Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 1927–1931, March 1994
Genetics

An *SRY*-related sequence on the marsupial X chromosome: Implications for the evolution of the mammalian testis-determining gene

(sex chromosomes/sex determination/chromosome evolution)

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Communicated by Mary F. Lyon, November 8, 1993 (received for review September 27, 1993)

ABSTRACT **The *SRY* gene on the human, mouse, and marsupial Y chromosomes is the testis-determining gene that initiates male development in mammals. The SRY protein has a DNA-binding domain (high mobility group or HMG box) similar to those found in the high-mobility-group proteins. SRY is specific for the Y chromosome, but many autosomal genes have been identified that possess a similar HMG box region; those with the most closely *SRY*-related box regions form a gene family now referred to as *SOX* genes. We have identified a sequence on the marsupial X chromosome that shares homology with *SRY*. Sequence comparisons show near-identity with the mouse and human *SOX3* gene (formerly called *a3*), the *SOX* gene which is the most closely related to *SRY*. We suggest here that the highly conserved X chromosome-linked *SOX3* represents the ancestral *SOX* gene from which the sex-determining gene *SRY* was derived. In this model *SOX3/SRY* divergence and the acquisition of a testis-determining role by *SRY* might have preceded (and initiated) sex chromosome differentiation or, alternatively, might have been a consequence of X chromosome–Y chromosome differentiation initiated at the locus of an original sex-determining gene(s), later superseded by *SRY*.**

Mammals have an XX female/XY male sex chromosome mechanism, in which a gene on the Y chromosome determines testis development, the first step in the male developmental pathway. *SRY* has been cloned from the sex-determining region of the human Y chromosome (1), and evidence from mutational analysis (2, 3) and transgenesis (4) confirms that it acts as the testis-determining gene. The predicted *SRY* gene product contains an HMG (high mobility group) box that binds to double-stranded DNA in a sequence-dependent manner and to cruciform DNA without sequence specificity (5, 6). Its similarity to a number of proteins that affect transcription suggests that the gene functions by activating or repressing other genes in the testis-determining pathway.

As would be expected of the mammalian testis-determining gene, homologues have been detected on the Y chromosomes of all eutherian and marsupial mammals tested (1, 7). The *SRY* gene is unique to the Y chromosome and is thus restricted to males. However, when used in Southern hybridization experiments, human SRY probes also detect sequences shared by males and females of many eutherian and marsupial species (1, 7, 8). These *SRY*-related sequences were thought to be members of a large family of related genes (8), originally termed a1, a2, etc. but now known as *SOX1*, *SOX2*, etc. (for *SRY*-related HMG box-containing). These *SOX* genes are highly conserved and have been demonstrated in other vertebrates (9). The relationships between the *SRY* and *SOX* genes are obviously of interest in considering the origin and evolution of the *SRY* gene in mammals.

Marsupials diverged from eutherian (placental) mammals 120–150 million years ago and monotremes (egg-laying mammals) diverged even earlier, so that comparisons between these three major mammalian groups may provide information about the function and early evolution of mammalian sex chromosomes and sex-determining genes. Eutherian, marsupial, and monotreme sex chromosomes have been found to differ in size and gene content, enabling the different evolutionary origins of regions of the human sex chromosomes to be deduced. The genes on the long arm and proximal short arm of the human X chromosome are present on the X chromosome in marsupial and monotreme mammals, and this region, therefore, represents a conserved, probably original, mammalian X chromosome (10, 11). The marsupial and monotreme X chromosome lacks genes borne on the short arm of the human X chromosome, suggesting that this region was originally autosomal and was added later to the eutherian X chromosome. The presence of several genes in this region with homologues on the Y chromosome implies that the region was added to both X and Y chromosomes, probably by recombination within an original pseudoautosomal region (10, 12). In marsupials, as in eutherian mammals, the Y chromosome is testis determining, but at least some sexual dimorphisms are sex hormone independent and seem to be a function of X chromosome dosage, rather than the presence or absence of a Y chromosome (13).

