Sry Expression Level and Protein Isoform Differences Play a Role in Abnormal Testis Development in C57BL/6J Mice Carrying Certain Sry Alleles

Kenneth H. Albrecht,1 Maureen Young, Linda L. Washburn and Eva M. Eicher

The Jackson Laboratory, Bar Harbor, Maine 04609

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ABSTRACT

Transfer of certain Mus domesticus-derived Y chromosomes (SryDOM alleles, e.g., SryPOS and SryAKR) onto the C57BL/6j (B6) mouse strain causes abnormal gonad development due to an aberrant interaction between the SryDOM allele and the B6-derived autosomal (tda) genes. For example, B6 XYPOS fetuses develop ovaries and ovotestes and B6 XYAKR fetuses have delayed testis cord development. To test whether abnormal testis development is caused by insufficient SryDOM expression, two approaches were used. First, gonad development and relative Sry expression levels were examined in fetal gonads from two strains of B6 mice that contained a single M. domesticus-derived and a single M. musculus-derived allele (B6-YPOSRIII and B6-YAKRRIII). In both cases, presence of the M. musculus SryDOM allele corrected abnormal testis development. On the B6 background, SryPOS was expressed at about half the level of SryDOM whereas SryAKR and SryDOM were equally expressed. On an F1 hybrid background, both SryPOS and SryDOM expression increased, but SryPOS expression increased to a greater extent. Second, sexual development and Sry expression levels were determined in XX mice carrying a transgene expressing SryPOS controlled by POS-derived or MUS-derived regulatory regions. In both cases one B6 transgenic line was recovered in which XX transgenic mice developed only testicular tissue but cord development was delayed despite normal transcriptional initiation and overexpression. For three transgenes where B6 XX transgenic mice developed as females, hermaphrodites, or males, the percentage of XX transgenic males increased on an F2 background. For the one transgene examined, Sry expression increased on an F1 background. These results support a model in which delayed testis development is caused by the presence of particular DOM SRY protein isoforms and this, combined with insufficient Sry expression, causes sex reversal. These results also indicate that at least one tda gene regulates Sry expression, possibly by directly binding to Sry regulatory regions.

Normally in mammals, XX individuals develop as females with ovaries, and XY individuals develop as males with testes. Although rare, complete sex reversal (SR) occurs in which XX individuals develop testes and XY individuals develop ovaries. In humans the easiest SR cases to explain are XY females who carry a nonfunctional SRY (sex-determining region, Y chromosome, symbolized as Sry in mice) gene and XX males who carry a normal SRY gene located on their paternally derived X chromosome due to an abnormal meiotic recombination event (reviewed in Schafer 1995; McElreavey and Fellous 1999). Several intriguing but unexplained SR conditions occur, however, including XY females and XY hermaphrodites who carry an apparently normal SRY gene and XY females who carry a mutated SRY gene inherited from their father who carried the same mutated SRY allele (reviewed in Schafer 1995; McElreavey and Fellous 1999). These cases are reminiscent of what occurs in mice when certain Mus domesticus-derived Sry genes are transferred onto specific inbred strains, such as C57BL/6j (B6).

Standard mouse inbred strains are a composite of two species, M. musculus and M. domesticus (reviewed in Bonhomme 1986). Most strains, such as B6, contain a M. musculus (MUS) Y chromosome, but a few, such as AKR/J, contain a M. domesticus (DOM) Y chromosome (Bishop et al. 1985; Nishihoka and Lamothe 1987; Tucker et al. 1992). Of interest to gonadal sex determination is the finding that transfer of some DOM Y chromosomes (i.e., Sry alleles) to the B6 strain interferes with testis development (Table 1). For example, B6 XYPOS mice carrying an SryDOM allele from wild-derived M. d. poschiavinus mice develop ovaries and ovotestes but not normal testes (referred to as B6-YPOS sex reversal; Eicher et al. 1982; Eicher and Washburn 2001). B6 XYAKR mice carrying an SryDOM allele from the AKR/J inbred strain have delayed testis cord development but do not develop ovarian tissue (Washburn and Eicher 1983, 1989; Eicher and Washburn 1986; Nagamine et al. 1987). Other SryDOM alleles do not interfere with normal testis development when present on B6, an example being B6 XYBLB mice (Sry allele from the BUB/BnJ inbred strain; Washburn et al. 2001). These phenomena are highly sensitive to genetic background. That is, an
**TABLE 1**

<table>
<thead>
<tr>
<th>Official designation (abbreviation)</th>
<th>Sry allele origin</th>
<th>Fetal gonad development</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6j (B6)</td>
<td>MUS</td>
<td>Normal</td>
<td></td>
</tr>
<tr>
<td>C57BL/6j-YPOS (B6-YPOS)</td>
<td>DOM</td>
<td>75% ovaries, 25% ovotestes</td>
<td>Eicher et al. (1982)</td>
</tr>
<tr>
<td>C57BL/6j-YAKR (B6-YAKR)</td>
<td>DOM</td>
<td>Delayed testis development</td>
<td>Washburn and  Eicher (1983)</td>
</tr>
<tr>
<td>C57BL/6j-YPOS,RIII (B6-YPOS,RIII)</td>
<td>DOM and MUS (RIII)</td>
<td>No ovarian tissue develops</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-YAKR,RIII (B6-YAKR,RIII)</td>
<td>DOM and MUS (RIII)</td>
<td>Normal</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-YAKR Tg(Sry129)2Ei (B6-YAKR Tg2)</td>
<td>MUS (Tg) 129 inbred strain</td>
<td>XX Tg2</td>
<td>Washburn et al. (2001)</td>
</tr>
<tr>
<td>C57BL/6j-Tg(SryPOS)83Ei (B6-Tg83)</td>
<td>DOM (Tg)</td>
<td>XXTg83</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-Tg(SryPOS)84Ei (B6-Tg84)</td>
<td>DOM (Tg)</td>
<td>XX Tg84</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-Tg(SryPOS)85Ei (B6-Tg85)</td>
<td>DOM (Tg)</td>
<td>XX Tg85</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-Tg(Sry129-POS)17Ei (B6-Tg17)</td>
<td>DOM (Tg)</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-Tg(Sry129-POS)28Ei (B6-Tg28)</td>
<td>DOM (Tg)</td>
<td>ND</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-Tg(Sry129-POS)94Ei (B6-Tg94)</td>
<td>DOM (Tg)</td>
<td>XX Tg94</td>
<td>This study</td>
</tr>
<tr>
<td>C57BL/6j-Tg(Sry129-POS)121Ei (B6-Tg121)</td>
<td>DOM (Tg)</td>
<td>ND</td>
<td>This study</td>
</tr>
</tbody>
</table>

**ND,** not determined.

*Sry*<sub>DOM</sub> allele that causes abnormal testicular development in B6 mice may not do so when present on another genetic background (Eicher *et al.* 1982; Eicher and Washburn 1986; Nagamine *et al.* 1987; Biddle and Nishioka 1988). For example, DBA/2J XY<sup>POS</sup> (D2 XY<sup>POS</sup>) and (D2 × B6)F<sub>1</sub> XY<sup>POS</sup> mice develop normal testes. These findings suggest that the ability of a specific Sry allele to function correctly is dependent on other genes, designated *tda* (*testis-determining autosomal*) genes, and genetic mapping experiments have supported this hypothesis (Eicher *et al.* 1996). If the proper functioning of the human Sry gene likewise is sensitive to genetic background, this could clarify the unexplained human XY SR conditions noted above.

