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A familial mutation in the testis-determining gene SRY shared by both sexes

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Abstract. A familial mutation in SRY, the gene coding for the testis-determining factor TDF, was identified in an XY female with gonadal dysgenesis, her father, her two brothers and her uncle. The mutation consists of a T to C transition in the region of the SRY gene coding for a protein motif known as the high mobility group (HMG) box, a protein domain known to confer DNA-binding specificity on the SRY protein. This point mutation results in the substitution, at amino acid position 109, of a serine residue for phenylalanine, a conserved aromatic residue in almost all HMG box motifs known. This F109S mutation was not found in 176 male controls. When recombinant wildtype SRY and SRY^{F109S} mutant protein were tested in vitro for binding to the target site AAC AAAG, no differences in DNA-binding activity were observed. These results imply that the F109S mutation either is a rare neutral sequence variant, or produces an SRY protein with slightly altered *in vivo* activity, the resulting sex phenotype depending on the genetic background or environmental factors.

Introduction

In mammals, sexual differentiation is triggered by the determination of gonadal sex. In the presence of a Y chromosome, the bipotential gonadal anlage develops into testes, whereas in its absence, ovaries are formed. It is not known how many genes are involved in either pathway. However, a key regulatory locus coding for a testis-determining factor (TDF) must be located on the Y chromosome. Analysis of sex reversed individuals has narrowed down the critical region for TDF to a 35-kb segment adjacent to the Y pseudoautosomal boundary (Palmer et al. 1989; Sinclair et al. 1990; Jäger et al. 1990a). A gene termed SRY (sex-determining region Y gene) has been identified within this segment (Sinclair et al. 1990). Strong evidence for equating SRY with TDF comes from studies in the mouse demonstrating expression of the mouse homologue Sry in the somatic compartment of the genital ridge at the time of testis formation (Koopman et al. 1990). More importantly, karyotypic female mice transgenic for a 14-kb fragment harbouring the Sry gene develop as males with azoospermic testes (Koopman et al. 1991).

SRY encodes a protein with a conserved sequence motif of about 80 amino acids known as the high mobitity group (HMG) box found in the high mobility group proteins and in an increasing number of transcription factors (Jantzen et al. 1990; Ner 1992). Recombinant SRY protein has been shown to bind to DNA target sequences recognized by some of these transcription factors (Nasrin et al. 1991; Harley et al. 1992; van de Wetering and Clevers 1992). *De novo* mutations within the HMG box region of SRY have been detected in a number of sex-reversed XY females with gonadal dysgenesis, supporting the notion of identity of SRY with TDF (Jäger et al. 1990b; Berta et al. 1990; Hawkins et al. 1992; Harley et al. 1992; McElreavey et al. 1992). In addition, two familial SRY mutations shared by fathers and daughters have been described (Berta et al. 1990; Harley et al. 1992; Vilain et al. 1992). Amino acid substitutions in mutant SRY proteins, and the introduction of such mutations at homologous positions within the HMG box of other proteins, lead to a reduction or loss of *in vitro* DNA-binding activity (Nasrin et al. 1991; Giese et al. 1991; Harely et al. 1992).

We have previously described a *de novo* frameshift mutation in the HMG box region of SRY in one out of 12 XY females analysed (Jäger et al. 1990b). We now report an inherited mutation in SRY, detected in one of three additional XY females studied, where one sex-reversed XY female shares the same amino acid substitu-

This paper is dedicated by G.S. to Professor Ulrich Wolf on the occasion of his 60th birthday

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tion with her XY male relatives. A preliminary report of this work has appeared elsewhere (Jäger et al. 1991).

Materials and methods

Case report

M. L. is the fourth out of five siblings. The parents, who originate from the Sudetenland, are healthy and unrelated. At her birth, paternal age was 28 years, maternal age 31 years. The pedigree is unremarkable with regard to infertility and gynaecological tumours; an undescended right testis has been diagnosed in the paternal uncle E. L. Two unmarried brothers are apparently normal. One brother died in early infancy because of cardiac failure. The physical and mental milestones of M. L. have been in the normal range. She was examined at the age of 17 and 28 years because of primary amenorrhea. Height is 180 cm. Prior to therapy, mammary development was at stage III (Tanner). No signs of virilisation were noticed.

