Published in final edited form as:
Genesis. 2000 ; 28(3-4): 111–124.

Related Function of Mouse SOX3, SOX9, and SRY HMG Domains Assayed by Male Sex Determination

David E. Bergstrom, **Maureen Young**, **Kenneth H. Albrecht**, and **Eva M. Eicher***
The Jackson Laboratory, Bar Harbor, Maine

Summary

Sox genes encode proteins related to each other, and to the sex determining gene *Sry*, by the presence of a DNA binding motif known as the HMG domain. Although HMG domains can bind to related DNA sequences, *Sox* gene products may achieve target gene specificity by binding to preferred target sequences or by interacting with specific partner proteins. To assess their functional similarities, we replaced the HMG box of *Sry* with the HMG box of *Sox3* or *Sox9* and tested whether these constructs caused sex reversal in XX mice. Our results indicate that such chimeric transgenes can functionally replace *Sry* and elicit development of testis cords, male patterns of gene expression, and elaboration of male secondary sexual characteristics. This implies that chimeric SRY proteins with SOX HMG domains can bind to and regulate SRY target genes and that potential SRY partner factor interactions are not disrupted by HMG domain substitutions.

Keywords

Sry; *Sox3*; *Sox9*; sex determination; testis determination; transgenic mice

INTRODUCTION

The *Sox* (*Sry*-box) gene family consists of genes that encode proteins with at least 50% amino acid identity to the 79 amino acid HMG-type DNA-binding motif found in the mammalian sex determining gene, *Sry* (sex determining region, Y chromosome) (Gubbay *et al.*, 1990; Laudet *et al*., 1993; Pevny and Lovell-Badge, 1997; Wegner, 1999). *Sox* genes are involved in a wide range of developmental processes, including chondrogenesis (Ng *et al*., 1997), hematopoiesis (Oosterwegel *et al*., 1993; Schilham and Clevers, 1998), neurogenesis (Hargrave *et al*., 1997; Rex *et al*., 1997; Uwanogho *et al*., 1995), lens development (Kamachi *et al*., 1995, 1998), and sex determination (Wegner, 1999). At least 30 family members have been identified in a diverse group of multicellular organisms, including 15 genes in mammals (Soullier *et al*., 1999; Wegner, 1999). Accumulating evidence from mutations in humans, mice, and *Drosophila* suggests *Sox* genes influence cell fate decisions during development (Pevny and Lovell-Badge, 1997).

The sequence-specific DNA binding, DNA bending, and transactivation properties of SOX proteins suggest they act as transcription factors with characteristics of both classical transcription factors and architectural chromatin factors. Studies in vitro demonstrate that the HMG domains of all SOX proteins tested to date bind with high affinity to a consensus DNA sequence (A/T A/T CAA A/T G) (Denny *et al*., 1992; Kanai *et al*., 1996; Mertin *et al*.,

*Correspondence to: Eva Eicher, The Jackson Laboratory, 600 Main Street, Bar Harbor, ME 04609. E-mail: E-mail: eme@jax.org.

1999). Several investigators have suggested that SOX proteins bind to this core motif with different affinities and that selectivity is determined by sequences adjacent to the core motif (Collignon *et al.*, 1996; Kamachi *et al.*, 1999; Mertin *et al.*, 1999). It is currently unclear if the affinity differences measured in vitro are sufficient to provide functional specificity in vivo. Further DNA binding selectivity and functional specificity may arise from interactions between SOX proteins and other cofactors (Kamachi *et al.*, 2000). For example, some SOX proteins require specific protein partners for high affinity, specific DNA binding (Yuan *et al.*, 1995; Ng *et al.*, 1997; De Santa Barbara *et al.*, 1998; Johnson *et al.*, 1998; Kamachi *et al.*, 1999). In some instances the protein-protein interaction domain within each SOX protein overlaps with its HMG domain, while in other cases the interaction and HMG domain map to distinct protein regions.

To investigate the functional relatedness and target specificity of HMG domains, we designed chimeric transgene constructs in which the HMG box of mouse *Sox3* or *Sox9* replaced the HMG box of mouse *Sry*. The *Sry* clone used for these experiments was a 14.6 kb genomic clone (Hacker *et al.*, 1995) that causes sex reversal in XX mice when present as a transgene (Koopman *et al.*, 1991; Eicher *et al.*, 1995).

The HMG boxes of *Sox3* and *Sox9* were chosen for study for several reasons. *Sox3* is the *Sox* gene most closely related to *Sry* (67% nucleotide identity within the HMG box; Fig. 1) and, like *Sry*, lacks introns. The strong sequence similarity and the fact that *Sox3*/SOX3 is X-linked in humans, mice and marsupials (Foster and Graves, 1994;Mumm *et al.*, 1997;Stevanovic *et al.*, 1993;Wright *et al.*, 1993) has prompted some investigators to propose that *Sox3* is the evolutionary precursor of *Sry*. The role of *Sox3/SOX3*, if any, in sex determination is unclear (Stevanovic *et al.*, 1993) although it is expressed in both XX and XY mouse gonads during the critical period of sex determination (Collignon *et al.*, 1996).

In contrast to *Sox3*, *Sox9* is more distantly related to *Sry* (59% nucleotide identity within the HMG box; Fig. 1). *Sox9* contains introns, is autosomal, and clearly plays an important role in testis development. In the human syndrome campomelic dysplasia, *SOX9* mutations cause skeletal dysmorphology and often lead to XY sex reversal (Foster, 1996;Foster *et al.*, 1994;Wagner *et al.*, 1994). In mice, SOX9 protein expression within the developing gonad is sexually dimorphic, being initially equal in male and female fetal gonads, while later increasing in XY gonads and decreasing in XX gonads (Kent *et al.*, 1996;Morais da Silva *et al.*, 1996;Wright *et al.*, 1995).

We used sexual development of XX mice carrying a chimeric *Sry/Sox* construct as a transgene to assay functional gene activity in vivo. Our results demonstrated that XX mice carrying correctly expressed chimeric transgenes with either the *Sox3* or *Sox9* HMG box develop testes and are phenotypically male. Furthermore, XX transgenic fetal gonads from these mice display testis-specific patterns of gene expression and develop normal-appearing testis cords. These results indicate that the *Sox3* and *Sox9* HMG boxes can functionally substitute for the *Sry* HMG box to initiate normal male sex determination.

RESULTS

Identification and Analysis of Founder Mice

To functionally test the biochemical similarity of the HMG domains of SOX3, SOX9, and SRY, we prepared DNA constructs (Fig. 2) in which the HMG box of *Sry* was replaced with the HMG box of *Sox3* or *Sox9*. We then used these constructs to create transgenic mice and analyzed these mice for presence of the transgene, presence of the Y Chromosome, sexual phenotype, and expression of male-specific molecular markers (Table 1). (The nomenclature used to designate each transgene is given in the Methods section.)

Initially, six founder mice (#31, a C57BL/6J female; and #32-36, five FVB/NJ males) were identified carrying the *SrySox3* construct, and 12 founder mice (#49 - 60, four females and eight FVB/NJ males) were identified carrying the *SrySox9* construct (Table 2). Two *SrySox9* founder males lacked a Y Chromosome and contained small testes, presumably because they contained two X chromosomes and no Y Chromosome. These two founders were not studied further. XX transgenic male offspring were obtained from two *SrySox3* founders (#32 and 36) and five *SrySox9* founders (#52, 56, 57, 58, and 59) (Table 2). Of these seven, four (#32, 36, 52, and 56) produced both XX transgenic females and XX transgenic males and three of these four (#32, 36, and 52) exhibited greater than 50% transmittance of the transgene, suggesting the occurrence of multiple integration events. Further phenotypic and genotypic analysis (of N2 and N3 backcross offspring) allowed us to establish two phenotypically distinct lines from founders 32, 33, 36, 52, and 56. A single line was established from the remaining founders with the exception of founder 54, who did not transmit the transgene.

Identification of Dominant and Partially Sex Reversing Transgenes

After establishing independent lines carrying single integrations, we analyzed each transgene for the ability to cause sex reversal in XX mice (Fig. 3A, B). Our analysis demonstrated that in several lines [Tg32 (*SrySox3*), and Tg52, Tg56, Tg57, Tg58, and Tg59 (*SrySox9*)] XX Tg/- offspring were externally invariably male and thus contained a dominant sex reversing transgene.

Sex reversal was not always complete in XX Tg/-offspring from lines Tg33, Tg36, Tg87 (*SrySox3*), and Tg79 (*SrySox9*) in that hermaphrodites and females were observed in some XX Tg/- mice.

Identification of Recessive Sex Reversing Transgenes

Twelve transgenes did not cause sex reversal of XX mice when present in the hemizygous state. Of these, line Tg78 was not further analyzed. For the remaining 11, intercrosses were established between Tg/- mice to determine if sex reversal occurred in XX Tg/Tg mice (Fig. 3C, D). Transgenic progeny were examined for external sexual appearance and presence of the Y Chromosome. At least 14 XX Tg females (in the absence of XX Tg males) were examined before concluding that homozygosity for the transgene did not cause sex reversal ($P < 0.005$). For eight lines, no indication of homozygous sex reversal was evident. But in three lines, Tg77, Tg33 (*SrySox3*), and Tg55 (*SrySox9*), approximately one-third of XX Tg offspring were male. This observation is consistent with the notion that sex reversal was caused by homozygosity of the transgene.