The simplest mechanism to explain B6-Y<sup>POS</sup> SR is that the Sry<sup>POS</sup> allele encodes a protein that does not interact correctly with downstream genes if they are derived from the B6 strain (Coward *et al.* 1994). However, DNA sequence analysis of several DOM Sry alleles revealed that this hypothesis alone is inadequate because no correlation between the open reading frame (ORF) sequence and SR was found (Carlisle *et al.* 1996; Albrecht and Eicher 1997). A second possible mechanism to explain B6-Y<sup>POS</sup> SR is that expression of the Sry<sup>POS</sup> allele is aberrant. Support for this hypothesis was obtained by Nagamine *et al.* (1999), who assayed Sry expression in mouse fetal gonads from three B6 consomic strains carrying different DOM Sry alleles, Sry<sub>FVB</sub>, Sry<sub>AKR</sub>, and Sry<sub>TIR</sub>. [Sequence analysis indicates that the Sry<sub>TIR</sub> and Sry<sub>POS</sub> ORFs are identical (Albrecht and Eicher 1997)]. They found that Sry expression was highest in B6 XY<sup>POS</sup> fetal gonads, which develop as normal testes, and lowest in B6 XY<sup>TIR</sup> fetal gonads, which develop as ovotestes or ovaries. Moreover, Sry expression was increased in (SWR × B6)F<sub>1</sub> XY<sup>TIR</sup> fetal gonads, which develop as normal testes.

Here we report results from two experimental ap-
proaches designed to further our understanding of Sry function in B6-YPOS SR. First, we determined Sry expression levels in fetal gonads from B6 mice carrying a Y chromosome containing both a MUS-derived Sry allele (SryPOS) and a DOM-derived Sry allele (SryDOM and SryAKR). This approach differed from that used by NAGAMINE et al. (1999) because it directly compared SryDOM and SryMUS expression within the same gonad so that the results were independent of the number of Sry-expressing cells. We found that SryPOS transcript levels were significantly reduced compared to SryDOM, whereas SryAKR and SryDOM transcript levels were equivalent. We then assayed Sry transcript levels on a (D2 × B6)F1 genetic background previously shown to allow normal testis determination in XYPOS mice (EICHER and WASHBURN 1986). Unexpectedly, we found that although both SryDOM and SryMUS transcript levels increased, SryDOM transcript levels increased more than SryAKR transcript levels. Together, these data suggest that B6 XYPOS SR is caused, at least in part, by insufficient Sry expression and that one or more tda genes directly regulate Sry expression. These results confirm and extend those of NAGAMINE et al. (1999).

Our second approach was based on the premise that if B6-YPOS SR is caused by insufficient Sry expression, then overexpression of SryPOS would rescue testis development in B6 mice. B6 transgenic mouse lines carrying either a chimeric Sry construct in which Sry129 expression was regulated by MUS regulatory regions or an SryPOS genomic DNA clone were produced. In two transgenic lines, B6 XX mice carrying either type of transgene developed testicular tissue exclusively. However, testis cord development was delayed despite normal transcriptional initiation and overexpression of Sry. These data suggest that delayed testis cord development is caused by SRY protein isoform differences that are exacerbated by insufficient Sry expression leading to ovarian tissue development in B6 XYPOS gonads. The above hypothesis is supported by the finding that the MUS SryB6 allele is expressed at relatively low levels (LEE and TAKETO 2001) without causing delayed testis cord development or SR.

In five Sry B6 transgenic lines, sex reversal was not complete. In two lines, XX transgenic mice developed as females, and in three lines XX transgenic mice developed as females, hermaphrodites, or males. However, for the three transgenes tested, the percentage of XX transgenic males increased on a (D2 × B6)F1 genetic background. In the one case assayed, transgene expression was increased in F1 gonads compared to B6 gonads, presumably due to the presence of an enhancer element responsive to genetic background.

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**MATERIALS AND METHODS**

**Creating B6-YPOS,B6 and B6-YAKR,B6 consomic strains:** The Sxr (sex-reversed) Y chromosome rearrangement [formally, Tp(Y)-IC1; CATTANACH et al. 1971] was used to generate mice biallelic for Sry carrying a single copy of a MUS and DOM Sry allele. (The Sxr Sry allele is MUS derived and from the RIII inbred strain.) The Sxr Y chromosome carries a duplication of most of the short arm (Yp), including the Sry gene, at the distal end of the long arm (Yq: EVANS et al. 1982; BISHOP et al. 1988). This duplicated copy of Sry is adjacent to the pseudoautosomal region and can be transferred to the X chromosome by homologous recombination.

The basic strategy to create the Sry biallelic strains was to first transfer the duplicated Sxr segment from the Sxr Y chromosome to an X chromosome and then to transfer it from the X chromosome onto the YPOS or YAKR chromosome. Because XX Swiss mice are sterile, we used the T(16:X)16H translocation (T16H) to cause preferential X inactivation of the XPOS chromosome: If X inactivation spreads to the Sry gene, these T16H/16H mice will develop as females (CATTANACH et al. 1982; SIMPSON et al. 1984). T16H/+ and EdaTa females were mated to XY males (EdaTa is ectodysplasin-A, also known as tabby). Non-tabby females (i.e., inherited T16H) that inherited Sry on their paternally derived inactive X chromosome were mated to B6 XYPOS hermaphrodites or to B6 XYAKR males to generate XPOS YPOS or XAKR YAKR males, respectively. XPOS YPOS and XAKR YAKR male offspring, respectively, were identified (i.e., the Sxr segment was transferred from the XPOS or YAKR chromosome by homologous recombination in the pseudoautosomal region). Therefore, XYPOS and XYAKR, respectively, to reflect the Sry alleles present.

**Sry transgene construction:** The Sry129POS chimeric transgene is based on a 14.6-kb MUS-derived genomic DNA fragment from a 129 inbred strain (GUBBAY et al. 1990) and previously shown to sex reverse XX mice carrying it as a transgene (KOOPMAN et al. 1991; EICHER et al. 1995; Figure 1). The Sry129 open reading frame and a portion of the 3' untranslated region were replaced by a homologous SryPOS DNA segment as follows: The replacement fragment was PCR amplified using high-fidelity Pfu DNA polymerase (Stratagene, La Jolla, CA), genomic DNA template containing the YPOS chromosome, and primers Sry-8316 (5'-CCATGTCAGCGCCCGCAATGC) and Sry-9816 (5'-AGCTGTGGTCGTGTAGTAGCC). (Sry primers are designated as 5' based on numbering in GenBank entry X67204.) The PCR product was gel purified and cloned into pCR-Script using the manufacturer's protocols (Stratagene). The DNA sequence of individual clones was confirmed prior to further use. The replacement fragment was inserted into the 14.6-kb Sry129 genomic DNA clone at the unique Hpal (base pair 8312) and SspI (base pair 9704) sites. (The Sry ORF is located between base pair 8304 and base pair 9491.) The nucleotide sequences 8304–8312 and 9491–9704 are identical in SryPOS and Sry129 (ALBRECHT and EICHER 1997).

