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Influence on spatiotemporal patterns of a male-specific *Sox9* activation by ectopic *Sry* expression during early phases of testis differentiation in mice

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Abstract

Testis induction is associated with gonadal *Sry* and *Sox9* expression in mammals. This study investigated whether *Sry* expression directly induces male-specific *Sox9* activation during early phases of testis differentiation. We have established an XX sex-reversal mouse line carrying the *Sry* transgene driven by a weak basal promoter of the *Hsp70.3* gene (*Hsp-Sry*), whereby the transgene was activated in the gonads along the entire anteroposterior axis from earlier stages. The effects of misexpression and overexpression of *Sry* on the spatiotemporal pattern of *Sox9* expression were examined using both XX and XY gonads of *Hsp-Sry* transgenic embryos. It was shown that ectopic expression of *Sry* transcripts in the entire gonadal area from earlier stages promotes neither any advance in the timing nor any appreciable ectopic activation of endogenous *Sox9* expression. Immediately after the onset of *Sox9* activation, however, both the level of *Sox9* expression and the number of SOX9-positive cells were significantly enhanced in *Hsp-Sry*/XY gonads, as compared with those in wild-type/XY and *Hsp-Sry*/XX gonads. These findings suggest that, although *Sry* is capable of up-regulating *Sox9* expression dose-dependently, *Sry* mRNA expression alone is not likely to provide positional or timing information needed for male-specific *Sox9* activation in developing XY gonads.

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Introduction

Sry (sex-determining region of the Y chromosome) is essential for initiating male sex differentiation in mammals. *Sry* is activated for a very short period in gonadal somatic cells to initiate Sertoli cell differentiation in mice. Several recent studies have demonstrated that *Sry* expression is first detected in the central region of the XY gonad at 11.0 dpc (12–14 tail–somite [ts] stage) and extends to both anterior and posterior ends by 11.5 dpc (approximately 17–18 ts) by using in situ hybridization (Bullejos and Koopman, 2001) and transgenic mouse approaches (Albrecht and Eicher, 2001). Thereafter, *Sry* expression is rapidly down-regulated in the middle region, becoming restricted to the posterior pole before it completely disappears at around 12.5 dpc (approximately 30 ts) (Bullejos and Koopman, 2001). This center-to-pole expression profile of *Sry* indicates that activation of the male-specific program may start in the gonadal somatic cells located in the middle portion of the XY genital ridge. This

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is also consistent with our previous report demonstrating a similar center-to-pole pattern in the potencies of both Sertoli cell differentiation and testis cord formation in the cultures of anterior, middle and posterior segments of the XY genital ridge (Hiramatsu et al., 2003).

Shortly after the onset of *Sry* expression, a *Sry*-related gene, *Sox9*, is up-regulated in mouse XY gonads (Kent et al., 1996; Morais da Silva et al., 1996). This male-specific activation has also been shown to occur in a center-to-pole pattern similar to that of the initial *Sry* expression profile (Moreno-Mendoza et al., 2003; Schepers et al., 2003; this study), suggesting that *Sox9* may be one of the first genes induced by *Sry* in the XY gonad. Human *SOX9* mutation causes abnormal skeletal development with XY sex reversal in most cases (Foster et al., 1994; Wagner et al., 1994). Moreover, misexpression of *Sox9* in XX gonads results in testis development, as demonstrated by the findings in *Odsex* mutant mice with a transgene inserted upstream of *Sox9* (Bishop et al., 2000) and transgenic mice ectopically expressing *Sox9* driven by the *Wt1* promoter (Vidal et al., 2001). With regard to such a close correlation between spatiotemporal expression patterns of *Sry* and *Sox9* in the XY gonad (Albrecht and Eicher, 2001; Kent et al., 1996; Morais da Silva et al., 1996), these reports suggest that *Sry* directly promotes the center-to-pole pattern of *Sox9* activation and that *Sox9* may be involved in the initiation and maintenance of Sertoli cell differentiation during testis differentiation.

Unfortunately, numerous intensive studies have failed to elucidate the mechanism by which *Sry* regulates *Sox9* expression. This may be mainly due to the fact that the regulatory region for the *Sox9* appears to extend through an interval of over 1 Mb in both human and mouse (Bishop et al., 2000; Kanai and Koopman, 1999; Pfeifer et al., 1999; Qin et al., 2004; Wunderle et al., 1998). On the other hand, Lovell-Badge et al. (2002) showed that the reporter construct of mouse *Sox9* gene containing approximately 70 kb 5′- and 30 kb 3′-flanking sequences can mimic its Sertoli cell-specific expression within the gonads of the transgenic mice. Although further deletion and sequence analyses of this region will possibly allow to define the SRY-acting *cis*-elements, a direct linkage between *Sry* and *Sox9* is unclear at present.

This study investigated whether *Sry* expression directly promotes the center-to-pole pattern of *Sox9* activation in the male genital ridge. We have established an XX sex-reversal transgenic mouse line carrying the *Sry* gene driven by a weak basal promoter of the *Hsp70.3* gene (*Hsp-Sry*), whereby the transgene-derived *Sry* transcripts were ectopically expressed throughout the entire gonadal area along the anteroposterior (AP) axis from earlier stages. We analyzed the effects of ectopic expression and overexpression of *Sry* transcripts on spatiotemporal patterns of *Sox9* activation during early phases of testis differentiation. As a result, here we show that *Sry* mRNA expression alone is not likely to provide positional or timing information needed for male-specific *Sox9* activation during early phases of testis differentiation.

