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Delayed *Sry* and *Sox9* expression in developing mouse gonads underlies B6-Y^{DOM} sex reversal

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Abstract

The phenomenon of B6-Y^{DOM} sex reversal arises when certain variants of the *Mus domesticus* Y chromosome are crossed onto the genetic background of the C57BL/6J (B6) inbred mouse strain, which normally carries a *Mus musculus*-derived Y chromosome. While the sex reversal has been assumed to involve strain-specific variations in structure or expression of *Sry*, the actual cause has not been identified. Here we used in situ hybridization to study expression of *Sry*, and the critical downstream gene *Sox9*, in strains containing different chromosome combinations to investigate the cause of B6-Y^{DOM} sex reversal. Our findings establish that a delay of expression of Sry^{DOM} relative to Sry^{B6} underlies B6-Y^{DOM} sex reversal and provide the first molecular confirmation that *Sry* must act during a critical time window to appropriately activate *Sox9* and effect male testis determination before the onset of the ovarian-determining pathway.
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Introduction

Genetics thrives on the principle that the exception proves the rule. Mutations and rearrangements of genes, when observable at the phenotypic level, provide some of the most powerful tools available for the identification of genes and the study of their functions, as evident from the history of developmental genetics in a number of model organisms. Less tractable and arguably more intriguing are phenotypic effects that arise not from defective genes but from abnormal combinations of genes, such as those seen in interstrain crosses in both plants and animals.

An intriguing example is the phenomenon known as B6-Y^{DOM} sex reversal. This situation occurs when certain variants of a *Mus domesticus* type Y chromosome are crossed onto the genetic background of the C57BL/6J (commonly abbreviated as B6) inbred mouse strain (Eicher et al., 1982). It has long been asserted that B6-Y^{DOM} sex reversal is caused by an abnormal interaction of B6-derived autosomal or X-linked loci with the *M. domesticus* type Y chromosome (Biddle et al., 1988; Eicher and Washburn, 1983, 1986; Eicher et al., 1982; Nagamine et al., 1987b), and over two decades of research has been directed at determining the molecular cause of this phenomenon.

Three groups of *M. domesticus* Y chromosomes have been identified, based on their differing ability to induce testes when present on the B6 genetic background (Biddle et al., 1991, 1988; Nagamine et al., 1987a,b, 1999). The first group, represented by Y^{POS} (derived from mice captured in the Val Poschiavo, Switzerland) and Y^{TIR} (from mice captured in Tirano, Italy), causes XY sex reversal with some 75% of gonads developing as ovaries, and the remainder as unilateral or bilateral ovotestes (Eicher and Washburn, 2001). A second group, represented by Y chromosomes in strains such as AKR and RF/J, causes a transient defect in which testis cord formation is delayed, but normal testes are present by the time of birth. Finally, Y chromosomes derived from strains such as FVB and SJL

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show no defect in testis development on a B6 genetic background.

An obvious candidate for the gene at the root of this phenomenon is the Y-linked gene *Sry*, which normally triggers differentiation of the bipotential gonad into a testis (Koopman et al., 1991). Indeed, it has been established that a *Mus musculus* type *Sry* transgene rescues B6-Y^{DOM} mice from sex reversal (Eicher et al., 1995). The *Sry* allele present in B6 (Sry^{B6}) must be different to the Sry^{DOM} allele because they have different testis-determining capabilities on the B6 genetic background, even though each allele is able to specify normal testis development on its native genetic background. However, the actual cause of the sex reversal, and why it is associated with Y chromosomes of some strains and not others, has not been established.

DNA sequence analysis has revealed that different SRY protein isoforms are encoded by various *Sry* alleles (Albrecht and Eicher, 1997; Carlisle et al., 1996; Coward et al., 1994; Nagamine et al., 1999). In several studies, no correlation was found between the predicted protein isoforms and the ability of a particular *Sry* allele to effect testis determination on the B6 genetic background (Albrecht and Eicher, 1997; Carlisle et al., 1996), although a recent analysis suggests that protein isoforms do play a role (Albrecht et al., 2003).

Other workers have focused on variant regulation of *Sry* gene expression as a likely cause of B6-Y^{DOM} sex reversal. Expression studies relating to *Sry* have been complicated by low levels of expression, small numbers of expressing cells in the embryo, and the unavailability to date of an antibody recognizing endogenous mouse SRY protein. For more than a decade, reverse transcriptase–polymerase chain reaction (RT-PCR) and ribonuclease protection studies provided the only means of assaying the expression of *Sry*. These studies established that mouse *Sry* expression begins in urogenital ridges around 10 days post coitum (dpc), peaks at 11.5 dpc, and then declines to undetectable levels by about 13 dpc (Hacker et al., 1995; Jeske et al., 1995; Koopman et al., 1990).

