Low Levels of Sry Transcripts Cannot Be the Sole Cause of B6-Y\textsuperscript{TIR} Sex Reversal

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Summary: Sry, a single-copy gene on the Y-chromosome, triggers the fetal gonad to begin testis differentiation in mammals. On the other hand, mutation or absence of Sry results in ovary differentiation and the female phenotype. However, cases of XY sex reversal in the presence of wild-type Sry exist in mice and man. One such example is the B6-Y\textsuperscript{TIR} mouse, whose autosomes and X-chromosome are from the C57BL/6J mouse (an inbred strain of Mus musculus molossinus), whereas the Y-chromosome is from a Mus musculus domesticus mouse originating in Tirano, Italy. The B6-Y\textsuperscript{TIR} mouse never develops normal testes and instead develops ovaries or ovotestes in fetal life. It has been suggested that low levels of Sry transcription may account for the aberrant testis differentiation in the B6-Y\textsuperscript{TIR} mouse. In this study, however, we observed relatively low levels of Sry transcripts not only in B6-Y\textsuperscript{TIR} but also in B6 mice, which develop normal testes. We conclude that low dosage of Sry transcripts cannot be the sole cause of sex reversal in the B6-Y\textsuperscript{TIR} gonad. genesis 30:7–11, 2001.

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Key words: Sry transcription; sex determination; XY sex reversal; testis

In eutherian mammals, sexual differentiation involves three hierarchical steps. First, genetic sex is determined by the sex chromosome that the sperm contributes to the zygote. Second, the bipotential gonadal primordium begins differentiation into a testis or an ovary according to its genetic sex. Third, phenotypic sex develops depending on the presence or absence of two testicular hormones: testosterone and Müllerian-inhibiting substance (MIS or AMH). Sry (sex-determining region of the Y) on the Y-chromosome dominantly triggers differentiation of a gonadal primordium into a testis, whereas its mutation or absence results in differentiation of an ovary (Berta et al., 1990; Gubbay et al., 1990; Sinclair et al., 1990; Koopman et al., 1991). Cases of XY sex reversal in the presence of wild-type Sry also exist in mice and man. When Y-chromosomes of certain Mus musculus domesticus variants are placed onto the C57BL/6J (B6) genetic background, the XY progeny (named B6-Y\textsuperscript{DOM} as a group or B6-Y\textsuperscript{POS}, B6-Y\textsuperscript{TIR}, etc. for specific M. m. domesticus variants) develop only ovaries or ovotestes in fetal life (Eicher et al., 1982; Nagamine et al., 1987; Biddle and Nishioka, 1988). These observations suggest that coordination between the Y-chromosome and autosomal genes, named testis-determining autosomal (tda), is critical for testis determination. Coordination most likely involves Sry, since a molossinus-type Sry transgene rescues B6-YPOS mice from sex reversal (Eicher et al., 1995). Sequence polymorphisms have been identified in the Sry open reading frame as well as regulatory regions among mouse strains (Albrecht and Eicher, 1997; Coward et al., 1994; Graves and Erickson, 1995). It was conceivable that structural differences of the Sry protein might result in variable coordination with tda. However, no correlation between a particular polymorphism and sex reversal has been identified (Carlisle et al., 1996; Albrecht and Eicher, 1997). As to the B6 genetic background, candidate tda\textsubscript{1}, tda\textsubscript{2}, and tda\textsubscript{3} genes have been localized to mouse chromosomes 2, 4, and 5, respectively (Eicher et al., 1996). The molecular mechanism of sex reversal in the B6-Y\textsuperscript{DOM} mouse is yet to be elucidated.

We have previously reported normal onset of Sry transcription in the B6-Y\textsuperscript{TIR} gonadal primordium (Lee and Taketo, 1994). In contrast, onset of MIS, 17\alpha-hydroxylase, and 3\beta-hydroxysteroid dehydrogenase transcription (all involved in testis differentiation) as well as downregulation of Sry were delayed or absent in the B6-Y\textsuperscript{TIR} fetal gonad. We speculated that the testis-determining pathway is impaired after initiation of Sry transcription in the B6-Y\textsuperscript{TIR} gonad. However, we could not exclude the possibility that the Sry transcription level may be suboptimal for testis determination since our RT-PCR method was not quantitative. Nagamine et al. (1999) have reported that the level of Sry transcripts in the B6-Y\textsuperscript{TIR} gonad is, indeed, lower than in other non-sex-reversed B6-Y\textsuperscript{DOM} mouse strains. In our study, we compared the levels of Sry transcripts in fetal gonads of B6 and B6-Y\textsuperscript{TIR} as well as other mouse strains. In agreement with the data of Nagamine et al. (1999), the level of Sry transcripts in the B6-Y\textsuperscript{TIR} gonad was lower than in

