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Interaction of Normal and Mutant SRY Proteins with DNA [and Discussion]

R. Rimini, A. Pontiggia, F. Spada, S. Ferrari, V. R. Harley, P. N. Goodfellow, M. E. Bianchi, J. A. M. Graves, A. McLaren and M. Fellows

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Interaction of normal and mutant SRY proteins with DNA

R. RIMINI,[1] A. PONTIGGIA,[1] F. SPADA,[2] S. FERRARI,[2] V. R. HARLEY,[3] P. N. GOODFELLOW[3] AND M. E. BIANCHI[1, 2]

[1] *DIBIT, San Raffaele Scientific Institute, I-20132 Milano, Italy*
[2] *Dipartimento di Genetica e di Biologia dei Microrganismi, Università degli Studi di Milano, I-20133 Milano, Italy*
[3] *Department of Genetics, Cambridge University, Cambridge CB2 3EH, U.K.*

SUMMARY

In mammals, sex determination is caused by the Y-chromosome gene *SRY*. The DNA-binding domain of human SRY protein is similar to those of the chromatin protein HMG1. Like HMG1, SRY binds to kinked DNA structures, and bends linear DNA sharply upon binding. We analysed the biochemical properties of mutant SRY proteins from five patients with complete gonadal dysgenesis: two bind and bend DNA almost normally, two bind inefficiently but bend DNA normally, and one binds DNA with almost normal affinity but produces a different angle. The mutations with moderate effect on complex formation can be transmitted to progeny, the ones with severe effects on either binding or bending are *de novo*. The angle induced by SRY depends on the exact DNA sequence, thus discriminating different target sites. We suggest that the exact spatial arrangement of the nucleoprotein complex organized by SRY in chromatin is essential for the expression of genes involved in testis differentiation.

1. INTRODUCTION

The Y chromosome gene *SRY* is required for normal male sexual differentiation in mammals. Its expression in the pre-Sertoli cells in a specific time window (between days 10.5 and 12 p.c. in the mouse) triggers the differentiation of the embryonic genital ridge into the testes (Gubbay *et al.* 1990).

SRY encodes a protein with a conserved DNA binding motif of about 70 amino acids known as the high mobility group (HMG) box, present in several eukaryotic proteins (for a review, see Bianchi 1995). HMG1, the most typical representative of the HMG box chromatin proteins, is indifferent to DNA sequence information but appears to have considerable affinity and specificity towards kinked DNA, such as four-way DNA junctions and DNA covalently modified by the drug cisplatin (Bianchi *et al.* 1989, 1992; Pil & Lippard 1992). In contrast, sequence specificity is shown by a large number of transcription regulators bearing an HMG domain, such as LEF-1 (Giese *et al.* 1991; Travis *et al.* 1991; van de Wetering *et al.* 1991) and SRY itself. SRY protein recognizes sites that conform to the sequence A/TAACAAA/T (Harley *et al.* 1992, 1994). Its interaction with DNA occurs mainly across the minor groove (van de Wetering & Clevers 1992), where there are relatively few hydrogen bond donors and acceptors. Its ability to recognize the identity of the bases is therefore restricted: numerous substitutions in the target site are allowed (Harley *et al.* 1992) and the binding specificity of SRY is rather low.

To find a common feature in the interaction of HMG boxes with DNA, we investigated the ability of human SRY protein to distort the DNA upon binding to linear target sequences, thus acting as an architectural component of stereospecific enhancer complexes (figure 1).

2. THE INTERACTION OF SRY PROTEIN WITH DNA

We obtained the HMG-box-coding sequence of *SRY* from a normal male by polymerase chain reaction (PCR), and cloned it in the expression vector pT7-7 (Ferrari *et al.* 1992). Upon induction with IPTG, the corresponding polypeptide (which we call hSRYbox) was expressed at high levels in *E. coli*, and was purified to homogeneity.

The binding ability of hSRYbox was analysed in comparison with HMG1. We first verified that HMG-box A of rat HMG1 does not recognize the GAACAAAG sequence, present in the enhancer of the CD3ϵ gene and previously reported as a preferential binding site for SRY protein (Harley *et al.* 1992). In contrast, hSRYbox is able to bind kined DNA molecules, irrespective of their sequences (Ferrari *et al.* 1992). Bandshift assays were performed using three different four-way junctions as probes, all of which formed well defined complexes with hSRYbox. In particular, we synthesized junction z, which contains sequences deliberately chosen as poor binding sites for SRY, to prove that hSRYbox recognizes the peculiar