Endocrinological studies showed normal amounts of prolactin, (free) testosterone, androstenedione, T3, and T4. Circadian output of cortisol and 17-hydroxyprogesterone were within the normal range. Dehydroepiandrosterone sulphate was slightly increased. Follicle-stimulating hormone and luteinising hormone (LH) were significantly increased. Results of adrenocorticotrophic hormone and LH tests suggested primary gonadal insufficiency. Objective olfactometry was normal. Bone density was decreased but improved after substitutional therapy. By laparotomy, bilateral streak gonads, atrophic Fallopian tubes and a rudimentary uterus with a narrow lumen were detected. Histological examination of the right streak gonad disclosed nests of large cells with alveolar cytoplasm and large nuclei separated by cysts, suggesting the diagnosis of gonadoblastoma. Routine cytogenetic studies revealed a 46,XY karyotype with no evidence for mosaicism or for structural anomalies of the sex chromosomes.

Amplification and sequence analysis of SRY

Polymerase chain reaction (PCR) amplification of genomic DNA samples (0.25–0.5 μg) isolated by standard methods (Sambrook et al. 1989) was performed in 50-μl reactions essentially as described previously (Jäger et al. 1990b). For amplification of the HMG box region of SRY, primers AP-1 and AP-2 were used (Jäger et al. 1990b; see Fig. 1). Additional primers used to amplify the remaining part of the SRY open reading frame plus some flanking sequences were: AP-3 (5′-tgcagaattcGGTGTTGAGGGCGGAGAA ATGC-3′, nucleotide position 271–302 according to Sinclair et al. 1990); AP-4 (5′-tgcaaagcttGTAGCCAATGTTACCCGATTGTC-3′, 1048–1026); AP-5 (5′-TGTCAGGGTACTAGGGGGTAG-3′, 228–248); AP-6 (5′-CACAACTTGTCTTGAAATTAAG-3′, 1205–1084) (nucleotides providing synthetic restriction sites are in lower case).

For sequence analysis (Sanger et al. 1977) of AP-1/AP-2 PCR products, six independent reactions were pooled and products were purified by spin dialysis using Centricon 100 columns (Amicon). Amplified DNA (0.25 pmol) was sequenced directly with sequencing primer SP-1 or SP-2 (10 pmol) (Fig. 1) as described by Casanova et al. (1990), using a labelling time of 60 s at 25°C. PCR products generated with other primer combinations were sequenced using amplification primers and sequencing primer SP-3 (5′-CAC GATGAATGCGTTCATGG-3′, 617–598). A USB Sequenase kit version 2.0 was used together with [α-^{35}S]dATP.

Dot blot analysis

AP-1/AP-2 PCR products of M. L., family members and unrelated male controls were spotted onto Hybond N^{+} (Amersham) using a Minifold I (Schleicher and Schüll). Blots were hybridised to 5′end-labelled (Sambrook et al. 1989) allele-specific oligonucleotide (ASO) probes corresponding either to the wildtype (WT) or mutant (M) sequence at nucleotide position 736. The oligonucleotides were designed to detect a C:A mismatch in each case. The sequence of WT-ASO (position see Fig. 1) was 5′-TCCTGGAAGAATGGC CATT-3′; the sequence of M-ASO was 5′-AATGGCCATCCT TCCAGGA-.3′. Hybridisation was for 12 h at 50°C in 10 ml of 4 × SSPE (1 × SSPE is 180 m*M* NaCl, 10 m*M* sodium phosphate buffer, pH 7.4, 1 m*M* EDTA), 7% SDS, using 2 pmol ASO probe (specific activity 1.5×10^6 dpm/pmol). Post-hybridisation washes were for 2 × 20 min at room temperature, 10 s at 53°C, and 5 min at room temperature in 5 × SSPE, 1% SDS. Blots were exposed to Fuji RX film for 30 min–14 h.

Expression of SRY and immunoblotting

For expression of SRY wildtype and SRY^{F109S} mutant protein in bacteria, DNA encoding the SRY open reading frame was amplified by PCR from genomic DNA and cloned into the bacterial expression vector pJLA503 (Schauder et al. 1987) as described (Harley et al. 1992). To confirm the presence of the F109S mutation and to exclude possible errors introduced during PCR, the entire SRY coding region was sequenced. Cytoplasmic extracts from the SRY^{F109S} mutant, wildtype SRY and control plasmid pJLA503 were prepared from induced cultures, glycerol was added to 10%, and aliquots were stored at −70°C (van de Wetering et al. 1991).

