In conclusion, a *de novo* mutation in the gene *SRY* is associated with sex reversal in an XY female. This provides compelling evidence that *SRY* is required for testis formation and male sex determination. □

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Expression of a candidate sex-determining gene during mouse testis differentiation

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THE development of a eutherian mammal as a male is a consequence of testis formation in the embryo, which is thought to be initiated by a gene on the Y chromosome. In the absence of this gene, ovaries are formed and female characteristics develop[1]. Sex determination therefore hinges on the action of this testis-determining gene, known as *Tdy* in mice and *TDF* in humans[2,3]. In the past, several genes proposed as candidates for *Tdy/TDF* have subsequently been dismissed on the grounds of inappropriate location or expression[4–9]. We have recently described a candidate for *Tdy*, which maps to the minimum sex-determining region of the mouse Y chromosome[10,11]. To examine further the involvement of this gene, *Sry*, in testis development, we have studied its expression in detail. Fetal expression of *Sry* is limited to the period in which testes begin to form. This expression is confined to gonadal tissue and does not require the presence of germ cells. Our observations strongly support a primary role for *Sry* in mouse sex determination.

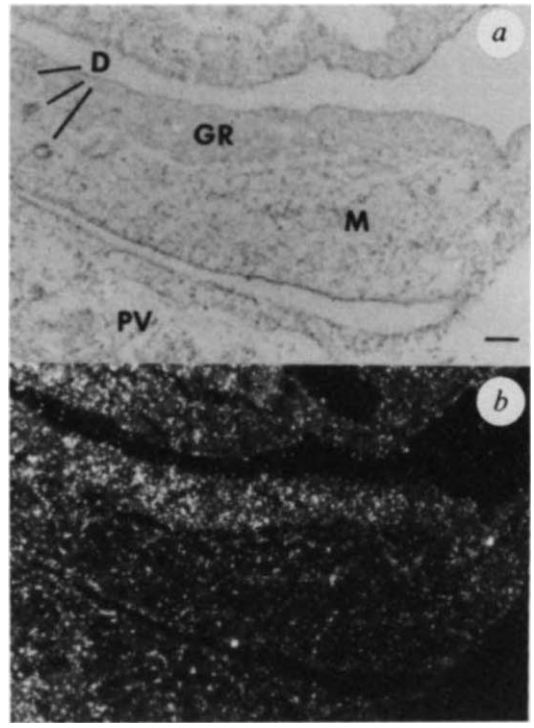


FIG. 2 *In situ* hybridization to a sagittal section of an 11.5-d.p.c. male embryo, using an anti-sense RNA probe for *Sry*. *a*, Bright-field illumination. *b*, Dark-field illumination. GR, genital ridge; M, mesonephros; D, mesonephric and paramesonephric ducts; PV, prevertebrae. Scale bar, 50 μm.
METHODS. *In situ* hybridization was carried out as described[22]. Embryos were sexed by staining for sex chromatin in amnion cells[23]. [^{35}S]UTP-labelled anti-sense RNA probe, corresponding to the 374-bp *Bgl*II-*Pst* I fragment of p. 4.2.2. (ref.10) was used. This probe showed no specific labelling to sections of female embryos, and control sense RNA probes did not show a specific signal on male and female sections (data not shown). Autoradiographic exposure was at 4 °C for 6 days.

Male and female mouse embryos are morphologically indistinguishable until about 11.5 days post coitum (d.p.c.). The first visible sign of male development, the formation of testes from the 'indifferent' genital ridges, occurs within 24 hours, with the alignment of Sertoli cells into cords. Any candidate for *Tdy* should therefore be expressed in male genital ridge at or before this stage of development. Our initial observations suggested this to be the case for *Sry* (ref. 10). We wished to determine whether *Sry* transcription in the genital ridge is part of a broader temporal or spatial profile of expression suggesting a general role, or whether it correlates more specifically with testis