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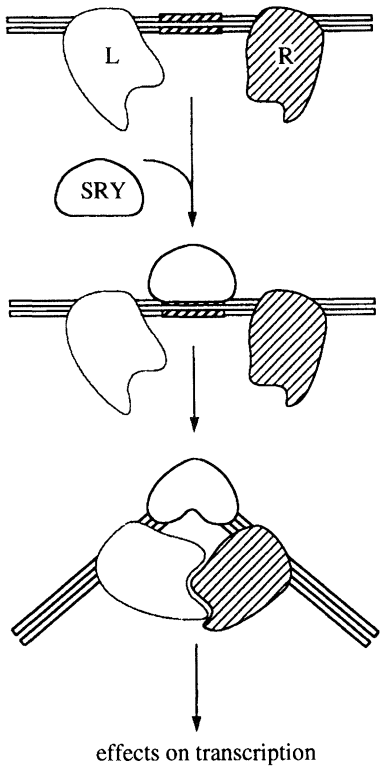

Figure 1. A model for the action of SRY protein. SRY bends the DNA considerably after binding to some sites, and this may promote protein–protein interactions between factors (L for 'left' and R for 'right') bound to either side of the SRY binding site. Thus, a mechanical action on DNA may induce the formation of a nucleoprotein complex endowed with high specificity (requiring at least three different sites bound by different proteins), high stability (the protein–protein and protein–DNA interactions would be cooperative and reinforce each other) and biological activity (through domains present on the L and R proteins). The deformation induced by SRY must be geometrically precise to serve as a genetic switch.

shape of four-way junctions and not sequence-specific binding sites adventitiously present in the junction. Four-way junctions and the GAACAAAG sequence compete with each other for binding to hSRYbox (Ferrari *et al.* 1992), suggesting that the polypeptide has a single DNA binding surface.

These results point to two distinct targets for hSRYbox: kinked DNA and linear DNA containing specific sequences. To find a possible connection between the two target DNAs, we investigated the ability of hSRYbox to induce large distortions on linear DNA. DNA fragments with a distortion in the middle of the molecule migrate in a polyacrylamide gel differently than DNA fragments of the same length and sequence with a distortion near one end (Wu & Crothers 1984). Although the relation between electrophoretic mobility and conformation is complex, the assay allows one to map the locus of protein-DNA interaction and to estimate the amount of distortion introduced in DNA (Wu & Crothers, 1984; Liu-Johnson *et al.* 1986; Thompson & Landy 1988). Figure 2 shows the result of the assay performed on hSRYbox: complexes with the CD3ϵ binding site in the middle of the fragment (probe D) migrate significantly slower than complexes with the site near the ends (probes A and G). Analysis of the data in terms of a simple geometric model (Ferrari *et al.* 1992) allowed us to mape the site of flexure of the DNA to the GAACAAAG sequence, and to demonstrate a large distortion of the DNA molecule ($\approx 76°$).

We further demonstrated that hSRYbox recognizes the GAACAAAG sequence in probe D by hydroxyl radical footprinting (figure 3). Free DNA and the DNA-hSRYbox complex were separated electrophoretically and cleaved with phenantroline-copper; the bands were then excised from the gel, the DNA was extracted and applied to a sequencing gel. The comparison of the patterns obtained with free and bound DNA indicates that seven consecutive deoxyribose moieties are protected from hydroxyl radical cleavage on each strand; there is no evidence for protein-induced enhancement of cleavage at any site. The seven based protected on the two strands are complementary to each other and correspond to the sequence GAACAAA.

3. DNA BINDING AND BENDING ABILITIES OF MUTANT HSRYBOXES

The discovery of natural mutations in *SRY* associated with sex-reversal in humans allowed us to test the biological role of SRY-induced DNA bending. The five mutations we considered were in patients with complete 46,XY gonadal dysgenesis, and show variable penetrance (table 1). Mutations F109S and I90M were also present in normal male relatives of the patients, including the father (Hawkins *et al.* 1992*b*; Jäger *et al.* 1992). The incomplete penetrance of these mutations can be ascribed to pleiotropic effects of other genes or to specific environments. Mutations M64I and G95R are *de novo* (Berta *et al.* 1990; Hawkins *et al.* 1992*a*); no male relatives were available for testing in the case of mutation K106I (Hawkins *et al.* 1992*b*).