For immunoblotting (Harlow and Lane 1988), extracts were subjected to 12% SDS-polyacrylamide gel electrophoresis. The polypeptide Ser-Ser-Pro-Gln-Gln-Arg-Asp-Arg-Tyr-Ser-His-Trp-Thr-Lys-Leu, corresponding to amino acid position 190 to the C-terminus of human SRY, was synthesised. Rabbit serum against this peptide was diluted 1:200 in phosphate-buffered saline/0.05% Tween 20, and blots were probed overnight at 4°C. Detection of anti-SRY antibodies was accomplished by a 2-h incubation of the immunoblot with horseradish-peroxidase-conjugated second antibody (Dako).

Gel retardation assay

The DNA probe used for gel retardation analysis was the 29-bp sequence containing the TCF-1 target site motif, AACAAAG, found 94 nucleotides upstream of the initiating ATG of human SRY (Harley et al. 1992). The bandshift reaction and gel electrophoresis conditions were as previously described (Harley et al. 1992), except that 1–8 μl bacterial extract (5–40 ng protein), 2 μg poly (dcI-dC), and 100 ng sonicated salmon sperm DNA were used.

Results

Using oligonucleotide primers flanking the HMG box in the SRY gene, genomic DNA from XY female M. L. and from a male control was amplified by PCR; the resulting PCR fragments were directly sequenced using two internal sequencing primers as indicated in Fig. 1. In this way, a point mutation was identified in DNA from M. L., viz. a T to C transition at position 736, leading to the substitution of a serine (S) residue for the phenylalanine (F) residue at amino acid position 109 (Figs. 1, 2). This F109S exchange is non-conservative, replacing the non-polar aromatic amino acid phenylalanine with the polar aliphatic serine residue. In comparison with other HMG box motifs, an aromatic amino acid, either phenylalanine, tyrosine or tryptophan, is found at the

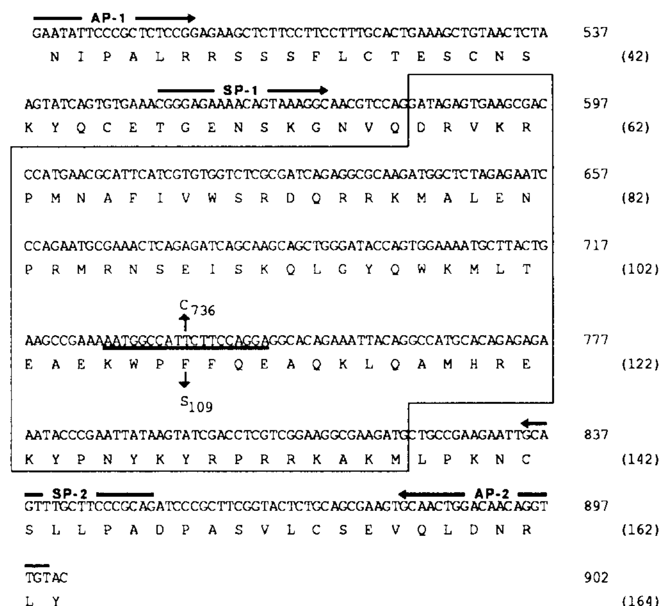

Fig. 1. Nucleotide and corresponding amino acid sequence of part of the open reading frame of the human SRY gene. Numbering of nucleotide and amino acid (single-letter code) positions (in *brackets*) is according to Sinclair et al. (1990) and Harley et al. (1992). *Arrows* labelled *AP-1*, *AP-2*, and *SP-1*, *SP-2* give the positions of the amplification and sequencing primers, respectively, used in PCR and direct sequencing. The HMG box motif comprising 80 amino acids is *framed*. The T to C transition in XY female M. L. at position 736 (Fig. 2) and the resulting substitution of serine (S) for phenylalanine (F) at position 109 are indicated. The *underlined* sequence was used for the design of ASOs