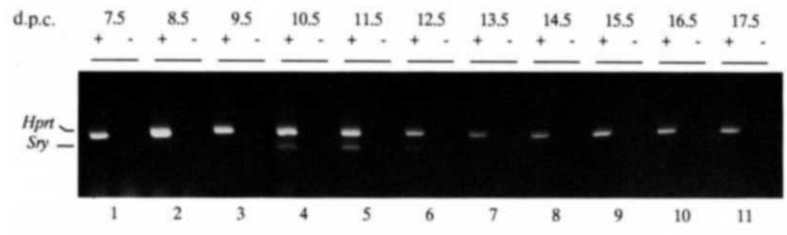


FIG. 1 Time course of fetal *Sry* expression. RNA from mouse embryos of various stages was added to a 'reverse transcription' reaction in the presence (+) or absence (−) of reverse transcriptase (RT). Subsequent PCR reactions contained oligonucleotide primers for hypoxanthine phosphoribosyltransferase (*Hprt*) and *Sry*. As expected, the 352-base-pair (bp) control *Hprt* band was seen in all +RT samples. The 266-bp band corresponding to *Sry* was only seen in 10.5–12.5-d.p.c. samples. As the *Sry* primers are capable of amplifying genomic DNA sequences, the absence of bands from all −RT samples confirms that any signal is due to *Sry* transcripts and not to DNA contamination.
METHODS. Small-scale RNA preparations were made, reverse transcribed and amplified as previously described[9,10], with the addition of Perfect Match (Stratagene) to PCR reactions. *Sry* primers 5′–3′ were GAGAGC ATGGAG GGCCAT and CCACTC CTCTGT GACACT. Annealing was at 53 °C. Samples, divided into + and − RT fractions, were from: 1, twelve whole embryos at 7.5 d.p.c.; 2, ten whole embryos at 8.5 d.p.c.; 3, 0.1 μg of poly(A)$^+$ RNA from pooled 9.5-d.p.c. embryos posterior to the forelimb-bud; 4, urogenital ridges from six 10.5-d.p.c. embryos; 5, urogenital ridges from a single 11.5-d.p.c. embryo; 6, single 12.5-d.p.c. testis; 7–11, 30, 25, 20, 15 and 12% of single testes from 13.5–17.5-d.p.c. fetuses, respectively. Parkes outbred mice were used unless otherwise stated.

