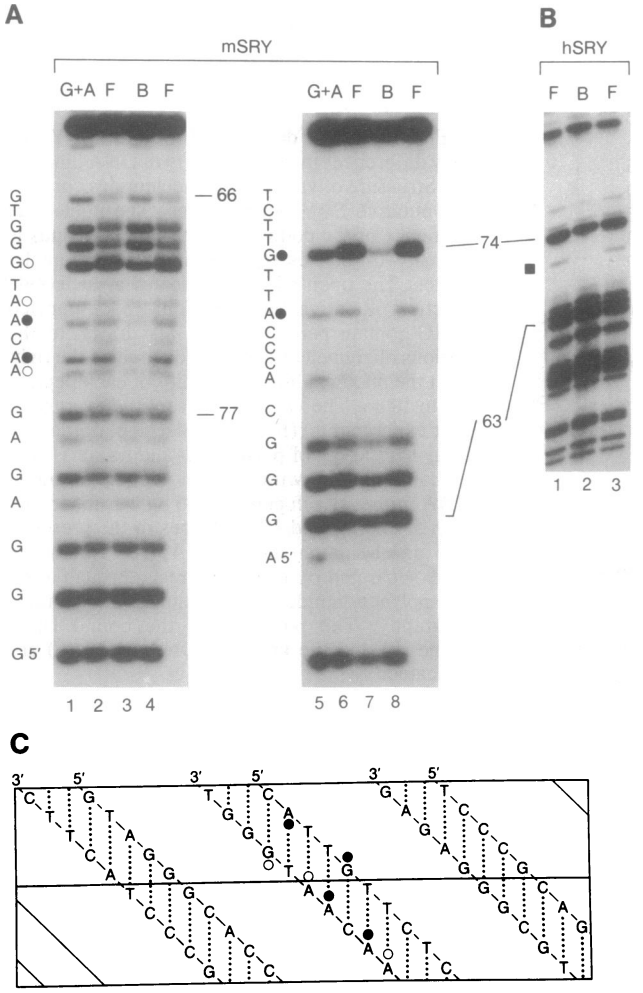

FIG. 2. Differences in sequence-specific DNA contacts by m- and hSRY. (*A* and *B*) Methylation interference analysis of binding of m- and hSRY HMG peptides to the TCR-11 oligonucleotide. The DNA probe was 5'-end-labeled on the noncoding strand (lanes 1–4) or coding strand (lanes 5–8), partially methylated with dimethyl sulfate, and incubated with the purified HMG-domain peptide of mSRY or of hSRY. Methylated adenosines and guanosines that interfered strongly (●, ■) or weakly (○) with binding of the peptide are indicated. (*C*) Summary of the methylation interference data of binding of the mSRY HMG peptide to the TCR-11 probe. The DNA double helix is shown in a planar representation. The dotted vertical lines represent the plane of base pairs and the diagonal lines represent the phosphate backbone. N-3 methyl groups of adenosines that interfered with binding are shown in the minor groove of the DNA helix. N-7 methyl groups of guanosines that interfered with binding of the HMG-domain peptide are shown in the major groove.

both minor and major groove contacts for DNA binding by the mSRY HMG domain (Fig. 2*C*).

The differences in the contributions of minor and major groove contacts by mSRY and hSRY were extended by the analysis of binding to synthetic TCR-11 oligonucleotides in which T·A base pairs were replaced by I·C base pairs (Fig. 3*A*). These substitutions alter the major groove without changing the minor groove (30). We replaced either the three lefthand A·T base pairs (probe IC-3) or the two righthand A·T base pairs (probe IC-2) in the SRY binding site with I·C base pairs. We also replaced all A·T base pairs with I·C base pairs (probe IC-5). As a control, we generated double-stranded oligonucleotides in which all A·T base pairs in the SRY binding site were replaced with G·C base pairs (probe GC-5). These latter oligonucleotides display alterations in both the major and minor grooves. Binding of the mSRY peptide to the various DNA probes was observed with the IC-2 DNA probe,