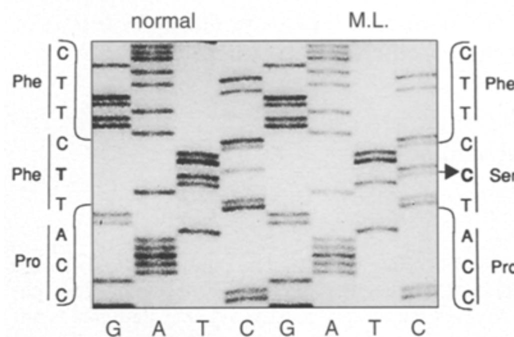

Fig. 2. Point mutation in the HMG box region of SRY in XY female M. L. Sequence autoradiograph obtained by direct sequencing of PCR products from DNA of a normal male control *(left)* and of XY female M. L. *(right)* using sequencing primer SP-1 (Fig. 1). *G*, *A*, *T*, and *C* indicate the sequencing lanes. The transition from T to C at position 736 is marked by an *arrow*, resulting in a substitution of serine (Ser) for phenylalanine (Phe)

corresponding position in almost all cases (Fig. 3). No further mutation was found in the SRY gene of M. L. when additional primers were used to amplify and sequence the remaining part of the open reading frame.

Sequence analysis and dot blot analysis using oligonucleotides specific for the wildtype and the mutant sequence at position 736 indicated that all accessible male relatives of M. L. were carriers of the same F109S mutation: her two living brothers, W. L. (IV-4 in Fig. 4) and H. L. (IV-5), her father G. L. (III-3), and her paternal