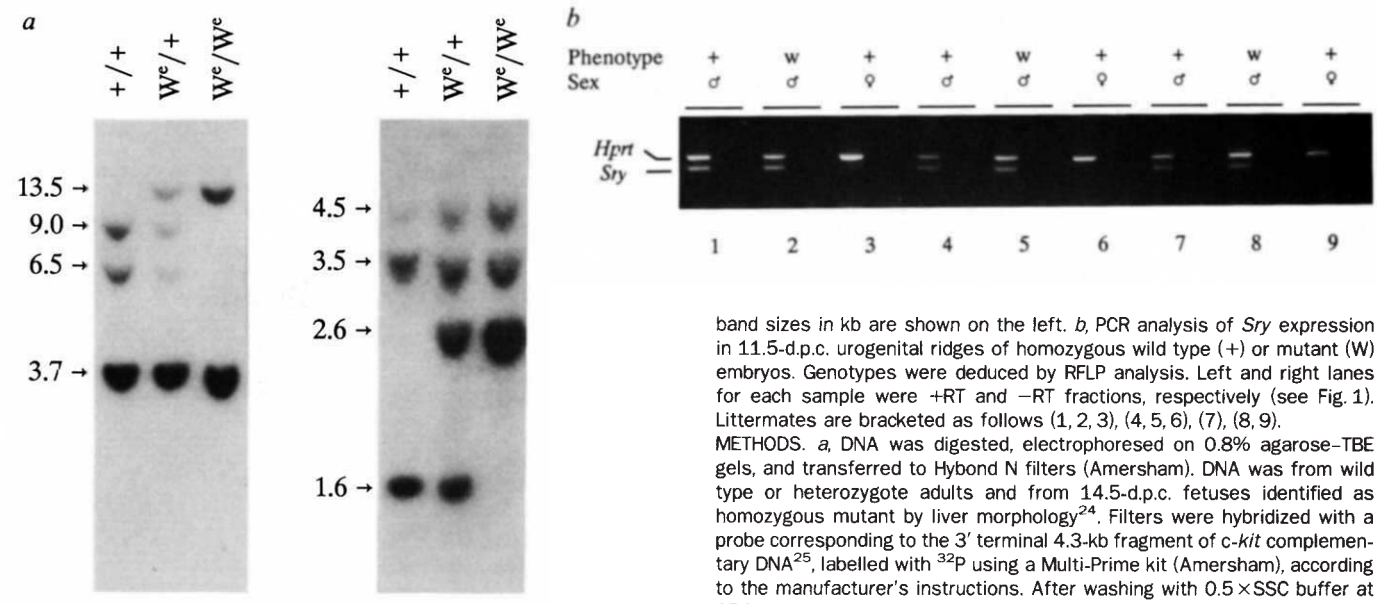


FIG. 3 Analysis of W^e embryos. *a*, Restriction fragment length polymorphism (RFLP) seen on Southern blots of DNA digested with *Bgl*II (left panel) or *Xba*I and *Pst*I (right panel) using a probe for c-*kit* (refs 16, 17); approximate band sizes in kb are shown on the left. *b*, PCR analysis of *Sry* expression in 11.5-d.p.c. urogenital ridges of homozygous wild type (+) or mutant (W) embryos. Genotypes were deduced by RFLP analysis. Left and right lanes for each sample were +RT and −RT fractions, respectively (see Fig. 1). Littermates are bracketed as follows (1, 2, 3), (4, 5, 6), (7), (8, 9).

METHODS. *a*, DNA was digested, electrophoresed on 0.8% agarose–TBE gels, and transferred to Hybond N filters (Amersham). DNA was from wild type or heterozygote adults and from 14.5-d.p.c. fetuses identified as homozygous mutant by liver morphology[24]. Filters were hybridized with a probe corresponding to the 3′ terminal 4.3-kb fragment of c-*kit* complementary DNA[25], labelled with ^{32}P using a Multi-Prime kit (Amersham), according to the manufacturer's instructions. After washing with 0.5 × SSC buffer at 65 °C, filters were autoradiographed for 16 h. Band sizes were estimated using λ–*Hin*dIII markers. *b*, Urogenital ridge RNA from individual progeny of C3H/H-101 $W^e/+$ matings was divided into +RT and −RT fractions and processed as described in Fig. 1 legend.

differentiation. In particular, we wished to determine whether *Sry* expression in the genital ridge is due to somatic or to germ cells, as it is known that the latter are not required for testis development[12,13].

RNA was extracted from mouse embryos and analysed for the presence of *Sry* transcripts by reverse transcription and polymerase chain reaction (PCR). No *Sry* expression was detected in 7.5-, 8.5- and 9.5-d.p.c. embryos, before genital ridge formation. Transcripts were first detected in the genital ridge at 10.5 d.p.c., were present at similar levels in the 11.5-d.p.c. urogenital ridge, and were less abundant in 12.5-d.p.c. testis (Fig. 1). *Sry* transcripts were not detected in testes from 13.5 to 17.5 d.p.c. Therefore, *Sry* expression corresponded precisely with the onset of testis differentiation, with a window of about one day either side.

In situ hybridization was used to examine the distribution of *Sry* transcripts in 11.5-d.p.c. embryos. Although the level of expression was low, the hybridization signal was clearly associated with the genital rather than the adjacent mesonephric component of the urogenital ridge (Fig. 2). The homogeneity of the signal over the genital ridge indicates that the cells expressing *Sry* represent a large population, not confined to any specific region of the genital ridge, and not organized into specific structures. It was not possible to detect hybridization