The Sry129 transgene was derived from a 13.5-kb genomic DNA clone (L961) isolated from a mouse carrying a YPOS chromosome (GUBBAY et al. 1992; Figure 1). Sequence analysis indicated that this clone extends from base pair 2355 to approximately base pair 15,825 relative to the 14.6-kb Sry129 clone described above and that a 36-bp deletion was present in the glutamine repeat region downstream of the DOM stop codon. The deleted region was replaced with a wild-type SryPOS DNA fragment as follows: The replacement fragment was PCR amplified using Pfu DNA polymerase, genomic DNA template containing the YPOS chromosome, and primers Sry-8366 (5'-CCAGCAGAACAAATACAGAGATCC) and Sry-9839 (5'-ATGGCATGCTGTATTGACCACAAAGC). The PCR product was digested with SphI, gel purified, and ligated into pBluescript II (Stratagene). Correct orientation was confirmed by restriction digestion and sequence analysis.
Sry transgenic mice: B6-Sry129POS and B6-SryPOS transgenic mice were produced by micro-injecting the constructs described above, without the plasmid backbone, into fertilized B6 eggs using standard methods (Wagner et al. 1981).

Four Sry129POS transgenic lines were recovered and formally designated C57BL/6J-Tg(Sry-129-POS)17Ei, . . . 28Ei, . . . 94Ei, and . . . 121Ei, hereafter referred to as Tg17, Tg28, Tg94, and Tg121. Three SryPOS transgenic founders were recovered and are formally designated C57BL/6J-Tg(Sry-POS)83Ei, . . . 84Ei, and . . . 85Ei, hereafter referred to as Tg83, Tg84, and Tg85.

Transgenic line C57BL/6J-Ei-YAKR Tg(Sry-129)2Ei (hereafter referred to as Tg2), carrying the original 14.6-kb Sry129 Tg (from which the Sry129POS Tg was derived), was used as a control for some analyses (Figure 1). All XX Tg2 animals present as males at weaning (Washburn et al. 2001).

Assessment of sexual phenotype in weaning-age mice: Animals were classified as weaning-age female, male, or hermaphrodite by the appearance of the external genitalia and by the presence of yellow pigmented hairs associated with the mammary glands. These pigmented hairs are present in B6 XX females, absent in B6 XY males, and present in most B6 XYPOS hermaphrodites and in all B6 XYPOS females (Eicher and Washburn 2001).

For histological analysis, gonads were dissected and fixed in Bouin’s fixative, embedded in paraffin, sectioned, and stained with hematoxylin and eosin using standard procedures.

Fetal gonad analysis: Fetuses were collected from overnight matings where noon on the day a vaginal plug was observed is designated day 0.5 or from timed early morning matings. For more precise staging of fetuses younger than E13.0 (E, embryonic day) the number of tail somites (ts) posterior to the hind-limb bud was determined: E10.5 is ~8 ts, E11.5 is ~18 ts, and E12.5 is ~30 ts (Hacker et al. 1995). E13.0–15.5 futzes were staged by fore-limb and hind-limb morphology (Theiler 1989).

To assess fetal gonad development morphologically, gonads with attached mesonephroi were dissected from E13.5–15.5 futzes and examined in whole mount using an inverted microscope and transmitted light. This developmental stage was chosen for analysis because a small amount of ovarian tissue is easily visualized in an ovotestis and after this stage the rapid growth of testicular tissue can obscure detection of ovarian tissue (Eicher et al. 1980). A tissue sample was saved from each fetus for genotype determination, as described below.

Genotyping: PCR was used to detect the presence of an SryPOS and/or SryDOM allele in genomic DNA using one of the following methods: (1) Primers Sry-8207 5′-AGATCTTGATT TTTAGTGTTC and Sry-8677 5′-GAGTACAGGTGTGCAGCT CTA were used to amplify a 470-bp DNA fragment (Gubbay et al. 1990) that was digested with MboI. Three fragments are diagnostic for SryDOM alleles (199, 187, and 84 bp) and two are diagnostic for SryPOS alleles (271 and 199; Eicher et al. 1995) and (2) primers Sry-9431 5′-TGTTGAGCATACACCATACC and Sry-9808 5′-TTGTGCTTCTTTGCTAGCC were used to amplify a 377-bp DNA fragment that, when digested with NalI, produces a 377-bp undigested fragment diagnostic for SryDOM alleles and two comigrating fragments diagnostic for SryPOS alleles (189 and 188 bp). The Y chromosome was detected by multiplex PCR using primers for the YMT/2B locus (5′-GTGGATCTTCTAATGTTAAG and 5′-CAGTTACAACTCAATCGATG and 5′-TGGGTCGCTGTAGTATAT) as a control (Capel et al. 1999).

The Sry129POS transgenes were detected by multiplex PCR using the YMT/2B and myogenin primers described above in conjunction with transgene specific primers (5′-GAGGGCAT GGTCAGTTGAAC and 5′-CTACGTCCATCATGATG and 5′-CAGTTACAACTCAATCGATG and 5′-TGGGTCGCTGTAGTATAT) as a control (Capel et al. 1999).

Genotyping was determined by semiquantitative PCR using B6 XYPOS transgenic genomic DNA and the Sry-9431 and Sry-9808 primers. The assay was similar to that described below for semiquantitative RT-PCR except that 20 PCR cycles were employed. The results from at least three independent DNA samples were averaged.

RT-PCR: Paired urogenital ridges or gonad/mesonephric complexes were dissected and nongonadal and nonmesonephric tissues were removed. The mesonephros was trimmed to the length of the gonad. The gonad and mesonephros were dissected apart in some later developmental stage samples. DNA was extracted from the dissected tissues using the RNaseasy mini kit (QIAGEN, Chatsworth, CA). Lysed tissue was stored at ~80° in RLT buffer (QIAGEN) until processed. The RNA was DNased during isolation using an on-column protocol (QIAGEN) or after elution from the column using the DNA-free protocol (Ambion, Austin, TX). After elution in 30 µl water, 2 µl of each RNA sample was tested for DNA contamination by PCR amplification (35 cycles) using the Sry-9431 and Sry-9808 primers. Any sample contaminated with DNA was reDNased, purified, and restested.