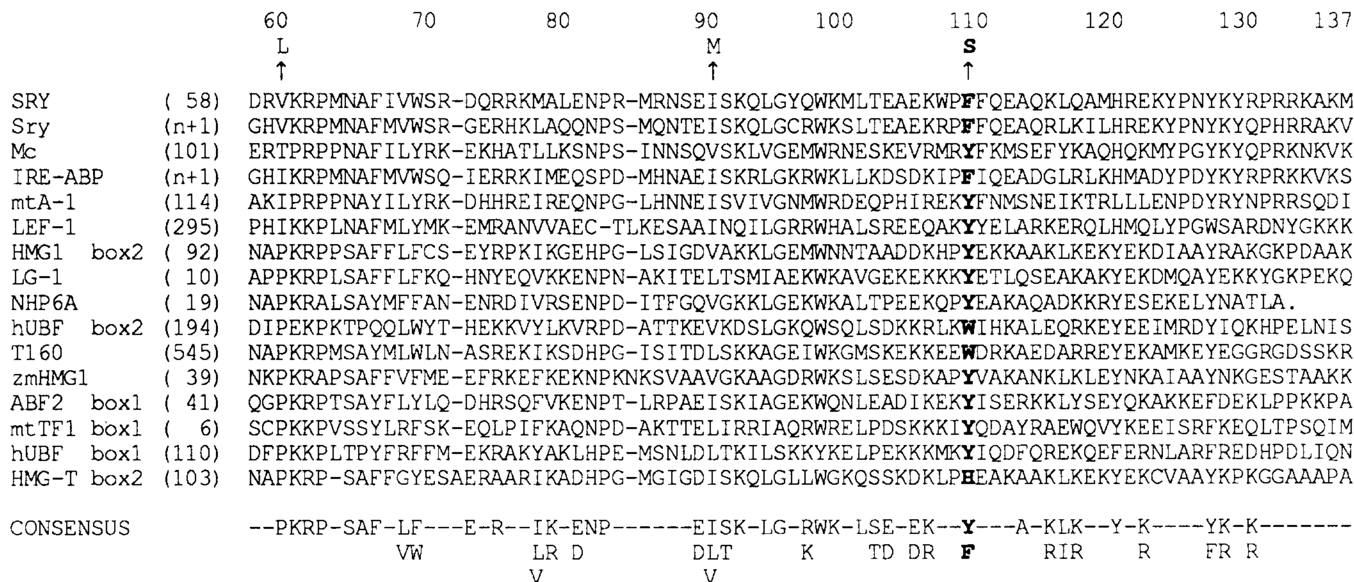

Fig. 3. The amino acid substitution in the HMG box motif of SRY observed in XY female M. L. and her XY male relatives occurs at a highly conserved residue. Amino acid sequences (single-letter code) of some members of the HMG box family are compared. Amino acid positions are indicated at the *beginning* of each *line*, and *above* the human SRY sequence. *Dashes* are introduced for optimal alignment. The F109S substitution found in XY female M. L. is indicated, homologous residues in other sequences are in *boldface*. V60L and I90M are familial SRY mutations identified in other pedigrees (Berta et al. 1990; Harley et al. 1992; Vilain et al. 1992). A consensus sequence shows identical or conserved amino acids in at least eight of the listed sequences. Conserved amino acids are grouped as follows: Y/F/W; I/V/L; S/T; K/R and D/E. Proteins aligned are: human SRY (Sinclair et al. 1990); mouse Sry (Gubbay et al. 1990); *S. pombe* Mc; rat IRE-ABP; *N. crassa* mt A-1; mouse LEF-1; pig HMG1; *Tetrahymena* LG-1; *S. cerevisiae* NHP6A; human hUBF; mouse T160; maize zmHMG1; *S. cerevisiae* ABF2; human mtTF1 [see Ner (1992) for references]; trout HMG-T (Pentecost et al. 1985)

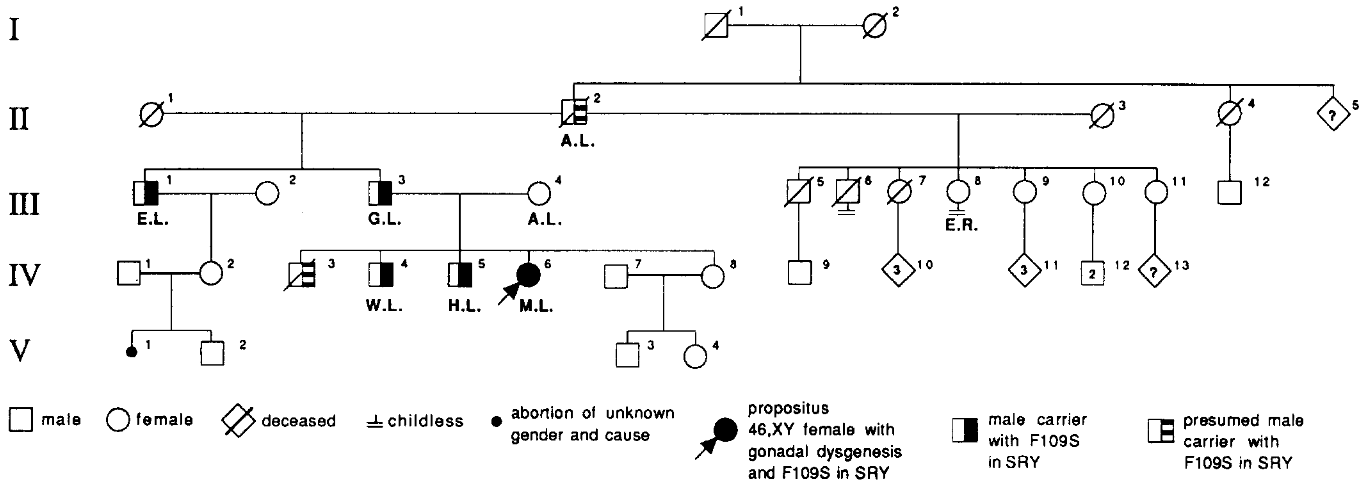

Fig. 4. Pedigree of family L. The index case, XY female M. L. (IV-6), is identified by an *arrow*. Males known or suspected to have the same F109S mutation as M. L. are symbolized by the *half-filled* or *half-stippled squares*, respectively. Female II-1 is A.L.'s second wife. Individuals appearing with their initials have been investigated or are discussed

uncle E.L. (III-1). By inference, her deceased brother (IV-3) and her paternal grandfather A.L. (II-2) also carried the same mutation. A sister (IV-8) and a first cousin (IV-2) are fertile females. We have investigated the possibility that one daughter from A.L.'s first marriage, E.R. (III-8 in Fig. 4), who remained childless throughout her life, is an undetected XY female. However, she has a normal female karyotype, and SRY sequences were not detectable by PCR.

To determine whether the F109S mutation observed in family L. may be a trivial polymorphism, we tested a total of 176 unrelated male controls by dot blot analysis. None of the male control DNA samples gave a hybridisation signal with the oligonucleotide specific for the mutant sequence at position 736 (data not shown).