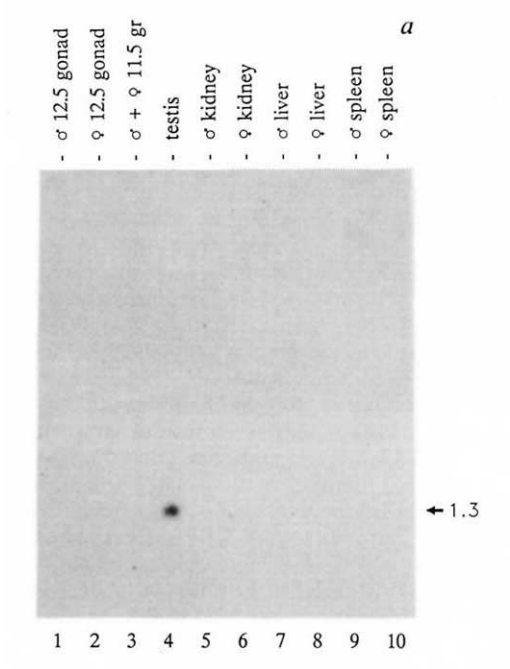
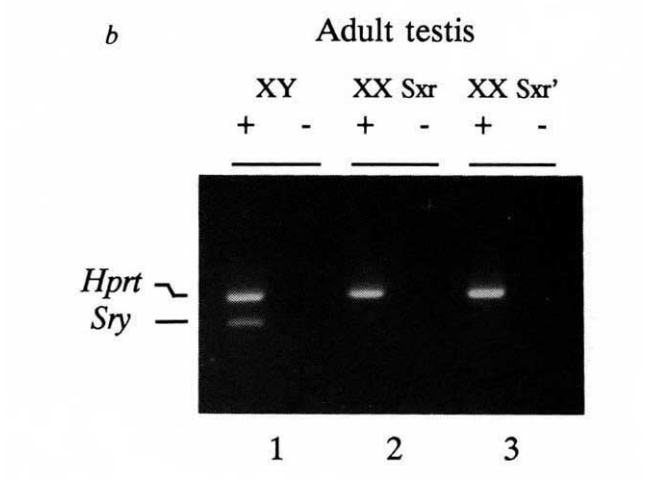


FIG. 4 Analysis of *Sry* expression in fetal and adult tissues. *a*, Northern blot analysis revealed a 1.3-kb transcript in adult XY testis RNA (lane 4), but not in other adult tissues or in 11.5-d.p.c. urogential ridge (lane 3), 12.5-d.p.c. male (or female) gonads (lanes 1 and 2), or other adult tissues (lanes 5–10).



Size (kb) on the right. *b*, PCR analysis of adult testis RNA. The 266-bp band corresponding to *Sry* transcripts was seen in the normal XY testis sample (1) but not XX Sxr or XX Sxr′ samples (2, 3), whereas the control *Hprt* band was seen in all samples. Reverse transcriptase was included (+) or omitted (−) as described in Fig. 1.

METHODS. *a*, 20 μg of total RNA extracted from pools of fetal or adult tissues was loaded in each lane, electrophoresed, transferred to Gene-Screen (Dupont), and probed with an RNA probe labelled with ^{32}P to a specific activity of 1×10^9 c.p.m. μg^{-1}. After hybridization, the filter was treated with RNase to reduce non-specific background, then exposed for 3 days. *b*, Total RNA (0.2 μg) was divided into +RT and −RT fractions and processed as described in Fig. 1 legend.

above background in 12.5-d.p.c. testes, after the formation of testis cords. The distribution of *Sry* transcripts in fetal tissues at 11.5 d.p.c. was further examined by PCR. When RNA was extracted from urogenital ridge, head, viscera and carcass fractions, there was no evidence for expression in any tissue other than urogenital ridge.

In mouse fetuses homozygous for mutations at the dominant white spotting (*W*) locus, testes form normally despite a lack of germ cells[12,14]. If *Sry* is to be considered a candidate for *Tdy*, it ought to be expressed in W/W as well as in wild-type genital ridges. We were able to identify embryos homozygous for the W^e allele[15] by virtue of a restriction fragment length polymorphism detected using a probe for c-*kit*[16,17] (Fig. 3*a*). In PCR analyses of *Sry* expression in genital ridge at 11.5 d.p.c., W^e/W^e fetuses proved indistinguishable from wild type (Fig. 3*b*).