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the B6-YSJL gonad that was not sex reversed. However, the level in the B6 gonad carrying its own Y-chromosome was as low as in the B6-YTIR gonad. We conclude that the low level of Sry transcripts cannot be the sole cause of B6-YTIR sex reversal.

Sry transcript levels were determined in each gonadal primordium at 11.5 days postcoitum (d.p.c.) by the primer-dropping method of semiquantitative RT-PCR (sqRT-PCR) (Wong et al., 1994). After cDNA synthesis, each sample was first amplified with primers specific to Sry for nine PCR cycles, and then primers for β-actin (internal standard) were introduced into each PCR reaction. The primer-dropping method decreases the competitive advantage of the more abundant template (β-actin) over the less abundant one (Sry) for PCR amplification. Amplification was continued for further 28 and 36 cycles for each fetal gonad. Examples of sqRT-PCR results are depicted in Figure 1. Comparative analyses between gonads and the different mouse strains examined in this study and their gonadal sex are listed in Table 1. Three classes of M. m. domesticus variants have been identified based on their Y-chromosomes' abilities to induce testicular differentiation in the B6 background (Biddle and Nishioka, 1988). In the first class, exemplified by FVB/N and SJL/J, all B6-YDOM mice develop as normal, fertile males. In the second class, transient sex reversal occurs with the Y-chromosome of the AKR/J (AKR) strain, in that some B6-YAKR mice develop ovotestes during fetal life but fertile testes by puberty. In the third class, the most severe and permanent sex reversal occurs with the Y-chromosome derived from M. m. domesticus variants found in Tirano, Italy, or Poschiavo, Switzerland. All B6-YTIR and B6-YPO mice develop ovaries or ovotestes during fetal life (Eicher et al., 1982; Nagamine et al., 1987; Biddle and Nishioka, 1988). When the YTIR chromosome is reintroduced onto an M. m. domesticus background (e.g., SJL), all mice develop normal testes in the first backcross generation (= F1). Thus, Sry transcript levels from the various Y-chromosomes can be compared in normal testes without involving sex reversal. As controls, the Y-chromosome of B6 on its “normal” and F1 genetic backgrounds (= B6-YB6 and SJLB6F1-YB6) were examined.

Nagamine et al. (1999) have reported that the level of Sry transcripts reaches a peak at 15–16 tail somite (t.s.) stage, which is typically seen at 11.5 d.p.c. Accordingly, we compared Sry transcript levels at 15–16 t.s. stage among mouse strains carrying various YDOM-chromosomes on the B6 background. Levels of Sry transcripts were the highest in B6-YSJL, intermediate in B6-YAKR, and the lowest in B6-YTIR mice (Fig. 2A). This order corresponds to the extent of sex reversal, in agreement with the data of Nagamine et al. (1999). While the level in B6-YSJL mice was significantly greater than in B6-YTIR and B6-YAKR (P < 0.001) mice, there was no significant difference between B6-YTIR and B6-YAKR mice. Unexpectedly, the lowest level among the different groups was found in B6-YB6 mice, which develop normal testes. This result suggests that the low level of SryB6 transcripts is sufficient to initiate testicular development in the B6 background.

Next, we compared Sry transcript levels between B6-YB6 and B6-YTIR fetal gonads at various developmental stages, covering 10.5, 11.5, and 12.0 d.p.c., which corresponded to 6–7, 13–20, and 21–26 t.s., respectively. In the B6-YB6 gonad, the Sry transcript level reached a peak at 15–16 t.s. stage and gradually declined thereafter (Fig. 3). In the B6-YTIR gonad, the peak level was also detected at 15–16 t.s. stage, but these maximal levels persisted up to 23–26 t.s. stage, the latest developmental stage examined. The difference in Sry transcript levels between B6-YB6 and B6-YTIR gonads at 23–26 t.s. stage was statistically significant (P < 0.001). These results are some-