Mutant hSRYboxes with single amino acid substitutions were prepared as described for wild-type hSRYbox in the previous paragraph. We measured the affinities of the normal and mutant hSRYboxes for a DNA fragment bearing the CD3ϵ site by titrating the polypeptides in bandshift assays with a limiting amount of labelled DNA. The binding ability of SRY mutants varied extensively: mutant F109S showed the same affinity for DNA as the wild type protein, mutants M64I and I90M showed a slightly reduced affinity, whereas the affinity of K106I was reduced by two orders of magnitude; mutant G95R did not bind DNA at all under our experimental conditions (table 1).

The bending capabilities of mutant hSRYboxes were compared by means of circular permutation assays (figure 2), using the set of probes described in the previous paragraph. Among the five mutants, only M64I induces a significantly different angle, measuring $\approx 56°$ (table 1). This difference, viewed against the uniform behaviour of the other mutants and of SRYboxes from several primates (Pontiggia *et al.* 1995), suggests a role for defective bending in the onset of sex-reversal. We also ruled out the possibility that mutation M64I modifies the binding specificity of SRY

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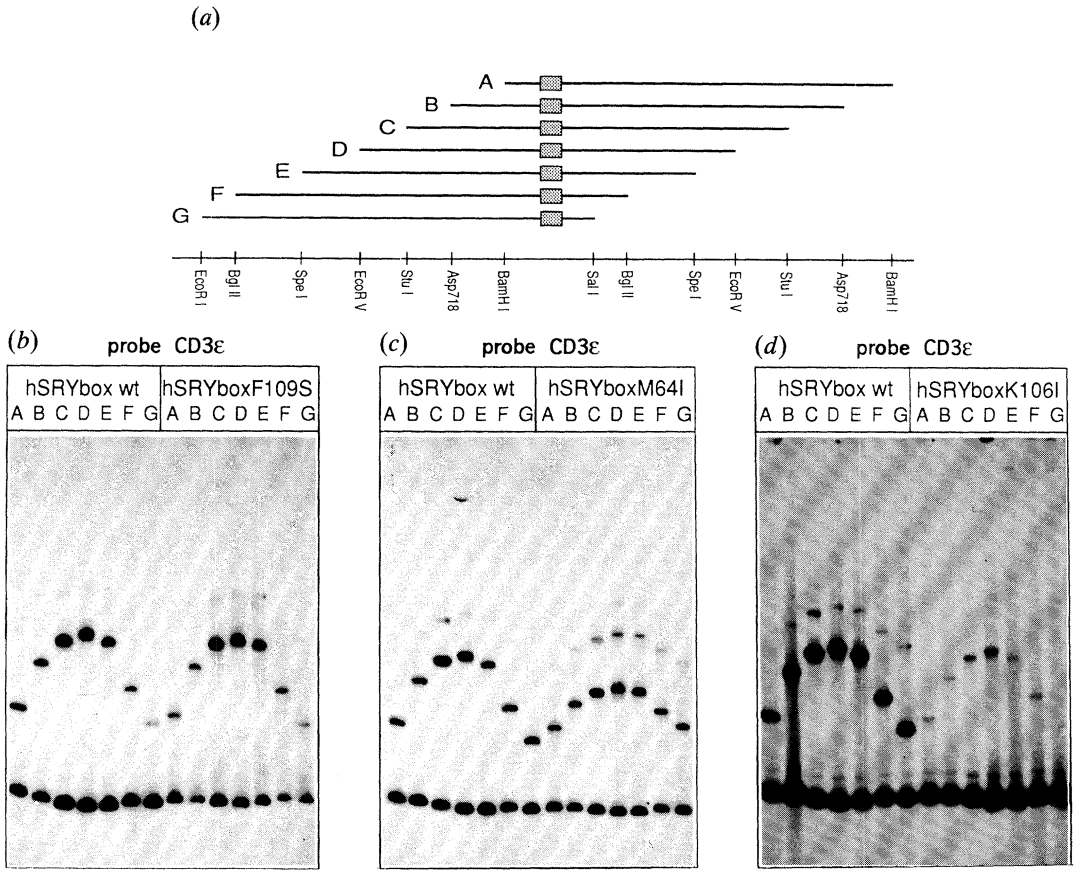