In an effort to illuminate the basis of B6-Y^{DOM} sex reversal, Lee and Taketo (1994) found that *Sry* expression was initiated at the normal time in B6-Y^{TIR} genital ridges, but that downstream events, including expression of several testis differentiation genes and the cessation of *Sry* expression, were delayed. Other studies revealed a delay of morphological differentiation of testis cords (Palmer and Burgoyne, 1991) and expression of anti-Mullerian hormone (Taketo et al., 1991) in B6-Y^{DOM} gonads, suggesting that a delayed molecular program of testis development attributable to *Sry* expression is the cause of B6-Y^{DOM} sex reversal.

On the other hand, Nagamine et al. (1999), in a semiquantitative RT-PCR study comparing *Sry* expression profiles from TIR (complete sex reversal), AKR (delayed cord formation), and FVB (no sex reversal) Y chromosomes, found normal timing but reduced levels of expression correlating with the degree of sex reversal in each strain. The data suggested that sex reversal could be caused by *Sry* expression levels occurring below a certain threshold. However, in further studies in which *Sry* expression from the B6 Y chromosome was compared with that from Y^{TIR}, low levels were observed in both cases, suggesting that low expression levels alone are not the cause of XY^{DOM} sex reversal (Lee and Taketo, 2001).

Recently, we succeeded in visualizing endogenous *Sry* expression in mouse genital ridges by in situ hybridization (Bullejos and Koopman, 2001), and others have studied *Sry* transgene expression by means of an enhanced green fluorescent protein (EGFP) marker under the control of mouse *Sry* regulatory sequences (Albrecht and Eicher, 2001). Similar results were obtained in both studies. *Sry* expression is spatially dynamic, occurring as a wave that emanates from the central region of the genital ridges, extends to the rostral and then caudal poles, and recedes to the caudal pole where it is then extinguished (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). Although *Sry* expression is seen within a window of about 3 days in the gonad, the duration of *Sry* expression in any given cell is likely to be much shorter, and *Sry* is expressed at different times in different regions of the developing testis. Aside from the biological significance of this observation, this phenomenon provides a firm basis for more accurately comparing the temporal and spatial expression patterns of *Sry* between different mouse strains.

In the present study, we analyzed the dynamics of expression of a number of *Sry* alleles by whole-mount in situ hybridization of mouse fetal gonads, with a view to establishing the cause of B6-Y^{DOM} sex reversal. To assess whether downstream events are affected, we also analyzed the expression pattern of *Sox9*. The results establish that a delay of expression of *Sry* and downstream genes, such as *Sox9*, underlies B6-Y^{DOM} sex reversal.

Materials and methods

Mouse strains

The origin and characteristics of the B6-Y^{POS} strain are described elsewhere (Eicher and Washburn, 1986; Eicher et al., 1982). B6-Y^{POS} embryos, derived from B6 female × B6-Y^{POS} male matings, were supplied by Drs. Eva Eicher and Linda Washburn, Jackson Laboratories, Bar Harbor, USA. At the time of our experiments, the Y^{POS} chromosome had been maintained on a C57BL/6J (B6) inbred background for over 40 generations. FVB/N and AKR/J mice were purchased from the Animal Resource Centre, Perth, Australia; males were mated with B6 females, and embryos from these matings are designated here as B6xFVB-Y^{FVB} and B6xAKR-Y^{AKR}, respectively. Swiss Quackenbush outbred females were obtained from the Central Animal Breeding House, University of Queensland, Brisbane,

Australia, and mated with males carrying an X-linked GFP marker (Hadjantonakis et al., 1998).

Dissection, staging, and genotyping of fetal mouse gonads

Fetuses were collected from timed matings and urogenital systems explanted. Staging was accomplished between 10.5 and 12.5 dpc (where 0.5 dpc is defined as noon on the day a mating plug was detected) by counting tail somites posterior to the hindlimb bud: 10.5 dpc correspond to approximately 8 tail somites (ts), 11.5 dpc to approximately 18 ts, and 12.5 dpc to approximately 28–30 ts (Bullejos and Koopman, 2001; Hacker et al., 1995). The chromosomal sex of each fetus was first determined by analyzing amniotic cells for the presence of Barr bodies, indicative of an inactive X chromosome in XX cells (Palmer and Burgoyne, 1991), and confirmed by PCR on tail DNA using primers to detect the *Smcx/Smcy* genes (5′-TGA AGC TTT TGG CTT TGA G-3′ and 5′-CCG CTG CCA AAT TCT TTG G-3′).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was carried out as described (Bullejos and Koopman, 2001; Hargrave and Koopman, 2000). Expression of *Sry* was detected using the *Sry* fragment p422.04 as a probe (Gubbay et al., 1990); probe Sox9.5a was used to detect *Sox9* transcripts (Wright et al., 1995). Numbers of samples analyzed at each time point are shown in Table 1.