One-third of the RNA sample (10 µl) was reverse transcribed at 42° for 1 hr in a 20-µl reaction using the RNA PCR kit (Applied Biosystems, Foster City, CA). Parallel reactions were
performed, one with reverse transcriptase (+RT) and one without (−RT). A no-template (H₂O) negative control was included in each experiment. The reverse transcription (RT) reaction (2 μl) was PCR amplified with primers specific for the Hprt gene (5'-CCTGGTGGATCATAAAGCAGT and 5'-CTCAAGCCATATCCACAAAC) as a positive control for the presence of intact RNA (Koopman et al. 1989).

Semiquantitative RT-PCR was used to determine the relative expression of the Sry⁰⁰ (Sry⁰⁰) vs. the Sry¹²⁹ (Sry⁰⁰ or Sry¹²⁹) alleles (Bergstrom et al. 2000). The reverse transcription reaction (4 μl) was amplified by PCR in the presence of [α-³²P]dCTP using the Sry-9431 and Sry-9808 primers and restriction digested with NdelI. The resulting fragments were separated on 2% agarose gels and Southern blotted using standard methods. The amount of radioactivity in each band was determined using Phosphor imaging plates and Image Gauge software (Fuji Medical Systems USA, Stamford, CT).

Sry expression levels were compared to the expression levels of Lhx1 (JIM homeobox protein 1) using a semiquantitative RT-PCR assay. Lhx1 was chosen as the control because it is expressed only in the mesonephric component of the genital ridge and expression is relatively constant during the developmental stages analyzed (Barnes et al. 1994; Fujii et al. 1994; Nagamine et al. 1999). Lhx1-specific primers were designed to amplify a 139-bp fragment that spanned a region with no NdelI restriction sites and contained a 97-bp intron (Lhx1-1660 5'-GGCAGAGGCTCTACATCATAG and Lhx1-1798 5'-CTTGGGATCCGGAGATAAAC). The Lhx1 primers were combined with Sry-9431 and Sry-9808 in a multiplex PCR reaction containing [α-³²P]dCTP and 2 μl of the RT reaction. The PCR reaction was digested with NdelI, separated on 3% agarose gels, Southern blotted, and analyzed as outlined above.

The number of PCR cycles corresponding to the exponential amplification phase was determined empirically for each RT-PCR assay (data not shown). Twenty-seven cycles were used for the Sry-only assay and 29 cycles were used for the multiplex Sry/Lhx1 assays. PCR used 1.5 mM MgCl₂ and a 57° annealing temperature.

Statistical analysis: A two-way analysis of variance (ANOVA) was used to determine if there was a significant effect of fetal age, genetic background, or interaction of these two variables on Sry expression. Analyses were performed using In-transformed data to better meet the assumptions of ANOVA. Scheffé’s F was used for post-hoc multiple comparisons when the ANOVA identified a significant effect. All effects were evaluated using α = 0.05.

RESULTS

To assess if ovarian tissue development in B6 XY⁺⁺ mice and delayed cord development in B6 XY⁺⁺ mice are caused by insufficient Sry expression, we developed two Sry biallelic B6 lines: One line carried a Y chromosome containing the MUS-derived Sry¹²⁹ allele and the DOM-derived Sry⁺⁺ allele (B6-Y⁺⁺,RII), and the other line carried a Y chromosome containing the Sry¹²⁹ allele and the DOM-derived Sry⁺⁺ allele (B6-Y⁺⁺,AKR). We reasoned that these B6 lines would allow a direct comparison of the relative expression of two Sry alleles within the same gonad and therefore the results would be independent of the number of Sry-expressing cells. In addition, these Sry biallelic lines would allow us to determine if a single copy of a MUS-derived Sry allele corrected testis development in B6 XY⁺⁺ and B6 XY⁺⁺ mice. Previous experiments demonstrated that the presence of a multi-copy MUS-derived Sry¹²⁹ transgene restored normal testis development in B6-Y⁺⁺ mice (Eicher et al. 1995).

A single copy of Sry¹²⁹ corrects testis development in B6 XY⁺⁺ and B6 XY⁺⁺ mice: All B6 XY⁺⁺,RII and B6 XY⁺⁺,AKR mice presented as normal males at weaning. Moreover, gonad differentiation in both types of Sry biallelic fetuses was normal at E13.5–15.5. The 16 B6 XY⁺⁺,RII fetuses analyzed had two normal testes whereas the 14 B6 XY⁺⁺ control sibs had ovaries (N = 22 gonads) or ovotestes (N = 6 gonads; Figure 2). In addition, the 19 B6 XY⁺⁺,AKR fetuses analyzed had two normal testes whereas 10 of 11 B6 XY⁺⁺ control sibs had testes with delayed cord differentiation. (One B6 XY⁺⁺ fetus had normal testis cord differentiation.) We conclude that the presence of a single copy of an endogenous MUS-derived Sry allele is sufficient to rescue testis differen-
at a significantly lower level than \( \text{SryPOS} \) and is expressed in a similar manner. We conclude that \( \text{SryRIII} \) to \( \text{Sry} \) is expressed at a significantly lower level than \( \text{SryPOS} \) vs. \( \text{SryRIII} \). Semiquantitative RT-PCR was used to determine \( \text{E13.0} \): expression levels of gonads from B6 XYPOS,RIII and B6 XYAKR,RIII gonads to examine relative \( \text{Sry} \) expression in gonads destined to develop as normal testes.

\( \text{Sry}^{\text{POS}} \) (DOM) transcript levels are reduced compared to \( \text{Sry}^{\text{III}} \) (MUS) transcript levels between \( \text{E10.5} \) and \( \text{E13.0} \): Semiquantitative RT-PCR was used to determine the relative expression of \( \text{Sry}^{\text{POS}} \) vs. \( \text{Sry}^{\text{III}} \) in urogenital ridges dissected from \( \text{E10.5} \)–\( \text{E13.0} \) B6 XYPOS,RIII fetuses. \( \text{Sry} \) expression normally is first detectable at \( \sim \text{E10.5} \) (\( \sim \text{8-ts} \) stage), peaks at \( \sim \text{E11.5} \) (18-ts stage), and is absent by \( \sim \text{E13.0} \). (Koopman et al. 1990; Hacker et al. 1995; Jeske et al. 1995). As indicated in Figure 3, the mean ratio (\( \pm 95\% \) confidence interval) of \( \text{Sry}^{\text{POS}}:\text{Sry}^{\text{III}} \) was 0.59 \( \pm 0.04 \) during this time, indicating that \( \text{Sry}^{\text{POS}} \) is expressed at a significantly lower level than \( \text{Sry}^{\text{III}} \). Statistical analysis using ANOVA indicates that this difference is constant between \( \text{E10.5} \) and \( \text{E13.0} \) (\( P = 0.958 \)), implying that during this time \( \text{Sry}^{\text{POS}} \) and \( \text{Sry}^{\text{III}} \) are temporally regulated in a similar manner. We conclude that \( \text{Sry}^{\text{POS}} \) is expressed at significantly reduced levels relative to \( \text{Sry}^{\text{III}} \) in B6 mice.