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**Table 1**

<table>
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<th>Mouse strain</th>
<th>Gonad morphology</th>
<th>Fetus</th>
<th>Postnate</th>
<th>Sex reversal</th>
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</tr>
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*The strains used in Nagamine et al. (1999) but not in this study.
**The strains used in both this study and Nagamine et al. (1999).
what different from the data of Nagamine et al. (1999), who detected a peak level of Sry transcripts in the B6-Y<sup>TR</sup> gonad at 19–20 t.s. stage and a decline thereafter. This difference may be attributable to the employment of different internal standards (β-actin versus Lim1). β-actin is ubiquitously expressed, while Lim1 is expressed in mesonephric tubules and ducts (Barnes et al., 1994). Our current results are consistent with our previous study that employed conventional RT-PCR and relied on the frequency of transcripts detection (Lee and Taketo, 1994). Overall, Sry transcription of B6 and TIR alleles in the B6 background appears to follow comparable time courses except for downregulation. We conclude that sex reversal in the B6-Y<sup>TR</sup> gonad cannot be solely attributed to low levels of Sry transcripts at any developmental stages examined.

We then compared Sry transcript levels on the SJLB6F1 background (Fig. 2B). All F1.XY progeny de-
velop normal testes in pre- and postnatal life. While the level from the Sry\textsuperscript{SUr} allele in the F1 background was as high as in the B6 background, the level from the Sry\textsuperscript{TIR} allele increased to an intermediate value in the F1 background compared with the low value in the B6 background. These results also agree with the data of Nagamine et al. (1999). However, the level from the Sry\textsuperscript{B6} allele was the lowest among the three alleles examined in the F1 background and as low as in the B6 background. These observations further support our hypothesis that the low level of Sry transcripts from the B6 allele is sufficient to initiate testicular differentiation. On the other hand, dosage of Sry transcripts from the TIR allele may be more critical for normal testis determination.

Polymorphisms have been documented in the Sry open reading frame in M. m. subspecies (Coward et al., 1994; Carlisle et al., 1996; Albrecht and Eicher, 1997). The most prominent polymorphism is an earlier stop codon in M. m. domesticus, which results in a shorter stretch of glutamine/histidine (Q/H) repeats in the carboxy terminus, compared with M. m. molossinus. It has been shown that polyQ/H regions of transcription factors are involved in interacting with other components important for transactivation (Perutz et al., 1994; Dubin et al., 1996). Furthermore, the carboxy region of the Sry transgene is essential for inducing testicular differentiation in XX embryos (Bowles et al., 1999). By comparing Sry sequence polymorphisms among the different M. m. domesticus variants, Coward et al. (1994) and Albrecht and Eicher (1997) concluded that the cause of sex reversal cannot be explained by Sry polymorphisms alone. We hypothesize that the longer form of Sry\textsuperscript{B6} is more efficient than the shorter form of Sry\textsuperscript{DOM} in initiating testis differentiation. Therefore, low dosages of Sry\textsuperscript{B6} are sufficient, whereas greater levels of Sry\textsuperscript{SUr} are required for effective initiation of testis differentiation. In addition, our results extend the hypothesis of Nagamine et al. (1999) in that the promoter/enhancer region of Sry is polymorphic among B6, TIR, and SJL alleles and regulated differently in various genetic backgrounds.

**MATERIALS AND METHODS**

**Mouse Strains**

B6, AKR, and SJL mice were obtained from Jackson Laboratory (Bar Harbor, ME). The consomic B6-Y\textsuperscript{TIR} strain was established by C. Nagamine (Vanderbilt University) and was received at our animal facility at the N8 strain was established by C. Nagamine (Vanderbilt University) and was received at our animal facility at the N8 backcross generation (Nagamine et al., 1999). Although all B6-Y\textsuperscript{TIR} gonads are either completely or partially sex reversed in fetal life, the fetal ovotestis usually develops into a small but fertile testis by puberty. Therefore, the B6-Y\textsuperscript{TIR} male with uni- or bilateral testes was used to propagate the B6-Y\textsuperscript{TIR} progeny. B6-Y\textsuperscript{SJL} and B6-Y\textsuperscript{AKR} strains were prepared in our animal facility by a series of backcrosses. B6-Y\textsuperscript{SJL}, B6-Y\textsuperscript{AKR} mice of the N35-N38, N10, and N10 backcross generations, respectively, were used for this study. SJL females were crossed with B6-Y\textsuperscript{B6}, B6-Y\textsuperscript{SJL}, B6-Y\textsuperscript{AKR}, and B6-Y\textsuperscript{TIR} males to place the Y-chromosomes on SJLB6F1 background.

