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Homology of a candidate spermatogenic gene from the mouse Y chromosome to the ubiquitin-activating enzyme E1

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THE *Sxr* (sex-reversed) region, a fragment of the Y chromosome short arm, can cause chromosomally female XX*Sxr* or X*Sxr*O mice to develop as sterile males[1–3]. The original *Sxr* region, termed *Sxr*^{a}, encodes: *Tdy*, the primary sex-determining gene; *Hya*, the controlling or structural locus for the minor transplantation antigen H–Y (ref. 4); gene(s) controlling the expression of the serologically detected male antigen (SDMA)[5]; *Spy*, a gene(s) required for the survival and proliferation of A spermatogonia during spermatogenesis[6,7]; *Zfy*-1/*Zfy*-2, zinc-finger-containing genes of unknown function[8]; and *Sry*, which is probably identical to *Tdy* (ref. 9). A deletion variant[10] of *Sxr*^{a}, termed *Sxr*^{b}, which lacks *Hya*, SDMA expression, *Spy* and some *Zfy*-2 sequences, makes positional cloning of these genes possible. We report here the isolation of a new testis-specific gene, *Sby*, mapping to the DNA deleted from the *Sxr*^{b} region (the Δ*Sxr*^{b} interval). *Sby* has extensive homology to the X-linked human ubiquitin-activating enzyme E1 (ref. 11). The critical role of this enzyme in nuclear DNA replication[12] together with the testis-specific expression of *Sby* suggests *Sby* as a candidate for the spermatogenic gene *Spy*.

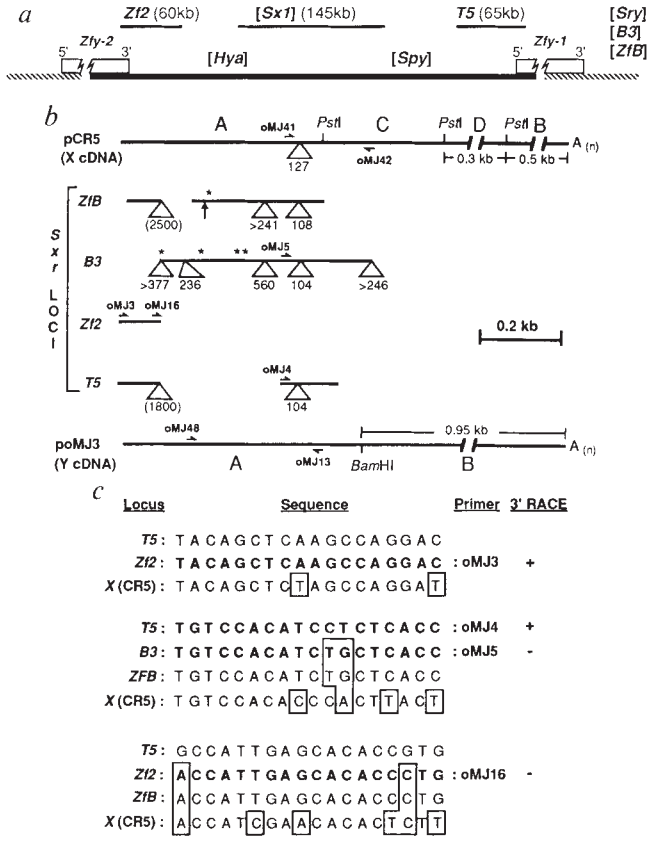

FIG. 1 The isolation of Δ*Sxr*^{b} DNA and the identification of exonic sequences on the Y chromosome. *a*, The Δ*Sxr*^{b} DNA is represented by the thick black line and the *Sxr* DNA remaining in the *Sxr*^{b} region by the broken line. The breakpoints of the deletion are shown by the gaps in the *Zfy* genes. The position of the breakpoints together with the indicated 3′-to-5′ orientation of the *Zfy* genes and their order with respect to *Hya*, *Spy* and *Sry* are as determined previously[19]. The positions of the *T5*, *Zf2* and *Sx1* contigs are shown at the top with the size of each in parenthesis[13]. Loci in square brackets have not been definitively mapped in relation to *Zfy* genes. *Hya*, *Spy* and *Sx1* must map between the *Zfy* genes and *Sry*, *B3* and *ZfB* must map outside the *Zfy* genes. *b*, Alignment of the *Sxr* genomic sequences with the pCR5 cDNA. The positions of the introns are indicated by triangles and their sizes (bp) where known are shown at the base of the triangle. Intron sizes in parentheses were calculated from PCR data (the difference in size between the genomic and cDNA products for a given pair of primers) and the others were obtained directly from the genomic sequence. The single X intron sequenced had no homology with the corresponding Y introns. Stop codons in the same frame as the CR5 open frame are marked by asterisks and the vertical arrow indicates a frameshift mutation indicating that the *B3* and *ZfB* homologues are pseudogenes. The *B3* sequence was derived from pY8 and is presented in Tucker *et al.