Results

A baseline of Sry expression

We previously published an analysis of *Sry* expression in fetal gonads of outbred Swiss mice, which have an *M. musculus* type Y chromosome (Bullejos and Koopman, 2001). These data are included in Fig. 1 for comparative purposes. To summarize, expression of *Sry* in Swiss embryos follows a wave pattern that is initiated in the central region of the gonad at 14 tail somites (ts) (Fig. 1t), extended to both poles by 16 ts (Fig. 1u), increases in strength by 18 ts (Fig. 1v), begins to diminish first in the central region by 20 ts (Fig. 1w) and next at the anterior pole by 22 ts, leaving strongest expression at the posterior pole at the 22 ts stage (Fig. 1x). Expression is completely extinguished by 24 ts (data not shown).

These findings were used as a baseline for further experiments to analyze *Sry* expression in genital ridges of B6 and the three *M. domesticus* Y-bearing strain combinations. To compare directly the expression patterns of Sry^{DOM} and Sry^{B6} alleles on a B6 genetic background, we used XY gonads from B6-Y^{B6} and B6-Y^{POS} fetuses. We also analyzed expression of other *M. domesticus Sry* alleles in XY B6xAKR-Y^{AKR} and B6xFVB-Y^{FVB} fetal gonads.

Sry is expressed briefly and early in B6 mice

In B6-Y^{B6} samples, no *Sry* expression was observed at 12 ts (Fig. 1a), but by 15 ts strong expression was noted throughout the genital ridges (Fig. 1b). This was maintained at 18 ts (Fig. 1c), after which expression rapidly declined, leaving only a very faint trace of expression at the posterior pole by 20 ts (Fig. 1d). Thus, *Sry* expression peaked some 2 ts (roughly 4.8 h) earlier in gonads of B6-Y^{B6} mice compared to gonads of Swiss and other strain combinations tested and was extinguished very rapidly, some 4 ts (roughly 9.6 h) earlier than in Swiss gonads. However, *Sry* expression did not begin correspondingly early: no expression was observed at 12 ts in B6 genital ridges (Fig. 1a). These observations suggest that *Sry* is expressed for a briefer period, and peaks earlier, in B6 than in other strains examined.

Our analyses also suggested that the B6 *Sry* allele is expressed at a lower level than other alleles examined because longer color substrate incubation times were invariably required for the B6 samples in in situ hybridization experiments, resulting in the higher background staining of these samples relative to others shown (Fig. 1). This conclusion is consistent with semiquantitative RT-PCR analyses suggesting lower levels of Sry^{B6} expression (Albrecht et al., 2003; Lee and Taketo, 2001; Nagamine et al., 1999).

Table 1
Numbers of samples analyzed

Probe	*Sry* allele	Stage (ts)						
		12	13/14	15/16	17/18	19/20	21/22	Total
Sry	Y^{B6}	2	–	1	2	1	4	10
	Y^{POS}	–	2	2	4	1	2	11
	Y^{AKR}	–	1	2	2	2	2	9
	$Y^{FVB/N}$	–	–	1	3	2	2	8
	Y^{SWISS}	3	7	8	8	8	8	42
Sox9	Y^{B6}	–	1	2	2	1	2	8
	Y^{POS}	–	–	3	1	1	2	7
	Y^{SWISS}	–	2	3	5	3	2	15

In cases where only a single sample was analyzed, the result obtained was in accord with the flanking time points. Where multiple samples were analyzed, representative samples are shown in Figs. 1 and 2.