An examination of the internal reproductive organs of adult mice from line Tg33 suggests that all XX Tg33/Tg33 mice develop as males and approximately half of XX Tg/-mice develop as hermaphrodites. No internal examination was performed on adult mice from lines Tg55 or Tg77.

In the Tg77 line, we also observed a dwarfism in all (nine) XX Tg males and in seven of 32 XY Tg males, that was strikingly similar to that observed in mice homozygous for the mutation achondroplasia (*cn*) (Lane, 1973; Lane and Dickie, 1968). Dwarfism was never observed in XX Tg/-, XY Tg/-, XX -/-, or XY -/- mice. We concluded that Tg77 homozygosity causes XX male sex reversal and a *cn*-like dwarfism. On rare occasion (0.5% of all progeny), hermaphroditism was evident among known or presumed Tg77 hemizygotes suggesting that some testicular tissue can develop in XX Tg77/- mice.

Copy Number

The copy number at each transgenic integration site was determined by quantitative PCR (Table 2). Integration sites contained from one (Tg78 and Tg88) to 40 (Tg58) transgene copies. All integration events with a copy number greater than 15 caused complete dominant sex reversal and, with one exception (Tg 52), all integration events with a copy number less than 15 caused incomplete or no sex reversal as hemizygotes. To the extent analyzed, a modest correlation (data not shown) was observed between increasing copy number, sex reversal, and transgene expression levels (see RT-PCR below).

Fetal Gonad Morphology

Gonad development was analyzed in fetal mice at 14.0 to 14.5 dpc, a time when both ovarian and testicular tissues are easily discerned within individual gonads (Table 3 and Fig. 4). For dominant sex reversing lines Tg32 and Tg 52, normal ovaries were present in all XX -/- mice and normal testes in all XY -/-, XY Tg/- and XX Tg/- mice.

For the recessive sex reversing lines Tg77 and Tg 55, normal ovaries were present in all XX -/- fetuses and normal testes in all XY -/- and XY Tg fetuses. In line Tg55, testes from XX Tg mice were normal in both appearance and developmental age. In line Tg77, testes from XX Tg mice appeared somewhat smaller and had less extensive cord development compared to testes from their XY fetal sibs (Fig. 4, arrow). For both recessive lines, transgenic XX mice with ovaries were presumably XX Tg/-and those with testes were presumably XX Tg/Tg. The frequency of XX Tg fetuses with ovaries or testes was in close accordance with results obtained from external genital morphology and segregation data (see Fig. 3C, D).

RT-PCR

To assess the temporal profile of transgene expression compared to endogenous *Sry* expression (Hacker *et al.*, 1995; Koopman *et al.*, 1990), we used RT-PCR to analyze RNA from paired XX Tg/- urogenital ridges (10.5 and 11.5 dpc) and gonads with attached mesonephroi (12.5 dpc, 14.5 dpc, and newborn) (Table 4). For comparison, *Sry* expression was determined in similarly staged XY -/- gonads. In control FVB/NJ XY fetal gonads, endogenous *Sry* expression was undetectable at 10.5 dpc, present at 11.5 and 12.5 dpc, and undetectable again at 14.5.

For lines Tg32, Tg52, and Tg55, no transgene expression was observed in XX Tg/- fetal gonads at 10.5 dpc; whereas, expression of Tg77 was observed at 10.5 dpc, just prior to the onset of endogenous *Sry* expression. At 11.5 dpc and 12.5 dpc, expression of each of the four transgenes in XX Tg/- fetal gonads was observed in parallel with expression of *Sry* in XY fetal gonads. At 14.5 dpc, continued expression of each transgene was observed in XX Tg/- fetal gonads, whereas expression of *Sry* in control XY fetal gonads was absent. To determine whether expression of a transgene persisted until birth, gonads from XX Tg/- newborn mice were analyzed. In contrast to *Sry* expression, which was absent, expression of the four transgenes was observed. Transgene expression in XX Tg77/Tg77 and XX Tg55/Tg55 homozygotes paralleled the expression patterns observed in gonads from their hemizygous counterparts.

We used quantitative RT-PCR to determine the expression level of each transgene relative to endogenous *Sry* transcripts in XY fetal gonads (Fig. 5A). In general, determinations were made at 11.5 dpc, the point at which *Sry* transcript accumulation is at its maximum (Hacker, *et al.*, 1995). Transgene transcript levels for Tg55/- and Tg77/- XY gonads were increased 1.15 and 1.75 fold, respectively, compared to *Sry* transcript levels. Not surprisingly, transgene transcript levels in Tg55/Tg55 and Tg77/Tg77 XY gonads were nearly double this level (2.40 and 3.60 fold, respectively). The dominant transgene Tg52 was expressed in hemizygotes at 2.77 fold increase over that of *Sry*. Expression of the dominant transgene Tg32 was only determined at

12.5 dpc, a point in time when *Sry* transcript levels are subsiding. At this time, Tg32 was expressed at a 10.13 fold increase compared to *Sry*.

The sex-specific expression patterns of anti-Müllerian hormone (*Amh*) and desert hedgehog homolog (*Dhh*), two molecular markers of Sertoli cell differentiation, were assayed by RT-PCR using RNA from gonad/mesonephros complexes from individual 14.5 dpc fetuses (Fig. 5B). In the dominant sex reversing lines Tg32 (*SrySox3*) and Tg52 (*SrySox9*), *Amh* and *Dhh* were expressed in XY -/-, XY Tg/- , and XX Tg/- gonads, but not in XX -/- gonads. In the recessive sex reversing lines Tg77 (*SrySox3*) and Tg55 (*SrySox9*), *Amh* and *Dhh* were not expressed in XX -/- or XX Tg/- fetal gonads; whereas, both were expressed in XX Tg/Tg, XY-/-, and XY Tg gonads. That is, *Amh* and *Dhh* expression was only observed if testicular tissue had developed despite the fact that transcription of Tg77 and Tg55 was detected in XX Tg/- fetal gonads.

Expression of two markers specific for Leydig cells were examined: cytochrome P450 cholesterol side-chain cleavage (*Cyp11a*); and cytochrome P450 17 alpha-hydroxylase/17,20 lyase (*Cyp17*) (Fig. 5C, D). Because these markers are sometimes detectable in XX fetal gonadal tissues (Greco and Payne, 1994), we used semiquantitative PCR to assay expression levels relative to *Hprt*. *Cyp11a* was strongly detected in XY -/- gonadal tissues (7.6 fold higher than *Hprt*) but only weakly detected in XX -/- gonadal tissues (0.02 fold as high as *Hprt*). Likewise, *Cyp17* was strongly detected in XY -/- gonadal tissues (11.4 fold higher than *Hprt*) but only weakly detected in XX -/- gonadal tissues (0.11 fold as high as *Hprt*). For the dominant lines Tg32 (*SrySox3*) and Tg52 (*SrySox9*), we observed high levels of *Cyp11a* and *Cyp17* expression (typical of XY controls) in XY Tg/-and XX Tg/- fetal gonads. For the recessive lines Tg77 (*SrySox3*) and Tg55 (*SrySox9*), weak expression of these genes (typical of XX controls) was observed in XX Tg/-fetal gonads, but strong expression of both (typical of XY controls) was observed in XY Tg and XX Tg/Tg fetal gonads. In these cases the upregulation of *Cyp11a* and *Cyp17* expression (typical of XY controls) was not activated by the lower levels of transgene expression present in hemizygotes but required the higher level of transgene expression present in XX Tg/Tg homozygous.

DISCUSSION

Functional Substitution of HMG Boxes

Sox genes encode proteins related to each other and to *Sry* by the presence of a DNA binding motif, known as the HMG domain (Gubbay *et al.*, 1990), that recognizes a short consensus DNA sequence containing significant degeneracy. Data obtained in vitro suggests that the DNA binding specificity of *Sox* proteins results from preferential binding to more specific expanded consensus sequences (Mertin *et al.*, 1999) or from interaction with specific partner proteins (Kamachi *et al.*, 2000), or perhaps both. The problem of DNA binding specificity among SOX proteins is especially interesting in the differentiating mammalian gonad where *Sry* and other *Sox* genes (including *Sox3* and *Sox9*) are expressed.

Our results demonstrate that *Sox3* and *Sox9* HMG boxes can functionally substitute for the *Sry* HMG box because both *SrySox3* and *SrySox9* transgenes caused sex reversal in XX transgenic individuals. Such sex reversal was apparent at the levels of primary gonadal development (formation of testicular cords), expression of testis specific markers (in fetal gonads), and secondary sexual differentiation (elaboration of adult male sexual characteristics).

Regardless of construct, we identified some lines in which all XXTg/- mice were male. In other cases, we identified lines in which XXTg/- mice were female but all XXTg/Tg mice were male. Not surprisingly, we also identified lines exhibiting incomplete sex reversal (XXTg/- hermaphrodites) or no sex reversal (XXTg/Tg females). This range of phenotypes is consistent

with the hypothesis that the degree of expression from a transgene depends on copy number and integration site.

It is possible that the transgenic constructs analyzed did not substitute for SRY but instead activated the male sex determination pathway at a point downstream of SRY, perhaps at the level of SOX9. This possibility seems unlikely given the differences in structure between the SRY and SOX9 proteins outside of the HMG domain. For example, the SOX9 protein contains a C-terminal transcriptional activation domain (Ng *et al.*, 1997) that may not have a functional equivalent in SRY. Although mechanisms can be invoked whereby the *SrySox3* and *SrySox9* transgene activate the male sex determination pathway downstream of SRY, the simplest explanation is that *SrySox3* and *SrySox9* transgenes act as functional replacements of SRY.