We have isolated an *SRY*-related sequence from the marsupial X chromosome, which is closely homologous to the mouse and human *SOX3* gene. This raises the possibility that X chromosome inactivation or gene dosage may play a role in sex determination in marsupials. However, we suggest that a more likely explanation for the presence of *SRY* homologues on marsupial, mouse, and human X chromosomes is that *SOX3* and *SRY* were originally alleles of a developmentally important gene shared by partly differentiated ancestral X and Y chromosomes.

MATERIALS AND METHODS

We used two marsupial species, representing the two major Australian orders that diverged about 50 million years ago, the striped-faced dunnart *Sminthopsis macroura* (Order Polyprotodonta, Family Dasyuridae) and the Tammar wallaby *Macropus eugenii* (Order Diprotodonta, Family Macropodidae). Tissue was originally provided by D. W. Cooper (School of Biological Sciences, Macqurie University) and L. Selwood (Department of Genetics and Human Vari-

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Abbreviation: HPRT, hypoxanthine phosphoribosyltransferase.

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ation, Latrobe University) and was retained under the provisions of permit RP 90-177 from the Victorian Department of Conservation and the Environment. Rodent–marsupial cell hybrids containing a marsupial X chromosome were obtained by fusing hypoxanthine phosphoribosyltransferase (HPRT)-deficient Chinese hamster cells with fibroblasts from *Planigale maculata*, a dasyurid marsupial related to *S. macroura* (14). Marsupial fibroblast and hybrid lines were cultured under standard conditions in Dulbecco's modified Eagle's medium/10% fetal calf serum.

DNA extraction and Southern blotting procedures were adapted from Reed and Mann (15). Ten micrograms of restricted DNA was electrophoresed through 0.8% agarose, transferred to Hybond-N+ (Amersham) with 20× standard saline citrate (SSC) and fixed with 0.4 M NaOH. A 0.9-kb *Hin*cII subclone of pY53.3, containing the human *SRY* gene (1), was labeled with [^{32}P]dCTP by random priming. The probe was hybridized in 5× SSPE [1× SSPE is 0.5% SDS/5× Denhardt's solution (0.18 M NaCl/10 mM phosphate, pH 7.4/1 mM EDTA)/10% (wt/vol) dextran sulfate/denatured salmon sperm at 100 μg/ml at 65°C]. Membranes were washed in 2× SSC/0.1% SDS at 65°C and autoradiographed for 6–10 days at −70°C. Fragment sizes are estimated relative to λ *Hin*dIII molecular-weight markers.

Genomic libraries were constructed from *S. macroura* liver DNA, partially restricted with *Sau*3AI and size-selected in 10–40% glycerol gradients. DNA was ligated to EMBL3A (BRL) and packaged with λ *in vitro* packaging reactions (Amersham). A total of 1.0 × 10^6 plaque-forming units were screened without amplification, using the 0.9-kb *Hin*cII fragment of pY53.3 as a probe. Positive clones were plaque-purified and subcloned into pUC vectors. Subclones were sequenced by the dideoxynucleotide chain-termination method (16) using Sequenase II (United States Biochemical) and *Taq* Track (Promega).

In situ hybridization was done as described (17). Probe was nick-translated with [^{3}H]dATP, [^{3}H]dCTP, and [^{3}H]dTTP to activities of 2–6 × 10^7 cpm/μg and was hybridized to formamide-denatured metaphase spreads over a range of concentrations. Slides were autoradiographed by using Amersham nuclear track emulsion, and autoradiographs with the highest signal/noise ratio were selected for scoring. The location of grains over at least 100 spreads was recorded by using a Leitz Dialux microscope fitted with a Sony video recorder. The grain distribution was analyzed by using the z_{max} statistic, which tests the overall distribution for departures from randomness, detects overlabeled sites, and attaches a level of significance to the localization (18).