\( \text{Sry} \) transcript level is affected by genetic background: B6 XYPOS fetuses develop ovaries or ovotestes whereas (\( \text{D2} \times \text{B6} \))F1 XYPOS fetuses develop testes (Eicher et al. 1982). If B6 XYPOS SR is caused by insufficient \( \text{Sry} \) expression, expression of \( \text{Sry} \) should be increased in (\( \text{D2} \times \text{B6} \))F1 XYPOS fetal gonads. We analyzed the relative expression of \( \text{Sry}^{\text{POS}} \) and \( \text{Sry}^{\text{III}} \) in urogenital ridges dissected from \( \text{E10.5} \)–\( \text{E13.0} \) (\( \text{D2} \times \text{B6} \))F1 XYPOS,RIII fetuses. If the expression of \( \text{Sry}^{\text{POS}} \) and \( \text{Sry}^{\text{III}} \) were increased equivalently, the ratio of \( \text{Sry}^{\text{POS}}:\text{Sry}^{\text{III}} \) would remain at 0.59. However, if the expression of one allele was increased relative to the other, the ratio would change. We found that the mean ratio (\( \pm 95\% \) confidence interval) of \( \text{Sry}^{\text{POS}}:\text{Sry}^{\text{III}} \) was \( 0.74 \pm 0.05 \) (Figure 3), indicating that \( \text{Sry}^{\text{POS}} \) is expressed at a significantly lower level than \( \text{Sry}^{\text{III}} \). The ANOVA indicates that this difference is constant from \( \text{E10.5} \) to \( \text{E13.0} \) (\( P = 0.958 \)). However, the ANOVA also indicates that the increased expression of \( \text{Sry}^{\text{POS}} \) on the F1 genetic background vs. the B6 background is significant (\( P < 0.0004 \)). We conclude that the expression of \( \text{Sry}^{\text{POS}} \) is increased relative to \( \text{Sry}^{\text{III}} \) on a hybrid genetic background.

\( \text{Sry}^{\text{POS}} \) expression is more sensitive than \( \text{Sry}^{\text{III}} \) to genetic background: To determine if the expression level of one or both \( \text{Sry} \) alleles is increased on the F1 background, the expression level of each allele and \( \text{Lhx1} \) were compared. The analysis was conducted using \( \text{E11.5} \) (16–20 ts) urogenital ridges because this is the time \( \text{Sry} \) normally is maximally expressed. As indicated in Figure 4, expression of both \( \text{Sry}^{\text{POS}} \) and \( \text{Sry}^{\text{III}} \) was increased relative to \( \text{Lhx1} \) in 16- to 18-ts gonads from F1 XYPOS,RIII compared to gonads from B6 XYPOS,RIII fetuses. The ANOVA indicates that the difference between the B6 and F1 genetic backgrounds is significant (\( P < 0.003 \)). This result, coupled with the finding that the ratio of \( \text{Sry}^{\text{POS}}:\text{Sry}^{\text{III}} \) was increased to 0.74 in F1 fetal gonads, suggests...
that Sry\textsuperscript{pos} is more sensitive to Sry\textsuperscript{ani} to genetic background. (These data also confirm that Sry\textsuperscript{pos} is expressed at lower levels than Sry\textsuperscript{ani}.) Additionally, the data suggest that peak Sry expression occurs at an earlier developmental stage (18 ts vs. 19 ts, or ~2 hr) in the F\textsubscript{1} background. Whether this small difference in timing is significant is unknown.

\textit{Sry\textsuperscript{AKR} and Sry\textsuperscript{ani}} are expressed at equivalent levels in E10.5–13.0 gonads: In contrast to B6 XY\textsuperscript{pos} gonads, B6 XY\textsuperscript{AKR} gonads develop as normal testes, but have delayed testis cord differentiation. The relative expression of Sry\textsuperscript{AKR} vs. Sry\textsuperscript{ani} was determined by semiquantitative RT-PCR using RNA from E10.5–13.0 B6 XY\textsuperscript{AKR,ani} urogenital ridges. The mean ratio (±95% confidence interval) of Sry\textsuperscript{AKR}:Sry\textsuperscript{ani} is 1.02 ± 0.1 (Figure 3), indicating that Sry\textsuperscript{AKR} and Sry\textsuperscript{ani} are expressed at equivalent levels. As indicated by the ANOVA, the relative expression ratio was constant throughout this time (P = 0.958). We conclude that Sry\textsuperscript{AKR} and Sry\textsuperscript{ani} are expressed at essentially equivalent levels and are similarly regulated temporally. These results suggest that delayed testis cord development in B6 XY\textsuperscript{AKR} fetuses is not caused by insufficient or delayed Sry expression.

\textit{Sry\textsuperscript{pos} expression is lower than Sry\textsuperscript{ani} expression:} Recent data indicated that Sry\textsuperscript{pos} (MUS) is expressed at lower levels than Sry\textsuperscript{ani} (DOM; Lee and Taketo 2001). We assayed the relative expression of Sry in E11.5 (18-ts stage) B6 XY\textsuperscript{pos} and B6 XY\textsuperscript{AKR} gonads using Lhx1 as a control. The average Sry:Lhx1 ratio was 0.95 in B6 XY\textsuperscript{AKR} gonads (N = 5 gonad pairs) and 0.58 in B6 XY\textsuperscript{pos} gonads (N = 10 single gonads). (D2 × B6)\textsubscript{F\textsubscript{1}} XY\textsuperscript{pos} gonads (N = 16 single gonads) the average Sry:Lhx1 ratio was 0.43. Thus, the expression of Sry\textsuperscript{pos} (MUS), like that of Sry\textsuperscript{ani} (MUS), is less sensitive to genetic background than the expression of Sry\textsuperscript{pos} (DOM). Our results confirm those of Lee and Taketo (2001) and emphasize the fact that Sry alleles are expressed at distinct levels.

\textit{Sry\textsuperscript{pos} transcripts are present at later developmental stages than Sry\textsuperscript{ani}:} Previous results indicated that expression of Sry persisted longer in B6 XY\textsuperscript{ani} gonads, which develop abnormally, than in B6 XY\textsuperscript{pos} (Lee and Taketo 1994) and B6 XY\textsuperscript{pos} (Nagamine et al. 1999) gonads, which develop normally. (\textit{Y\textsuperscript{pos}} is DOM derived.) However, in an \textit{F\textsubscript{1}} hybrid background where XY\textsuperscript{ani} gonads develop into normal testes, Sry\textsuperscript{ani} expression was not prolonged. Between E10.5 and E13.0 Sry\textsuperscript{pos} is present at ~60% of the Sry\textsuperscript{ani} level in B6 XY\textsuperscript{ani,ani} gonads. However, after E13.0, the situation is reversed and Sry\textsuperscript{pos} is present at higher levels than Sry\textsuperscript{ani}. For example, Sry\textsuperscript{pos}, Sry\textsuperscript{ani}, and Lhx1 transcript levels were determined by RT-PCR in seven E13.5 B6 XY\textsuperscript{pos,ani} gonads with attached mesonephroi (three pairs and four single complexes). The average Sry\textsuperscript{pos},Lhx1 ratio was 0.03 whereas the average Sry\textsuperscript{ani},Lhx1 ratio was 0.007, indicating that at this stage Sry\textsuperscript{pos} is present at about four times the level of Sry\textsuperscript{ani}. These data suggest that expression of Sry\textsuperscript{pos} persists longer than expression of Sry\textsuperscript{ani} in B6 XY\textsuperscript{pos,ani} gonads.