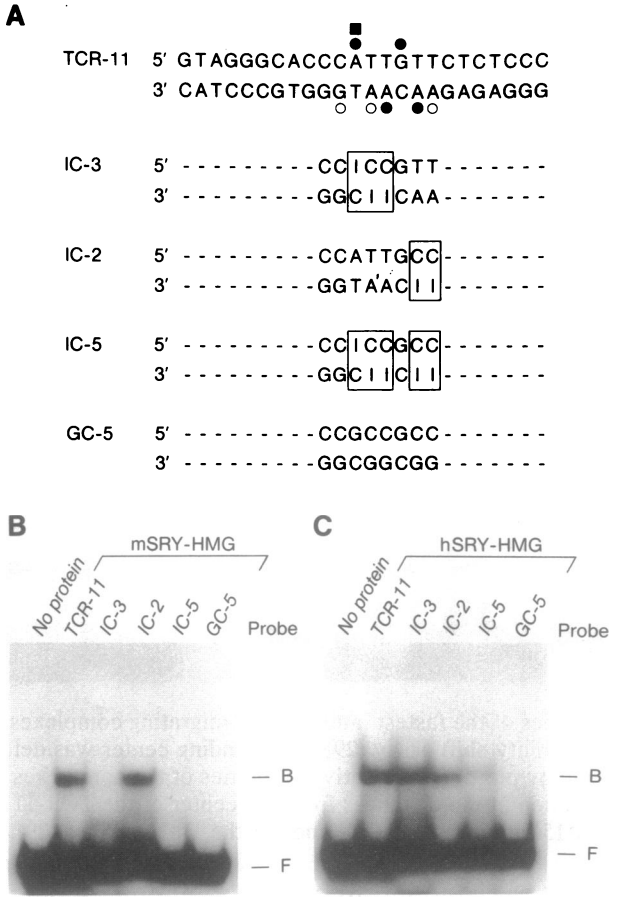

FIG. 3. Replacements in the SRY binding site of A·T base pairs with I·C base pairs differentially influence binding by the HMG domains of m- and hSRY. (*A*) Structure of double-stranded TCR-11 oligonucleotides containing a binding site for SRY (ref. 21 and this paper). The solid circles and the square indicate methylation interference with binding of the HMG-domain peptides of m- and hSRY, respectively. The open circles represent partial methylation interference with protein binding. Below, the structures of I·C- and G·C-substituted oligonucleotides are shown. The nucleotide substitutions are highlighted by a box. Dashes indicate identical nucleotides. (*B* and *C*) Electrophoretic mobility shift assay of purified m- and hSRY HMG-domain peptides with various oligonucleotide probes. Each peptide (1 ng) was incubated with labeled DNA probes and binding was analyzed as in Fig. 1*B*. The positions of the peptide–DNA complex (B) and the unbound DNA probe (F) are indicated.

whereas no binding was detected to the I·C-substituted DNA probes IC-3 and IC-5 (Fig. 3*B*). In contrast, incubation of the substituted DNA probes with a purified hSRY HMG-domain peptide showed binding not only to the substituted probe IC-2 but also to IC-3 and, to a weaker extent, to the probe IC-5 (Fig. 3*C*). Together with the methylation interference analysis, these data suggest that the HMG domains of mSRY and hSRY recognize the TCR-11 binding site through many minor groove contacts, although the extent of major groove contacts differs for both proteins. The HMG domain of mSRY displays several major groove contacts consistent with its relatively high sequence specificity of DNA recognition.

Previously, we have shown that both HMG-domain proteins LEF-1 and mSRY induce sharp bends into the DNA helix (21). Likewise, the hSRY HMG domain was shown to bend DNA (23). Although a functional role of DNA bending for eukaryotic gene expression has not yet been established, these observations raised the interesting possibility that altered conformations of DNA induced by proteins may contribute to their DNA binding affinity and specificity. To examine DNA bend-