*[20]. The locations of primers are represented by the horizontal arrows. The subsequently cloned Y cDNA poMJ3 is shown at the bottom of the lineup. The origins of pCR5 subclones A–D and poMJ3 subclones A and B are indicated as are the positions of the primers used in later expression studies (oMJ13, 48, 41 and 42). *c*, Primer sequences used in 3′ RACE experiments aligned with the corresponding sequences from the other loci. In each block all sequences are compared with the *T5* DNA and differences are boxed. The locus of origin of each sequence is indicated. Sequences from which primers were synthesized have the primer's name to their right and are in bold. The ability of a primer to generate a specific 3′ RACE product is indicated by + or −.

METHODS. The X cDNA CR5 was isolated from an adult testis library made from 129/sv/*Pas* testis poly(A) RNA inserted into the *Eco*RI site of λNM1149 and subcloned into pBluescript II (Stratagene). The phage library was constructed by conventional methods and screened with pY8. To isolate potential exons, cloned DNA from the *ZfB*, *Zf2* and *T5* loci was restricted, Southern blotted and hybridized with pCR5.A. Positive fragments were excised from gels purified by freeze-fracture in liquid nitrogen with an equal volume of equilibrated phenol and subcloned into pBluescript II. Smaller fragments of ~0.3 kb were similarly subcloned from these recombinants. These latter subclones, together with pY8 (which was assigned to the *B3* locus[13]) and pCR5, were sequenced directly from double-stranded plasmid by the dideoxy chain termination method[21] using Sequenase version 2.0 (USB) and a combination of nested deletion sets and oligonucleotide primers. Sequence comparison was performed using the University of Wisconsin genetics computer group sequence analysis software package[22]. The 3′ RACE[23] was done essentially as in ref. 24 using total RNA from C57BL/6 adult testis, prepared according to ref. 25. Different conditions were used for the PCR reaction which was run in 10 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dNTP and 25 pmol each primer. Only the annealing temperature was varied in the cycling parameters. Specific CR5.C/D homologous products were observed for oMJ3 and oMJ4 at 62 °C but not for oMJ5 or oMJ16 at temperatures above 56 °C. No products were amplified with any primers when the reverse transcriptase was omitted from the first strand synthesis.