Materials and methods

Generation of transgenic mouse lines

It was previously shown that the 5′-flanking region of the mouse *Sry* gene includes the regulatory elements that mimic the spatiotemporal pattern of the initial *Sry* expression (Albrecht and Eicher, 2001). Since only a low level of *Sry* expression in the genital ridge is required for testis differentiation (Koopman et al., 2001), we used the murine *Hsp70.3* (also known as *Hsp68*) promoter as a weak basal promoter to ubiquitously express *Sry* in gonadal tissues. We constructed the *Hsp-Sry* transgene by replacing the entire 5′-flanking region of the murine *Sry* gene with the mouse *Hsp70.3* promoter sequences (Fig. 2A). The DNA fragments containing *Hsp70.3* promoter (Kothary et al., 1989; kindly provided by Dr. H. Fujimoto, The Mitsubishi-Kagaku Life Science Institute) were subcloned into *Mun*I site at SryStuI constructs (Bowles et al., 1999; kindly provided by Dr. J. Bowles, The University of Queensland). The *Sal*I-cut DNA of *Hsp-Sry* construct was microinjected into fertilized eggs of C57BL/6 (B6) mice (Japan SLC, Hamamatsu, Japan), followed by transfer of viable eggs into the oviducts of pseudopregnant Slc:ICR mice (Japan SLC). Of the 111 viable mice born, we obtained four F0 male mice (three XY and one XX sex-reversal) carrying a full-length *Hsp70.3-Sry* transgene. We then established two sex-reversal (#40 and #46) and one non-sex-reversal (#44) lines (adult male mice of all three lines were viable and fertile with no obvious abnormalities) (Fig. 2B). Approximately 80% of the #46 transgenic offspring were embryonic lethal before 10.5 dpc. Since the transgene was transmitted at a ratio of approximately 1:1 to the offspring at an expected Mendelian ratio (*HSP-Sry* vs. wild type: 130 vs. 141 in XY, 104 vs. 128 in XX/total 503) in the mating between the *HSP-Sry* #40 male and a wild-type female, we used this #40 line (simply called “*HSP-Sry*”) in this study. In addition, no appreciable up-regulation of *Sry* by heat-shock treatment (42°C, 15 min) was detected in the genital ridge of the *Hsp-Sry* #40 line.

In the following experiments, the male *HSP-Sry* B6 mice were mated with ICR female mice (Japan SLC), and the embryos were collected at 10.5–13.5 dpc. Before 12.5 dpc, we carefully counted the tail somite number (from the caudal proximal end of the genital tubercle to the tail end) of each embryo. The animal experiments were conducted in accordance with the Guidelines for Animal Use and Experimentation as set out by The University of Tokyo.

Genomic Southern blot and PCR analyses

Genomic DNA of the tail tip of mice was isolated using a Wizard genomic DNA purification kit (Promega

Co., Ltd., WI). For embryonic DNA, part of the head region was dissected from each embryo, and genomic DNA was prepared using the same kit. To determine the genotype by Southern blot analysis, DNAs were digested with *Eco*RI, electrophoresed, and transferred to a nylon membrane. The blots were hybridized with a ^{32}P-labeled probe of the 520-bp *Pvu*II fragment corresponding to the 3′-UTR region (probe A; position indicated as bold bar in Fig. 2A). To analyze the genotype of the embryo by PCR, the *Hsp-Sry* transgene was amplified using the following primers: *Hsp70.3*-promoter forward, 5′-AAA GGC GCA GGG CGG CGA GCA GGC CAC-3′ (at −26 to +1 bp from transcription start site); transgene-specific *Sry* reverse, 5′-GCC CTC CAT GCT CTC TAG ACA ATT CAC-3′ (X67204: 8312 to 8289 plus the 3 nt specific for the *Hsp-Sry* transgene, which is indicated by underline). The genetic sex of each embryo was determined by PCR using *Zfy*-specific primers as described previously (Bowles et al., 1999).

Semiquantitative and quantitative RT-PCR analyses

Total RNA was extracted from the genital ridges of each embryo using an Isogen RNA isolation kit (Nippon Gene, Tokyo, Japan). After DNase I treatment for 30 min, each RNA was reverse-transcribed using an oligo(dT) primer with a Superscript II cDNA synthesis kit (Invitrogen Life Technologies, CA) following the manufacturer's instructions. A reverse transcriptase-free reaction was performed as a control experiment.

For semiquantitative RT-PCR for *Sry* expression, *Sry* and *G3pdh* (internal control) were amplified in the same tube by the following primers: *Sry* forward primer: 5′-CAG TTC CAC GAC CAG CAG CTT ACC TAC-3′ [X67204: 9384 to 9410], *Sry* reverse primer: 5′-AGC CAG GCA TCT AGT AAG AGT CCT TGA CC-3′ [X67204: 10127 to 10099] (their positions are indicated by arrowheads in Fig. 2A); *G3pdh* forward primer: 5′-TGA AGG TCG GTG TGA ACG GAT TTG GC-3′ and *G3pdh* reverse primer: 5′-CAT GTA GGC CAT GAG GTC CAC C-3′. PCR was performed with 36 cycle amplifications of 95°C for 1 min, 58°C for 1 min, 72°C for 1 min. Five microliters of each sample was electrophoresed in 2% agarose gel.