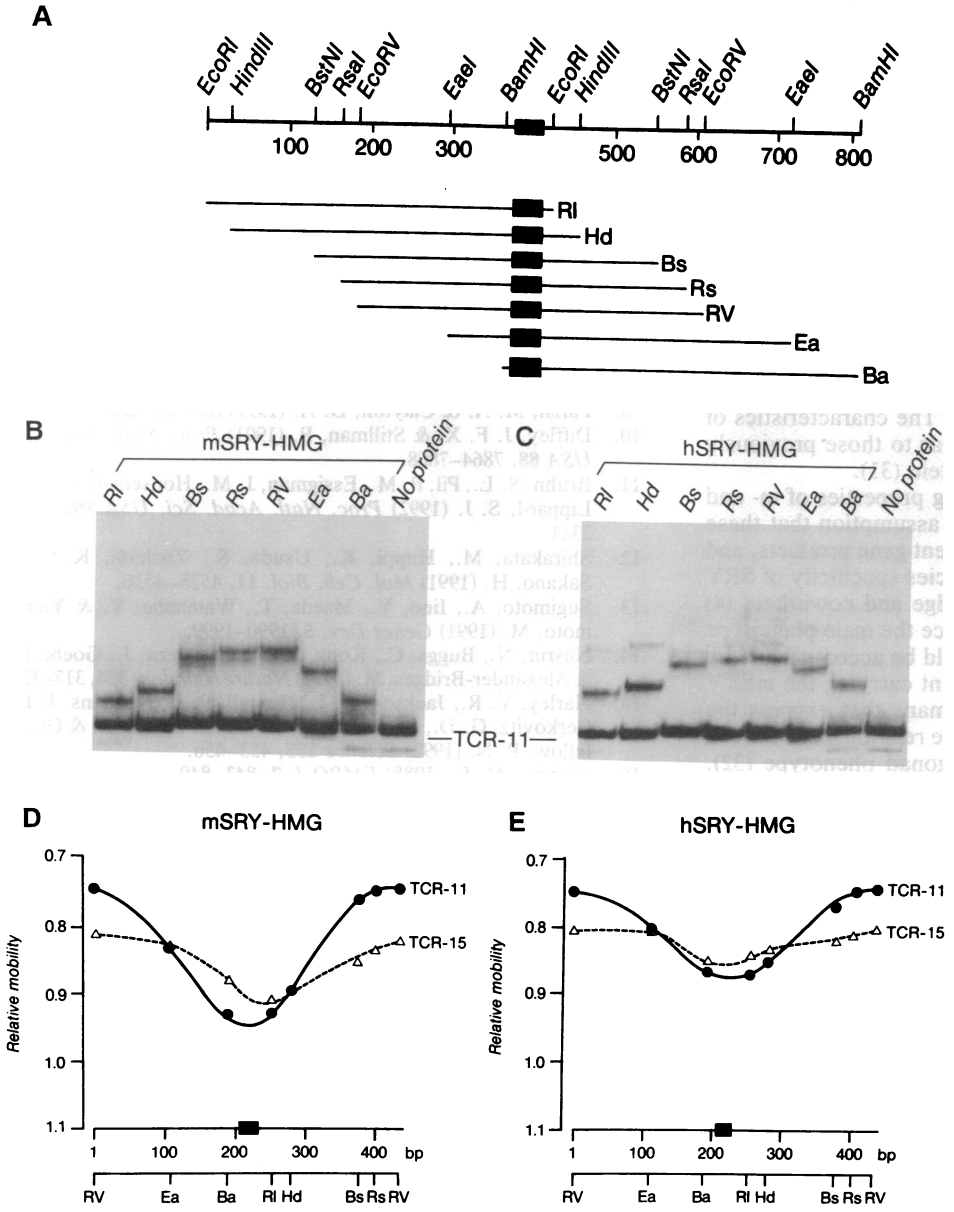

FIG. 4. Circular permutation analysis of DNA flexure induced by binding of the purified HMG-domain peptides of m- and hSRY. (*A*) Structure of the restriction DNA fragments used for the circular permutation analysis. The DNA fragments contain a SRY binding site at various positions relative to the molecular ends. Circularly permuted DNA probes of identical length (440 bp) were generated by cleavage with one of the enzymes indicated. The SRY binding site in a DNA probe is indicated as a solid box. The probes are designated according to the restriction enzymes used for their preparation: RI, *Eco*RI; Hd, *Hin*dIII; Bs, *Bst*NI; Rs, *Rsa* I; RV, *Eco*RV; Ea, *Eae* I; Ba, *Bam*HI. (*B* and *C*) Electrophoretic mobility shift assay of binding of m- and hSRY HMG-domain peptides to circularly permuted TCR-11 DNA probes. Each SRY HMG-domain peptide (10 ng) was incubated with DNA probes in the presence of 100 ng of salmon sperm DNA. The peptide–DNA complexes were analyzed by electrophoresis through a native polyacrylamide gel and were visualized by autoradiography. (*D* and *E*) Determination of the center of bending of the different binding sites TCR-11 and TCR-15. The relative mobilities of the protein–DNA complexes as shown in *B* and *C* (and data not shown) were plotted as a function of the position of the binding site relative to the probe ends. The solid box represents the SRY binding site and the numbers below indicate the distance from the left *Eco*RV site of the DNA fragment to the right *Eco*RV site. The bend centers were estimated by extrapolating the linear portions of the curve to a position on the DNA fragment.

ing induced by mSRY and hSRY, we generated circularly permuted DNA probes containing the SRY binding site in the context of the oligonucleotides TCR-11 and TCR-15, both of which are recognized by mSRY with high affinity (see Fig. 1). The circularly permuted DNA binding fragments were incubated with the mSRY HMG-domain peptide and analyzed in an electrophoretic mobility shift assay. As expected from our previous analysis (21), the mobilities of the mSRY–TCR-11 complexes differed for the individual DNA probes. Interestingly, the mobilities of the mSRY–TCR-15 complexes differed from those of the mSRY–TCR-11, indicating differences in the extent of DNA bending (Fig. 4 *B* and *D*). The extent of the DNA distortion was calculated to be approximately 85° and 60° for the TCR-11 and TCR-15 DNA fragments, respectively. For comparison, we incubated the same set of circularly permuted DNA fragments with the HMG-domain peptide of hSRY. Analysis of protein-induced DNA bending by a gel mobility shift assay and calculation of the bending angles indicated that the hSRY HMG-domain peptide bends the TCR-11 and TCR-15 DNA fragments by approximately 60° and 30°, respectively (Fig. 4 *C* and *E*).