\textit{Transgenic overexpression of Sry\textsuperscript{pos} rescues testis determination:} The comparative Sry expression results suggested that B6-Y\textsuperscript{pos} SR is caused, at least in part, by insufficient Sry\textsuperscript{pos} expression. If this hypothesis is correct, transgenic overexpression of Sry\textsuperscript{pos} in B6 XX mice should initiate normal testis determination. Two different Sry\textsuperscript{pos} transgenic constructs were employed to test this hypothesis (Figure 1). The first was a genomic DNA clone isolated from the \textit{M. d. poschiavinus} Y chromosome. Analyses of three B6 transgenic lines (Tgs 83–85, Table 2) carrying this construct (Sry\textsuperscript{pos}) are presented. The second construct was derived from the original 14.6-kb Sry clone but contained the DOM Sry\textsuperscript{pos} ORF in place of the MUS Sry\textsuperscript{ani} ORF so that expression of Sry\textsuperscript{pos} was controlled by MUS-derived regulatory regions. Analyses of four B6 transgenic lines (Tgs 17, 28, 94, and 121, Table 2) carrying this construct (Sry\textsuperscript{ani}) are presented.

### TABLE 2

<table>
<thead>
<tr>
<th>Transgene designation</th>
<th>B6</th>
<th>(D2 × B6)</th>
<th>(C3H × B6)</th>
<th>Tg copy number</th>
<th>Expression level (Tg:B6)</th>
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</thead>
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<tr>
<td>Sry\textsuperscript{pos}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg85</td>
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<td>NA</td>
<td>17</td>
<td>3:1</td>
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<td>0 (N = 84)</td>
<td>80 (23:30:20:9:5)</td>
<td>83 (1:2:5:9:7:5)</td>
<td>3</td>
<td>1:1</td>
</tr>
<tr>
<td>Tg84</td>
<td>0 (N = 71)</td>
<td>0 (23:30:20:9:5)</td>
<td>61 (7:11:9:7:5)</td>
<td>9</td>
<td>2:1</td>
</tr>
<tr>
<td>Sry\textsuperscript{ani}</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tg94</td>
<td>100 (N = 33)</td>
<td>NA</td>
<td>ND</td>
<td>11</td>
<td>5:1</td>
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<td>Tg17</td>
<td>56 (20:19:2:4:5)</td>
<td>ND</td>
<td>ND</td>
<td>12</td>
<td>ND</td>
</tr>
<tr>
<td>Tg28</td>
<td>9 (49:199:4:4)</td>
<td>53 (8:19:2:4:5)</td>
<td>ND</td>
<td>14</td>
<td>ND</td>
</tr>
<tr>
<td>Tg121</td>
<td>18 (37:199:7:7)</td>
<td>ND</td>
<td>ND</td>
<td>8</td>
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</tbody>
</table>

NA, not applicable because 100% of B6 XX Tg offspring are sex reversed. ND, not determined.
Figure 5.—Both Tg85 and Tg94 are overexpressed relative to the endogenous SryB6 allele in E11.5 urogenital ridges. Representative radioactive semiquantitative RT-PCR results. Lanes labeled + and − represent samples with and without reverse transcriptase, respectively. Tg85 is expressed three times and Tg94 five times greater than the endogenous SryB6 allele.

At weaning, 75% of B6 XYPOS mice present as normal females and 25% present as hermaphrodites (Eicher and Washburn 2001). In contrast, for both the SryPOS and Sry129POS constructs, one transgenic line from each was identified in which all XX transgenic (XX Tg) mice presented as normal males at weaning (SryPOS-Tg85 and Sry129POS-Tg94). Histological examination of testes from three XX Tg85 and seven XX Tg94 adult males demonstrated the presence of Sertoli and Leydig cells, lack of ovarian tissue, and absence of germ cells (data not shown). (The absence of germ cells is expected in XX males due to the lack of a Y chromosome and presence of two X chromosomes.)

We then examined gonads from B6 XX Tg85 and XX Tg94 E14.5–15.5 fetuses to determine if ovarian tissue was present during fetal development. All XX Tg85 (N = 24) and XX Tg94 (N = 26) gonads developed testicular tissue exclusively (Figure 2). In contrast, ovarian tissue is readily visible in all gonads from E14.5–15.5 B6 XYPOS fetuses (Eicher and Washburn 2001).

Semi quantitative RT-PCR analysis revealed that Tg85 and Tg94 were overexpressed relative to SryB6 in B6 XY Tg fetal gonads at the 18-ts stage (E11.5), the timepoint when Sry is normally maximally expressed: Tg85 was expressed threefold greater and Tg94 fivefold greater than the endogenous SryB6 allele (Figure 5). We conclude that overexpression of SryPOS allows normal testes to develop in E14.5 B6 fetuses.

Testis cord development is delayed in XX Tg85 and XX Tg94 fetal gonads: At E14.5–15.5 B6 XYAKR fetal gonads are normal appearing testes but at developmental stages prior to E14.5, testis cord development is delayed relative to B6 XYB6 gonads. We examined testis cord differentiation in E13.5 XX Tg85 and XX Tg94 fetuses to determine if testis development was normal. Similar to B6 XYAKR, all XX Tg85 (N = 10) and XX Tg94 (N = 14) gonads had delayed testis cord development (Figure 6).

Because delayed testis cord development could be caused by delayed initiation of transgene expression, we assayed transgene expression in urogenital ridges from fetuses at E10.5, the time when endogenous Sry expression is first initiated (Koopman et al. 1990; Hacker et al. 1995; Jeske et al. 1995). Tg94 transcripts were detected in 7-ts-stage XY Tg fetuses whereas endogenous Sry transcripts were not yet detectable (three pairs of urogenital ridges, 35 PCR cycles). Tg94 expression was clearly higher than in the endogenous SryB6 in the 11-ts XY Tg sample. We conclude that testis cord development is delayed in XX Tg85 and XX Tg94 fetal gonads despite normal transcriptional initiation and overexpression of the transgenes.

Because the Sry Tg constructs might be missing regulatory elements necessary for the initiation of normal, nondelayed testis cord development, we examined testis cord development in E13.5 B6 XX Tg fetuses from an Sry transgenic line, Tg2, carrying an intact 14.6-kb Sry129 (MUS) Tg. As shown in Figure 6, in contrast to the delayed testis cord development observed in E13.5 B6 XX Tg85 (DOM), B6 XX Tg94 (DOM), and B6 XYAKR
fetuses, testis cord development in E13.5 B6 XX Tg2 (MUS) and B6 XYSR Tg2 fetuses was complete (N = 12 gonads). Because the Tg2 and Tg94 constructs contain the same MUS-derived regulatory regions, we conclude that delayed testis cord development in B6 XX Tg94 fetuses is not caused by the absence of a critical regulatory region(s).