**Isolation of Fetal Gonads**

The pregnant females were killed by cervical dislocation, their fetuses removed at 10.5 to 12.0 d.p.c., and fetal urogenital complexes were harvested and stored at -80°C. Noon of the day when the copulation plug was found was defined as 0.5 d.p.c. Developmental stages were assessed by counting the tail somite number beginning at the genital tubercle (Nagamine et al., 1999). The chromosomal sex of each fetus was determined by PCR amplification of the Y-encoded Zfy (Nagamine et al., 1989). Part of the fetal body was lysed in a buffer (100 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0; 0.5% Tween 20; 0.5% NP-40; 0.4 mg/mL proteinase K) overnight, and proteinase K was inactivated by heating at 90°C for 5 min. A 100-fold diluted lysate was subjected to 35 cycles of PCR amplification using the conditions described in Nagamine et al. (1989). Ten microliter aliquots of PCR products were run on 2% agarose gels with Tris/acetate EDTA (TAE) buffer and visualized with ethidium bromide fluorescence (Sambrook et al., 1989).

**Semiquantitative RT-PCR (sqRT-PCR)**

cDNA synthesis.

Total RNA was isolated from each gonad using the method described by Chomczynski and Sacchi (1987) with modifications (Lee and Taketo, 1994), and subjected to two positive and one negative cDNA synthesis reactions as follows. Total RNA from each gonad was suspended in 19 μL of DEPC-treated ddH₂O. After denaturation at 90°C for 5 min, each RNA solution was divided into three equal aliquots. Two of the aliquots were subjected to cDNA synthesis (RT) with 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 1.6 U/μL RNA Guard (Amersham-Pharmacia), 1 mM each dNTPs (Amersham-Pharmacia), 5 μM random hexamers (Amersham-Pharmacia), and 10 U/μL M-MLV reverse transcriptase (Life Technologies). With the third aliquot, RT was performed without reverse transcriptase (RT-). The reaction mixture was incubated at 25°C for 10 min and then at 42°C for 60 min, after which the enzyme was inactivated at 99°C for 5 min.

**PCR amplification.**

cDNA sample was processed for PCR amplification in a total volume of 100 μL with 40 μM each of the sense (5’-GGGACAGAGTTGAGACCC-3’) and antisense (5’-TGTGAGTACAGGTGTGAGCTTAC-3’) Sry primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 3.5 mM MgCl₂, 200 μM each dNTPs, and 0.025 U/μL Taq DNA polymerase. The mixture was first amplified for 9 cycles. Each cycle included denaturation at 94°C for 20 s, annealing at 52°C for 15 s, and extension at 72°C for 30 s, except that denaturation during the first cycle was 5 min. After the last extension step, 40 μM each of the sense (5’-CCTAGGCACAGGTTGTGAGCTTAC-3’) β-actin primers and an additional 2.5 U Taq DNA polymerase were added to each reaction.
mix at 72°C. Each 100 μL reaction was divided in half. Thereafter, for each gonad, one set (2 RT+ and 1 RT−) of reactions was PCR amplified for further 28 cycles while the other set was amplified for 36 cycles.

Quantitation.
Ten microliters of each PCR reaction was electrophoresed on a 4% NuSieve 3:1 agarose (BioWhittaker Molecular Applications, Rockland, ME) gel in TAE buffer and visualized with ethidium bromide fluorescence. The intensity of each band was quantified using AlphaImager Visual Applications, Rockland, ME) gel in TAE buffer and visualized with ethidium bromide fluorescence. The intensity of each band was quantified using AlphaImager Visual Applications, Rockland, ME) gel in TAE buffer and visualized with ethidium bromide fluorescence. The intensity of each band was quantified using AlphaImager Visual Applications, Rockland, ME) gel in TAE buffer and visualized with ethidium bromide fluorescence. The intensity of each band was quantified using AlphaImager Visual Applications, Rockland, ME) gel in TAE buffer and visualized with ethidium bromide fluorescence. 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