For real-time quantitative RT-PCR for *Sox9* expression, specific primers and the fluorogenic probe for *Sox9* were designed using Primer Express 1.5 software as follows: *Sox9* forward primer, 5′-CGT GGA CAT CGG TGA ACT GA-3′ [NM011448: 1119 to 1138]; *Sox9* reverse primer: 5′-GGT GGC AAG TAT TGG TCA AAC TC-3′ [NM011448: 1202 to 1180]; Taqman probe, 5′-AGC GAC GTC ATC TCC AAC ATT GAG ACC T-3′ [NM011448: 1141 to 1168]. Amplification of the *G3pdh* gene was used to standardize the amount of RNA in each reaction mixture (Taqman control reagents). PCR was performed using an ABI Prism 7900 HT sequence detector with 40 cycle amplifications of 95°C for 15 s, 60°C for 1 min followed by enzyme activation at 95°C for 10 min. All reagents for real-time PCR were purchased from Applied Biosystems.

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as previously described by Kanai-Azuma et al. (1999). In short, the embryos were fixed in 4% PFA-PBS for 4 h then dehydrated in methanol. The samples were rehydrated, pretreated with 10 μg/ml proteinase K in PBST for 30 min, and then hybridized with digoxigenin (DIG)-labeled RNA probes in a solution containing 50% formamide, 10% dextran sulfate, 5× SSC, 1% SDS, 50 μg/ml heparin and 50 μg/ml denatured yeast RNA at 70°C for 16 h. After treatment with RNase A (100 μ g/ml; Sigma) at 37°C for 15 min, they were washed twice with 2× SSC/5× SSC at 65°C for 1 h. The signals were detected by an immunological method using alkaline phosphatase (ALP)-conjugated anti-DIG antibody (Roche Molecular Biochemicals) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP)/nitro blue tetrazolium (NBT) (Roche) as the chromogen. In some wild-type/XY and *Hsp-Sry*/XX samples, all procedures for fixation, hybridization, staining and sectioning were performed as carefully as possible under the same condition in order to evaluate the relative signal intensities. For two-color in situ hybridization, fluorescein isothiocyanate (FITC)-labeled and DIG-labeled probes were used (Hecksher-Sorensen et al., 1998). ALP-conjugated anti-DIG was used for the first detection with BCIP/NBT, and then ALP-conjugated anti-FITC (Roche) was used for the second detection with BCIP/iodonitrotetrazolium violet (INT) as described previously (Hauptmann, 2001). The ALP for the first step was inactivated by the incubation at 65°C for 2 h. RNA probes for *Sry* (Bullejos and Koopman, 2001) and *Sox9* (Kent et al., 1996) (provided by Dr. P. Koopman, The University of Queensland) were used in this study. All samples were photographed using a dissecting microscope, and then 10-μm frozen sections were prepared.

Immunohistochemistry

Anti-SOX9 Ab [rabbit polyclonal Ab against a synthetic peptide corresponding to the C-terminal region of SOX9 (Kent et al., 1996), kindly provided by Dr. P. Koopman] and anti-SF1/Ad4Bp Ab (Hatano et al., 1994; Ikeda et al., 2001; kindly provided by Dr. Ken-ichirou Morohashi, the National Institute for Basic Biology, Japan) were used in this study. Deparaffinized sections were incubated with anti-SOX9 Ab (2 ng/ml) at 4°C for 12 h. For comparative immunostaining of SOX9 and SF1/Ad4Bp, two consecutive 4-μm-thick sections were separately incubated with anti-SOX9 (2 ng/ml) or anti-SF1/Ad4Bp (1/1000 dilution) Ab at 4°C for 12 h. After washing in TBS, its reaction was visualized with HRP-labeled secondary Ab in combination with a Tyramide Signal Amplification kit (NEN Life Science Product) as described previously (Noma et al., 2002). After staining with anti-

SOX9 Ab, the germ cells were visualized by alkaline phosphatase activity staining. In addition, a nonspecific reaction could not be detected in the germ cells and gonadal somatic cells when the sections were incubated with control IgG instead of anti-SOX9 or anti-SF1/Ad4Bp Ab.

For quantitative analysis of the number of SOX9-positive cells and ALP-positive germ cells, cell numbers were counted separately in three longitudinal sagittal sections per gonad [the section containing the largest area in the middle position of the gonad, and two sections before and behind it at an interval of approximately 40 μm (i.e., each 8th–12th section in 4-μm serial sectioning)] (four embryos from each genotype). After counting cell numbers in each section, the section image of each gonad was taken with a FUJIX digital camera (Fujifilm, HC-300Z), and then the whole gonadal area was measured using the Scion image program (Ver. 4.02). Finally, the cell number per area (cell number per mm^2) was calculated separately in XY wild-type, *Hsp-Sry*/XX and *Hsp-Sry*/XY gonads, and the data were statistically analyzed by Student's *t* test.