Binding Specificity of SOX3 and SOX9 HMG Domains

Analysis of the human SOX9 protein in vitro has demonstrated a DNA binding preference for the sequence AGAACAATGG (preferred flanking sequences underlined). The human SRY protein, however prefers the expanded consensus TAAACAATAG (Mertin *et al.*, 1999). Although the increased specificity afforded by expanded consensus sites observed in vitro may play a role in vivo, this remains to be tested. The interchangeable nature of the HMG boxes demonstrated here suggests that potential binding site specificity does not completely preclude interactions at presumably noncognate binding sites in mice. Thus, a mouse *Sry* gene containing a *Sox3* or *Sox9* HMG box can direct male sexual differentiation presumably by activating the same target sequences that *Sry* does.

The dissimilarity of the *Sox3* and *Sox9* HMG boxes at the level of sequence relatedness may not indicate functional dissimilarity with regard to DNA binding site preference in vivo. Our data suggest that the more divergent HMG box of *Sox9* functions as well as (if not better than) the HMG box of *Sox3* as a substitute for the HMG box of *Sry*. It does seem, however, that some degree of over-expression of *SrySox3* and *SrySox9* transgenes in vivo is necessary to cause male sex reversal. For example, TgN-(*SrySox3*)77 caused sex reversal at an expression threshold somewhere between 1.75 and 3.60 fold above *Sry* and TgN(*SrySox9*)55 caused sex reversal at a threshold somewhere between 1.15 and 2.40 fold above *Sry*. Neither the 1.15 fold overexpression of *SrySox9* (observed in Tg 55 hemizygotes) nor the 1.75 fold overexpression of *SrySox3* (observed in Tg 77 hemizygotes) was sufficient to cause sex reversal. Parallel experiments expressing *SrySox3*, *SrySox9*, and *Sry* (as a control) as single copy transgenes (Bronson *et al.*, 1996) would be informative.

A substantial body of work indicates that at least some *Sox* gene products achieve high affinity DNA binding specificity by interacting with specific partner proteins (Ambrosetti *et al.*, 1997; De Santa Barbara *et al.*, 1998; Fraidenraich *et al.*, 1998; Kamachi *et al.*, 1999; Kamachi *et al.*, 1995; Kamachi *et al.*, 1998; Lamb and Rizzino, 1998; Li *et al.*, 1997; Ng *et al.*, 1997; Nishiguchi *et al.*, 1998; Yuan *et al.*, 1995). These interactions may or may not involve the HMG domain. In chicken SOX1, the protein-protein interaction domain maps within the proximal portion of the C-terminal domain but is distinct from the HMG box. In chicken SOX9, the interaction domain maps to a similar location but involves the HMG box as well. Our finding that the HMG boxes of mouse *Sox3* and *Sox9* can replace the HMG box of *Sry* and elicit male sexual differentiation in vivo suggests that any potential partner protein interactions critical for high affinity binding to *Sry* target genes were unperturbed by the HMG domain substitutions.

Transcriptional Regulation of the *SrySox3* and *SrySox9* Transgenes

For the four lines we analyzed in detail, the transcriptional regulation of *SrySox3* and *SrySox9* parallels that of *Sry* with two notable exceptions. First, these four transgenes continued

to be transcribed after *Sry* RNA expression was extinguished. We suggest that the transgene constructs used lack a *cis*-acting regulatory element necessary for transcriptional silencing.

Second, the *SrySox9* transgene is prematurely expressed (at l0.5 dpc) in line Tg77. This misexpression could be explained if the transgene were responding to the endogenous regulatory elements of another gene at the site of integration. The *cn*-like dwarfism exhibited by Tg77 homozygotes may represent the inactivation of just such a gene. Another intriguing possibility is that temporal misexpression of Tg77 at 10.5 dpc (or perhaps an ectopic spatial expression of Tg77) leads to dwarfism by interfering with other SOX proteins.

The Role of *Sox3* in the Sex Determination Pathway

It has been proposed that *Sox3* acts as a repressor of *Sox9* expression during sex determination in females and that *Sry* expression relieves *Sox9* repression by acting as a repressor of *Sox3* in males (Graves, 1998a, 1998b). One means by which *Sry* could repress *Sox3* activity would be by competing for DNA binding sites. Our results demonstrate that the HMG domain of *Sox3* can interact with genes in the sex-determining pathway when expressed appropriately and as thus our data is compatible with this hypothesis.

METHODS

Nomenclature

Founders are referred to by number (e.g., founder #32). Individual transgenic lines or transgene integration sites are referred to by official designation (e.g., TgN[*SrySox3*]32Ei) or an abbreviation thereof (e.g., Tg-[*Sox3*]32 or Tg32). Homozygosity of a transgene is designated as Tg/Tg (e.g., Tg55/Tg55). Hemizygosity of a transgene is designated as Tg/- (e.g., Tg55/-). Absence of a transgene is denoted as -/-. Uncharacterized transgene carriers (Tg/Tg or Tg/-) are designated as Tg (e.g., Tg55). Transgenes that cause complete sex reversal of XX mice when hemizygous are referred to as “dominant” sex reversing transgenes. Transgenes that cause complete sex reversal of XX mice when homozygous are referred to as “recessive” sex reversing transgenes.

Oligonucleotides

Oligonucleotides used for PCR and sequencing (Table 1) were purchased from Great American Gene Company (Ramona, CA).

Transgenesis

Plasmid p741ΔCS (containing the 129 strain-derived 14.6 kb *Sry* genomic DNA insert from λ741 [Gubbay *et al.*, 1990] in a modified pBluescript I vector) provided the backbone into which the *Sox3* and *Sox9* HMG boxes were cloned (Fig 2). A 467 bp *Eco*RV fragment from p741ΔCS, which contains the HMG box of *Sry* and flanking DNA (nucleotides 8247 to 8714, GenBank entry X67204 [Gubbay, *et al.*, 1990]), was subcloned into the *Eco*RV site of pBluescript II SK+ and used as a template for inverse PCR using primers *Sry* HMG 5'R and *Sry* HMG 3′F. The HMG boxes of mouse *Sox3* and *Sox9* (nucleotides 221 to 457 [Collignon *et al.*, 1996] and nucleotides 608 to 844 [Wright *et al.*, 1995], respectively) were amplified from their respective cDNAs using the *Sox3* HMG 5′F/*Sox3* HMG 3′R or *Sox9* HMG 5′F/*Sox9* HMG 3′R primer pairs, and the DNA polymerase *Pfu*.

Each *Sox* HMG box PCR product was ligated into the inverse PCR product described above. *Eco*RV fragments containing the substituted HMG boxes were then substituted for the corresponding *Eco*RV fragment in p741ΔCS to form the *pSrySox3* and *pSrySox9* transgene constructs.

Purified DNA was injected at a concentration of 10 ng/μl into fertilized FVB/NJ or C57BL/6J oocytes using standard methodologies (Hogan *et al.*, 1994). Founders were identified by PCR using the genotyping protocols described below. To establish independent transgenic lines, founders were mated to mice of the same inbred strain. In some instances, two independent lines were established from a single founder.

Genotyping

Animals were genotyped for the presence of a transgene and a Y Chromosome using DNA prepared from tail-tip biopsies (modified from Higuchi, 1989). All genotyping was performed by multiplex PCR using primers 1887 and 1888 (designed from the myogenin gene) as a control to verify that amplification occurred (Capel *et al.*, 1999). Amplification of the *SrySox3* transgene utilized primers *Sry*8045F and *Sox3*-357R or *Sry*8041F and *Sox3*-361R. Amplification of the *SrySox9* transgene utilized primers *Sry*8045F and *Sox9*-744R. Thermocycling parameters were: 94°C for 2 min 30 s; 40 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min; 72°C for 9 min; and holding at 4°C. Amplification of the Y Chromosome used primers 1889 and 1890 (Capel *et al.*, 1999) designed from the Y-specific repeat, pYMT2/B (Bishop and Hatat, 1987). Thermocycling parameters were: 95°C for 2 min; 35 cycles of 96°C for 10 s, 60°C for 30 s, 72°C for 30 s; 72°C for 5 min; and holding at 4°C.

Determination of Transgene Homozygosity/Hemizygosity in Recessive Sex Reversing Lines

For 11.5 and 12.5 dpc XX Tg fetuses, Tg hemizygosity versus homozygosity was determined by semiquantitative PCR. Aliquots of tail lysate were amplified for 20 cycles as described above (see Genotyping) with the addition of α-[^{32}P]-dCTP (0.1-0.2 μl/reaction [10μCi/μl, 3,000 Ci/mmol], 24 μM nonradioactive dNTPs). The amplification products were resolved on 2.5% agarose gels, alkaline blotted onto positively charged nylon membranes, exposed on phosphoimaging plates (Fuji), and quantitated using ImageGauge software.

For 14.5 dpc XX Tg fetuses and newborn XX Tg mice, hemizygosity versus homozygosity of a transgene was inferred by observing gonad morphology: Those containing ovaries were assumed to be Tg/- and those containing testes were assumed to be Tg/Tg.