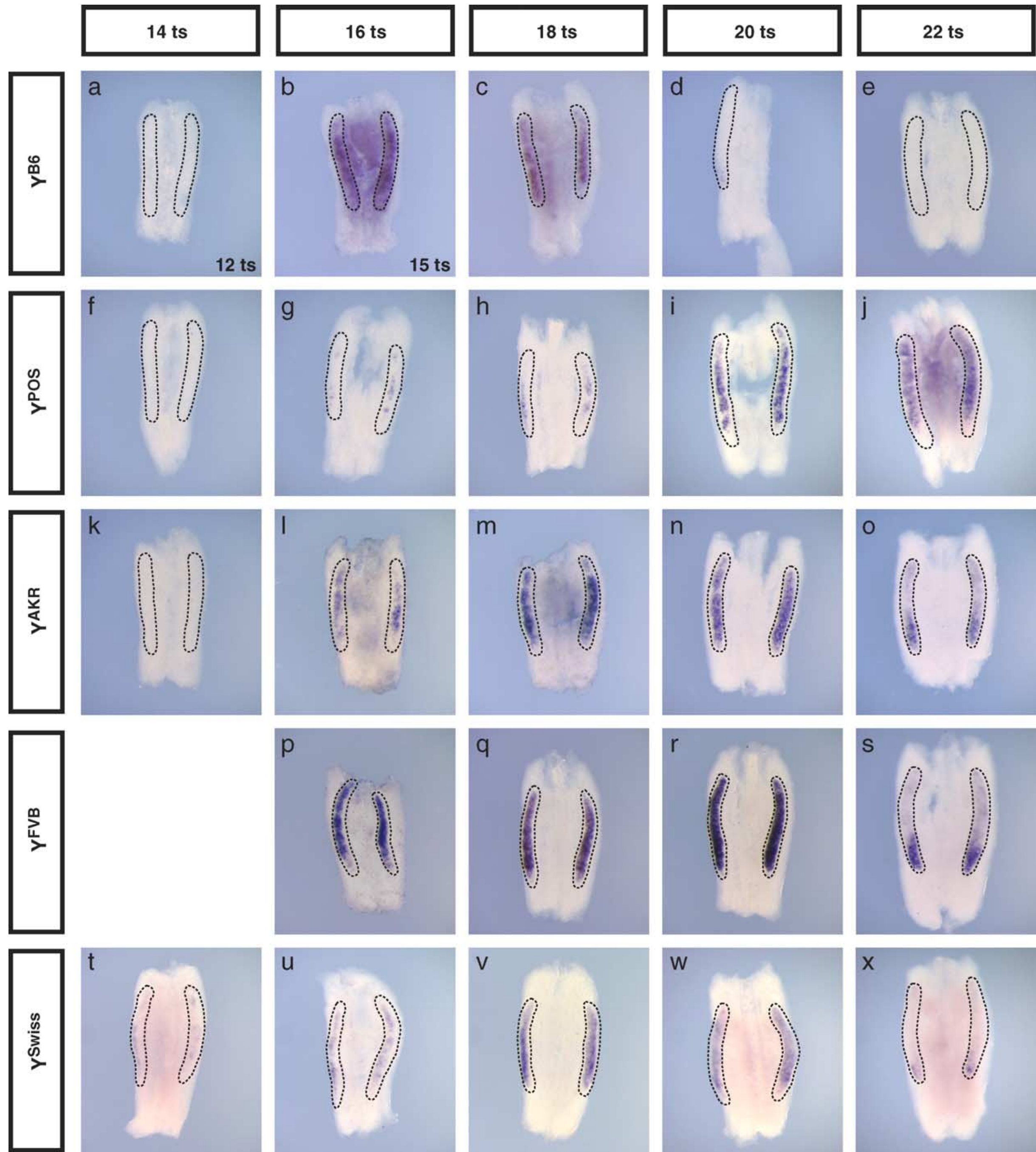

Fig. 1. Developmental time course of *Sry* expression in male embryonic urogenital ridges from 14 to 22 tail somites in C57BL/6J (Y^{B6}) (a–e), B6-Y^{POS} (Y^{POS}) (f–j), B6xAKR-Y^{AKR} (Y^{AKR}) (k–o), B6xFVB-Y^{FVB} (Y^{FVB}) (p–s), and Swiss Quackenbush (Y^{Swiss}) (t–x) mice. Dotted lines show outlines of the genital ridges in each sample. The comparison of the expression pattern between different *Sry* alleles reveals an early and brief expression profile in B6 mice and a delayed expression profile in B6-Y^{POS} mice.

The Sry expression profile is delayed in B6-Y^{POS} mice

The major focus of the present study was to address the question of whether, and in what way, expression of *Sry* might be altered in XY B6-Y^{DOM} sex reversal, particularly in its most severe form, exemplified by B6-Y^{POS}. When *Sry* expression was analyzed in B6-Y^{POS} gonads, we found no expression at 14 ts (Fig. 1f); expression became evident only at 16 ts (Fig. 1g). Maximal extent and levels of *Sry* expression were reached by 20 ts (Figs. 1i and j). These findings show that the onset of *Sry* expression in XY B6-Y^{POS} gonads (Figs. 1g–j) is delayed by up to 2 ts (some 4.8 h) compared to expression in Swiss gonads (Figs. 1t–w). Certainly, peak levels of Sry^{POS} expression were reached only after expression of Sry^{B6} had all but ceased (Figs. 1b–e, g–j); this represents a delay of 4 ts (approximately 9.6 h)

for Sry^{POS}. These observations provide clear evidence of a delay in expression associated with the *POS* allele of *Sry* on a B6 genetic background.

