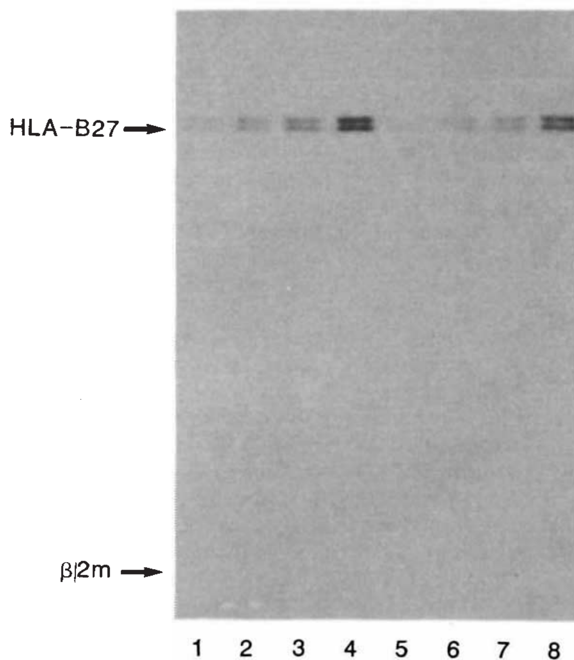

FIG. 4 *In vitro* translated HLA-B27 heavy chain assembles with endogenous microsomal β2m. Messenger RNA (50 ng) for HLA-B27 heavy chain was translated in the presence of Raji microsomes (3 μl) at different concentrations of NP 384-394 peptide; lanes 1 and 5, absence of peptide; lane 2 and 6, 1 μM; lanes 3 and 7, 4 μM; lanes 4 and 8, 16 μM. Immunoprecipitations were carried out with monoclonal antibody W6/32 (lanes 1-4), and BBM.1 (lanes 5-8). *In vitro* translations were as described in the legend to Fig. 1.

amounts of HLA-B27 heavy chains when the concentration of NP 384-394 is increased (Fig. 4, lanes 1-4). Thus, we observe enhanced assembly, even without translation of β2m mRNA. The antibody BBM.1 confirms this result (Fig. 4, lanes 5-8); it is possible to immunoprecipitate the HLA-B27 heavy chain, and to observe enhanced assembly with peptide NP 384-394, with a monoclonal antibody directed against β2m.

Here, we have shown that a small peptide can stimulate assembly of HLA-B27 class I heavy chain with β2m. This occurs in the lumen of microsomal vesicles that mimic the ER compartment. Thus, it seems that assembly of MHC class I molecules can occur in this compartment. The increase of assembly is roughly 3-8-fold (when analysed by densitometry) over that observed in the absence of the specific peptide. Overexposure of the x-ray films indicates that some HLA-B27-β2m assembly occurs in the absence of the NP 384-394 peptide. This is probably due to stimulation either by endogenous peptides, or spontaneous association of the chains without peptides involved. Such 'empty' MHC class I antigens have recently been demonstrated[17,18].

Our results confirm the findings of Townsend and colleagues, who found that assembly and cell surface expression of murine H-2 antigens can be stimulated by specific peptides in the cell line RMA-S (ref. 9). This implies that our system, supplemented with human microsomes, probably resembles the *in vivo* assembly process. Assembly of MHC class I antigens can occur in the lysate of detergent-solubilized cells, and this process is dependent on both peptide and β2m concentrations[19]. In our system we were unable to detect assembly after solubilization (data not shown). The peptide concentrations used in this study are similar to those used by Townsend *et al.*[19]. As the different components of our system can easily be varied, it will be useful in elucidating the complicated nature of MHC class I antigen assembly. □

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Genetic evidence equating *SRY* and the testis-determining factor