Figure 2. DNA bending activity of normal and mutant SRY HMG-boxes on the CD3ϵ sequence. (*a*) DNA probes used for the circular permutation analysis. Plasmid pBend2CD3ϵ, containing the CD3ϵ site (hatched box) flanked by tandemly repeated DNA sequences, was cleaved at the restriction sites indicated in the map. The DNA fragments obtained in this way (designated A–G) all contain circular permutations of the same sequence of 141 b.p. (*b*, *c*, *d*) Circular permutation analysis of DNA bending induced by mutant hSRYboxes F109S, M64I and K106I. The letter above each lane identifies the probe used (see panel *a*). Three fmol of labelled DNA probes were mixed in 9 µl of standard DNA binding buffer (8% Ficoll, 100 mM NaCl, 10 mM HEPES pH 7.9) with 1 µl of purified protein ($\approx$ 2 ng of hSRYbox wild type, $\approx$ 2 ng of hSRYbox F109S, $\approx$ 10 ng of hSRYbox M64I, $\approx$ 25 ng of hSRYbox K106I). After incubation for 10 min on ice, samples were applied to vertical 10% polyacrylamide gels in 0.5 × TBE and electrophoresed at 11 V/cm. The gels were dried and autoradiographed; the gel in panel (*d*) was overexposed to detect the faint bands of DNA complexed to hSRYbox K106I. The retarded bands running behind the principal protein–DNA complex band are complexes containing more than one polypeptide molecule per DNA molecule. No significant difference in the mobility of the free DNA probes can be observed, indicating that the GAACAAAG sequence does not distort DNA on its own.

protein. After five cycles of selection and amplification of double-stranded DNA from a population of molecules initially displaying all possible sequence variation at the central ten nucleotides, we obtained a collection molecules that are bound by M64I considerably better than the initial population. These were cloned, sequenced and aligned (Pontiggia *et al.* 1994). The consensus sequence we obtained exactly matches that obtained by Harley *et al.* (1994) performing a similar experiment with wild type SRY protein.

4. THE SEQUENCE OF THE TARGET SITE AFFECTS DNA BENDING BY SRY

We next investigated the role that the DNA sequence plays in the formation of the SRY-DNA nucleoprotein complex. Permutation assays were performed on wild type and mutant hSRYboxes using as target sites two sequences slightly diverged from CD3ϵ (table 2). The sequence TAACAATG (mut11) has been reported to bind SRY protein with higher affinity than the GAACAAAG sequence present in the enhancer of the CD3ϵ gene, while the sequence GAACACAG (mut0) was reported to be a poorer binding site (Giese *et al.* 1992). Every hSRYbox variant bound the mut11 fragments with higher affinity than CD3ϵ fragments, but induced a slightly smaller angle. The mut0 fragments were bound almost six-fold less efficiently than the CD3ϵ fragments. Bending was dramatically reduced with wild type hSRYbox, whereas mutant M64I did not produce any measurable distortion.

These data suggest that the correct geometrical configuration of the nucleoprotein complex depends not only on the protein but also on the sequence of the target site and its intrinsic bendability.

5. DISCUSSION

The results presented in the previous sections reconcile the apparently disparate ability of different

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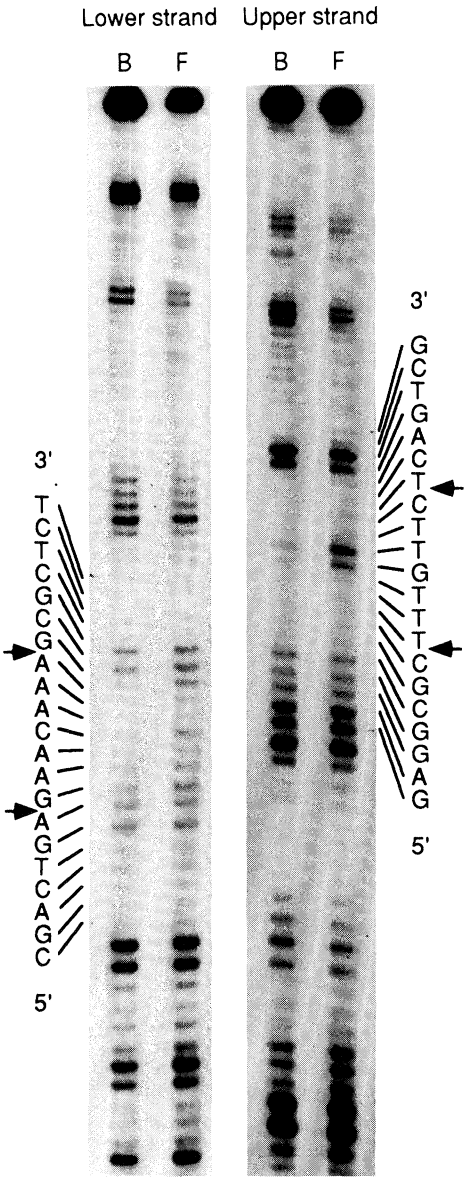