Results

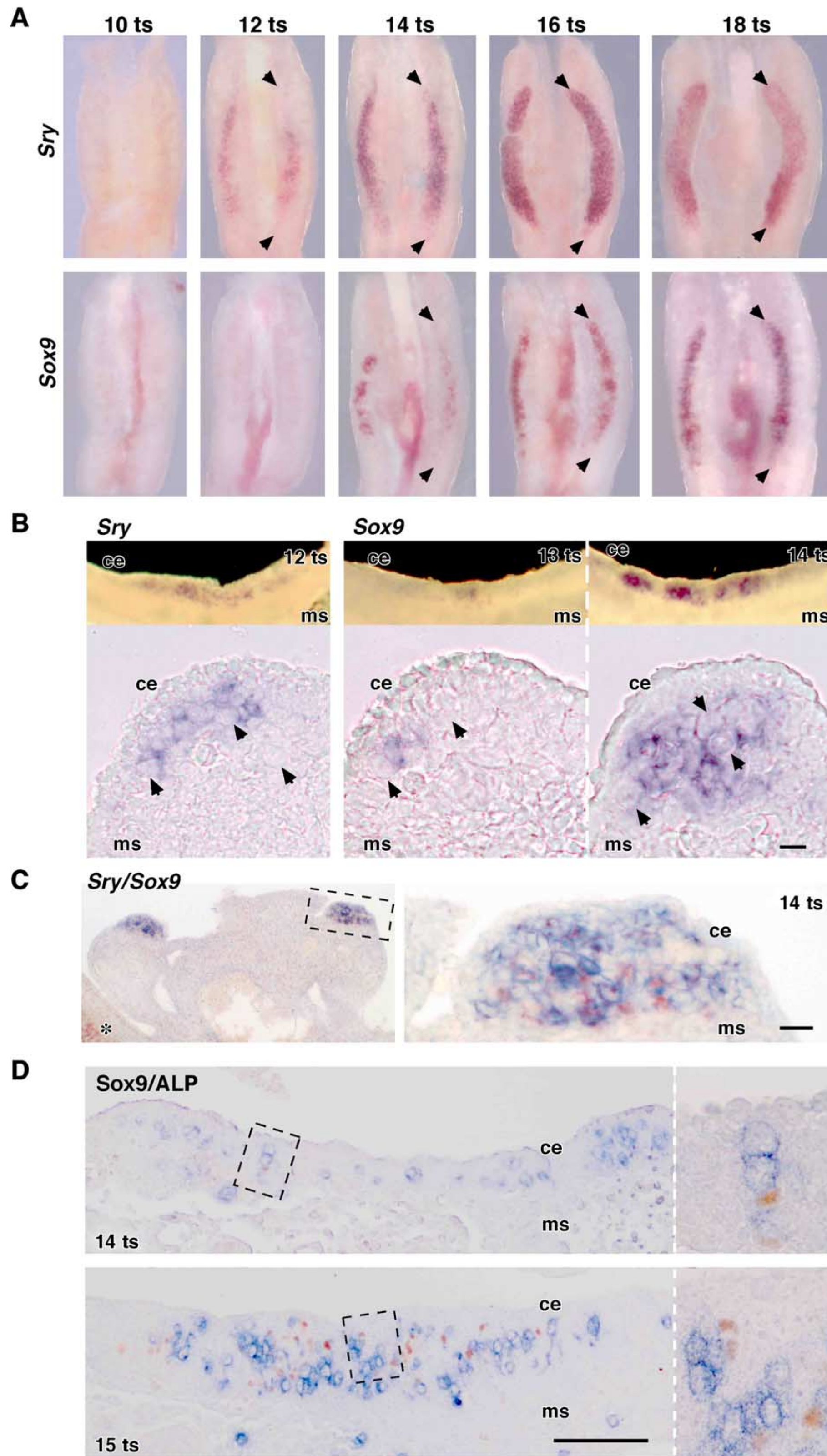
A close correlation between the spatiotemporal expression patterns of Sry and Sox9 at early phases of testis differentiation

Previous studies have suggested that initial *Sry* expression is first detected in the central region of XY gonads (Albrecht and Eicher, 2001; Bullejos and Koopman, 2001). To confirm the spatiotemporal expression patterns of *Sry* and *Sox9* in normal testis differentiation, we performed whole-mount in situ hybridization using XY wild-type genital ridges isolated at various stages. *Sry* expression was first detected in the central region at 12 ts and, subsequently, its expression expanded to the entire gonadal area during 14–15 ts (Figs. 1A, B). Moreover, sectioning analysis of the stained genital ridges revealed that *Sry*-positive cells are located in the cell layer beneath the coelomic epithelium, and that they are often located near the large round cells of presumptive germ cells. Similar results have previously been obtained by Albrecht and Eicher (2001) and Bullejos and Koopman (2001). Similarly, *Sox9* expression was first detected in the central portion of the XY genital ridge at 13 and 14 ts and, subsequently, its expression domain extended to the anterior and posterior ends by 15–16 ts (Figs. 1A, B). This finding suggests a delay of only 1–2 ts (2–4 h) in the timing of *Sox9* activation after the onset of *Sry* expression. Since no appreciable *Sox9*-positive signal was detected in the XY gonads before 12 ts (Fig. 1A) or in the XX gonads at any stage ("XX Wt" in Fig. 5), only *Sox9* transcripts at a sufficient expression level may be detectable under our experimental condition of in situ hybridization (Morais da Silva et al., 1996). Double-labeling in situ hybridization analysis using DIG-labeled *Sry* and FITC-labeled *Sox9* probes showed that male-specific *Sox9* expression likely occurs in parts of *Sry*-positive cells located in the inner area of the XY gonads at 14 ts (Fig. 1C). Section analysis also showed that *Sox9* expression was frequently found in the somatic cells adjacent to presumptive germ cells, similar to that for *Sry* expression (Fig. 1B). To confirm a close association between *Sox9*-positive cells and germ cells at the initial phase of testis differentiation, we performed a double staining of anti-SOX9 Ab and alkaline phosphatase (ALP) activity (Fig. 1D). Immunohistochemical analysis revealed that SOX9-positive cells were first detected in XY gonads at 14 ts. At this stage, they were already located near the ALP-positive germ cells. Furthermore, in the XY gonads isolated at 15 ts, the number of SOX9-positive cells had increased and were predominantly located near the germ cells. Therefore, these findings emphasize a close correlation between the initial expression patterns of *Sry* and *Sox9* in presumptive pre-Sertoli cells located near the germ cells. These results clearly agree with the model of cellular events relating to *Sry* and *Sox9* expressions as illustrated by Lovell-Badge et al. (2002) and Sekido et al. (2004).