RESULTS

In our original experiments to identify a male-specific *SRY* gene in marsupials, Southern analysis was done on DNA from male and female animals of the two marsupial species, probed with the human *SRY* gene. This procedure identified a single male-specific restriction fragment in marsupial DNA, which was subsequently cloned and shown to be homologous to *SRY* (7). In addition, this probe revealed several bands shared by males and females and thought to be the equivalent of the mouse *SRY*-like HMG box-containing genes *a1–4* (since named *Sox* genes) described by Gubbay *et al.* (8).

One of the male/female-shared bands from each species showed a very clear 1:2 male/female dosage effect characteristic of X chromosome-linked genes (Fig. 1*a*). Experiments were undertaken to clone and characterize the 2.3-kb *Eco*RI fragment from *S. macroura* that showed dosage differences and to determine its location on marsupial chromosomes.

The human SRY probe was used to screen *S. macroura* genomic phage libraries. Several genomic clones were isolated, three of which (SOXX1, -2, and -3) were found to have internal 2.3-kb *Eco*RI fragments. Restriction mapping showed

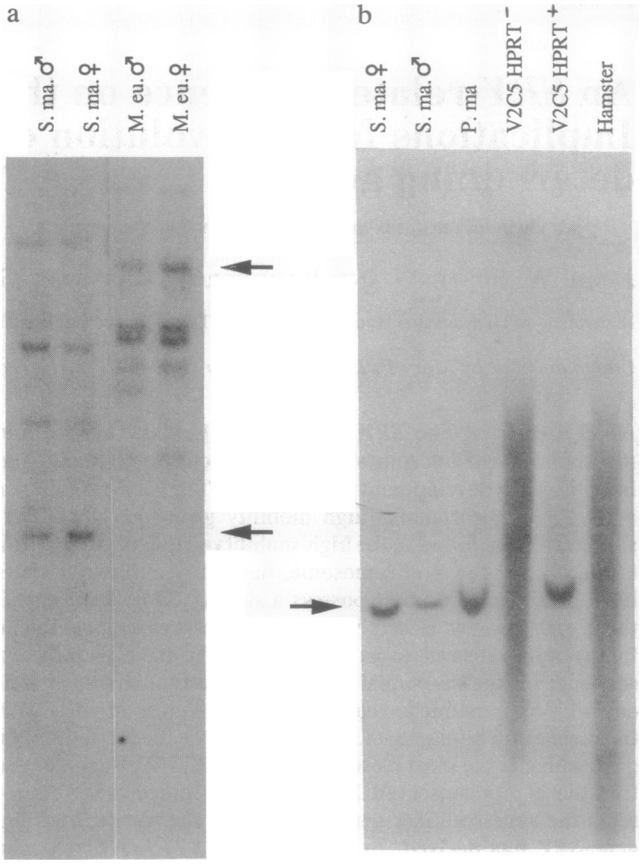
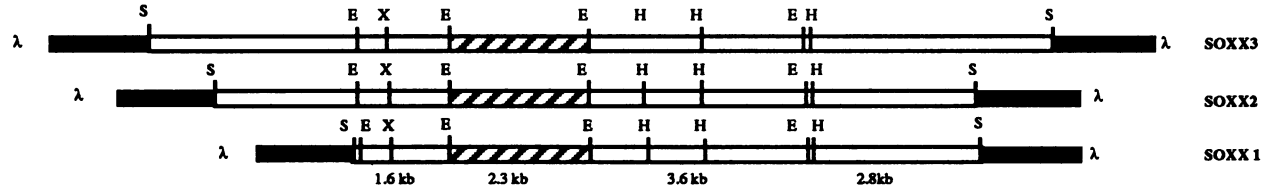