Figure 3. Identification of the site of interaction of hSRYbox with probe D. *In situ* copper-phenantroline footprinting was performed as described by Papavassiliou (1993). Five fmol of probe D from pBend2CD3ϵ (see figure 2*a*) was 5′-labelled with ^{32}P-γATP and mixed to 100 fmol wild type hSRYbox. Free and bound DNA molecules were separated electrophoretically and the whole gel was equilibrated with 0.18 mM 1,10-phenantroline, 41 μM $CuSO_4$, 10 mM Tris pH 8.0. Cleavage was started by adding 3-mercaptopropionic acid to 4.8 mM and stopped after four minutes with 2.2 mM 2,9-dimethyl-1,10-phenantroline. Free (lanes F) and bound DNA (lanes B) was recovered from individual bands and applied to a sequencing gel alongside G+A sequencing reactions of the relevant strand. The arrows bracket the protected bases.

HMG boxes to recognize distorted DNA exclusively (for example, those in HMG1), or to recognize specific sequences (as those in SRY and other transcriptional regulators). In all cases, the DNA complexed to HMG boxes has a structure that diverges considerably from regular B-form DNA.

Circular permutation assays indicate that SRY bends the target site in the CD3ϵ promoter considerably, and hydroxyl radical footprinting indicates

Table 1. *Summary of SRY mutation characteristics and properties of the SRY HMG-boxes*

(The mutations in *SRY* we have considered were described by Berta *et al.* (1990), Hawkins *et al.* (1992*a*, *b*) and Jäger *et al.* (1992). The dissociation constants for complexes between the various hSRYboxes and DNA were calculated by titrating the polypeptides against 0.25 nM DNA. Samples were electrophoresed at 4 °C in 10% polyacrylamide gels as described in figure 2. The radioactivity present in the bands was measured by exposing the wet gel to Phosphorlmager screens. Under conditions of protein excess, the dissociation constant is equivalent to the concentration of polypeptide where half of the input DNA is taken up in the complexes and half is free. 'Linear' refers to the D probe bearing the CD3ϵ site, 'angle' refers to the angle calculated by the algorithm of Ferrari *et al.* (1992) from the circular permutation assays on the set of probes bearing the CD3ϵ site.)

mutation	type	K_D CD3ϵ site	angle
wt		2×10^{-8} M	76°
F109S	familial	2.5×10^{-8} M	76°
I90M	familial	4×10^{-8} M	75°
M64I	*de novo*	6.5×10^{-8} M	56°
K106I	relatives unavailable	2×10^{-6} M	73°
G95R	*de novo*	$\geqslant 10^{-5}$ M	

that it protects only seven base pairs. Molecular modelling suggests that DNA can be kinked in the required way if the minor groove is widened, the major groove is compressed and the plane of the bases moves away from the perpendicular to the helix axis. Such models are compatible with the observations that SRY interacts with the minor groove of DNA (van de Wetering & Clevers 1992) and that an isoleucine residue of SRY wedges between successive A·T base pairs and disrupts their stacking (King & Weiss 1993).

The X-ray structure of cisplatinated DNA, a preferential target for HMG-box proteins, is available (Sherman *et al.* 1985, 1988). In the covalent adduct there is a bend towards the major groove of 32° and the minor groove is correspondingly expanded. Likewise, a wide narrow groove is also present at the crossover point in four-way junction DNA (Lilley & Clegg 1993). A wide minor groove may therefore be the most salient feature of the interaction of HMG-boxes with DNA: when the minor groove is already wide, HMG-boxes will bind irrespective of the sequence of bases in the DNA, whereas linear DNA will be distorted to a wide minor groove conformation only if the HMG box can establish specific contacts with a particular succession of bases.

Because the DNA bending properties of HMG boxes are so prominent and unusual, one can predict that they will be critical for the biological function of HMG-box proteins. In the case of SRY, other considerations support this hypothesis: (i) SRY has a limited sequence selectivity, indistinguishable from that of other proteins with similar DNA binding domains but very different biological functions (Denny *et al.* 1992*a*, *b*); (ii) the only conserved segment in SRY proteins from closely related species is the HMG box, while the rest of the protein diverges (Tucker &

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Table 2. *Influence of the DNA sequence in the amplitude of the angle induced by SRY proteins*

(DNA fragments containing two variations of the CD3ϵ sequence, designated mut11 and mut0, were generated as described in figure 2*a*. The dissociation constants for complexes between hSRYboxes and DNA were calculated as described in table 1. 'Angle' refers to the angle calculated by the algorithm of Ferrari *et al.* (1992).)

		wt		M64I	
probe	sequence	K_D	angle	K_D	angle
CD3ϵ	GAACAAAG	2×10^{-8} M	76°	6.5×10^{-8} M	56°
mut11	TAACAATG	10^{-8} M	73°	3×10^{-8} M	55°
mut0	GAACACAG	10^{-7} M	35°	3.5×10^{-7} M	< 10°

Lundrigan 1993; Whitfield *et al.* 1993); (iii) SRYboxes from different primates bend the CD3ϵ site in a very similar way to human SRY protein, despite several variations in their amino acid sequences (Pontiggia *et al.* 1995); and (iv) the angle produced by mouse SRY is quite different from that produced by human SRY (Giese *et al.* 1992) and this correlates with the inability of transgenic human SRY to cause male sex differentiation in mice (Koopman *et al.* 1991).