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THE testis-determining factor gene (*TDF*) lies on the Y chromosome and is responsible for initiating male sex determination. *SRY* is a gene located in the sex-determining region of the human and mouse Y chromosomes and has many of the properties expected for *TDF*[1-3]. Sex reversal in XY females results from the failure of the testis determination or differentiation pathways. Some XY females, with gonadal dysgenesis, have lost the sex-determining region from the Y chromosome by terminal exchange between the sex chromosomes[4] or by other deletions[5]. If *SRY* is *TDF*, it would be predicted that some sex-reversed XY females, without Y chromosome deletions, will have suffered mutations in *SRY*. We have tested human XY females and normal XY males for alterations in *SRY* using the single-strand conformation polymorphism assay[6,7] and subsequent DNA sequencing. A *de novo* mutation was found in the *SRY* gene of one XY female: this mutation was not present in the patient's normal father and brother. A second variant was found in the *SRY* gene of another XY female, but in this case the normal father shared the same alteration. The variant in the second case may be fortuitously associated with, or predisposing towards sex reversal; the *de novo* mutation associated with sex reversal provides compelling evidence that *SRY* is required for male sex determination.

SRY sequences were amplified by the polymerase chain reaction from DNA of XY females, as well as normal male controls. The amplified products were cleaved with restriction enzymes

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and run on non-denaturing polyacrylamide gels to detect single-strand conformational polymorphisms (SSCP) (Fig. 1). Three patterns were detected: one pattern common to all of 50 normal male controls and the majority of patient samples; a second pattern for XY female individual (A.A.) and a third pattern for XY female (J.N.). The normal father and brother of A.A. were tested and found to give the same pattern as the controls, implying that A.A. has suffered a *de novo* mutation. In contrast, DNA from father (N.) and his XY daughter (J.N.) showed the same pattern. The amplified products (609 base pairs) from both families were cloned and sequenced (Fig. 2*a*). As predicted by the SSCP assay, A.A. has a *de novo* mutation G→A that is not shared with her father and brother. For J.N., a G→C change was shared with her father. Other than these observed base changes, the XY females J.N. and A.A. had complete sequence identity to *SRY* over the amplified 609 base pairs (bp) (Fig. 2*a*). Paternity in both families was confirmed by Southern blotting with minisatellite probes[8,9].

The *de novo* mutation in A.A. causes a conservative change from methionine to isoleucine at a residue that lies within the putative DNA-binding motif of *SRY* and is identical in all *SRY* and *SRY*-related genes (Fig. 2*b*). The association of a *de novo* mutation with a new phenotype provides evidence that the phenotype and mutation are related, and by inference that *SRY* is required for male sex determination. Formally this does not exclude the possibility that other genes on the Y chromosome are required for sex determination, but previous work suggests that, if they exist, these genes must be located adjacent to the pseudoautosomal boundary, close to *SRY*[1,10].

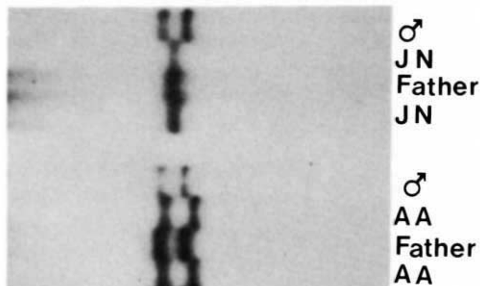

FIG. 1 SSCP analysis of *SRY* in XY females. On the left is family N: ♂, DNA from the normal 46XY male cell line PGF[12]; DNA from the father is flanked on each side by DNA of his XY daughter (J.N.). On the right is family A: again DNA from the father is flanked by that of his XY daughter (A.A.). In total, 11 XY females were tested by SSCP. These individuals: P.F., J.A., A.M., R.B., GM2598, A.S., M., A.A., I.D., J.N. and K.L. all have gonadal dysgenesis, are karyotypically normal and positive both for the Y-pseudoautosomal boundary and *ZFY*. A.A. and J.N. both have pure gonadal dysgenesis: A.A. has a streak gonad with ovarian stroma and no germ cells; J.N. has a cystic gonad with ovarian stroma and no germ cells. The fathers, A. and N. are both normal, fertile males. Fifty normal males were tested as controls and no variation detected.