We were unable to detect the *Sry* transcripts present in 11.5-d.p.c. genital ridge or 12.5-d.p.c. testis by northern blotting, presumably because of the low level of expression. Northern analysis of adult testis RNA revealed *Sry* transcripts of a similar size to those seen in adult human testis (Fig. 4*a*). In mouse testis, the transcripts are about 1.3 kilobases (kb) and run as a tight doublet under some conditions. To investigate whether the expression seen in adult testis was somatic, we used PCR to analyse RNA from XX Sxr and XX Sxr′ adult mouse testes, which lack germ cells[18,19]. In contrast to the situation in fetal gonads, *Sry* expression in adult testis is dependent on germ cells (Fig. 4*b*). We are now addressing the question of whether this expression is a property of germ cells themselves, or a result of somatic–germ cell interaction. It is also unclear whether the adult and fetal transcripts are physically or functionally distinct. A number of genes suspected of having a role in directing developmental events in the embryo show reactivation in adult testis, but in no case has their function there been defined[20].

Sry has been shown to meet several criteria that can be applied to any candidate for *Tdy*. It maps to the appropriate area of the Y chromosome, is conserved in a wide range of mammals, is deleted in a line of XY female mice known to be mutant in *Tdy*, and encodes a product consistent with a regulatory role[10,11,21]. The present results show that *Sry* follows a tightly controlled spatial and temporal profile of expression that correlates well with testis differentiation. As this did not depend on the presence of germ cells, we conclude that *Sry* is expressed in one of the somatic cell lineages present in the developing gonad, as must be the case if *Sry* is to be considered a candidate for *Tdy*. The rapid cessation of *Sry* transcription after testis cord formation suggests that *Sry* initiates a cascade of gene expression, but is not required for the maintenance of gene activity in the developing testis. It will be interesting to determine the nature of the genes involved in subsequent steps of the pathway. □

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A human XY female with a frame shift mutation in the candidate testis-determining gene *SRY*

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THE primary decision about male or female sexual development of the human embryo depends on the presence of the Y chromosome[1,2], more specifically on a gene on the Y chromosome encoding a testis-determining factor, TDF. The human sex-determining region has been delimited to a 35-kilobase interval near the Y pseudoautosomal boundary[3]. In this region there is a candidate gene for TDF, termed *SRY*, which is conserved and specific to the Y chromosome in all mammals tested[3]. The corresponding gene from the mouse Y chromosome is deleted in a line of XY female mutant mice, and is expressed at the expected stage during male gonadal development[4]. We have now identified a mutation in *SRY* in one out of 12 sex-inversed XY females with gonadal dysgenesis who do not lack large segments of the short arm of the Y chromosome[5–8]. The four-nucleotide deletion occurs in a sequence of *SRY* encoding a conserved DNA-binding motif and results in a frame shift presumably leading to a non-functional protein. The mutation occurred *de novo*, because the father of the sporadic XY female that bears it has the normal sequence at the corresponding position. These results provide strong evidence for SRY being TDF.

We have characterized a sample of XY females with gonadal dysgenesis for the presence or absence of sequences from interval 1 to 7 of the Y chromosome, including the former TDF candidate gene *ZFY*[9] (our unpublished data). We selected 10 sporadic and two sib cases to study from this sample, because they did not have deletions in the short arm of the Y chromosome[5–8], nor were they familial cases from pedigrees indicating X-linked recessive or male-limited autosomal recessive inheritance[10]. These XY females were therefore suited for analysis of mutations in *SRY*.

The sequences of the human *SRY* gene that have been published are from an exon whose limits are not so far precisely known[3,4]. The open reading frame in this sequence is predicted to encode a sequence with an 80-residue stretch that is characteristic of a DNA-binding motif known as the HMG box (boxed in Fig. 1). This motif, first recognized by comparison of the human transcription activator protein UBF with the high-mobility-group protein HMG1[11], is conserved in other or suspected DNA-binding proteins[3,4,11]. Using oligonucleotide primers flanking the HMG box in the *SRY* sequence, genomic DNA from the 12 XY females and controls was amplified by the polymerase chain reaction (PCR), and the resulting PCR fragments were directly sequenced using two internal sequencing primers as indicated in Fig. 1. In this way, the entire sequence of the 422-base-pair (bp) PCR products could be determined.

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