Sex reversal caused by the *M. d. poschiavinus*-derived Y chromosome represents the severest class of B6-Y^{DOM} sex reversal. We wished to determine whether the delayed expression of *Sry* was related to the ability of Y^{POS} to cause severe XY sex reversal, or whether it might be a feature of all *M. domesticus* type Y chromosomes. To this end, we examined the expression pattern of *Sry* in B6xAKR-Y^{AKR} and B6xFVB-Y^{FVB} fetuses. A wavelike profile of expression was observed in both (Figs. 1k–o, p–s). Significantly however, the delay evident in B6-Y^{POS} mice was not observed in B6xAKR-Y^{AKR} or B6xFVB-Y^{FVB}, and expression profiles in both cases were more similar to that seen in gonads from Swiss outbred mice (Fig. 1, compare c, h, m, q and v). While Sry^{AKR} and Sry^{FVB} expressions were studied on an F1 background, it can be concluded that delayed expression of *Sry* is not associated with all *M. domesticus* type Y chromosomes.

Sox9 expression is initiated in a wave in XY genital ridges

To examine how genes downstream of *Sry* are affected in B6-Y^{DOM} sex reversal, we next examined the dynamics of *Sox9* expression by whole-mount in situ hybridization. In

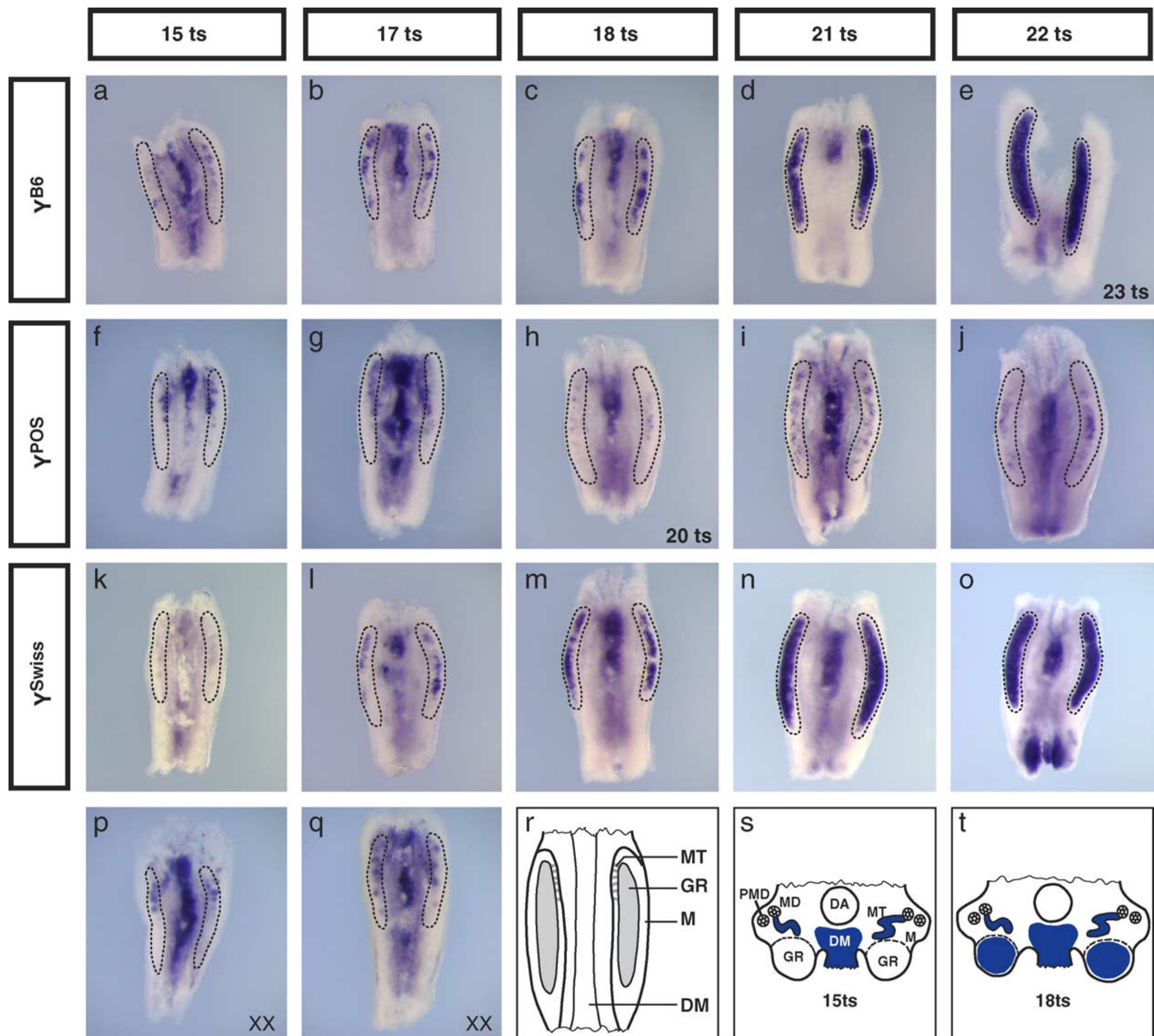

Fig. 2. Developmental time course of *Sox9* expression in male embryonic urogenital ridges from 15 to 22 tail somites in C57BL/6J (Y^{B6}) (a–e), B6-Y^{POS} (Y^{POS}) (f–j), and Swiss Quackenbush (Y^{Swiss}) (k–o) mice. *Sox9* expression in female (XX) embryonic urogenital ridge explants at 15 (p) and 17 (q) tail somites is shown for comparison; *Sox9* in female samples is expressed in the mesonephric tubules and dorsal mesentery, but not in the genital ridges themselves (Kent et al., 1996; Sainio et al., 1997). Dotted lines show outlines of the genital ridges in each sample. *Sox9* expression is delayed in B6-Y^{POS} mice; staining observed in B6-Y^{POS} gonads at 15 and 17 ts stages (f and g) corresponds to the caudal mesonephric tubules lying dorsal to the genital ridges, which are positive in both male and female samples (compare f, g, p, and q). The structures present in the urogenital ridge explants are shown (r), along with diagrams showing in blue the structures staining for *Sox9* expression in cross sections of Swiss XY urogenital explants at 15 (s) and 18 (t) ts stages. GR, genital ridge; M, mesonephros; DM, dorsal mesentery; MT, mesonephric tubule; MD, mesonephric duct; PMD, paramesonephric duct; DA, dorsal aorta.