METHODS. Polymerase chain reactions were performed with the primers XES7 and XES2 located within the *SRY* open reading frame, amplifying a 609-bp fragment. The primer sequences are: XES7, 5'CCCGAATTCGACAATGCAATCATATGCTTCTGC3'; XES2, 5'CTGTAGCGGTCCCGTTGCTGCGGTG3'. PCRs were performed with ~100 ng of genomic DNA, 200 μM each dNTP, 0.5 μM each primer, 1.5 mM $MgCl_2$, 10 mM Tris (pH 8.3), 50 mM KCl, 0.01% (w/v) gelatin, 0.25 U of Taq polymerase and 0.5 μl of [α-^{32}P]dCTP (3,000 Ci mmol^{-1}, 10 mCi ml^{-1}) in a volume of 10 μl. Reactions were cycled for 1.2 min at 94 °C, 1.2 min at 65 °C and 2 min at 72 °C for 35 cycles. One μl of the product was digested with *Hin*fI and *Taq*I in the presence of 4 mM spermidine hydrochloride in a 10 μl volume. The digested DNA was diluted 1:10 in 0.1% SDS, 10 mM EDTA, followed by a 1:2 dilution in 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol. Samples of 1–3 μl were heated at 80 °C for 5 min to denature the DNA, then loaded onto 6% acrylamide, 10% glycerol non-denaturing gels using a sequencing-gel apparatus. Electrophoresis was carried out at 25 mA, with a fan heater set on cold directed at the gel as a cooling device. Autoradiography of the dried gels was for 3 days without an intensifying screen.

The variant found in J.N. causes a conservative change from valine to leucine (Fig. 2*b*). This residue is conserved amongst *SRY* and *SRY*-related sequences with the exception of a mouse autosomal gene (autosomal-4), which has a conservative change to isoleucine at this position[2]. There are several possible explanations for this variant. First, the variant could cause conditional sex reversal depending on other genetic or environmental factors. A similar observation has been described in the mouse, where the ability of some alleles of *Tdy* to induce testis formation depends on the genetic background[11]. Second, the variant could be fortuitously found in a family segregating for an autosomal or X-linked sex reversing gene. Finally, the variant could cause sex reversal and the father is mosaic for wild-type and variant sequences.

The *SRY* gene of the majority of the XY females we have tested appears normal by the SSCP assay. It is possible that these individuals have mutations in *SRY* that are not detected by the assay we have used, either because they do not cause a band shift or because they fall outside the region tested. Alternatively, these individuals may have mutations in another part of the sex-determining pathway.