FIG. 1. Southern blot analysis of *SRY*-related genes in marsupials and cell hybrids. (*a*) Southern analysis of *Eco*RI-restricted DNA from male and female *S. macroura* (S. ma., lanes 1 and 2) and *M. eugenii* (M. eu., lanes 3 and 4), hybridized with a 0.9-kb *Hin*cII fragment of p53.3, containing the human *SRY* open reading frame. The probe detects a male-specific fragment (7) in both species, as well as several fragments shared between males (♂) and females (♀). The shared bands at 2.3 kb and 7.5 kb in *S. macroura* and *M. eugenii*, respectively (arrows), show a 1:2 male/female intensity difference, suggesting X chromosome linkage. (*b*) Southern analysis of *Eco*RI-restricted DNA from Chinese hamster–dasyurid marsupial somatic cell hybrid. Lanes: 1 and 2, *S. macroura* (S. ma.) female and male; 3, *P. maculata* (P. ma.) marsupial parent cell line; 4, V2C5 HPRT$^-$-deficient revertant cell line lacking the marsupial X chromosome; 5, V2C5 HPRT$^+$ hybrid retaining only the marsupial X chromosome; 6, Chinese hamster parent cell line. The membrane was hybridized with the 1.6-kb *Eco*RI fragment of SOXX2, which detects a 1.6-kb fragment (arrow) common to *P. maculata* and *S. macroura*. This band shows a 1:2 dosage between male and female *S. macroura* and is also present in the X-bearing hybrid but not in the hamster parent or the revertant that lacks the marsupial X chromosome. Reprobing the blot with the human *ZFY* gene probe (which is autosomal in marsupials) detected a strong hamster-specific band in lanes 4–6 and marsupial-specific band in lanes 1–3 (data not shown). This reprobing revealed that lanes 4 and 6 were overloaded with respect to the other lanes, which may account for the smearing in these lanes.

that these independent clones shared 1.6-, 2.3-, and 3.6-kb *Eco*RI fragments of DNA, arranged as shown in Fig. 2*a*.

To determine the chromosomal origin of the fragments in these phage clones, the 1.6- and 2.3-kb *Eco*RI and the 2.8-kb *Eco*RI/*Sal* I restriction fragments from SOXX2 were used to probe Southern blots containing DNA from HPRT$^+$ rodent–marsupial somatic cell hybrids that retain a dasyurid X chromosome, and HPRT$^-$ revertants that have lost it. Both the 2.3- and 2.8-kb fragments contained repetitive elements and produced uninformative smears with rodent DNA. The 1.6-kb cloned marsupial fragment detected only a marsupial-specific band in the hybrid DNA, which was absent from the

a



b

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Sma  SOXX  AAGCGAGGCTCACCCGCCGCGGCCGCGGCACAGGCGGAGGGCGAAAAGTTGCTGCAGTCTCCGGGACCTCGGACTCTGCCC

Sma  SOXX  GCCTCCCTCCCACCGATCCCCGGGCGCTCGTTTCTTTCTCTTCCCTTCCCGCCTCGCGAGCGTCCCACAGGCAAACTGGCG

           CACCTCCTGCCAGCGCCTGCCATGTACAGTCTCCTGGAGAGTGAGATGAAAAGCCCGGTGGTCCAG---GGGCCTCCGGCG
Sma  SOXX                      MetTyrSerLeuLeuGluSerGluMetLysSerProValValGln - GlyProProAla
Hsa  SOX3                      MetTyrSerLeuLeuGluThrGluLeuLysAsnProValGlyThrProThrGlnAlaAla

           GGAGCGGGCGGCGCCGCGGCCCCCGGGGGCACGGGCAAGAGCGGGGCGAACGCGGGAGGCGGGGGAGGGGGCGCCGGCGGC
Sma  SOXX  GlyAlaGlyGlyAlaAlaAlaProGlyGlyThrGlyLysSerGlyAlaAsnAlaGlyGlyGlyGlyGlyAlaGlyGly
Hsa  SOX3  GlyThrGlyGlyProAlaAlaProGlyGlyAlaGlyLysSerSerAlaAsnAlaAlaGlyGlyAlaAsnSerGlyGlyGly