With the expectation that an *Sxr* gene would be conserved on other mammalian Y chromosomes, 270 kilobases (kb) of ΔSxr^b DNA, previously isolated by chromosomal walking[13], was used to screen 'zoo' blots of DNA from a male and female human, horse, pig and rabbit. A 5-kb *Eco*RI fragment, termed icZf.1.8.F, mapping about 20 kb 3′ to the zinc-finger exon of *Zfy*-2, detected male-specific fragments in all species tested, with the exception of human (data not shown). Analysis of the region into which icZf.1.8.F maps (*Zf2*) (Fig. 1*a*) shows it to be complex, containing dispersed sequences that are present in at least five copies in *Sxr*. Contig sequences have been established from four of these related loci[13]. A homologue of the multi-copy *Sxr* probe pY8 (ref. 2), previously shown to detect a 3.5-kb transcript in adult testis[14], also maps into the *Zf2* contig. Screening an adult testis complementary DNA library with pY8 resulted in the isolation of pCR5, a 1.6-kb partial cDNA. This cDNA was mapped, using somatic cell hybrids, to the proximal portion of the mouse X chromosome (C. E. B. and P. Avner, unpublished results). The hybridization pattern of pCR5 to the zoo blots also revealed male-specific fragments and thus pCR5 represents the X homologue of a new Y gene and could be used to isolate exons rapidly from the cloned *Sxr* DNA. The contig DNA was, therefore, screened with pCR5 and homologous fragments were isolated and sequenced from two ΔSxr^b loci, *Zf2 and T5* and two Sxr^b loci, *B3* and *ZfB*. A comparison of these genomic Y sequences with the pCR5 cDNA sequence revealed regions of homology adjacent to regions lacking homology, probably representing exons and introns, respectively (Fig. 1*b*).

The sequence information was used to design primers from each locus for the isolation of specific transcripts using the 3′ RACE technique (3′ rapid amplification of cDNA ends). These

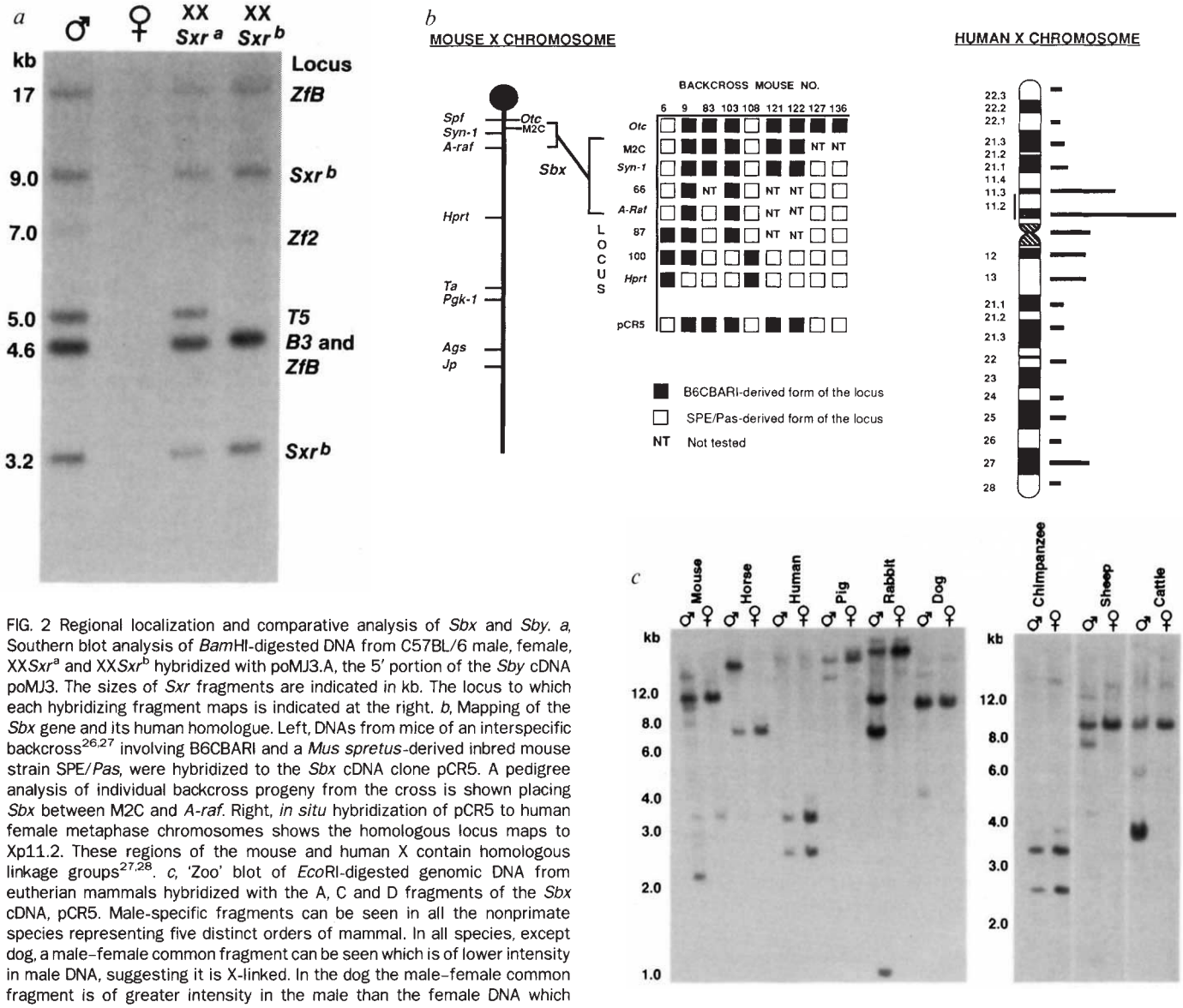

FIG. 2 Regional localization and comparative analysis of *Sbx* and *Sby*. *a*, Southern blot analysis of *Bam*HI-digested DNA from C57BL/6 male, female, XXSxr^a and XXSxr^b hybridized with poMJ3.A, the 5′ portion of the *Sby* cDNA poMJ3. The sizes of *Sxr* fragments are indicated in kb. The locus to which each hybridizing fragment maps is indicated at the right. *b*, Mapping of the *Sbx* gene and its human