Determination of Transgene Copy Number

The number of copies of construct DNA incorporated into each integration site was determined by quantitative PCR. Duplicate samples of genomic DNA (25 ng, chromosomally XY, hemizygous for the transgene) were amplified in the presence of α-[^{32}P]-dCTP using the *Sry*-specific primers *Sry*9431F and *Sry*9808R. Thermocy-cling parameters were: 95°C for 2 min; 20 cycles of 94°C for 30 s, 57°C for 30 s, and 72°C for 30 s; 72°C for 5 min; and holding at 4°C. Following amplification, reaction products were digested with *Nla*IV (1U/reaction, 2 h at 37°C). *Nla*IV does not cleave the 378 bp transgene product but digests the FVB/NJ-derived *Sry* product into two comigrating bands of approximately 190 bp. Following digestion, the amplification products were resolved on 1.5% agarose TAE gels, and alkaline blotted onto positively charged nylon membranes. After a 1 h exposure to imaging plates, a Fuji Phosphor imaging system equipped with ImageGauge software was used to quantitate the intensity of each transgene derived band relative to the single copy *Sry* band. Transgene copy number was defined by the average of two determinations using DNA from different mice.

Timed Matings

Urogenital ridges or gonads with attached mesonephroi were dissected from fetuses at 10.5, 11.5, 12.5, and 14.5 dpc. The developmental age of fetuses was confirmed by analysis of somite number posterior to the hind limb bud (10.5 dpc = 8 ± 3 somites, 11.5 dpc = 18 ± 3 somites, 12.5 dpc = 28 ± 3 somites) and/or foot plate morphology (Kaufman, 1995; Rugh, 1968).

Assessment of Gonad Morphology

Gonads from at least 75 fetuses (with a minimum of 14 being XX Tg) per line were dissected in a buffered EDTA solution, observed under brightfield optics, and photographed as previously described (Eicher and Washburn, 1978). Gonads were scored as testes or ovaries based on the presence of testicular cords or reticular tissue, respectively. Gonads were scored as ovotestes if both tissues were present.

RT-PCR

RNA was prepared from pairs of urogenital ridges (10.5 and 11.5 dpc) or gonads with attached mesonephroi (12.5 dpc, 14.5 dpc, and newborn pups) using the RNeasy Miniprep protocol (Qiagen, Inc., Hercules, CA) and eluted in a volume of 30 μl. Reverse transcription (RT) reactions (PE Applied Biosystems, Inc, Riverside, CA) were carried out in 15-30 μl volumes containing 5-10 μl of RNA (as described above), 1 X Buffer II, 5 mM $MgCl_2$, 1.0 U/μl of RNase inhibitor, 2.5 U/μl of MuLV reverse transcriptase, 2.5 μM oligo $d(T)_{16}$, and 1 mM dNTPs (Amersham Pharmacia Biotech, Piscataway, NJ). Incubation was at 25°C for 10 min, 42°C for 60 min, 99°C for 5 min, and holding at 4°C. PCR amplification of RT reactions was carried out in reactions containing: 1 X Buffer II, 1.5 mM $MgCl_2$, 200 μM dNTPs and 200 nM primers.

Qualitative analyses of *Sry* and transgene expression utilized primers *Sry*10105F and *Sry*10279R (specific for *Sry* and the transgenes) and primers oMJ41 and oMJ42 (internal positive control primers for ubiquitin-activating enzyme E1, Chr X; *Ube1x*) (Mitchell *et al.*, 1991). Cycling parameters for *Sry* and a transgene were: 95°C for 2 min; 43 cycles 94°C for 30 s, 58°C for 1 min, 72°C for 90 s; 72°C for 10 min; and holding at 4°C. Qualitatitive analyses of anti-Müllerian hormone (*Amh*, primers oIMR501F and m*Amh*1262R) and desert hedgehog homolog (*Dhh*, primers m*Dhh*-F and m*Dhh*-R) expression were performed identically (in multiplex reactions with *Ube1x*) except that the annealing temperature was raised to 62°C and the number of cycles was reduced to 29. For qualitative analyses, gels were stained with ethidium bromide and photographed.

Quantitative analysis of transgene expression was performed on urogenital ridge (11.5 dpc) and gonadal (12.5 dpc, with attached mesonephroi) RNA from XYTg/+ or XY Tg/Tg embryos, using RT-PCR and the *Nla*IV assay described above except that the number of cycles was increased to 27. Semiquantitative analysis of cytochrome P450, 17 (*Cyp17*) expression was performed on 14.5 dpc gonadal RNA from XY Tg/+ or XY Tg/Tg embryos using *Cyp17*-specific primers m*Cyp17F* and m*Cyp17R* with *Hprt*-specific primers *Hprt*F and *Hprt*R (Koopman *et al.*, 1989). Cycling parameters were: 95°C for 2 min; 23 cycles of 94°C for 30 s, 62°C for 1 min, 72°C for 1 min; 72°C for 5 min; and holding at 4°C. Semiquantitative analysis of cytochrome P450, 11a, cholesterol side chain cleavage (*Cyp11a*) expression was performed as described for *Cyp17* except the gene specific primers m*Cyp11a-F* and m*Cyp11a-R* were used and the number of cycles was increased to 25. For quantitative analyses, DNA was alkaline blotted, UV cross-linked, exposed to phosphoimaging plates, and quantified as described above.

ACKNOWLEDGMENTS

We thank Karin Shuster-Gossler of the Cell Biology and Microinjection Service of The Jackson Laboratory (TJL) for preparing transgenic mice, and Doug McMinimy, Amy Lambert, and Andrea Ried of the Microchemistry Service of TJL for DNA sequencing. We thank Linda L. Washburn, Leona H. Gagnon, Barbara K. Lee, and Elaine P. Shown for technical assistance. Appreciation is expressed to Robin Lovell-Badge (MRC, NIMR, London, U.K.) for providing *Sox3* and *Sox9* cDNA clones and p741, Andrew McMahon (Harvard University, Cambridge, MA) for *Dhh* sequence information, and Peter Schweitzer (TJL) for *Amh* primers. We thank Gregory A. Cox and Timothy P. O'Brien (TJL) for critical review of the manuscript.

LITERATURE CITED

Ambrosetti DC, Basilico C, Dailey L. Synergistic activation of the fibroblast growth factor 4 enhancer by *Sox2* and *Oct-3* depends on protein-protein interactions facilitated by a specific spatial arrangement of factor binding sites. Mol Cell Biol 1997;17:6321–6329. [PubMed: 9343393]

Bishop CE, Hatat D. Molecular cloning and sequence analysis of a mouse Y Chromosome RNA transcript expressed in the testis. Nucleic Acids Res 1987;15:2959–2969. [PubMed: 3562242]

Bronson SK, Plaehn EG, Kluckman KD, Hagaman JR, Maeda N, Smithies O. Single-copy transgenic mice with chosen-site integration. Proc Natl Acad Sci U S A 1996;93:9067–9072. [PubMed: 8799155]

Capel B, Albrecht KH, Washburn LL, Eicher EM. Migration of mesonephric cells into the mammalian gonad depends on *Sry*. Mech Dev 1999;84:127–131. [PubMed: 10473126]

Collignon J, Sockanathan S, Hacker A, Cohen-Tannoudji M, Norris D, Rastan S, Stevanovic M, Goodfellow PN, Lovell-Badge R. A comparison of the properties of *Sox-3* with *Sry* and two related genes, *Sox-1* and *Sox-2*. Development 1996;122:509–520. [PubMed: 8625802]

De Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Sudbeck P, Scherer G, Poulat F, Berta P. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Mullerian hormone gene. Mol Cell Biol 1998;18:6653–6665. [PubMed: 9774680]

Denny P, Swift S, Connor F, Ashworth A. An *SRY*-related gene expressed during spermatogenesis in the mouse encodes a sequence-specific DNA-binding protein. Embo J 1992;11:3705–3712. [PubMed: 1396566]

Eicher EM, Shown EP, Washburn LL. Sex reversal in C57BL/6JYPOS mice corrected by a *Sry* transgene. Philos Trans R Soc Lond B Biol Sci 1995;350:263–268. [PubMed: 8570690]discussion 268-269

Eicher EM, Washburn LL. Assignment of genes to regions of mouse chromosomes. Proc Natl Acad Sci U S A 1978;75:946–950. [PubMed: 273256]

Foster JW. Mutations in *SOX9* cause both autosomal sex reversal and campomelic dysplasia. Acta Paediatr Jpn 1996;38:405–411. [PubMed: 8840554]

Foster JW, Dominguez-Steglich MA, Guioli S, Kowk G, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, Brook JD, Schafer AJ. Campomelic dysplasia and autosomal sex reversal caused by mutations in an *SRY*-related gene. Nature 1994;372:525–530. [PubMed: 7990924]

Foster JW, Graves JA. An *SRY*-related sequence on the marsupial X chromosome: implications for the evolution of the mammalian testis-determining gene. Proc Natl Acad Sci U S A 1994;91:1927–1931. [PubMed: 8127908]

Fraidenraich D, Lang R, Basilico C. Distinct regulatory elements govern *Fgf4* gene expression in the mouse blastocyst, myotomes, and developing limb. Dev Biol 1998;204:197–209. [PubMed: 9851853]

Graves JA. Evolution of the mammalian Y Chromosome and sex-determining genes. J Exp Zool 1998a; 281:472–481. [PubMed: 9662834]

Graves JA. Interactions between *SRY* and *SOX* genes in mammalian sex determination. Bioessays 1998b; 20:264–269. [PubMed: 9631654]

Greco TL, Payne AH. Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3 beta-hydroxysteroid dehydrogenase, P450 17 alpha-hydroxylase/C17-20 lyase, and P450 aromatase in fetal mouse gonads. Endocrinology 1994;135:262–268. [PubMed: 8013361]

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P, Lovell-Badge R. A gene mapping to the sex-determining region of the mouse Y Chromosome is a member of a novel family of embryonically expressed genes. Nature 1990;346:245–250. [PubMed: 2374589]

Hacker A, Capel B, Goodfellow P, Lovell-Badge R. Expression of *Sry*, the mouse sex determining gene. Development 1995;121:1603–1614. [PubMed: 7600978]

Hargrave M, Wright E, Kun J, Emery J, Cooper L, Koopman P. Expression of the *Sox11* gene in mouse embryos suggests roles in neuronal maturation and epithelio-mesenchymal induction. Dev Dyn 1997;210:79–86. [PubMed: 9337129]

Higuchi, R. Simple and rapid preparation of samples for PCR. In: Erlich, HE., editor. PCR Technology: Principles and Applications for DNA Amplification. Stockton Press; New York: 1989. p. 31-38.