Swiss outbred mice, *Sox9* expression in the gonads was found to be male specific at all stages examined (13–27 ts). These observations support the male-specificity reported previously by Kent et al. (1996), but not the observations of Morais da Silva et al. (1996), who reported expression of *Sox9* in the genital ridges of both sexes at 10.5 dpc. Additional *Sox9* expression was detected in male and female mesonephric tubules, and in dorsal mesentery attached to the urogenital explants (Fig. 2), as reported previously (Kent et al., 1996; Morais da Silva et al., 1996); this staining can be seen in both XX and XY samples (Figs. 2p–t).

Typically, *Sox9* expression was not detected in XY genital ridges at 15 ts (Fig. 2k), although expression in a small number of cells was sometimes seen, in agreement with the observations of Moreno-Mendoza et al. (2003). By 16 ts, *Sox9* expression was detected in the central portion of the XY genital ridge (data not shown and Schepers et al., 2003). The expression domain expanded to include the anterior pole by 17 ts (Fig. 2l), and the anterior 3/4 of the genital ridge at 18 ts (Fig. 2m). By the 21 ts stage, *Sox9* expression was seen throughout the genital ridges (Fig. 2n), continuing in male gonads until birth (Fig. 2o and data not shown).

Thus, we find that *Sox9* expression is not initiated synchronously throughout the length of mouse genital ridges, but instead as a cumulative wave in the order central–anterior–posterior. This mimics the wave of *Sry* expression, suggesting that *Sox9* is a downstream target of SRY. However, *Sox9* expression persists once initiated whereas *Sry* expression is extinguished in a wave.

Sox9 expression is delayed in B6-Y^{POS}

When *Sox9* expression was compared in B6-Y^{POS} and B6-Y^{B6} XY gonads, major differences were observed. Very low levels of *Sox9* expression were detected at 15 ts in B6-Y^{B6} (Fig. 2a), which is before the onset of expression in Swiss genital ridges (Fig. 2k). This result is consistent with our observation that *Sry*^{B6} expression reaches a peak earliest among the *Sry* alleles analyzed in this study. At later stages, the *Sox9* expression profile in B6-Y^{B6} (Figs. 2b–e) was essentially similar to that observed in gonads from Swiss fetuses.

In B6-Y^{POS}, however, a marked delay in the onset of *Sox9* expression was observed. No expression was detected until 20 ts (Fig. 2h). Staining observed at 15 and 17 ts (Figs. 2f and g) corresponds to the caudal mesonephric tubules, dorsal to the gonad itself, which are *Sox9* positive both in males and females (Figs. 2p–t) (Sainio et al., 1997). Thus, in these gonads, the onset of *Sox9* expression is delayed by a period corresponding to 6 ts (approximately 14.4 h) compared with B6-Y^{B6}, or 5 ts (approximately 12 h) compared with Swiss. The domain of expression expanded at 21 ts (Fig. 2i) and 22 ts (Fig. 2j) stages, by which time expression was still confined to the central part of the gonad (Fig. 2j), analogous to *Sox9* expression in B6-Y^{B6} and Swiss genital ridges at 17 ts (Figs. 2b and l).