**External sexual phenotype of XX Tg mice and transgene expression level are sensitive to genetic background:** Of the seven Sry transgenes analyzed, Tg83 and Tg94 were the only ones in which 100% of the B6 XX Tg offspring were completely sex reversed (Table 2). In contrast, at weaning 56% of B6 XX Tg17 mice, 9% of B6 XX Tg28 mice, and 18% of B6 XX Tg121 mice presented as male. No B6 XX Tg83 (N = 84) or B6 XX Tg84 (N = 71) mice presented as males.

Because the XX Tg females are fertile, we intercrossed hemizygotes from the Tg28, Tg83, and Tg84 lines to determine if these transgenes caused XX SR when homozygous. Insertion of the transgene created recessive lethal mutations in the Tg28 and Tg83 lines (as suggested by underrepresentation of transgenic offspring in the intercross) so that the phenotype of Tg homozygotes could not be examined. From Tg84 intercrosses, two XX Tg SR males were present among the 37 XX Tg offspring. Because known XX Tg84/ + mice are not sex reversed, we conclude that two copies of Tg84 can cause XX sex reversal. The homozygous phenotypes for Tg17 and Tg121 were not examined because B6 XX Tg heterozygotes are sometimes sex reversed.

Because B6-YPOS SR is highly sensitive to genetic background, we produced F1 hybrid Tg mice by mating B6 Tg carriers to D2 and C3H/HeSnJ (C3H) mice and examined the external sexual phenotype of XX Tg mice at weaning (Table 2). In the three transgenic lines tested (both SryPOS and Sry129POS), the phenotype of XX Tg mice was modulated by genetic background. For example, at one extreme, 81% (13/16) of the (C3H or D2 × B6)F1 XX Tg83 mice presented as males whereas all (N = 84) B6 XX Tg83 mice presented as females. For Tg84, different F1 hybrid backgrounds gave different results: All 23 (D2 × B6)F1 XX Tg84 mice were female whereas 7 (C3H × B6)F1 XX Tg84 mice were female and 11 were male. Surprisingly, none of the F1 XX Tg84 mice were obvious hermaphrodites. We conclude that the external sexual phenotype and, by inference, testis determination of XX Tg mice is sensitive to genetic background.

Semiquantitative RT-PCR was used to determine if an increase in transgenic RNA transcript levels correlated with sex reversal in F1 XX Tg mice. We analyzed (D2 × B6) Tg83 E11.5 gonads because Tg83 seemed to be the most sensitive to genetic background. Tg83 expression was compared to Lhx1 expression in gonads from 16- to 21-ts fetuses. As illustrated in Figure 7, initial (16- to 17-ts) expression was similar in both backgrounds. However, at the 18- to 21-ts developmental stage, Tg83 expression was increased in the F1 background. The data presented in Figure 7 represent average expression, and not all of the XX Tg 83 gonads are destined to develop as testes. Therefore, the difference in expression between the B6 and F1 genetic backgrounds probably is greater than represented. The number of gonads analyzed for each stage is indicated.

**DISCUSSION**

Transfer of certain *M. domestica*s-derived (DOM) Y chromosomes (*SryDOM* alleles) onto specific inbred strains, such as B6, causes abnormal testis determination (Eicher et al. 1982; Eicher and Washburn 1986; Nagamine et al. 1987; Biddle and Nishioka 1988). The degree of abnormality depends on the particular Y chromosome transferred. The experiments presented were designed to elucidate the mechanism of *SryDOM* misfunction when present on the B6 genetic background.

To determine if DOM Sry alleles are expressed at different levels or in different temporal patterns from those of MUS Sry alleles, we developed two B6 mouse lines that each carry a single DOM Sry allele (POS or AKR) and a single MUS Sry allele (RII). Gonads in B6 XYSR and B6 XYAKRII mice are phenotypically normal testes. This finding confirms and extends results demonstrating that transgenic overexpression of a MUS Sry129 allele rescues SR in B6-YPOS mice (Eicher et al. 1995) and delayed testis cord development in B6-YAKRII mice (data presented here). Also, the data indicate that the DOM SryPOS and SryAKR alleles do not act as dominant-negative
alleles. It is interesting to note that, to our knowledge, all Sry transgenic lines capable of sex reversing XX mice contain multiple insertions of the Sry gene (Koopman et al. 1991; Eicher et al. 1995; Washburn et al. 2001). The reasons for this are not clear, but it may be that expression of a transgene is dependent on the chromosomal site of insertion and on the presence of critical cis-acting regulatory elements. In the case of the B6 XY<sup>POS</sup>R<sup>III</sup> and B6 XY<sup>AKR</sup>R<sup>III</sup> mice, a single copy of a MUS-derived Sry gene was present and able to correct testicular abnormalities. We suggest that the reason this single copy of Sry functioned normally was that it was present on a segment of the mouse Y chromosome that normally contains it.

If the misexpression hypothesis is correct, expression of the Sry<sup>POS</sup> allele should be more “abnormal” than that of the Sry<sup>AKR</sup> allele. This was, in fact, the case: On the B6 background, the DOM Sry<sup>POS</sup> allele was expressed at ∼59% of the MUS Sry<sup>RII</sup> allele whereas the DOM Sry<sup>AKR</sup> allele and the MUS Sry<sup>RII</sup> allele were expressed at equal levels. Moreover, if the misexpression hypothesis is correct, expression of Sry<sup>POS</sup> would be more “normal” on a hybrid genetic background known to rescue B6-Y<sup>POS</sup> sex reversal. This, too, was the case: The Sry<sup>POS</sup> allele was expressed at ∼74% of the MUS Sry<sup>RII</sup> allele on a (D2 × B6)F<sub>1</sub> genetic background. Because relative Sry expression was measured in genital ridges destined to develop as normal gonads and independent of the number of Sry-expressing cells, we conclude that Sry expression per cell is reduced. The results, however, do not exclude the possibility that the number of Sry-expressing cells also is reduced.

The fact that the relative expression of Sry<sup>POS</sup>/Sry<sup>RII</sup> and Sry<sup>AKR</sup>/Sry<sup>RII</sup> was constant between E10.5 and E13.0 suggests that the temporal expression of DOM and MUS alleles is similar during this time. Therefore, it is unlikely that delayed Sry expression is responsible for either SR in B6 XY<sup>POS</sup> gonads or delayed testis development in B6 XY<sup>AKR</sup> gonads. These results are consistent with those of Lee and Takeo (1994) and Nagamine et al. (1999).

After E13.0, expression of Sry<sup>POS</sup> persisted longer than expression of Sry<sup>RII</sup>. This result implies that Sry<sup>POS</sup> expression is downregulated more slowly than Sry<sup>RII</sup> expression. However, we cannot exclude the possibility that the Sry<sup>POS</sup> transcript is more stable than the Sry<sup>RII</sup> transcript. We suggest that if persistent expression is due to inefficient downregulation of Sry<sup>POS</sup> expression, then the same regulatory elements that prevent efficient upregulation of Sry<sup>POS</sup> expression may be identical to those that prevent efficient downregulation.