Hogan, B.; Beddington, R.; Constantini, F.; Lacy, E. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Press; Plainview, NY: 1994. p. 497

Johnson LR, Lamb KA, Gao Q, Nowling TK, Rizzino A. Role of the transcription factor *Sox-2* in the expression of the *Fgf-4* gene in embryonal carcinoma cells. Mol Reprod Dev 1998;50:377–386. [PubMed: 9669521]

Kamachi Y, Cheah KS, Kondoh H. Mechanism of regulatory target selection by the SOX high-mobility-group domain proteins as revealed by comparison of SOX1/2/3 and SOX9. Mol Cell Biol 1999;19:107–120. [PubMed: 9858536]

Kamachi Y, Sockanathan S, Liu Q, Breitman M, Lovell-Badge R, Kondoh H. Involvement of SOX proteins in lens-specific activation of crystallin genes. Embo J 1995;14:3510–3519. [PubMed: 7628452]

Kamachi Y, Uchikawa M, Collignon J, Lovell-Badge R, Kondoh H. Involvement of *Sox1, 2* and *3* in the early and subsequent molecular events of lens induction. Development 1998;125:2521–2532. [PubMed: 9609835]

Kamachi Y, Uchikawa M, Kondoh H. Pairing SOX off: with partners in the regulation of embryonic development. Trends Genet 2000;16:182–187. [PubMed: 10729834]

Kanai Y, Kanai-Azuma M, Noce T, Saido TC, Shiroishi T, Hayashi Y, Yazaki K. Identification of two *Sox17* messenger RNA isoforms, with and without the high mobility group box region, and their differential expression in mouse spermatogenesis. J Cell Biol 1996;133:667–681. [PubMed: 8636240]

Kaufman, MH. The atlas of mouse development. Academic Press; San Diego, CA: 1995.

Kent J, Wheatley SC, Andrews JE, Sinclair AH, Koopman P. A male-specific role for *SOX9* in vertebrate sex determination. Development 1996;122:2813–2822. [PubMed: 8787755]

Koopman P, Gubbay J, Collignon J, Lovell-Badge R. *Zfy* gene expression patterns are not compatible with a primary role in mouse sex determination. Nature 1989;342:940–942. [PubMed: 2480529]

Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R. Male development of chromosomally female mice transgenic for *Sry*. Nature 1991;351:117–121. [PubMed: 2030730]

Koopman P, Munsterberg A, Capel B, Vivian N, Lovell-Badge R. Expression of a candidate sex-determining gene during mouse testis differentiation. Nature 1990;348:450–452. [PubMed: 2247150]

Lamb KA, Rizzino A. Effects of differentiation on the transcriptional regulation of the *FGF-4* gene: critical roles played by a distal enhancer. Mol Reprod Dev 1998;51:218–224. [PubMed: 9740330]

Lane PW. Achondroplasia (cn). Mouse News Lett 1973;49:33.

Lane PW, Dickie MM. Three recessive mutations producing disproportionate dwarfing in mice: achondroplasia, brachymorphic, and stubby. J Hered 1968;59:300–308. [PubMed: 5713631]

Laudet V, Stehelin D, Clevers H. Ancestry and diversity of the HMG box superfamily. Nucleic Acids Res 1993;21:2493–2501. [PubMed: 8506143]

Li X, Cvekl A, Bassnett S, Piatigorsky J. Lens-preferred activity of chicken delta 1- and delta 2-crystallin enhancers in transgenic mice and evidence for retinoic acid-responsive regulation of the delta 1-crystallin gene. Dev Genet 1997;20:258–266. [PubMed: 9216065]

Mertin S, McDowall SG, Harley VR. The DNA-binding specificity of SOX9 and other SOX proteins. Nucleic Acids Res 1999;27:1359–1364. [PubMed: 9973626]

Mitchell MJ, Woods DR, Tucker PK, Opp JS, Bishop CE. Homology of a candidate spermatogenic gene from the mouse Y Chromosome to the ubiquitin-activating enzyme E1. Nature 1991;354:483–486. [PubMed: 1684224]

Morais da Silva S, Hacker A, Harley V, Goodfellow P, Swain A, Lovell-Badge R. *Sox9* expression during gonadal development implies a conserved role for the gene in testis differentiation in mammals and birds. Nat Genet 1996;14:62–68. [PubMed: 8782821]

Mumm S, Zucchi I, Pilia G. *SOX3* gene maps near *DXS984* in Xq27.1, within candidate regions for several X-linked disorders. Am J Med Genet 1997;72:376–378. [PubMed: 9332676]

Ng LJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles J, Wright E, Bell DM, Tam PP, Cheah KS, Koopman P. SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol 1997;183:108–121. [PubMed: 9119111]

Nishiguchi S, Wood H, Kondoh H, Lovell-Badge R, Episkopou V. *Sox1* directly regulates the gamma-crystallin genes and is essential for lens development in mice. Genes Dev 1998;12:776–781. [PubMed: 9512512]

Oosterwegel M, van de Wetering M, Clevers H. HMG box proteins in early T-cell differentiation. Thymus 1993;22:67–81. [PubMed: 8209398]

Pevny LH, Lovell-Badge R. *Sox* genes find their feet. Curr Opin Genet Dev 1997;7:338–344. [PubMed: 9229109]

Rex M, Uwanogho DA, Orme A, Scotting PJ, Sharpe PT. *cSox21* exhibits a complex and dynamic pattern of transcription during embryonic development of the chick central nervous system. Mech Dev 1997;66:39–53. [PubMed: 9376322]

Rugh, R. The mouse: its reproduction and development. Burgess Publishing Co; Minneapolis, MN: 1968. p. 430

Schilham MW, Clevers H. HMG box containing transcription factors in lymphocyte differentiation. Semin Immunol 1998;10:127–132. [PubMed: 9618758]

Soullier S, Jay P, Poulat F, Vanacker JM, Berta P, Laudet V. Diversification pattern of the *HMG* and *SOX* family members during evolution. J Mol Evol 1999;48:517–527. [PubMed: 10198118]

Stevanovic M, Lovell-Badge R, Collignon J, Goodfellow PN. *SOX3* is an X-linked gene related to SRY. Hum Mol Genet 1993;2:2013–2018. [PubMed: 8111369]

Uwanogho D, Rex M, Cartwright EJ, Pearl G, Healy C, Scotting PJ, Sharpe PT. Embryonic expression of the chicken *Sox2, Sox3* and *Sox11* genes suggests an interactive role in neuronal development. Mech Dev 1995;49:23–36. [PubMed: 7748786]

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, Wolf U, Tommerup N, Schempp W, Scherer G. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the *SRY*-related gene *SOX9.* Cell 1994;79:1111–1120. [PubMed: 8001137]

Wegner M. From head to toes: the multiple facets of Sox proteins. Nucleic Acids Res 1999;27:1409–1420. [PubMed: 10037800]

Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A, Koopman P. The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos. Nat Genet 1995;9:15–20. [PubMed: 7704017]

Wright EM, Snopek B, Koopman P. Seven new members of the *Sox* gene family expressed during mouse development. Nucleic Acids Res 1993;21:744. [PubMed: 8441686]

Yuan H, Corbi N, Basilico C, Dailey L. Developmental-specific activity of the *FGF-4* enhancer requires the synergistic action of *Sox2* and *Oct-3.* Genes Dev 1995;9:2635–2645. [PubMed: 7590241]