Production of a sex-reversal transgenic line where Sry is ectopically expressed in the entire gonadal area across a broad span of developmental stages

The close correlation between the spatiotemporal expression patterns of *Sry* and *Sox9* suggests that *Sry* expression

Fig. 1. A close correlation between the spatiotemporal expression patterns of *Sry* and *Sox9* at early phases of testis differentiation. (A) Whole-mount in situ hybridization analyses showing both *Sry* and *Sox9* are activated in a center-to-pole pattern during early stages of testis differentiation. *Sry* expression starts in the middle region of XY gonad at around the 12-tail somite (ts) stage, while *Sox9* expression is similarly found in the middle region at around the 13–14 ts stage. Anterior is shown on the top in each plate, while anterior and posterior edges of the gonadal area are indicated by arrowheads. (B) Whole-mount in situ hybridization analyses showing the expression sites of *Sry* and *Sox9* in the XY genital ridges immediately after the onset of each expression. Upper plates show lateral views of the XY genital ridges (anterior is shown on the left in each plate), showing *Sry* expression at 12 ts (left) and *Sox9* expression at 13 (middle) and 14 ts (right). Each lower plate exhibits the transverse section at the middle portion of the genital ridge shown in each upper plate. Both *Sry*- and *Sox9*-positive cells are frequently found near large round cells of presumptive germ cells (arrowheads). (C) Double-labeling in situ hybridization analysis showing a direct comparison between the expression domains of *Sry* and *Sox9* in the XY genital ridges at 14 ts. The embryos were hybridized with FITC-labeled *Sox9* (brown) and DIG-labeled *Sry* (blue) probes. Transverse sections were prepared in the middle position of the gonads of the stained embryos. *Sox9* expression tends to occur in parts of *Sry*-positive cells located in the inner area of the XY gonads at 14 ts. (D) Immunohistochemical staining of anti-SOX9 Ab (brown) with alkaline phosphatase (ALP; blue) activity staining, showing a close association between SOX9-positive cells and germ cells within the XY gonads isolated at 14 ts (upper) and 15 ts (lower). Right panels show a higher magnification of the area surrounded by the broken lines in left panels. Embryos at approximately 11.0 or 11.5 dpc show 12 or 18 ts, respectively. Asterisk in C, developing cartilage; ce, coelomic epithelium of the gonadal area; ms, mesonephros. Scale bars in the sectioning images indicate 10 μm in B and C, and 100 μm in D.



A
10 ts
12 ts
14 ts
16 ts
18 ts
Sry
Sox9
B
Sry
Sox9
ce
12 ts
13 ts
14 ts
ms
C
Sry/Sox9
14 ts
D
Sox9/ALP
14 ts
15 ts

directly induces the center-to-pole pattern of *Sox9* activation in the XY gonad. To test this hypothesis, we examined the effects of misexpression and overexpression of *Sry* transcripts on endogenous *Sox9* expression using a transgenic mouse line. Since the 5′-flanking region of the mouse *Sry* gene has previously been shown to include the regulatory *cis*-elements that mimic the initial *Sry* expression pattern (Albrecht and Eicher, 2001), we have constructed a *Hsp-Sry* transgene, which replaces the entire 5′-flanking region of the murine *Sry* gene with the *Hsp70.3* promoter sequences, a weak basal promoter (Fig. 2A), and then generated four types of transgenic mice carrying a full-length *Hsp-Sry* transgene (Fig. 2B). Three of the four transgenic lines