The relative expression results were confirmed by measuring expression of the individual Sry alleles against expression of a control gene (Lhx1). These data indicated that expression of both the DOM and MUS Sry alleles was increased on the hybrid genetic background, but the expression of the DOM allele was increased to a greater extent. This result suggests that the Sry<sup>POS</sup> allele is more sensitive to genetic background than the Sry<sup>RII</sup> allele. It is likely, therefore, that at least one tda gene affects Sry expression and that this interaction is direct. The simplest model is that one or more tda genes is a transcription factor that controls Sry transcription by directly interacting with the Sry promoter. However, other models are possible. For example, a tda gene could interact with the Sry transcript and affect its stability or localization. Further functional studies are needed to test these models.

We found that in B6 XY<sup>AKR</sup>R<sup>III</sup> fetal gonads the DOM Sry<sup>AKR</sup> and MUS Sry<sup>RII</sup> alleles were expressed at equal levels. The question of whether the Sry<sup>RII</sup> allele initiates normal testis determination is complicated by the fact that we analyzed testis development in XX<sup>SR</sup> fetuses where random X inactivation can affect the expression of the Sry<sup>RII</sup> allele. However, 32 of the 40 B6 XX<sup>SR</sup> gonads examined between E13.25 and E14.5 were normal testes without delayed testis cord development. (The remaining 8 gonads were ovotestes.) This result suggests that in the absence of significant inactivation of the X<sup>SR</sup> chromosome, the Sry<sup>RII</sup> expression level is sufficient to initiate normal testis development on the B6 background. Because Sry<sup>AKR</sup> and Sry<sup>RII</sup> are expressed at equivalent levels yet B6 XY<sup>AKR</sup> gonads have delayed testis cord development, delayed testis cord development cannot be attributed solely to insufficient Sry<sup>AKR</sup> expression. Rather, delayed testis cord development probably is caused by reduced translation of the Sry<sup>AKR</sup> transcript, by reduced stability of the SRY<sup>AKR</sup> protein isoform, or by reduced ability of the SRY<sup>AKR</sup> protein isoform [which is approximately half the size of the MUS SRY protein isoform (Coward et al. 1994)] to initiate testis development. These results are consistent with the finding that Sry<sup>POS</sup> (MUS) is expressed at lower levels than Sry<sup>AKR</sup> yet testis development in B6 XY<sup>SR</sup> mice is normal (Lee and Takeo 1994 and results herein). Furthermore, these results suggest that at least one tda gene participates in the sex determination cascade downstream of or in parallel with Sry.

Overall, the Sry expression analysis indicates that B6-Y<sup>POS</sup> SR is caused by insufficient Sry<sup>POS</sup> expression and that delayed testis cord development in B6 XY<sup>AKR</sup> mice is caused by reduced efficiency of the SRY<sup>AKR</sup> isoform. If this model is correct, then overexpression of Sry<sup>POS</sup> in B6 mice would rescue SR but might not rescue delayed testis cord development. Two different transgenic constructs were used to test this hypothesis: an Sry<sup>POS</sup> genomic DNA clone and a chimeric construct in which expression of the Sry<sup>POS</sup> ORF was controlled by MUS regulatory regions (Sry<sup>POS</sup>MUS). Two B6 transgenic lines, one from each type of construct, were established in which all XX transgenic progeny developed testes. However, testis cord development was delayed in both lines despite overexpression of Sry<sup>POS</sup> and normal transcriptional initiation from the transgenes. These results suggest that testes develop when Sry<sup>POS</sup> is expressed at
relatively high levels; however, overexpression is not sufficient to correct delayed testis cord development. The transgenic results support a model where delayed testis cord development is caused by the presence of particular DOM SRY protein isoforms that cause SR when underexpressed. The fact that (D2 × B6)F1, XYPOS fetuses develop normal testes without evidence of delay (Eicher et al. 1996) indicates that delayed testis cord development requires that at least one tda gene be homozygous for the B6 allele.

To our knowledge, all SryDOM ORFs analyzed have a stop codon in the glutamine repeat region downstream of the HMG box, which means that SRYDOM proteins are about half the size of SRYMus proteins (Coward et al. 1994; Carlisle et al. 1996; Albrecht and Eicher 1997). However, the predicted “half-size” SRYDOM protein isoform alone is not sufficient to account for either sex reversal or delayed testis development when present on the B6 genetic background because some SryDOM alleles, such as SryYAB and SryYPB, initiate normal testis development when on the B6 background (Biddle and Nishihora 1988; Nagamine et al. 1999; Washburn et al. 2001). Different SryDOM protein isoforms that differ in the number of glutamines encoded by the third glutamine repeat cluster (GRC-3) have been identified. No correlation is found between the number of glutamines in GRC-3 and sex reversal (Carlisle et al. 1996; Albrecht and Eicher 1997). However, it is possible that the number of glutamines in GRC-3 plays a role in whether a given SryDOM allele causes delayed testis development when on the B6 background. SryYAB, which causes delayed testis development, has 13 glutamines in GRC-3 while SryYPD and SryYPB, which initiate normal testis development, have 12. The situation is complicated by the fact that different Sry alleles are expressed at different levels. For example, the SryYAB allele is expressed at higher levels than the SryYAB allele is (Nagamine et al. 1999).

Not all of the transgenes produced exclusively male XX Tg progeny, and for several the percentage of male XX Tg progeny was increased on a hybrid genetic background. For the one transgene examined, the increase in male XX Tg progeny was correlated with increased expression of the Sry transgene. The results indicate that the transgenes contain a DNA element that controls Sry expression level and is sensitive to genetic background. We suggest that this element is likely to directly interact with a tda gene. Furthermore, this control element is present in the region of minimal overlap between the two types of transgenes (i.e., between ~2355 bp and ~14,625 bp). Future experiments are focused on identifying the Sry expression control element.

We are intrigued by the finding that all (D2 × B6)F1, XX Tg84 mice are female whereas approximately half of the (C3H × B6)F1, XX Tg84 mice are female and the remainder are male. This result nicely illustrates the fact that sex determination in mice is exquisitely sensitive to genetic background. We do not know if the difference between the D2 and C3H inbred strains is due to different alleles of the tda genes previously mapped or to differences in novel tda genes. Molecular identification of the tda genes will clarify this.

As noted in the Introduction, several intriguing but unexplained SR conditions are found in humans, including XY females and XY hermaphrodites who carry an apparently normal SRY gene and XY females who carry a mutated SRY gene inherited from their carrier father. We hypothesize that these human SR conditions are like B6-YPOS SR and are caused by conditionally insufficient SRY expression. Therefore, it is possible that the human homologs of tda genes implicated in B6-YPOS SR play a role in these and other human SR conditions.

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