To substantiate the prediction of a critical role for DNA bending in SRY function we analyzed five SRY mutations associated with sex-reversal and complete gonadal dysgenesis in humans. The DNA binding and bending abilities of mutant hSRYboxes I90M and F109S do not significantly differ from that of wild type SRY protein. The corresponding mutations are transmissible and present indeed also in normally differentiated males; they can be considered conditional mutations whose phenotypic expression depends on the interaction with different genetic backgrounds and the environment. Mutations G95R and K106I significantly reduce the binding affinity of the hSRYbox, so that *in vivo* SRY may be unable to recognize the target site; mutation G95R is *de novo*. Mutant M64I shows an unimpressive three-fold reduced binding affinity with respect to the wild type but a significant reduction in the deformation of the binding site. Most likely, residue methionine 64 belongs to the surface of interaction between the protein and the DNA and is directly involved together with isoleucine 68 (King & Weiss 1993) in disrupting the stacking of the bases in the binding site. The inappropriate bending of the bound DNA is probably the key molecular defect of mutant M64I, because we have excluded the possibility that mutant SRYbox may recognize a different repertoire of sequences.

Finally, we investigated the contribution of the DNA sequence in the correct spatial organization of nucleoprotein complex with SRY. We have shown that target sites with altered sequences can still be recognized by SRY, but are bent differently. This implies that different DNA sequences can be discriminated on the basis of the geometry of the complex they support rather than on the probability of their occupancy. This possibly justifies the limited sequence-specificity of SRY protein as opposed to the sequence-specificity required by its action.

In conclusion, we have demonstrated that the DNA binding ability of SRY protein is correlated with a peculiar feature, DNA bending. The DNA bending activity of SRY protein is affected by the sequence of the target site, and the alteration of bending may lead to sex reversal. Our results suggest a direct role of SRY as an architectural component of stereospecific enhancer complexes (figure 1) and, more generally, a direct role of protein-induced DNA deformation for transcription control and gene expression.

The research efforts of R. Rimini, A. Pontiggia, F. Spada, S. Ferrari and M. E. Bianchi are supported by Associazione Italiana per la Ricerca sul Cancro (AIRC), those of V. Harley and P. N. Goodfellow are supported by the Wellcome Trust.

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Discussion

J. A. M. Graves (*University of La Trobe, Melbourne, Australia*). Do regions of SRY (mouse or human) outside the box interact with DNA or other bound proteins?

M. E. Bianchi. So far we have not found any protein interacting with mouse or human SRY. Contact with DNA depends only from the HMG box, with no contribution from other regions of the protein.

A. McLaren (*Wellcome/CRC Institute, Cambridge, U.K.*). What happens at DNA replication? Do transcription factors in general, and SRY protein in particular, detach totally from the DNA before replication occurs, or do they remain attached to one of the two DNA strands?

M. E. Bianchi. The general consensus is that transcription factors detach from DNA at replication. We do not know in particular whether replication requires SRY to detach because the rigorous experimental test is difficult to carry out. However I would be surprised if the protein stayed attached to a single DNA strand.

I have to add that we know from immunofluorescence studies that SRY is not attached to DNA in metaphase chromosomes; since there is no nuclear membrane during this stage of cell cycle, SRY diffuses to the cytoplasm, and has to be reimported inside the nucleus when the nuclear membrane reforms.

M. Fellows (*Pasteur Institute, Paris*). Did Dr Bianchi observe SRY *de novo* mutation which has a normal binding and bending?