Recombinant wildtype SRY protein produced in bacteria binds to the sequence AACAAAG or TTCAAAG, whereas mutant SRY protein carrying amino acid substitutions found in XY females fails to bind, or binds with reduced activity (Nasrin et al. 1991; Harley et al. 1992). We cloned and expressed the SRY open reading frame of M. L., and of a normal male control, in the expression vector pJA503 (Schauder et al. 1987). The SRY^{F109S} mutant protein appeared, like wildtype SRY, as a 26-kDa band following SDS-polyacrylamide gel electrophoresis and immunoblotting; this band was absent in the pJLA503 control plasmid extract (Fig. 5, upper panel). Levels of mutant and wildtype SRY, as judged from twofold dilutions of bacterial extracts, were comparable. We tested for binding of SRY to an oligonucleotide containing the target sequence AACAAAG. Radiolabelled double-stranded oligonucleotides were incubated with the extracts, and the resulting complexes were analysed in a gel retardation assay. Both wildtype SRY and SRY^{F109S} protein formed complexes with the

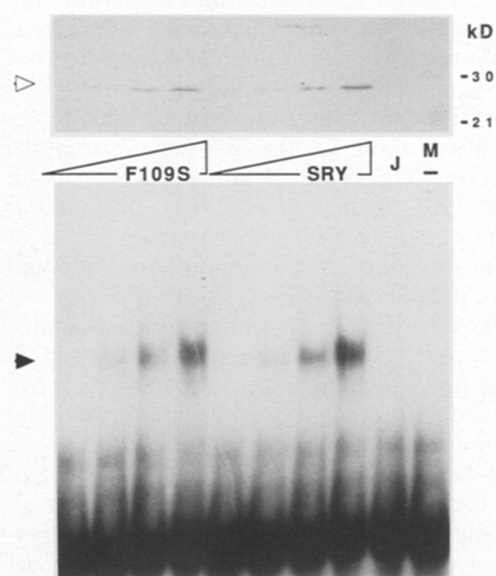

Fig. 5. DNA-binding activity of F109S mutant. Expression and DNA-binding activity of SRY^{F109S} mutant and normal SRY recombinant proteins. *Upper panel* An immunoblot stained with anti-SRY serum. Increasing volumes of bacterial extracts (1 µl, 2 µl, 4 µl, 8 µl) were analysed by immunoblot following SDS-polyacrylamide gel electrophoresis. *Lane J* shows 8 µl control pJLA extract. *Lane M* shows rainbow molecular weight markers (Sigma). *Open arrow* indicates the position of SRY. *Lower panel* Gel retardation analysis of the oligonucleotide probe containing the TCF-1 motif AACAAAG by mutant and normal SRY. Lane assignment and volumes of bacterial extracts are as shown in the *upper panel*, except for lane "–" which contains no extract. *Closed arrow* indicates the position of the SRY-DNA complex

probe and no complex was apparent in the control pJLA503 extract (Fig. 5, lower panel). Thus, the F109S mutation does not appear to affect DNA-binding activity under our standard conditions.

We have previously observed that SRY-DNA complex formation has reached equilibrium by 5 min (V. Harley, unpublished results). This equilibrium is not disturbed for the F109S mutant, even under conditions where the probe is no longer in excess, suggesting that the "on" rate of the mutant protein for DNA is normal (results

not shown). To assess whether SRY^{F109S} is thermo-labile, incubations were carried out at either 25°C, 37°C, 50°C, or 55°C prior to electophoresis run at room temperature. Above 37°C, SRY complex formation was reduced in SRY^{F109S} to the same extent as wildtype SRY (data not shown).

Discussion

We have identified a patient, M.L., with XY gonadal dysgenesis who has a point mutation in the HMG box region of the SRY gene, viz. a C to T transition at nucleotide position 736, resulting in the substitution of serine for phenylalanine at amino acid positon 109. The same F109S exchange in SRY was present in four living and, by inference, in two deceased XY male relatives of M. L. Thus, one out of seven carriers of the F109S mutation shows sex reversal. The male individual IV-9 (Fig. 4) could be an additional carrier of the F109S mutation but is unavailable to us. The F109S mutation is not a polymorphism at it was not found in 176 unrelated males studied by us, nor in about 50 other XY females analysed for SRY mutations (Jäger et al. 1990b; Berta et al. 1990; Hawkins et al. 1992; Pivnick et al. 1992). In addition, sequencing the complete SRY open reading frame of M. L. ruled out a second, de novo mutation that could account for her phenotype.