homologue. Left, DNAs from mice of an interspecific backcross[26,27] involving B6CBARI and a *Mus spretus*-derived inbred mouse strain SPE/*Pas*, were hybridized to the *Sbx* cDNA clone pCR5. A pedigree analysis of individual backcross progeny from the cross is shown placing *Sbx* between M2C and *A-raf*. Right, *in situ* hybridization of pCR5 to human female metaphase chromosomes shows the homologous locus maps to Xp11.2. These regions of the mouse and human X contain homologous linkage groups[27,28]. *c*, 'Zoo' blot of *Eco*RI-digested genomic DNA from eutherian mammals hybridized with the A, C and D fragments of the *Sbx* cDNA, pCR5. Male-specific fragments can be seen in all the nonprimate species representing five distinct orders of mammal. In all species, except dog, a male–female common fragment can be seen which is of lower intensity in male DNA, suggesting it is X-linked. In the dog the male–female common fragment is of greater intensity in the male than the female DNA which suggests the presence of an additional male-specific fragment of the same size.

METHODS. *a*, Restricted DNA (8 μg) was run in each lane of a 0.8% agarose gel in 1×TAE (0.04 M Tris-acetate, 0.001M EDTA). The electrophoresed DNA was transferred to Hybond-N membrane (Amersham) and fixed at 80 °C for 2 h. The membrane was prehybridized and hybridized in 0.5 M $NaPO_4$, pH 7.2; 7% SDS[29] at 68 °C for 36 h. After hybridization, the membranes were washed 3×20 min in 1×SSC, 0.2% SDS at 65 °C. The probe was labelled with ^{32}P using random hexamer primers[30]. *b*, For the backcross mapping of *Sbx*, DNAs restricted with *Taq*I to reveal a B6CBARI/SPE/*Pas* RFLP were hybridized with pCR5. The *M. spretus*- or B6CBARI-derived allele was identified and compared with previously typed markers to arrive at a map position. *In situ* hybridization, using cells collected according to the bromodeoxyuridine method[31] was done using ^3H-labelled pCR5 essentially as previously described[32]. One hundred metaphase spreads were scored and a total of 412 grains counted of which 84 (20.4%) were on the X chromosome with a peak at Xp11.2. No significant hybridization was seen on any of the autosomes (the Y was not available for scoring as female cells were used). *c*, Southern blot performed as in *a* except hybridization was done at 58 °C and the filters washed at 2×SSC, 0.2% SDS at 55 °C.