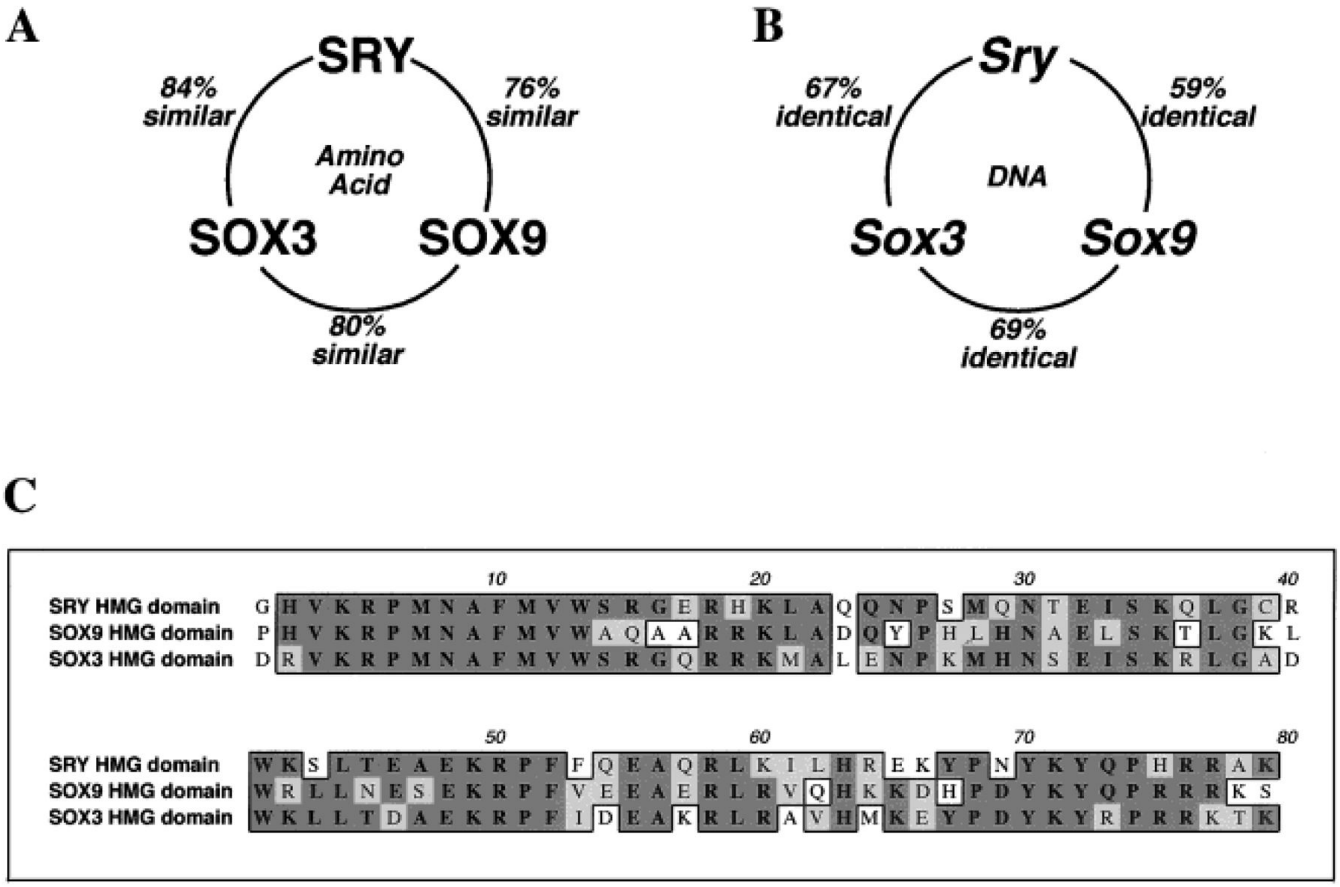

FIG. 1.

The HMG box DNA binding domains of mouse *Sry*, *Sox3*, and *Sox9*. Relatedness of the HMG domains of SRY, SOX3, and SOX9 as defined by amino acid similarity (**A**) and nucleic acid identity (**B**). (**C**) Amino acid alignment of the HMG domains of SRY, SOX3, and SOX9. Dark gray, amino acid identities; light gray, amino acid similarities.

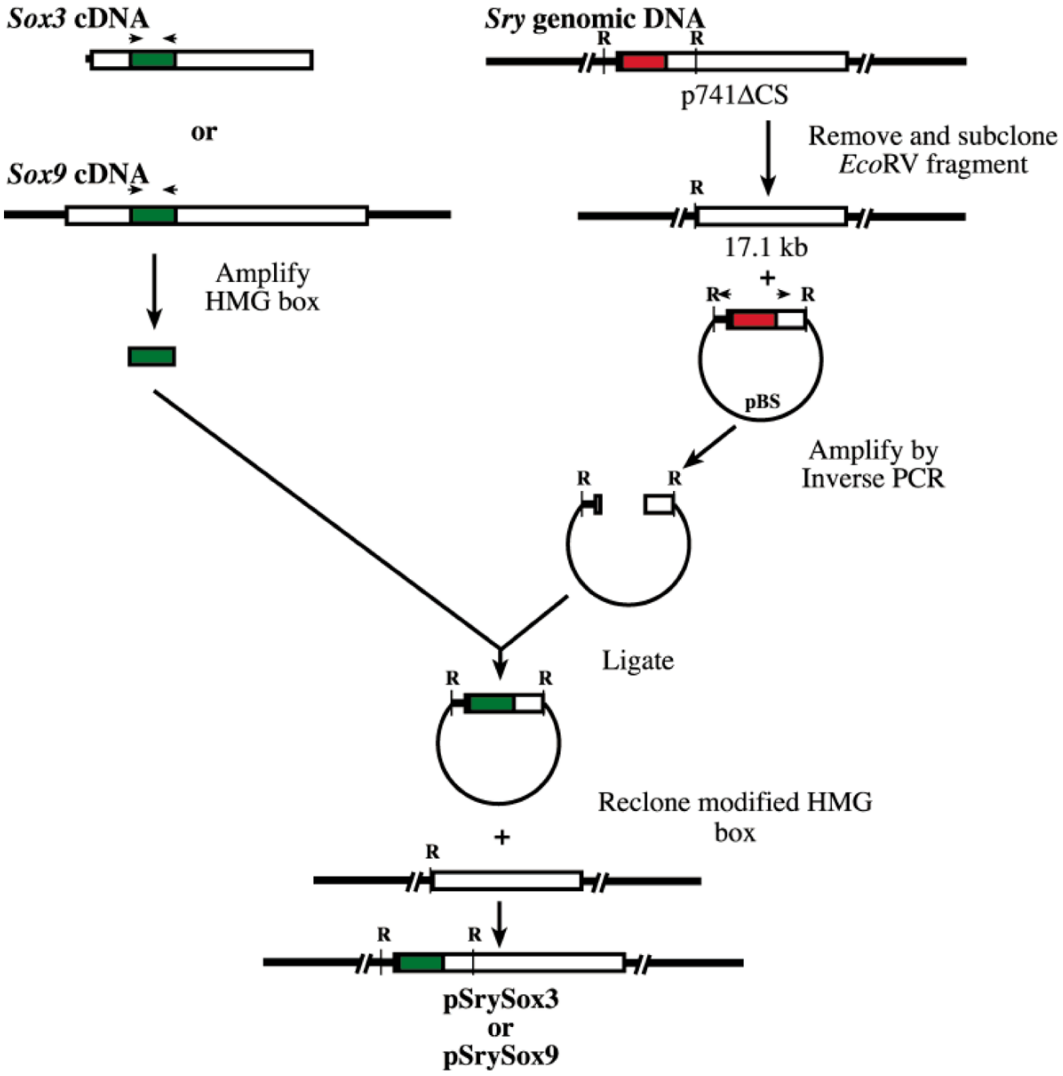

FIG. 2.

Construction of *SrySox3* and *SrySox9* transgenes. The HMG boxes of *Sox3* and *Sox9* were amplified by PCR and cloned into an inverse PCR product containing sequences immediately flanking the HMG box of *Sry*. The replaced HMG boxes and immediate flanking DNA were then subcloned into the larger genomic clone of *Sry* to form the *SrySox3* and *SrySox9* transgenes. White rectangles, open reading frames; green rectangles, *Sox* HMG boxes; red rectangles, *Sry* HMG box; small arrows, PCR primers; solid lines, untranslated DNA; R, *Eco*RV sites.