M. E. Bianchi. Not so far. I am disinclined to think there is one, but negative evidence is always weak.

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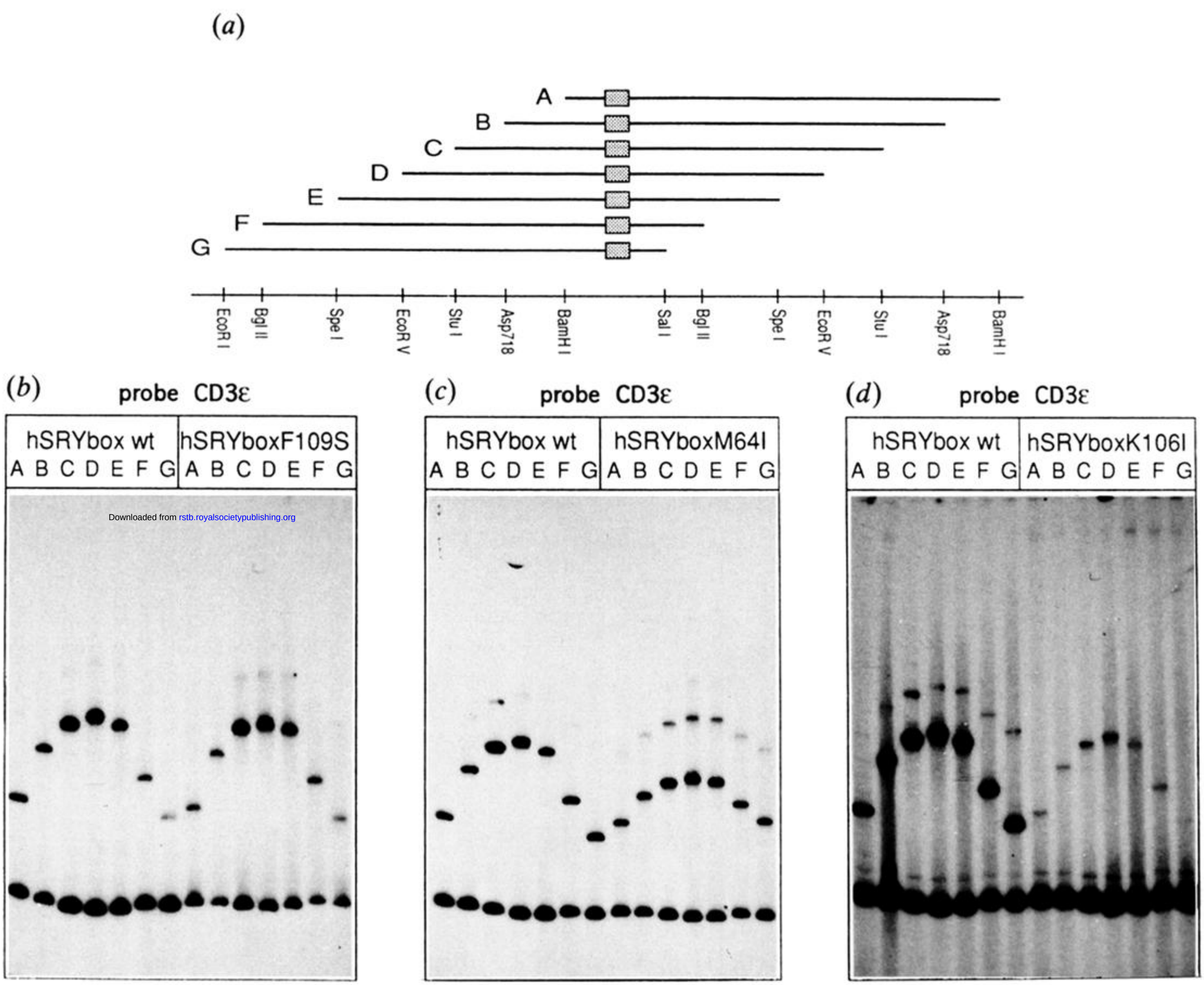
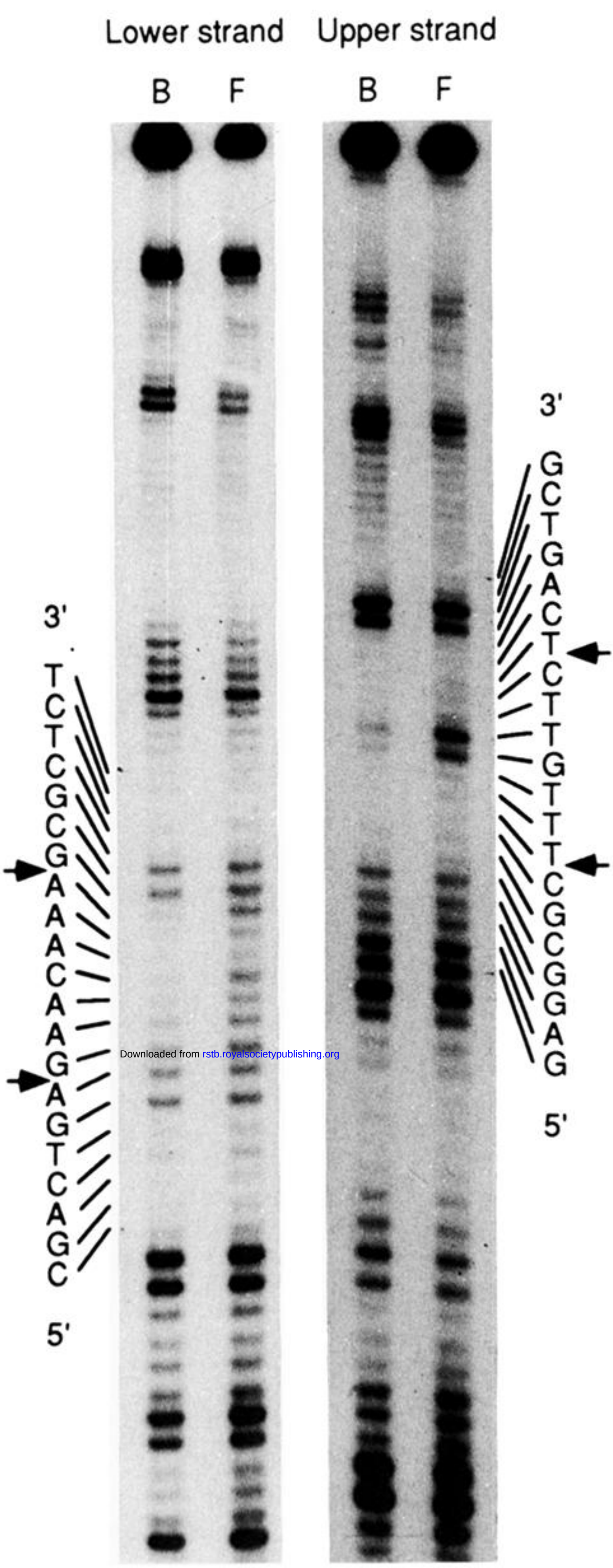