Extrapolation of the gel mobility shift data obtained with the TCR-11 DNA fragments indicated that the bending centers mapped to the SRY binding site (Fig. 4*D* and *E*). However, the center of the mSRY-induced bend in the TCR-15 DNA fragments was shifted toward the 3′ end of the SRY binding site (Fig. 4*D*). The significance of this shift in the bend center is unclear but may reflect the differences in the DNA interaction by m- and hSRY. Thus, these data indicate that the HMG domains associated with m- and hSRY induce different DNA bends at the same nucleotide sequences.

DISCUSSION

Our study shows that the DNA binding properties of the HMG domains of m- and hSRY can be distinguished by various criteria. (*i*) The specificity of DNA binding by mSRY is significantly greater than that of hSRY. (*ii*) The mode of DNA recognition appears to differ for both proteins. Methylation interference analysis and A·T → I·C substitution experiments indicated prominent major groove contacts for mSRY but predominant minor groove recognition for hSRY. In particular, both methylation of the N7 position of G74 in the TCR-11 oligonucleotide and replacement of the A·T base pairs at positions 71–73 in the TCR-11 oligonucleotide with I·C base pairs interfered with DNA binding by the HMG

domain of mSRY but not the HMG domain of hSRY. Finally, the degree of DNA bending at the nucleotide sequence TCR-11 was calculated to approximate 85° for mSRY and 60° for hSRY. In principle, this difference in the extent of DNA bending could be explained by variations in the binding affinity or in the mode of DNA recognition. Consistent with the former explanation, determination of the equilibrium dissociation constants for the interaction of the HMG domains of m- and hSRY with the TCR-11 oligonucleotide indicated half-maximal binding at 3 nM and 50 nM, respectively (data not shown). Moreover, G+C-rich sequences flanking the SRY binding site can influence the DNA bending angle. Deletion of G77 in the TCR-15 oligonucleotide, which did not significantly change the relative levels of DNA binding by either m- or hSRY, markedly decreased the extent of bending induced by both proteins. The characteristics of SRY-induced DNA bending are similar to those previously observed with the bacterial CAP protein (31).

The differences in the DNA binding properties of m- and hSRY are quite surprising, given the assumption that these proteins represent genetically equivalent gene products, and may explain the previously noted species specificity of SRY function. Experiments by Lovell-Badge and coworkers (4) indicated that hSRY is unable to induce the male phenotype in genetically female mice, which could be accomplished by gene transfer of a 14-kb DNA fragment carrying the mSRY gene. Moreover, analysis of XX humans that express the male phenotype failed to show a simple relationship between the presence of SRY and the male gonad phenotype (32). Pedigree analysis of XX sex reversal indicated the possibility of a male gonad phenotype in the absence of SRY, suggesting that these individuals are defective in a putative autosomal gene encoding the negative regulator of male sex determination, the *Z* gene (32). According to this view, mammalian sex determination would be determined by a regulatory cascade in which SRY negatively regulates the expression of this *Z* gene. Consistent with such a model of SRY function, sex determination in the yeast *Schizosaccharomyces pombe* was found to be governed by a regulatory hierarchy in which the HMG-domain protein Ste11 regulates the transcription of another HMG-domain gene, termed *MatMc*, which is also required for sexual development (33).

The observed differences in the DNA binding properties and function of h- and mSRY suggest that these proteins either recognize distinct sequences or associate with distinct nuclear factors to regulate gene expression. In particular, the lower specificity of DNA binding by hSRY raises the possibility of an interaction with a protein that would aid in the recognition of a specific target site by hSRY. Interestingly, the amino acid sequences of SRY proteins of primates differ substantially outside the HMG domain (34, 35). By analogy, the HMG-domain protein UBF was shown to interact with the SL-1 protein complex to regulate RNA polymerase I transcription in a species-specific manner (19). The identification of functional target sites for m- and hSRY and isolation of putative accessory proteins should shed light on the species-specific differences in the DNA binding properties and function of these proteins.

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