           GGCGGCGGCGGC----------------------------------------GACCAGGACCGGGTCAAGCGGCCCATGAAC
Sma  SOXX  GlyGlyGlyGly - - - - - - - - - - - - - - - AspGlnAspArgValLysArgProMetAsn
Hsa  SOX3  SerSerGlyGlyAlaSerGlyGlyGlyGlyGly - - - - - - ThrAspGlnAspArgValLysArgProMetAsn

           GCTTTCATGGTGTGGTCCCGGGGGCAGAGACGCAAGATGGCCCTGGAGAACCCCAAGATGCACAACTCGGAGATCAGCAAG
Sma  SOXX  AlaPheMetValTrpSerArgGlyGlnArgArgLysMetAlaLeuGluAsnProLysMetHisAsnSerGluIleSerLys
Hsa  SOX3  AlaPheMetValTrpSerArgGlyGlnArgArgLysMetAlaLeuGluAsnProLysMetHisAsnSerGluIleSerLys

           CGGCTGGGCGCCGACTGGAAGCTGCTCACCGACGCCGAGAAGCGGCCCTTCATCGACGAGGCCAAGCGGCTGCGCGCCGTG
Sma  SOXX  ArgLeuGlyAlaAspTrpLysLeuLeuThrAspAlaGluLysArgProPheIleAspGluAlaLysArgLeuArgAlaVal
Hsa  SOX3  ArgLeuGlyAlaAspTrpLysLeuLeuThrAspAlaGluLysArgProPheIleAspGluAlaLysArgLeuArgAlaVal

           CACATGAAGGAGTACCCGGACTACAAGTACCGGCCCCGGCGGAAGACCAAGACGCTGCTCAAGAAGGACAAGTACTCCCTG
Sma  SOXX  HisMetLysGluTyrProAspTyrLysTyrArgProArgArgLysThrLysThrLeuLeuLysLysAspLysTyrSerLeu
Hsa  SOX3  HisMetLysGluTyrProAspTyrLysTyrArgProArgArgLysThrLysThrLeuLeuLysLysAspLysTyrSerLeu

           CCCGGCAACCTGCTCCCCCCCGGCACCGCGGCC---------------------------------GTCAACAGCCCCGTG
Sma  SOXX  ProGlyAsnLeuLeuProProGlyThrAlaAla - - - - - - - - - - - - - ValAsnSerProVal
Hsa  SOX3  ProSerGlyLeuLeuProProGlyAlaAlaAlaAlaAlaAlaAlaAlaAlaAlaAlaAlaAlaSerSerProVal

           GGCGTGGGCCAGAGGCTGGACACGTACGCGCACATGAACGGCTGGGCCAACGGGGCGTACTCGCTGATGCCCGAGCAGCTC
Sma  SOXX  GlyValGlyGlnArgLeuAspThrTyrAlaHisMetAsnGlyTrpAlaAsnGlyAlaTyrSerLeuMetProGluGlnLeu
Hsa  SOX3  GlyValGlyGlnArgLeuAspThrTyrThrHisValAsnGlyTrpAlaAsnGlyAlaTyrSerLeuValGlnGluGlnLeu

           AGCTACGGCCAGCACCCGGGCATGAACGGC------------CCGCAGCTGCAGCAGATGCACCGCTACGACATGGCCGGC
Sma  SOXX  SerTyrGlyGlnHisProGlyMetAsnGly - - - - ProGlnLeuGlnGlnMetHisArgTyrAspMetAlaGly
Hsa  SOX3  GlyTyrAlaGlnProProSerMetSerSerProProProProProAlaLeu - - - HisArgTyrAspMetAlaGly