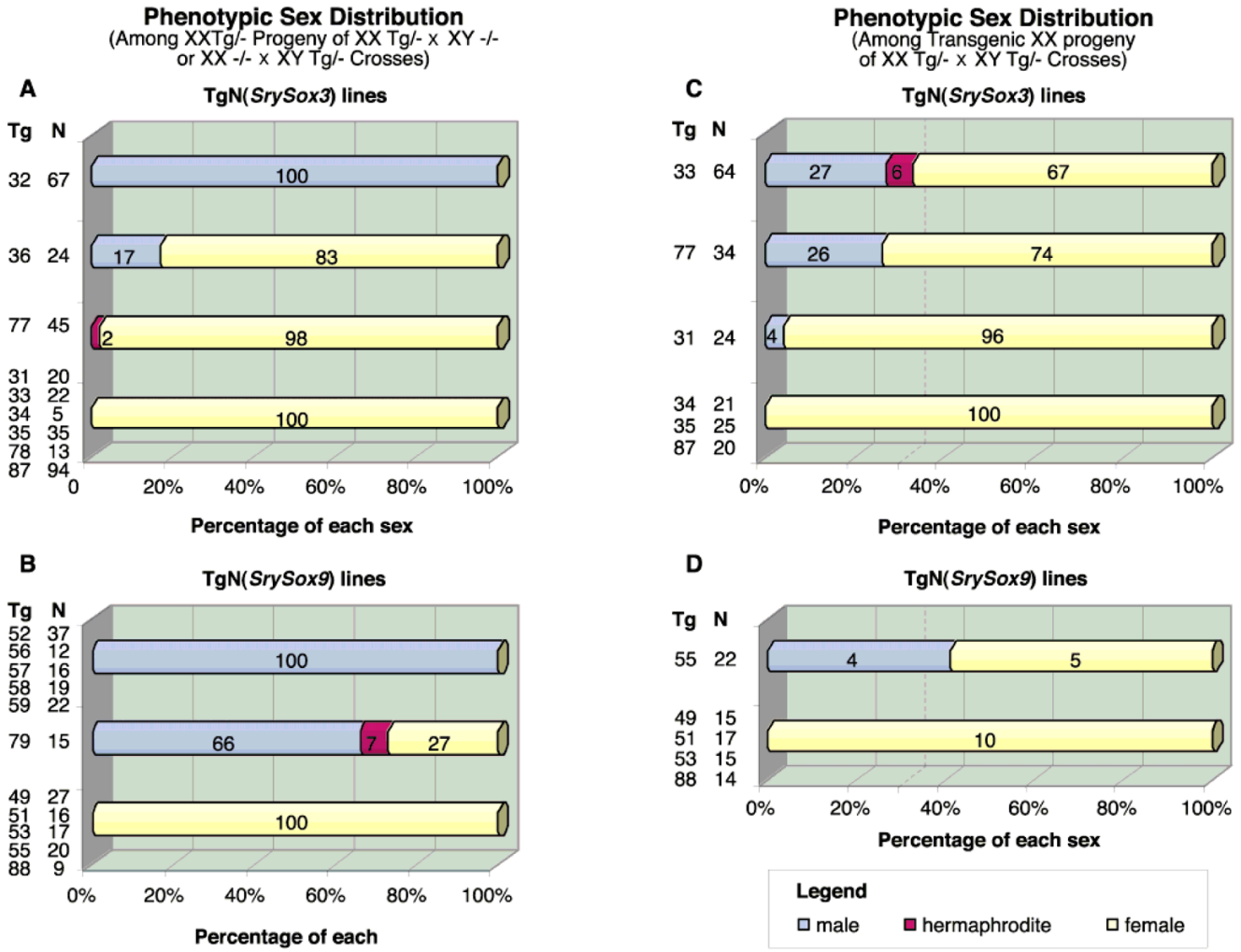

FIG. 3.

Phenotypic sex distribution among transgenic progeny. Percentages of each sex as scored externally at weaning are shown. (**A,B**) Phenotypic sex distribution among XXTg/- progeny of XX Tg/-3 XY -/- or XX -/- 3 XY Tg/- crosses. Transgene lines 32, 52, 56, 57, 58, and 59 displayed complete sex reversal among XX Tg/- progeny. Sex reversal within transgene lines 36, 77, and 79 was only partially penetrant. (**A**) TgN(*SrySox3*) lines. (**B**) TgN(*SrySox9*) lines. (**C,D**) Phenotypic sex distribution among transgenic XX progeny of XX Tg/- × XY Tg/- crosses. Transgene lines 33, 55, and 77 displayed complete sex reversal of presumed XX Tg/Tg progeny. (**C**) TgN(*SrySox3*) lines. (**D**) TgN(*SrySox9*) lines. Tg, transgene number; N, number of animals scored.

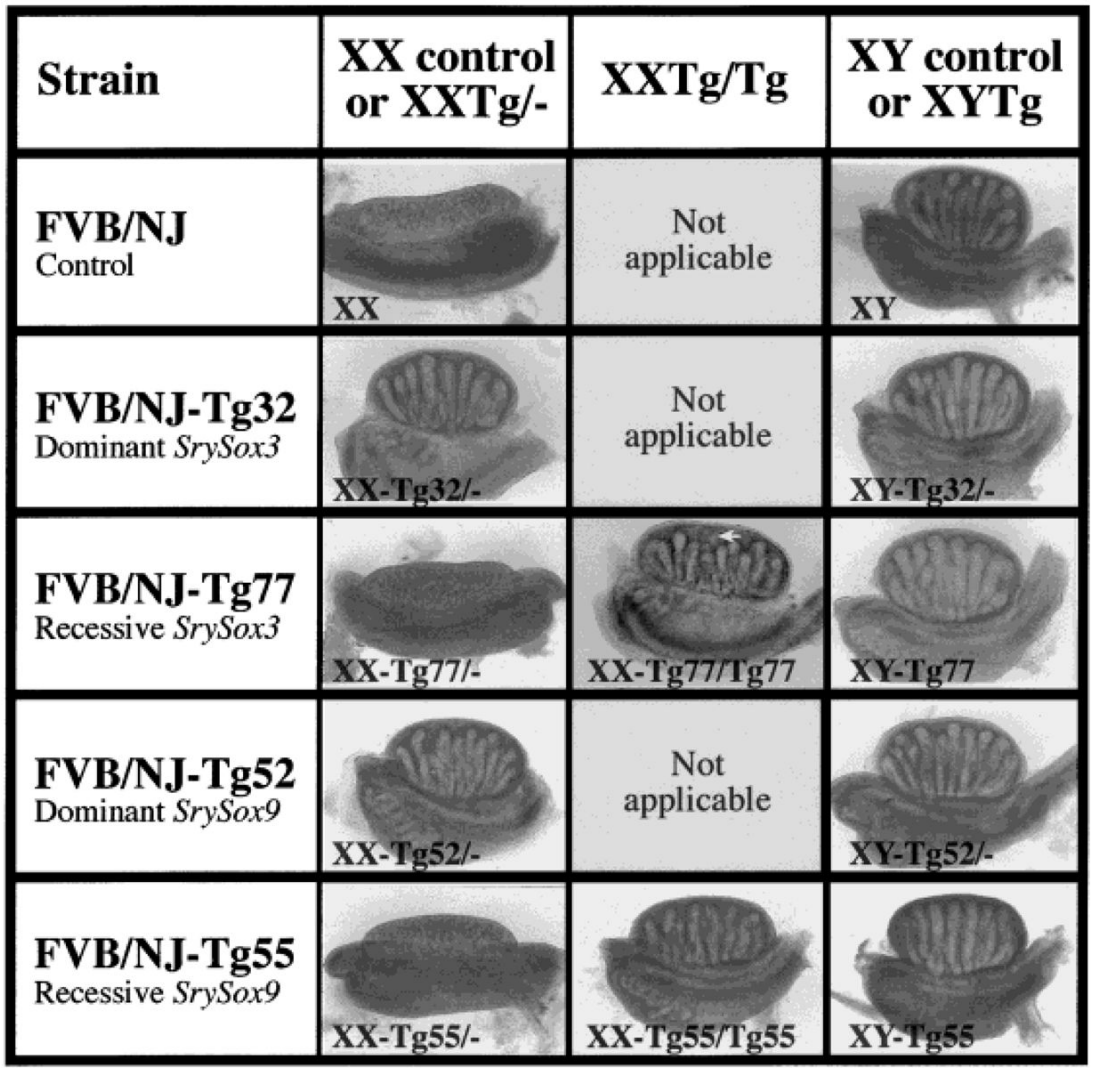

FIG. 4.

Morphology of *SrySox3* and *SrySox9* fetal gonads. Shown are representative fetal gonads from the dominant line Tg(*Sox3*)32, the recessive line Tg(*Sox3*)77, the dominant line Tg(*Sox9*)52, the recessive line Tg(*Sox9*)55, and nontransgenic controls at 14.5 dpc. A minimum of 75 fetuses per line were analyzed. The less extensive cord development of a XX Tg77/Tg77 fetal testis is shown by an arrowhead.

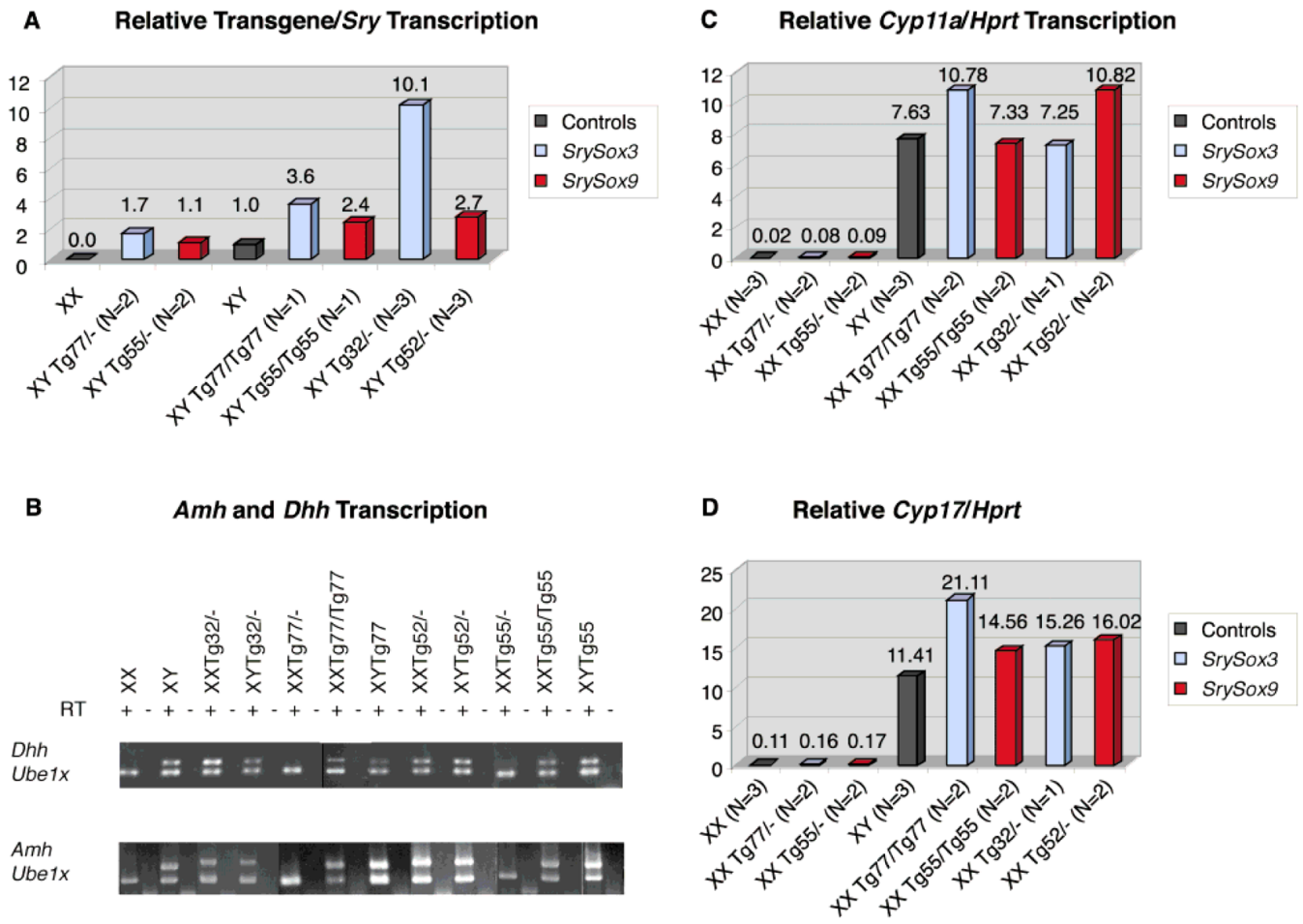

FIG. 5.