igure 2. DNA bending activity of normal and mutant SRY HMG-boxes on the CD3ϵ sequence. (*a*) DNA probes sed for the circular permutation analysis. Plasmid pBend2CD3ϵ, containing the CD3ϵ site (hatched box) flanked by andemly repeated DNA sequences, was cleaved at the restriction sites indicated in the map. The DNA fragments btained in this way (designated A–G) all contain circular permutations of the same sequence of 141 b.p. (*b*, *c*, *d*) ircular permutation analysis of DNA bending induced by mutant hSRYboxes F109S, M64I and K106I. The letter bove each lane identifies the probe used (see panel *a*). Three fmol of labelled DNA probes were mixed in 9 µl of andard DNA binding buffer (8% Ficoll, 100 mM NaCl, 10 mM HEPES pH 7.9) with 1 µl of purified protein ($\approx$ 2 ng f hSRYbox wild type, $\approx$ 2 ng of hSRYbox F109S, $\approx$ 10 ng of hSRYbox M64I, $\approx$ 25 ng of hSRYbox K106I). After ncubation for 10 min on ice, samples were applied to vertical 10% polyacrylamide gels in 0.5 × TBE and lectrophoresed at 11 V/cm. The gels were dried and autoradiographed; the gel in panel (*d*) was overexposed to etect the faint bands of DNA complexed to hSRYbox K106I. The retarded bands running behind the principal rotein–DNA complex band are complexes containing more than one polypeptide molecule per DNA molecule. No gnificant difference in the mobility of the free DNA probes can be observed, indicating that the GAACAAAG equence does not distort DNA on its own.



gure 3. Identification of the site of interaction of hSRYbox ith probe D. *In situ* copper-phenantroline footprinting was erformed as described by Papavassiliou (1993). Five fmol of obe D from pBend2CD3ϵ (see figure 2*a*) was 5′-labelled ith ^{32}P-γATP and mixed to 100 fmol wild type hSRYbox. ree and bound DNA molecules were separated electro-horetically and the whole gel was equilibrated with 0.18 mM 10-phenantroline, 41 μM $CuSO_4$, 10 mM Tris pH 8.0. leavage was started by adding 3-mercaptopropionic acid to 8 mM and stopped after four minutes with 2.2 mM 2,9-methyl-1,10-phenantroline. Free (lanes F) and bound NA (lanes B) was recovered from individual bands and pplied to a sequencing gel alongside G+A sequencing actions of the relevant strand. The arrows bracket the rotected bases.