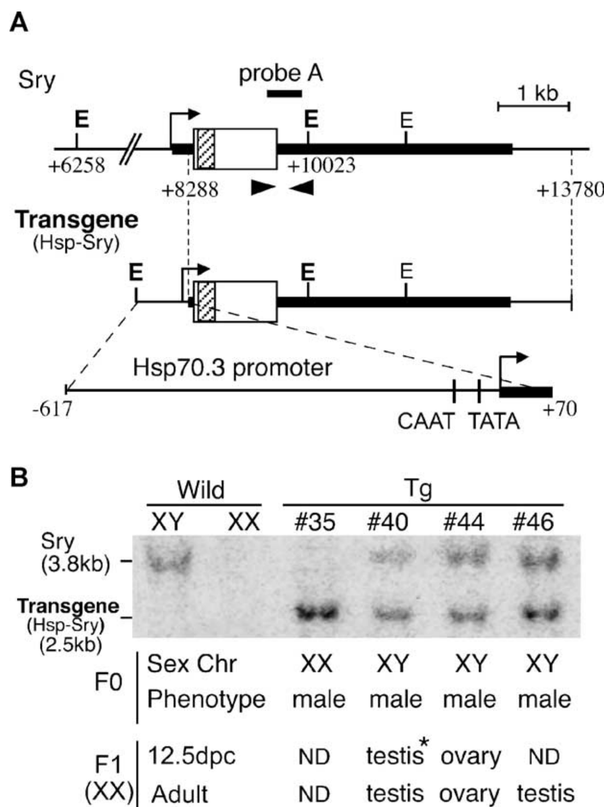


Fig. 2. Generation of the transgenic mice carrying the *Sry* transgene driven by the *Hsp70.3* promoter (*Hsp-Sry*). (A) A schematic representation of the *Hsp-Sry* transgene. Restriction site of *Eco*RI is indicated by E. The bold line of probe A indicates the position of the probe for genomic Southern blot hybridization using *Eco*RI-digested samples (transgene, 2.5 kb; wild allele, 3.8 kb) (genotyping results of adult F0 mice are shown in B), while the arrowheads show the primer positions for semiquantitative RT-PCR of *Sry* (results are shown in Fig. 3). The box indicates the open-reading frame (hatched box, HMG box), while the solid bold bars indicate the noncoding region of *Sry* cDNA. The nucleotide numbers of the deposited *Sry* genomic sequences (accession no. X67204) are shown in the figure, while the nucleotide numbers from the transcription start site of the *Hsp70.3* gene and the positions of the CAAT and TATA boxes are shown in the *Hsp70.3* promoter. (B) Southern blot hybridization analysis showing four types of male transgenic mice (Tg) carrying the full-length *Hsp-Sry* transgene (F0, adult). The sex chromosome type of F0 and the phenotype of 12.5-dpc XX embryos and adult F0 or F1 mice in each line are also shown. Asterisk means a delay in testis differentiation at the posterior pole, found in a part of the testes at 12.5–13.5 dpc. ND, not determined.

showed XX sex reversal at the adult stage. All XX offspring of one *Hsp-Sry* line (#40 in Fig. 2B; hereafter *Hsp-Sry*) showed testis development at embryonic stages. Most XX gonads at 12.5–13.5 dpc exhibited proper testis development, although testis cord formation was delayed in the posterior edges of some XX testes (4 of 24 XX testes, data not shown).

Semiquantitative RT-PCR analysis in the XX embryos of the *Hsp-Sry* line at 11.5 dpc showed moderate expressions of the transgene in various tissues including the genital ridges (arrow in Fig. 3A). Moreover, in contrast to transient expression of the endogenous *Sry* gene during 14–21 ts, constitutive expression of the transgene was detected in the *Hsp-Sry*/XX genital ridges at all developmental stages examined (at least, from 9 to 28 ts and 13.5 dpc; arrow in Fig. 3B). Whole-mount in situ hybridization analysis of dissected urogenital ridge tissue also confirmed non-stage-specific expression of *Sry* in the *Hsp-Sry*/XX gonads (Fig. 4A). In contrast to the center-to-pole wave of *Sry* expression in XY wild-type gonads during 12–24 ts, *Sry* transcripts derived from the transgene were expressed in the entire gonadal area of the *Hsp-Sry*/XX genital ridges at least from 9 to 30 ts. In order to evaluate *Sry* expression level in each gonadal position between wild-type/XY and transgenic XX genital ridges at 13 ts (just before the onset of *Sox9* expression), all procedures for fixation, hybridization, staining and sectioning were performed as carefully as possible under the same condition. As a result, signal intensities of the *Sry*-positive cells in *Hsp-Sry*/XX gonads at 13 ts showed similar to those in wild-type/XY gonads at the same stage (Fig. 4B). A sagittal sectioning analysis also revealed that no appreciable differences in their signal intensities were detected between the middle and pole positions of the *Hsp-Sry*/XX gonads. This confirmed the sufficient expression levels of the transgene-derived *Sry* transcripts in the pole positions of *Hsp-Sry*/XX genital ridges just before the onset of male-specific *Sox9* up-regulation. Although we could find some differences in their signal intensities among the gonadal cells, its expression was widely detected in the gonadal area including coelomic epithelium at 9 and 13 ts (Fig. 4B; upper plates in Fig. 4C). In 12.5 dpc (30 ts) XX testis, the transgene was strongly expressed in the Sertoli cells within the newly formed testicular cords (lower plates in Fig. 4C), suggesting that the transgene is properly activated in pre-Sertoli cell lineage.

Spatiotemporal patterns of Sox9 expression and developmental changes in expression level and positive cell numbers in the XX and XY genital ridges of Hsp-Sry embryos

First, we examined the influence of misexpression of *Sry* on male-specific activation of the endogenous *Sox9* gene during early phases of testis differentiation. In XY wild-type embryos, *Sox9* expression was first detected in the central region of the gonads at 13–14 ts and, subsequently, its