Sox9 expression in B6xFVB-Y^{FVB} and B6xAKR-Y^{AKR} genital ridges was essentially similar to the profile observed in Swiss outbred genital ridges (data not shown). Thus, a delay in *Sox9* expression is not a general response to the presence of an *M. domesticus* type Y chromosome on a B6 background but instead is correlated with the sex reversal seen in B6-Y^{POS} gonads.

Discussion

Discordance between sex chromosome constitution and phenotypic sex provides a powerful system with which to study the genetics of sexual development. For this reason, the genetic basis of B6-Y^{DOM} sex reversal in mice has been the focus of extensive study and speculation since its discovery over two decades ago (Eicher et al., 1982). Evidence continues to be presented arguing for or against an involvement of SRY protein structure (isoform efficiency and protein interaction models) or of *Sry* gene regulation (delayed or reduced expression models) (Albrecht et al., 2003; Lee and Taketo, 2001; Nagamine et al., 1999; Xian et al., 2001).

A number of previous studies point to the importance of *Sry* expression levels for proper initiation of male sex determination in mammals. XY sex reversal due to reduced expression of *Sry* has been observed as a result of long-range regulatory mutations of *Sry* (Capel et al., 1993), and targeted mutation of the *Fog2* gene (Tevosian et al., 2002), insulin receptor family genes (Nef et al., 2003), and the +KTS isoform of *Wt1* in mice (Hammes et al., 2001). Consistent with these observations, a study by Nagamine et al. (1999) of B6-Y^{DOM} sex reversal identified reduced *Sry* expression as a contributing factor. However, the choice of *Sry*^{FVB}, which appears to be expressed at atypically high levels, as a benchmark for comparison of *Sry*^{TIR} expression, and the omission of a comparative analysis of *Sry*^{B6} led these authors to focus on levels rather than timing issues. Reexamination of their data reveals a 2 ts (approximately 4.8 h) delay in the peak of expression of *Sry*^{TIR} relative to *Sry*^{FVB}, consistent with our current findings.

Lee and Taketo (2001) compared expression of *Sry*^{TIR} and *Sry*^{B6} directly by semiquantitative RT-PCR and concluded that expression levels were similar at the time points examined. Their samples were pooled from relatively broad time intervals, obscuring the subtleties of the dynamic profile of *Sry* expression. In 13–18 ts pooled gonads, the level of *Sry*^{B6} expression was higher than that in *Sry*^{TIR} gonads, whereas the situation was reversed in 19–26 ts pools. Allowing for differences in strains studied and methods used, those data also are consistent with our findings. Further, an earlier study by the same authors reported normal onset, but prolonged expression of *Sry* in B6-Y^{TIR} (Lee and Taketo, 1994). It is not clear from that

study whether the onset of *Sry* expression is truly coincident or delayed in certain strains because samples were grouped in days post coitum rather than by tail somite number. In terms of *Sry* function, this issue is not as relevant as the issue of when expression reaches threshold levels, as we argue below.

A recent study using semiquantitative RT-PCR found that temporal expression of Sry^{DOM} and Sry^{MUS} are indistinguishable between 10.5 and 13.0 dpc (Albrecht et al., 2003), although it is possible that differences in sensitivity or ability to resolve spatial information may underlie the apparent discrepancy.

The observation from in situ hybridization experiments that *Sry* is expressed in a dynamic and reproducible wave during XY mouse genital ridge development (Bullejos and Koopman, 2001), combined with close attention to staging of embryos by tail-somite number (each additional tail somite pair corresponding to an interval of approximately 2.4 h), allowed us to conduct a more precise study of the contribution of *Sry* expression to B6-Y^{DOM} sex reversal. This method allows us to draw conclusions relating to the timing of *Sry* expression based not only on the presence or absence of signal, but also on the position of the expression domain within each sample.

Using this assay, we provide the first clear indication that delayed expression of *Sry* is associated with B6-Y^{POS} sex reversal. We found that downstream molecular events, exemplified by *Sox9* expression, are similarly delayed in B6-Y^{POS} relative to other strains tested. These findings have important implications for our understanding of the function of *Sry*, of windows of competence to respond to *Sry* during male sex determination in mammals, and of the contribution of inappropriate *Sry* expression to XY sex reversal.