primers are shown in Fig. 1*c* compared with the corresponding sequences from the other X and Y loci where known. Only primers oMJ3 and oMJ4, which are identical to sequences from the *T5* locus, amplified 3′ RACE products, of 1.6 kb and 1.2 kb, respectively. Primers identical to sequences from the *ZfB* and *Zf2* loci (oMJ16) or the *B3* locus (oMJ5), but not the *T5* locus, failed to amplify a specific product. The cDNA for oMJ3 and oMJ4 products were cloned and named poMJ3 and poMJ4. Southern analysis with a 600-base pair (bp) fragment from poMJ3, poMJ3.A, revealed an almost exclusively *Sxr* hybridization pattern (Fig. 2*a*) and the bands were further assigned to individual loci by hybridizing poMJ3.A to the contig DNA from the *B3, ZfB, T5* and *Zf2* loci (data not shown). Thus, we have identified and cloned a transcript which is identical to sequences in the *T5* locus and conclude that this putative gene maps to the ΔSxr^b region. We have termed this gene *Sby* (for ΔSxr^bY homologue) and the X homologue represented by cDNA pCR5, *Sbx*. Two nondeleted, Sxr^b fragments or 3.2 and 9.0 kb (see Fig. 2*a*) do not map to the *B3, ZfB, T5* of *Zf2* contigs. Thus the *T5* locus may not be the only *Sxr* locus from which an *Sby* transcript is expressed. *Sbx* was mapped in the mouse to the M2C/*A-raf* subcentromeric interval and in the human to the homologous Xp11.2 interval (Fig. 2*b*).

The *Sbx* cDNA detects homologues on the sex chromosomes of other mammals more effectively than the *Sby* cDNA and when used to analyse DNA from a male and a female mouse, human, horse, pig, rabbit, dog, chimpanzee, sheep and cow it can clearly be seen that CR5 hybridizes to male-specific restriction fragments in all but the primate DNA (Fig. 2*c*). Male-female common fragments can also be detected in all species tested including primates. These fragments appear fainter in males than females consistent with X-linkage. We are now attempting to screen for human Y homologues at reduced stringencies but it remains a strong possibility that, in primates, the homologous X gene compensates for the lack of the Y-linked homologue.

The *Sby* cDNA, poMJ3, was sequenced and found to be 1,511 bp long with an open reading frame (ORF) across nucleotides 1–1,326. The sequence was 100% identical to the putative exons previously sequenced from the *T5* genomic DNA but

```
            1
Hum-E1/A1S9 TESYSSSQDPPEKSIPICTLKNFPNAIEHTLQWARDEFEGLFKQPAENVNQYLTDPKFVERTLRLAG.TQPLEVLEAVQR
Mus-Sbx     TESYSSSQDPPEKSIPICTLKNFPNAIEHTLQWARDEFEGLFKQPAENVNQYLTDSKFVERTLRLAG.TQPLEVLEAVQR
Mus-Sby        YSSSQDPPEKSIPICTLKNFPNAIEHTVQWARDEFEGLFKQSAENVNQYLTDPKFMERTLQLAG.TQPLEVLEAIHC
Yeast-E1    TESYSSSRDPPEKSIPLCTLRSFPNKIDHTIAWAKSLFQGYFTDSAENVNMYLTQPNFVEQTLKQSG..DVKGVLESISD