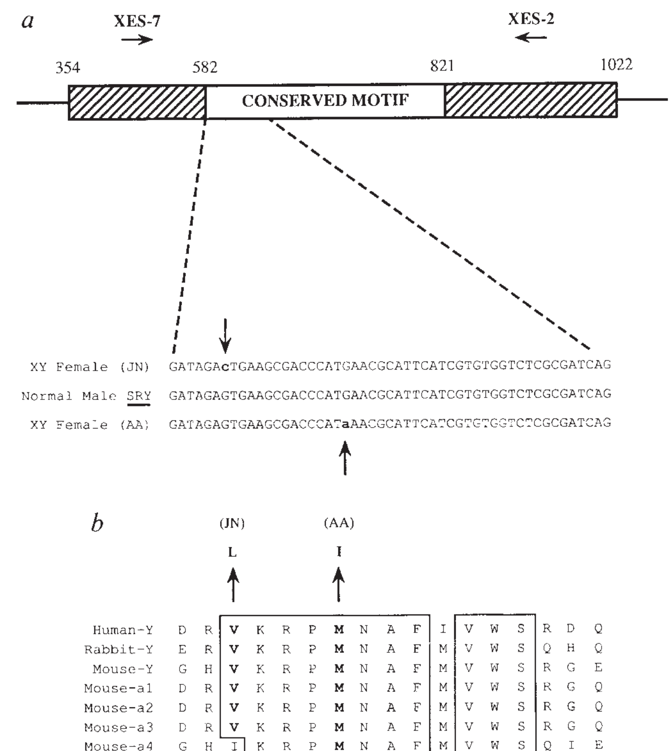

FIG. 2 *a*, The open reading frame of the genomic clone pY53.3 (*SRY*) is shown extending from 354–1,022 bp[1]. The conserved motif, which encodes a potential DNA-binding protein, extends from 582 bp to 821 bp[1]. The primers XES-7 and XES-2 were used to amplify a 609-bp region. The entire amplification product was sequenced, but only the small region containing the changes in the XY females is displayed. The dotted line indicates the location within the open reading frame of the nucleotide sequence shown. Top line, nucleotide sequence for XY female (J.N.) with base changes from G→C indicated by arrow. Middle line, normal male *SRY* nucleotide sequence. Bottom line, XY female (A.A.) with a base change from G→A indicated by arrow. *b*, The amino-acid sequence of *SRY* for the human-Y, rabbit-Y, mouse-Y and mouse autosomal *SRY*-like genes a_1, a_2, a_3, a_4[1,2]. Boxed shaded regions show identical amino acids conserved across these species. In the XY female J.N. the variant causes a change from valine to leucine, whereas in the XY female A.A. the mutation causes a change from methionine to isoleucine.

METHODS. PCR products were subcloned in pUC18 vectors (NEB). Six independent subclones from individuals (A.A.) and (J.N.) were sequenced on both strands. Double-stranded DNA was sequenced by the dideoxy chain termination method[13] using synthetic oligonucleotide primers and Sequenase (USB).

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.

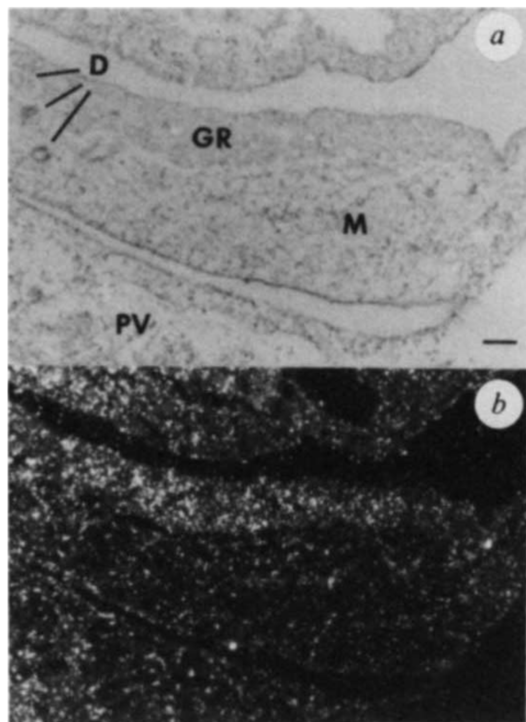

FIG. 2 *In situ* hybridization to a sagittal section of an 11.5-d.p.c. male embryo, using an anti-sense RNA probe for *Sry*. *a*, Bright-field illumination. *b*, Dark-field illumination. GR, genital ridge; M, mesonephros; D, mesonephric and paramesonephric ducts; PV, prevertebrae. Scale bar, 50 μm.
METHODS. *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

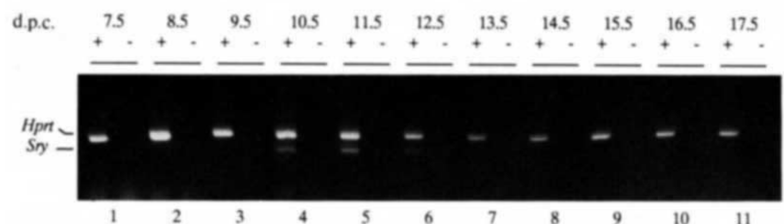

FIG. 1 Time course of fetal *Sry* expression. RNA from mouse embryos of various stages was added to a 'reverse transcription' reaction in the presence (+) or absence (−) of reverse transcriptase (RT). Subsequent PCR reactions contained oligonucleotide primers for hypoxanthine phosphoribosyltransferase (*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.