           CCTGCAGTACAGCCCCATGATGCCGGCCGC---CCAGACCTACATGAACTCCTC.....
Sma  SOXX  LeuGlnTyrSerProMetMetPro - AlaAlaGlnThrTyrMetAsnSerSer.....
Hsa  SOX3  LeuGlnTyrSerProMetMetProProGlyAlaGlnSerTyrMetAsnValAlaAlaAlaAlaAlaAlaAlaSerGlyTyr
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FIG. 2. Restriction maps and sequences of SOXX. (*a*) Restriction maps of phage clones SOXX1, -2, and -3, isolated from independent *S. macroura* genomic libraries. Maps show position of *SRY* homologous region (hatched) and the size and location of *Eco*RI fragments used as probes (see text for details). E, *Eco*RI; H, *Hin*dIII; S, *Sal* I; X, *Xba* I. (*b*) Nucleotide sequence of the *S. macroura* (marsupial) *Sox3* homologue SOXX2 clone, showing the predicted amino acid sequence. Alignment of the amino acid sequence of human (Hsa, ref. 19), is shown beneath the *S. macroura* (Sma) *SOX3* sequence. The HMG box is underlined. Dashes were inserted to maintain alignment, which is disrupted by the variable number of repeats within each sequence. The marsupial sequence is incomplete at the 3′ end, and the human sequence has been truncated at this point.

revertant (Fig. 1*b*). Thus, somatic cell genetic analysis assigned the SOXX sequence to the X chromosome in this dasyurid marsupial.

To confirm the assignment of this SOXX sequence to the X chromosome and map it relative to other markers in normal cells of the two test species, clone SOXX2 (excluding the 2.3-kb region that contains the conserved cross-hybridizing sequences) was used for *in situ* hybridization to metaphase chromosomes of *S. macroura* and *M. eugenii*. The grain distributions each showed a single site of hybridization on the X chromosome in both species (Fig. 3); statistical analysis confirmed that these sites alone were significant.

The 2.3-kb *Eco*RI fragment of SOXX2 was subcloned into pUC19, the region with homology to *SRY* was sequenced, and an open reading frame of >800 bp was identified. The protein predicted by the SOXX open reading frame contained the 79-amino acid HMG box sequence, the DNA-binding motif typical of eutherian *SRY* and *SOX* genes (Fig. 2*b*). Within the conserved motif, SOXX was found to share 84% homology with the *S. macroura SRY* sequence, 84% homology (including 9 conservative amino acid changes) with human *SRY*, and 81% homology with mouse *Sry*. Outside the HMG box, no homology with *SRY* genes was detectable, and there was only limited homology with the closely related mouse autosomal *Sox1* and *Sox2* genes. Most striking, however, was the 100% homology between SOXX and the mouse and human *SOX3* genes within the HMG box. Homology outside the box extended in the N-terminal direction to the putative initiation of translation methionine and in the C-terminal direction to the end of available sequence (Fig. 2*b*).

DISCUSSION

We have demonstrated that an *SRY*-homologous sequence SOXX lies on the marsupial X chromosome. In the Tammar wallaby, the SOXX sequence maps to the same region of the X chromosome as does the coagulation factor gene *F9*. The sequence of SOXX is most closely homologous to that of

FIG. 3. Mapping of SOXX to marsupial chromosomes using *in situ* hybridization. Ideograms of *S. macroura* and *M. eugenii* chromosomes showing the location of silver grains after *in situ* hybridization with 1.6- and 2.8-kb fragments of SOXX2. There are sites of hybridization over the X chromosomes of both *S. macroura* [analysis using the z_{max} test indicates a significant site of hybridization on the X chromosome ($k = 14$ segments, $n = 676$ grains; $z_{max} = 10.864$, 0.1% significance level = 3.80)] and *M. eugenii* Y [analysis indicates a significant site of hybridization on chromosome Xq ($k = 14$ segments, $n = 601$ grains; $z_{max} = 8.953$, 0.1% significance level = 3.80)].

mouse and human *SOX3*. The human homologue *SOX3* has subsequently been cloned and mapped to human Xq27 near to *F9* based upon deletion mapping in a hemophilia B patient (19). The mouse *Sox3* gene has now been mapped to the mouse X chromosome to a position similar to that of the human gene, a region of conserved synteny (19). Thus, it appears that a highly conserved *SOX* gene maps to a conserved region of the X chromosome, at least in therian mammals. From its sequence similarity and conserved position on the X chromosome, we conclude that SOXX is the marsupial homologue of the *SOX3* gene and will refer to it by this name.