Wheat-E1    TENYGASRDPPEKQAPMCTVHSFPHNIDHCLTWARSEFEGLLEKTPTEVNAFLSNPTTYISAARTAGDAQARDQLERVIE

            81
Hum-E1/A1S9 SLVLQRPQTWADCVTWACHHWHTQYSNNIRQLLHNFPPDQLTSSGAPFWSGPKRCPHPLTFDVNNPLHLDYVMAAANLFA
Mus-Sbx     SLVLQRPQTWGDCVTWACHHWHTQYCNNIRQLLHNFPPDQLTSSGAPFWSGPKRCPHPLTFDVNNTLHLDYVMAAANLFA
Mus-Sby     SLVLQRPQTWADCVTWAYQHWHTQYSHNIQQLLHNFPPAQLTSSGALFWSGPKRCPHPLTFDINNPLHLDYVMAAANLFA
Yeast-E1    SLS.SKPHNFEDCIKWARLEFEKKFNHDIKQLLFNFPKDAKTSNGEPFWSGAKRAPTPLEFDIYNNDHFHFVVAGANLRA
Wheat-E1    CLDRDKCETFQDSITWARLKFEDYFSNRVKQLTFTFPEDSMTSSGAPFWSAPKRFPRPVEFSSSDQSQLSFILAAAILRA

            161
Hum-E1/A1S9 QTYGLTGSQDRAAVATF.....LQSVQVPEFTPKSGVKIHVSDQELQSANAS.VDDSR.LEELKATLPSPDKLP..GFKM
Mus-Sbx     QTYGLTGSQDRAAVASL.....LQSVQVPEFTPKSGVKIHVSDQELQSANAS.VDDSR.LEELKATLPSPDKLP..GFKM
Mus-Sby     QTYGLGGSQDCAVVAKL.....LQSLPVPKFAPKSGIRIHVSEQELQSTSATTIDDSH.LEELKTALPTPDKLL..GFKM
Yeast-E1    YNYGIKSDDSNSKPNVDEYKSVIDHMIIPEFTPNANLKIQVNDDDPDPNANAANGSDE.IDQLVSSLPDPSTLA..GFKL
Wheat-E1    ETFGIPIPEWAKTPNKLAAEA.VDKVIVPDFQPKQGVKIVTHEKATSLSSAS.VDDAAVIEELIAKLEEVSKTLPSGFHM

            241
Hum-E1/A1S9 YPIDFEKDDDSNFHMDFIVAASNLRAENYDIPSADRHKSKLIAGKIIPAIATTTAAVVGLVCLELYKVVQGHRQLDSYKN
Mus-Sbx     YPIDFEKDDDSNFHMDFIVAASNLRAENYDISPADRHKSKLIAGKIIPAIATTTAAVVGLVCLELYKVVQGHQQLDSYKN
Mus-Sby     YPIDFEKDDDSNFHMDFIVAASNLRAENYGISPADRHKSKLIAGKIIPAIATTTSAIVGLVCLELYKVVQGHQQLESYKN
Yeast-E1    EPVDFEKDDDTNHHIEFITACSNCRAQNYFIETADRQKTKFIAGRIIPAIATTTSLVTGLVNLELYKLIDNKTDIEQYKN
Wheat-E1    NPIQFEKDDDTNFHMDVIAGFANMRARNYSIPEVDKLKAKFIAGRIIPAIATSTAMATGLVCLELYKALAGGHKVEDYRN

            321
Hum-E1/A1S9 GFLNLALPFFGFSEPLAAPRHQYYNQEW.TLWDRFEVQGLQPNGEEMTLKQFLDYFKTEHKLEITMLSQGVSMLYSFFMP
Mus-Sbx     GFLNLALPFFGFSEPLAAPRHQYYNQEW.TLWDRFEVQGVQPNGEEMTLKQFLDYFKTEHKLEITMLSQGVSMLYSFFMP
Mus-Sby     SFINLALPLFSFSAPLAPECHQFYDQEW.TLWDRFDVQGLQPSGEEMTLKQFLDYFKTEHKLEVIMLSQGVSMLYSVFMP
Yeast-E1    GFVNLALPFFGFSEPIASPKGEYNNKKYDKIWDRFDIKG......DIKLSDLIEHFEKDEGLEITMLSYGVSLLYASFFP
Wheat-E1    TFANLAIPLFSIAEPVPPKTIKHQELSW.TVWDRWTVTG......NITLRELLEWLK.EKGLNAYSISCGTSLLYNSMFP

            401
Hum-E1/A1S9 AAKLKERLDQPMTEIVSRVSKRKLGRHVRALVLELCCNDESGEDVEVPYVRYTIR*
Mus-Sbx     AAKLKERLDQPMTEIVSRVSKRKLGRHVRHWCLSCAATMKAARTSRSLMSDIPFADLRAFP*
Mus-Sby     ASKLKERLDQPMTEIVSCVSKQKLGHHVKSLVFELCCNSDSGDDIEVPYVRYIIR*
Yeast-E1    PKKLKERLNLPITQLVKLVTKKDIPAHVSTMILEICADDKEGEDVEVPFITIHL*
Wheat-E1    ..RHKERLDRKVVDVAREVAKMEVPSYRRHLDVVVACEDDDDNDVDIPLVSVYFR*