In the F109S substitution, an aromatic phenylalanine residue is replaced by a non-aromatic serine residue. This particular phenylalanine residue resides at a position where an aromatic residue, phenylalanine, tyrosine, or tryptophan, is typically found in other HMG box motifs (Fig. 3; Ner 1992). The sequence of one out of several trout testis HMG-T proteins (Pentecost et al. 1985) has a histidine at this position (Fig. 3), a conservative substitution as histidine can be grouped with the aromatic residues. The second exception is yeast ACP2 with glutamine and glycine at the corresponding positions in the first and second HMG boxes, respectively (Haggren and Kolodrubetz 1988). Diffley and Stillman (1991) have noticed homology of HMG box motifs with a conserved element in the heat shock protein hsp70 family. The phenylalanine residue is present at the homologous position even in such distantly related proteins. The high degree of evolutionary conservation suggests an important structural function for the phenylalanine residue at position 109 in the HMG box of SRY, as opposed to a role in sequence-specific DNA binding. We note that the F109S exchange leads to interrution of a predicted (Garnier et al. 1978) alpha helical stretch from amino acid 99 to 128 in SRY, a structural feature consistently predicted at corresponding positions in other HMG box motifs (Shirakata et al. 1991; van de Wetering and Clevers 1992).

The presence of DNA-binding activity in the SRY^{F109S} mutant protein is consistent with the incomplete penetrance of this mutation. We did not, however, expect apparently normal activity given the nature of the amino acid substitution and in view of our previous observation of reduced or negligible binding of SRY mutant protein from two other familial cases (Harley et al. 1992). Furthermore, our results contrast with those of Giese et al. (1991) who have constructed a tyrosine to serine mutation at the corresponding position in the HMG box of LEF-1 (Fig. 3), and reported a 10-fold reduction in DNA-binding activity of this mutant. At present, we do not know the in vivo target sequence of SRY. It is therefore possible that the oligonucleotide containing the TCF-1 target site motif, AACAAAG, used in our assay is inappropriate for SRY. However, we find this unlikely given that the sequence AACAAAG is a high affinity site, commonly selected by SRY when presented with a pool of random oligonucleotides (V. Harley, unpublished results). Although SRY^{F109S} shows normal DNA-binding activity in vitro, the DNA sequence specificity might be slightly altered in vivo so that its true in vivo target sequence is less favoured. Such an alteration might be a consequence of a conformational change and/or of the ability of SRY^{F109S} to interact with some crucial cofactor(s). Alternatively, the DNA bending capability recently described for wildtype SRY (Giese et al. 1992) might be altered in SRY^{F109S}.

Vilain et al. (1992) described a three-generation family with the XY individuals carrying the same V60L substitution in SRY (Berta et al. 1990), of which three were sex-reversed females. The SRY^{V60L} mutant protein showed negligible DNA-binding (Nasrin et al. 1991; Harley et al. 1992). As in the case of the V60L mutation, several hypotheses can be put forward to explain the incomplete penetrance of the F109S mutation. First, SRY^{F109S} could be a rare neutral variant, XY female M.L. carrying a de novo mutation at an autosomal or X-linked sex reversing gene, or within a regulatory sequence of the SRY gene. Secondly, the in vivo activity of SRY^{F109S} could be around a threshold level, the critical amount present depending on physiological or environmental conditions. One such parameter, temperature, has been tested in vitro, with SRY^{F109S} being no more thermolabile than wildtype SRY. Finally, the functional status of SRY^{F109S} may depend on the genetic background, generating two sexual phenotypes by interaction with two different alleles of a second non-Y-linked locus. This could be a target sequence for SRY in a gene downstream in the sex-determination pathway, or a protein cofactor that intertacts with SRY, as mentioned above. This has an analogy in the mouse, where the ability of some Tdy alleles to induce testis formation depends on particular alleles at autosomal loci (Eicher and Washburn 1986).

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