It is widely believed that the testis-determining pathway is normally activated before, and actively represses, the ovarian-determining pathway, ensuring testis development in XY individuals (Eicher and Washburn, 1986). Thus, *Sry* must be activated by a certain time point, otherwise the ovarian pathway will initiate and the testis-determining pathway will "miss the boat" (Palmer and Burgoyne, 1991). According to this model, Y^{POS} has been postulated to act late and/or the ovarian-determining pathway in B6 early, such that the combination B6-Y^{POS} leads to XY sex reversal—the so-called timing mismatch hypothesis (Burgoyne, 1988; Burgoyne and Palmer, 1991; Eicher and Washburn, 1986). The present data provide the first molecular confirmation of the timing mismatch hypothesis.

It also is commonly postulated that *Sry* expression must reach a threshold level in cells of the supporting cell lineage within a certain developmental window of time to initiate testis determination. Our data suggest that the threshold is exceeded too late or not at all in the 75% of B6-Y^{DOM} gonads that develop as ovaries, or is reached during the window of competence in some gonadal cells in the remaining 25% that develop as ovotestes. In this light, the matter of when *Sry* expression is initiated in B6-Y^{POS} may be irrelevant, and we propose that the time when *Sry* expression reaches the threshold level is likely to be the critical factor in whether it is or is not able to specify testis development.

Similarly, the prolonged *Sry* expression reported by Lee and Taketo (1994) in B6-Y^{TIR} and by Albrecht et al. (2003) in B6-Y^{POS} is unlikely to be a causal factor in B6-Y^{DOM} sex reversal. We have shown experimentally that prolonging *Sry* expression in the gonads of transgenic mice has no bearing on sex determination (J. Bowles and PK, unpublished data). Instead, the prolonged expression of Sry^{TIR} on the B6 background most likely results from the failure of normal feedback mechanisms that repress *Sry* expression after testis determination is initiated (Albrecht and Eicher, 2001; Albrecht et al., 2003; Koopman, 1999; Lee and Taketo, 1994).

As a consequence of the wavelike profile of *Sry* expression during mouse genital ridge development, cells in the different regions of the genital ridges are exposed to *Sry* mRNA at different times, and for different lengths of time. In B6-Y^{POS}, it is likely that the *Sry* threshold is met in a proportion of supporting cells in the central region only, accounting for the observed activation of *Sox9* limited to this region. A similar restricted pattern of *Sox9* activation has recently been noted in B6-Y^{TIR} fetal testes (Moreno-Mendoza et al., 2004). Interestingly, the delay of 12–14 h in *Sox9* expression that we observed in B6-Y^{POS} gonads corresponds with the 13-h delay in cord formation identified by Palmer and Burgoyne (1991) for Y^{POS} chromosomes independent of genetic background.

As has been noted previously (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001), the wave of *Sry* expression is likely to explain the histology of ovotestes in B6-Y^{DOM} sex reversal, in which testicular material is usually found in the central region and ovarian tissue at the poles (Albrecht et al., 2000; Eicher, 1982; Eicher et al., 1995; Nagamine et al., 1999). Because Sry^{POS} is expressed at low levels only in the central region at a time (18 ts) when other alleles are at or close to their maximal levels of expression along the entire length of the genital ridge, the threshold of SRY protein might be reached in the central region of the B6-Y^{POS} gonad only, if at all.

We have shown that Sry^{POS} acts late, presumably causing a critical time window to be missed in all or most supporting cells of B6-Y^{POS} genital ridges. However, Sry^{POS} is able to specify testis development in 100% of cases on its native (*M. domesticus*) background. Because Y^{DOM} sex reversal occurs only on a B6 background, it has been proposed that the ovarian determination pathway is activated early in this strain relative to other *Mus musculus* strains (Eicher and Washburn, 1986; Palmer and Burgoyne, 1991). We found that the *Sry* expression wave is completed earlier in B6-Y^{B6}, providing the first experimental evidence that molecular events relating to sex determination occur earlier in this strain relative to other mouse strains. This early expression pattern of testis-determining genes may

have allowed the coevolution of new variants of ovary-determining genes that act early in the B6 strain. Whether the ovarian-determining pathway is activated early in B6 remains to be determined, and the use of early ovarian somatic cell markers such as *Foxl2* (Loffler et al., 2003) and *Fst* (Menke and Page, 2002) will be required to resolve this issue.

In summary, we have shown that experimentally crossing the *Sry*^{POS} allele onto a B6 background results in a delayed *Sry* expression profile that ultimately results in XY sex reversal. We suggest that regulatory mutations affecting timing or levels of *SRY* expression may represent a more common mechanism of human *SRY*-positive XY sex reversal than previously suspected. Further studies will allow more precise definition of the molecular and cellular events that determine the threshold level SRY must reach, and of the time window within the threshold must be attained, in order to engage the testis-determining pathway.

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