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FIG. 3 Comparison of the partial deduced amino-acid sequence (single-letter code) of *Sby* to those of *Sbx*, the A1S9 cDNA[15] and the ubiquitin-activating enzymes (E1) from human[11], wheat[16] and yeast[17]. The human E1 sequence includes the corrections published[33]. As A1S9 and the corrected human E1 sequences encode identical peptides in this region, they are presented together as hum-E1/A1S9. Positions of identity with *Sby* and conservative substitutions are shaded. Underlined, *Sbx* differs from *Sby* through the loss of a single base from the CR5 cDNA. This is an accurate representation of the sequence of the CR5 clone but may represent a rare cloning artefact. This will be clarified by the sequencing of further cDNA clones. Dots represent gaps introduced to maintain alignment. The nucleotide sequences from which the *Sby* and *Sbx* amino-acid sequences were deduced have been deposited in the Genbank database accession numbers in the EMBL data library: *Sbx*, X62580; *Sby*, X62581.

METHODS. The *Sby* cDNA poMJ3 was subcloned into the *Sma*I and *Sal*I sites of pBluescript II and sequenced as described in the legend to Fig. 1. Comparisons at the nucleotide and amino-acid level were done using the University of Wisconsin GCG sequence analysis software package[22].

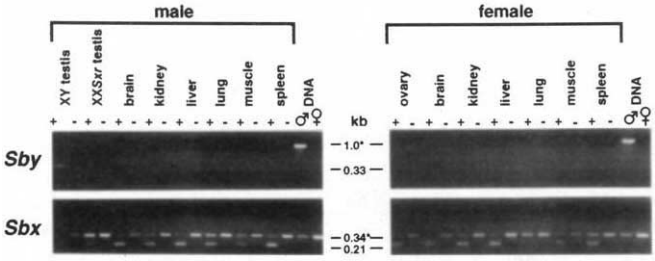

FIG. 4 Expression patterns of *Sby* and *Sbx* in adult tissues. Expression was studied using RT-PCR from total RNA from a variety of male and female tissues and XX*Sxr*^{a} testis. +, −, Presence or absence of reverse transcriptase during the first strand cDNA synthesis respectively. The sizes of fragments amplified are indicated in (kb) and the products amplified from genomic DNA are indicated with an asterisk. The RT-independent fragment amplified from the RNA samples by oMJ41/42 arises from DNA contamination. The lack of an RT-dependent product with the *Sbx* primers in female lung is probably due to degradation of the RNA in this sample as the same primers amplify a product in male lung.
METHODS. Total RNA was prepared from tissues following the protocol in ref. 25. The RNA was not DNase treated. RT-PCR was done essentially as for 3′ RACE[24], except that after first strand synthesis a primer pair (25 pmol each) specific for either *Sbx* or *Sby* was added. The cycle parameters used were 95 °C (5 min); 72 °C to add 1.25 units of *Taq* polymerase (Perkin-Elmer Cetus); 60 °C (30 s); 72 °C (90 s); and then 95 °C (30 s); 60 °C (30 s); 72 °C (30 s) for 34 cycles followed by 72 °C (15 min). The *Sbx* primers were oMJ41: 5′-TGTCCACACCCACTTACT-3′ and oMJ42: 5′-GCACTCTGCAACTCCTGG-3′. The *Sby* primers were oMJ13: 5′-CCTCCTAGTCCGTATGTC-3′ and oMJ48: 5′-GACCCCAAGTTCATGGAG-3′. The first strand reaction, containing 3–7 μg total RNA, was diluted to 1 ml and 0.5 μl used for the amplification. For the controls, 50 ng genomic DNA was amplified. Amplified products were electrophoresed on a 2% Nusieve agarose gel in 1 × TAE.