Qualitative and quantitative RT-PCR. (**A**) Relative transgene/*Sry* transcription levels as determined by quantitative RT-PCR. The values of 0 for XX gonads and 1 for XY gonads are assigned. All other levels are measured relative to endogenous *Sry* expression. All values are for gonads at 11.5 dpc except for gonads from Tg32, which were 12.5 dpc. N, number of gonad/mesonephros pairs assayed. (**B**) *Dhh* and *Amh* expression in *SrySox3* and *SrySox9* fetal gonads. Composite figure of representative RT-PCR assays of *Dhh* and *Amh* expression in 14.5 dpc *SrySox3*, *SrySox9*, and control fetal gonads. +RT, experimental reactions containing reverse transcriptase; -RT, control reactions without reverse transcriptase; *Ube1x*, internal control. Each assay is representative of duplicate experiments. (**C,D**) Relative *Cyp11a* and *Cyp17/Hprt* expression in *SrySox3* and *SrySox9* fetal gonads. Shown are the gonadal/mesonephric expression levels of *Cyp11a* (**C**) and *Cyp17* (**D**) relative to *Hprt* as determined by semiquantitative RT-PCR. All values are for gonads at 14.5 dpc. N, number of gonad pairs assayed.

Table 1

Oligonucleotides

Forward primer	**Sequence (5′-3′)**	**Reverse primer**	**Sequence (5′-3′)**	**Use**
A. *SrySox3* and *SrySox9* transgene construction				
Sry HMG 5′R	CTCCATGCTCTCTAGACAATTGTCACCAGTC	*Sry* HMG 3′F	GTGTCACAGAGGAGTGGCATTTTACAGCCT	*SrySox3* and *SrySox9*
Sox3 HMG 5′F	GACCGCGTCAAGCGACCCATG	*Sox3* HMG 3′R	CTTGGTCTTGCGGCGGGGC	*SrySox3*
Sox9 HMG 5′F	CCACACGTCAAGCGACCCAT	*Sox9* HMG 3′R	CGACTTCCTCCGCCGGG	*SrySox9*
B. *SrySox3* and *SrySox9* ORF sequencing				
Sry 8064F	CTTTAAGTTTTGACTTCTGTATC	*Sry* 9432 R	CAGTGATGTCAGCTGTTAGTA	Sequencing *SrySox3* and *SrySox9* ORFs
Sry 8612F	GTGGGACAGGAACCCACAT			
C. Y Chromosome, *SrySox3* and *SrySox9* genotyping				
1887	TTACGTCCATCGTGGACAGCAT	1888	TGGGCTGGGTGTTAGTCTTAT	*Myog*[a]
1889	CAGTTACCAATCAACACATCAC	1890	CTGGAGCTCTACAGTGATGA	Y Chr (YMT2/B gene)
Sry 8045F	GTGGCTTTTAGCTCTTACACT	*Sox3*-357R	TCGGTCAGCAGTTTCCAGTC	*SrySox3* transgene
Sry 8041F	GTCAGTGGCTTTTAGCTCTTACAC	*Sox3*-361 R	CGCATCGGTCAGCAGTTT	*SrySox3* transgene
Sry 8045F	GTGGCTTTTAGCTCTTACACT	*Sox9*-744R	TCGTTCAGCAGCCTCCAGA	*SrySox9* transgene
D. Quantitative PCR and RT-PCR				
Sry 9431F	TGGTGAGCATACACCATACC	*Sry* 9808 R	TTGCTGTCTTTGTGCTAGCC	*Sry* and transgenes
E. RT-PCR				
oMJ41	TGTCCACACCCACTTACT	oMJ42	GCACTCTGCAACTCCTGG	*Ubelx*[b]
*Sry*10105F	GGACTCTTACTAGATGCCTGGC	*Sry*10279R	GTGTTTTTGCCTGCTCAGAA	*Sry* and transgenes
oIMR501F	GGAACACAAGCAGAGCTTCC	*mAmh1262R*	CGCTGGTCCAGAGTATAGCAC	*Amh*
mDhh-F	GGATTCGACTGGGTCTACTACG	*mDhh-R*	GGGTGTGAGCAACAGTTTGC	*Dhh*
F. Semiquantitative RT-PCR				
HprtF	CCTGCTGGATTACATTAAAGCACTG	*HprtR*	GTCAAGGGCATATCCAACAACAAAC	*Hprt*[c]
mCyp17-F	CCAGCCTGACAGACATTCTG	*mCyp17-R*	TATCGTGATGCAGTGCCCA	*Cyp17*
mCyp11a-F	AGGACTTTCCCTGCGCT	*mCyp11a-R*	GCATCTCGGTAATGTTGG	*Cyp11a*

[a] Used in multiplex reactions as an internal control in conjunction with all genotyping assays.

[b] Used in multiplex reactions as an internal control in conjunction with all RT-PCR assays.

[c] Used in multiplex reactions as a quantitation standard in conjunction with all semiquantitative RT-PCR assays.

Table 2

Segregation Data for SrySox3 and SrySox9 Founders

Founder[a]	Sex	Sex Chrom. Const.	Transgenic offspring (%) XX female	XX male	XY male	Total number offspring (N)	Tg transmittance (%)	Lines derived from founder	Hemizygous copy number
A. *SrySox3*									
31	Female	XX	9	0	15	47	23	Tg31	n.d.
32[b]	Male	XY	15	20	28	46	63		
								Tg32	32
								Tg77	8
33[b]	Male	XY	12	0	21	42	33		
								Tg33	4
								Tg87	n.d.
34	Male	XY	3	0	0	40	3	Tg34	n.d.
35	Male	XY	7	0	11	45	18	Tg35	3
36[b]	Male	XY	28	5	19	93	57		
								Tg36	4
								Tg78	1
B. *SrySox9*									
49	Male	XY	21	0	27	48	48	Tg49	3
50[c]	Male	XX						none	
51	Male	XY	2	0	2	48	4	Tg51	13
52[b]	Male	XY	27	15	38	34	79		
								Tg52	8
								Tg88	1
53	Female	XX	30	0	21	44	50	Tg53	3
54	Male	XY	0	0	0	46	0	none	
55	Female	XX	17	0	17	36	33	Tg55	4
56[b]	Male	XY	6	15	21	47	43		
								Tg56	19
								Tg79	13
57	Male	XY	0	21	26	38	47	Tg57	25

Founder[a]	Sex	Sex Chrom. Const.	Transgenic offspring (%) XX female	XX male	XY male	Total number offspring (N)	Tg transmittance (%)	Lines derived from founder	Hemizygous copy number
58	Female	XX	0	7	5	42	12	Tg58	40
59	Female	XX	0	23	50	26	73	Tg59	22
60[c]	Male	XX						none	

n.d., not determined

[a] All founders and lines were of the strain FVB/NJ except for Tg31 which was of the strain C57BL/6J.

[b] Founder carried more than one transgene.

[c] Sex reversed.

Table 3

Fetal Gonad Morphology Among Mice From Two SrySox3 (Tg32, Tg77) and Two SrySox9 (Tg52, Tg55) Transgenic Lines

		Fetal gonad morphology (N)	
Mating female × male	**Genotype of offspring**	**Ovary**	**Testis**
A. -/- × Tg32/-			
	XX	16	0
	XX Tg/-	0	14
	XY	0	28
	XY Tg/-	0	26
B. Tg77/- × Tg77/-			
	XX	14	0
	XX Tg	22	6
	XY	0	10
	XY Tg	0	26
C. -/- × Tg52/-			
	XX	22	0
	XX Tg/-	0	35
	XY	0	26
	XY Tg/-	0	22
D. Tg55/- × Tg55/-			
	XX	10	0
	XX Tg	21	13
	XY	0	11
	XY Tg	0	24

Table 4

Sry and Transgene Transcription Profiles

Genotype	10.5 dpc	11.5 dpc	12.5 dpc	14.5 dpc	newborn
XY *Sry*	-	+	+	-	-
XX Tg(*Sox3*)32/-	-	+	+	+	+
XX Tg(*Sox9*)52/-	-	+	+	+	+
XX Tg(*Sox3*)77/-	+	+	+	+	+
XX Tg(*Sox3*)77/Tg(*Sox3*)77	n.d.	+	+	+	+
XX Tg(*Sox9*)55/-	-	+	+	+	+
XX Tg(*Sox9*)55/Tg(*Sox9*)55	n.d.	+	n.d.	+	+

n.d., not determined.