There are two alternative interpretations of the discovery of an X chromosome-borne *SRY*-related gene. One possibility is that *SOX3*, as well as *SRY*, is involved in sex determination in mammals. If these genes are functionally equivalent, they may act together to determine sex according to the dosage of active alleles, rather than by the unique action of the Y chromosome-linked allele, as originally proposed by Chandra (21), and applied to *ZFX/ZFY*, previously proposed to be involved in sex determination (22). In marsupials, but not eutherians, there is some evidence that development of scrotum or mammary gland is controlled by the dosage of a gene(s) borne on the X chromosome (13). However, testis determination in both eutherians and marsupial mammals seems independent of the numbers of X chromosomes, so it is hard to see how *SOX3* could be directly involved in testis determination.

The expression patterns of *Sox3* in mouse and human tissues are also inconsistent with a role in testis determination. *Sox3* is expressed broadly in the central nervous system of the mouse embryo, although it also shows significant expression in the indifferent gonad in both sexes (19). Human *SOX3* is expressed in several fetal tissues, including brain and spinal cord, and several adult tissues (19). Its lack of involvement in human testis determination is shown by the deletion of a portion of the X chromosome that includes it from a male hemophilia patient.

A more likely explanation for the occurrence of an X chromosome-linked homologue of *SRY* may be found in the evolution, rather than the functions, of *SOX3* and *SRY*. The *SOX3* gene is the most closely related to *SRY* of any of the HMG box-containing gene family (8, 9), suggesting that the two genes shared a common ancestor more recently than did *SRY* and other *SOX* genes.

Mammalian X and Y chromosomes were long ago proposed to have differentiated from a homologous chromosome pair in an ancestral mammal (23). This hypothesis receives support from the observation that the conserved region of the mammalian X chromosome contains genes that are also present on the Y chromosome. A sperm motility factor *UBE1* is shared on the X and Y chromosomes in marsupial as well as mouse (11), and *RPS4* has copies on Xq and Y chromosomes in human (24). There is also a lengthening list of genes cloned from the differentiated regions of the human or mouse Y chromosome, almost all of which have homologues on the X chromosome (for review, see ref. 12). Some of these genes (*STS*, *ZFX*, *ADMLX*, *AMG*) lie in the recently added region of the X chromosome (10), which was autosomal until relatively recently.

Comparisons in different species show that the X chromosome- and Y chromosome-borne homologues of some of these genes have diverged in sequence and function. The X chromosome- and Y chromosome-borne zinc finger genes *ZFX* and *ZFY* have a similar gene structure and sequence (25), and, because *ZFX* is included in the recently added region of the human X chromosome (being autosomal in marsupials and monotremes; see refs. 1, 10, and 26), it must have been located on an ancestral autosome 130 million years ago. The sequence of *ZFY* has diverged more rapidly than that of *ZFX* (27, 28).

For many of these X chromosome–Y chromosome-shared genes, the Y chromosome-borne homologue is transcribed at a lower level [e.g., amelogenin *AMGY* and the Kallman syndrome gene *ADMLY* (29, 30)] or not expressed at all (e.g., the Y chromosome-linked pseudogene *STSP*, homologous to the steroid sulfatase gene; see ref. 31). Some Y chromosome-linked genes have assumed a testis-specific expression pattern and have perhaps taken on a specialized role in male fertility, whereas their X chromosome-linked homologues are ubiquitously expressed. *Zfy* is testis specific in mouse but not in man (25), and the testis-specific *Ubely* is a sperm motility factor in mouse (32).