only 95–98% identical to those from the *B3, ZfB* or *Zf2* DNA. The *Sbx* cDNA, pCR5 is 1,603 nucleotides long with an ORF across nucleotides 3–1,359. Both cDNAs have poly(A) tails at their presumed 3′ ends. They only share homology in the ORFs, where they are 83% identical at the nucleotide level and 85% identical and 92% similar (85/92% id/sim) at the amino-acid level. Database searches revealed that *Sbx* and *Sby* are highly homologous with the human ubiquitin-activating enzyme (E1)[11] and the almost identical A1S9 human cDNA which complements defective DNA replication in the tsA1S9 mutant cell line[15]. At the amino-acid level, *Sby* is 84/92% id/sim to the human E1/A1S9 and *Sbx* is 97/98% id/sim. *Sby* and *Sbx* are also homologous with other cloned E1 genes from wheat[16] and yeast[17] (Fig. 3). As A1S9 and human E1 are virtually identical, A1S9 is probably E1 and *Sbx* represents the mouse homologue of the E1/A1S9 gene. As A1S9 has been localized to Xp11.2-4 in the human[18] the mapping of an *Sbx* homologue to human Xp11.2 (Fig. 2*b*) is consistent with this interpretation.

The expression patterns of *Sbx* and *Sby* were analysed using the reverse transcriptase-dependent polymerase chain reaction assay (RT-PCR). The results show that *Sbx* is expressed in all male and female tissues tested, consistent with its predicted housekeeping role as a ubiquitin-activating enzyme (Fig. 4). *Sby* expression is testis-specific and its absence in XX*Sxr*^{a} testes, which lack germ cells[6], implies that *Sby* expression is germ-cell dependent. This excludes *Sby* as a candidate for *Hya* as H-Y antigen is known to be expressed in virtually all male tissues.

Sby shows good homology to the human ubiquitin-activating enzyme and the (probably identical) A1S9 cDNA both of which have been shown to be essential to normal cell-cycle progression[12,15]. The loss of *Spy* function in the testis of X*Sxr*^{b}O mice has been similarly correlated with a reduced mitotic index in the T1 prospermatogonia and the differentiating A spermatogonia[7]. *Sby* may thus encode a testis-specific Y chromosomal E1 enzyme acting to augment insufficient levels of *Sbx* expression in the spermatogonial stages of spermatogenesis. Alternatively, *Sby* could control a specific ubiquitin-dependent pathway in spermatogenesis. To define further its function it will be important to determine whether *Sby* has ubiquitin-activating properties and if it can complement the loss of function in mutant E1 cell lines. Its map position in the *Sxr*^{b} deletion, its testis-specific expression, and its homology with the E1 enzyme are all consistent with *Sby* being *Spy*. Definitive proof of this, however, can only be obtained by reintroducing *Sby* into X*Sxr*^{b}O mice to see if it can complement the deleted *Spy* function. □

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A candidate spermatogenesis gene on the mouse Y chromosome is homologous to ubiquitin-activating enzyme E1

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THE human X-linked gene *A1S9* (refs 1–3) complements a temperature-sensitive cell-cycle mutation in mouse L cells[4], and encodes the ubiquitin-activating enzyme E1 (refs 5–7). The gene has been reported to escape X-chromosome inactivation[8], but there is some conflicting evidence[9]. We have isolated part of the mouse *A1s9* gene, mapped it to the proximal portion of the X chromosome and shown that it undergoes normal X-inactivation. We also

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