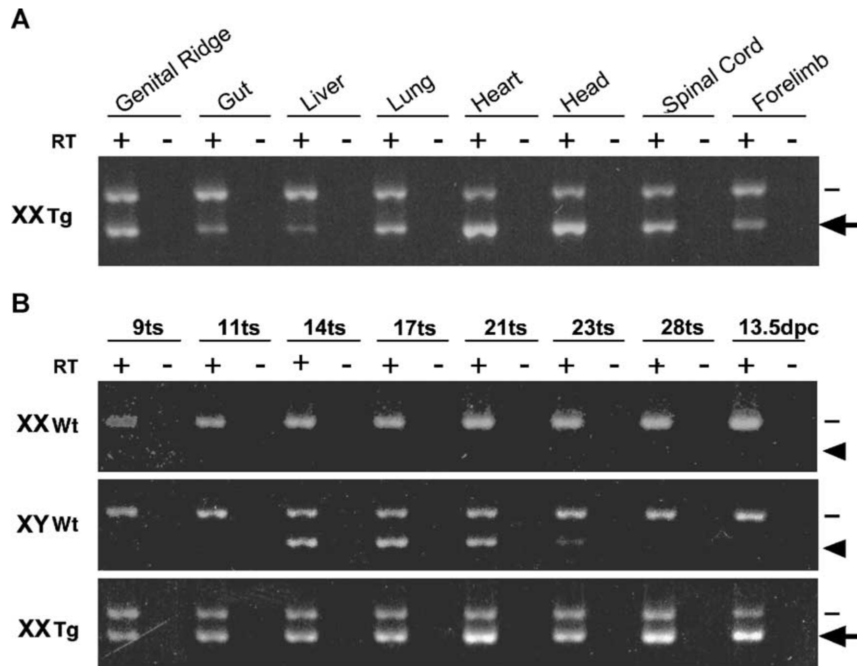
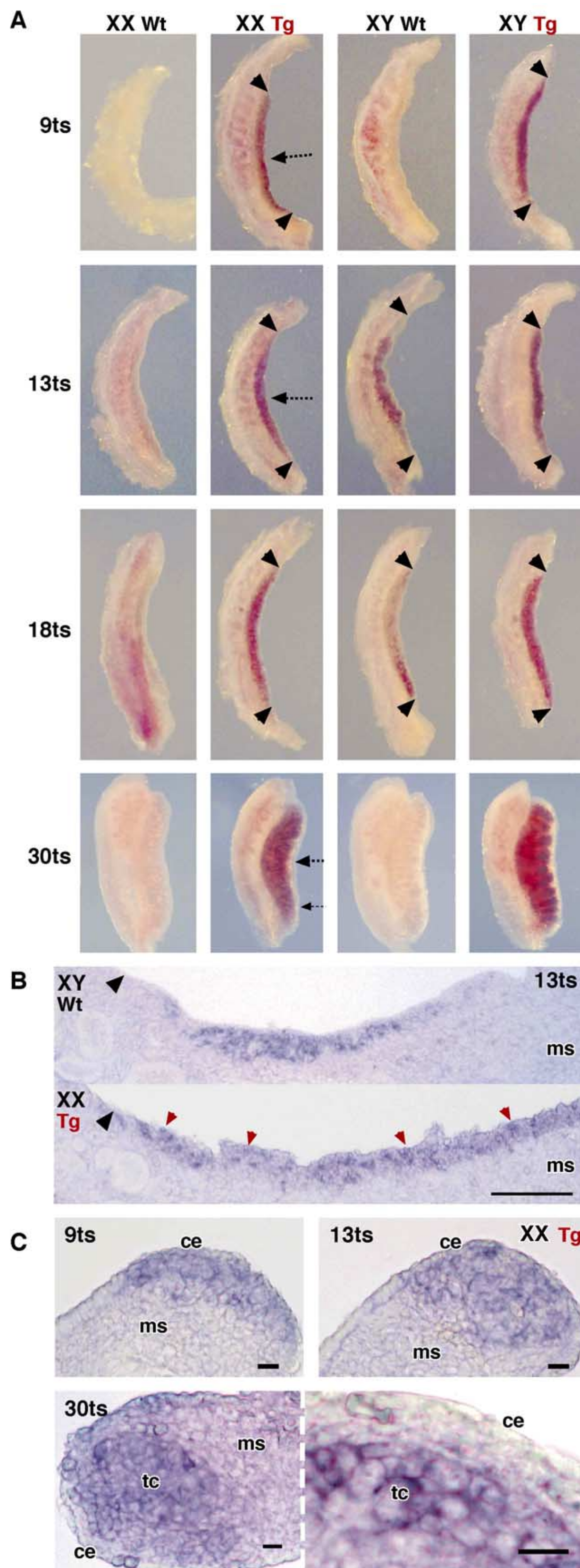


Fig. 3. Semiquantitative RT-PCR analyses on XX embryos of a sex-reversal *Hsp-Sry* #40 transgenic line showing ectopic expression of the transgene-derived *Sry* transcripts in various tissues isolated at 11.5 dpc and in developing genital ridges isolated at 10.5–13.5 dpc. (A) Semiquantitative RT-PCR analysis showing *Sry* expression in various tissues of *Hsp-Sry*/XX (XX Tg) embryos isolated at 18 ts (11.5 dpc). The *Hsp-Sry* transgene is activated in various tissues including the genital ridges at a moderately high level (arrow). (B) Semiquantitative RT-PCR analysis showing expression pattern of *Sry* in the developing genital ridges of *HSP-Sry*/XX (XX Tg) and wild type/XY(XY Wt) embryos. In the genital ridge, the *Hsp-Sry* transgene is continuously activated across a broad span of developmental stages (arrow), in contrast to transient expression of the endogenous *Sry* gene during 14 to 23 ts (arrowhead). RT + or − in each line indicates that RT was added or omitted, respectively, to the PCR reaction sample. Embryos at approximately 10.5, 11.0, 11.5 and 12.5 dpc show 8, 12, 18 and 30 ts, respectively. Each arrow or arrowhead indicates the position of *Sry* products, while each bar shows the position of *G3pdh* products as an internal control.

expression expanded to both anterior and posterior ends during 15 to 17 ts (“XY Wt” in Fig. 5). In *Hsp-Sry* embryos, *Sox9* expression in both XY and XX *Hsp-Sry* gonads showed a similar pattern to that found in wild-type XY gonads (“Tg” in Fig. 5), despite ectopic *Sry* expression in the entire gonadal area from earlier stages (Figs. 3B and 4). In short, no signal was detected in either XY or XX gonads of the *Hsp-Sry* embryos isolated before 12 ts (figure not shown) and positive signals were first detected in the central region of *Hsp-Sry* gonads at 13–14 ts (Fig. 5). Thereafter, *Sox9* expression expanded to both anterior and posterior ends at the same timing as that in XY wild-type gonads.