There are obvious parallels between these X chromosome–Y chromosome-shared genes and *SOX3/SRY*, and we suggest that *SOX3* and *SRY* diverged in sequence and function in a similar way to *ZFX/ZFY* and *Ubelx/Ubely* (Fig. 4). *SOX3* is widely expressed in both sexes, whereas *Sry* expression is restricted to male genital ridge and adult testis. *SOX3* is highly conserved in evolution, whereas *SRY* has diverged markedly in sequence, even within orders (20, 33).

We propose that *SOX3* and *SRY* were originally alleles of a *SOX* gene coding for a DNA-binding protein with a general

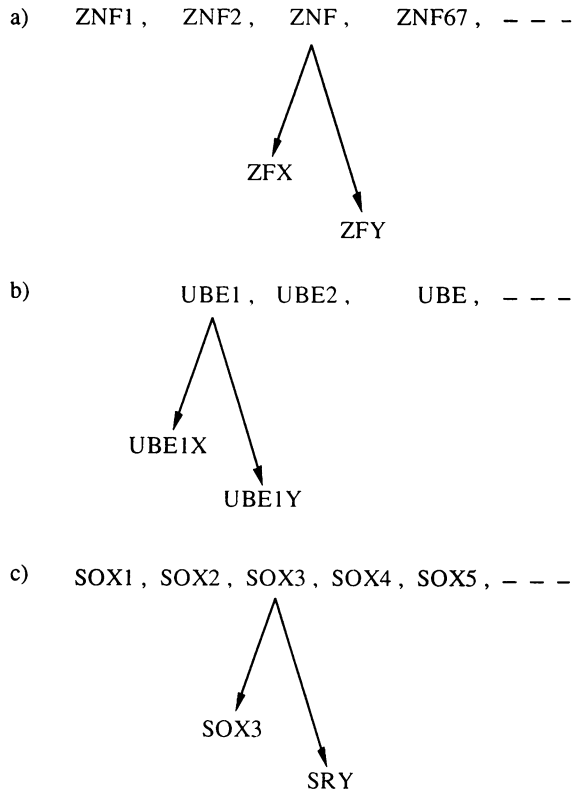

FIG. 4. Divergence of alleles on the X and Y chromosomes. The undifferentiated region of the proto-X and proto-Y chromosomes includes members of the zinc finger (*ZF*), the ubiquitin-activating enzyme (*UBE1*), and the *SOX* gene families. As the X and Y chromosomes are progressively differentiated and prevented from recombining, X- and Y-borne alleles of each diverge in sequence and function; the X-borne alleles (*ZFX*, *UBE1X*, and *SOX3*) retain a general role in both sexes, and the Y-borne alleles (*ZFY*, *UBE1Y*, and *SRY*) acquire male-specific patterns of expression and function.

function in both male and female embryos, perhaps involved in development of central nervous system or the indeterminate gonad. This ancestral *SOX3* gene was located on the undifferentiated or partially differentiated proto-X and proto-Y chromosomes of an ancestral mammal. *SRY* arose as variant form of *SOX3*, which evolved a male-specific function in testis determination. We cannot tell whether *SOX/SRY* divergence and the acquisition of a testis-determining role by *SRY* preceded (and initiated) mammalian X and Y chromosome differentiation, or whether it was a consequence of X chromosome–Y chromosome differentiation initiated at the locus of an original sex-determining gene(s), later superseded by *SRY*.

We thank Prof. P. N. Goodfellow and Dr. A. H. Sinclair for their encouragement during the early phases of this study and Prof. Goodfellow and Dr. R. Lovell-Badge for making sequence data available before publication. This study was supported by grants to J.A.M.G. from the Australian Research Council and the Australian National Health and Medical Research Council.

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