In order to examine the spatial patterns of male-specific *Sox9*-activation among the cells expressing *Sry* transcripts in the *Hsp-Sry*/XX genital ridges, we directly compared the expression domains of *Sry* and *Sox9* in the middle position of both wild-type/XY and *Hsp-Sry*/XX gonads by using double-labeling in situ hybridization. In the wild-type/XY genital ridges at 16 ts, *Sox9* expression was found in parts of *Sry*-positive cells located in the inner area of the gonad (left in Fig. 6A). At 18 ts, most of *Sox9*-positive cells decreased to express *Sry* transcripts, and several *Sry*-positive/Sox9-negative cells were detected outside of *Sox9*-positive domain (left in Fig. 6B). In the *Hsp-Sry*/XX gonads at 16–18 ts (right in Figs. 6A, B), *Sox9* expression domains clearly exhibit similar to those in wild-type/XY gonads at the same stages, despite having a wider distribution of *Sry*-positive cells throughout the gonadal area including the coelomic epithelium and the outer gonadal area beneath it. Moreover, all *Sry*-positive cells located within and just beneath the coelomic epithelium showed no *Sox9* expression, even though some of them highly express *Sry* transcripts. Therefore, these findings suggest that misexpression of *Sry* transcripts is not capable of inducing male-specific *Sox9* activation in the ectopic sites of not only the pole positions but also the coelomic epithelium and the layer just beneath it.

In order to compare *Sox9* expression levels among XX transgenic, XY transgenic and XY wild-type male genital ridges, we quantitatively examined the *Sox9* transcript level in each type of genital ridge by real-time RT-PCR (Fig. 7). No significant differences in *Sox9*-expression level were detected among the four genital ridges at 10 and 12 ts. After 14 ts, *Sox9* expression was up-regulated in the XY wild-type genital ridges. Interestingly, quantitative analysis revealed that the expression level of *Sox9* transcripts was significantly ($P < 0.01$) higher in the *Hsp-Sry*/XY genital ridges at 14 ts (immediately after the onset of *Sox9* activation in the XY wild-type gonad), as compared to the levels in wild-type/XY and *Hsp-Sry*/XX genital ridges at the same stage. At 16 ts, its expression level was significantly ($P < 0.01$) higher in the *Hsp-Sry*/XY and lower in the *Hsp-Sry*/XX genital ridges than that of wild-type/XY genital ridge. At 18 ts (when endogenous *Sry* expression level is



maximal), however, the *Sox9* expression level was similar in both *Hsp-Sry*/XY and wild-type/XY genital ridges. In the genital ridge isolated at 23 ts (at the point of disappearance of *Sry* transcripts in both anterior and middle portions), no significant differences of the *Sox9*-expression levels were found among all three male genital ridges. Since the *Hsp-Sry*/XY gonad expresses both endogenous *Sry* gene and *Hsp-Sry* transgene (data not shown), these findings suggest that *Sry* is capable of enhancing *Sox9* expression level dosage-dependently for a short period after the onset of *Sox9* activation.

By using immunohistochemical staining with anti-SOX9 Ab, we examined the distribution of the SOX9-positive cells in three male gonads from 13 to 18 ts (Fig. 8). Immunohistochemical analysis revealed that no positive reaction with anti-SOX9 staining was detected in any of the three male gonads at 13 ts (figure not shown). The protein was first detected in both XY and XX gonads of *Hsp-Sry* embryos at 14 ts with the same timing as that in wild-type XY embryos (Fig. 8A). In *Hsp-Sry*/XY gonads isolated at 15 and 16 ts, SOX9-positive cells appeared to have rapidly increased in number, as compared with those in wild-type/XY and *Hsp-Sry*/XX gonads at the same stages. Moreover, at these stages, the distribution of SOX9-positive cells in *Hsp-Sry*/XY gonads appeared to have expanded into the gonadal area where few germ cells existed (arrows in Fig.

Fig. 4. Whole-mount in situ hybridization analyses of XX embryos in a sex-reversal *Hsp-Sry* #40 transgenic line, showing misexpression of the transgene-derived *Sry* transcripts in the entire gonadal area across a broad span of developmental stages of testis differentiation. (A) Whole-mount in situ hybridization analysis showing *Sry* expression in the XX and XY genital ridges of wild type (Wt) and *Hsp-Sry* (Tg) mice. In contrast to the center-to-pole wave of endogenous *Sry* expression during 12 to 24 ts (XY Wt), the transgene-derived *Sry* transcripts are continuously detected in the entire gonadal area from at least 9 to 30 ts (XX Tg). No positive signal is detected in XX wild-type gonads at any stage. However, nonspecific reactions were observed in the mesonephros of some genital ridges. Anterior is shown on the top in each plate, while anterior and posterior edges of the gonadal area are indicated by arrowheads. (B) Sagittal sections showing region-dependent differences of *Sry* expression along AP axis of the genital ridges in wild-type/XY (upper) and *Hsp-Sry*/XX (lower) embryos at 13 ts. The samples were isolated from the same littermates, and treated identically including hybridization, staining and sectioning. The transgenic expression was found at similar levels in both middle and pole positions, and their signal intensities in the *Hsp-Sry*/XX gonad also show similar to those in the middle position of the wild type/XY gonad. Small red arrowheads show the *Sry*-positive cells located within coelomic epithelium, while anterior edges of the gonadal area are indicated by large black arrowheads. (C) Transverse sections of the *Hsp-Sry*/XX genital ridge stained with *Sry* antisense probe, showing transgenic expression at 9, 13 and 30 ts. The broken arrows in *Hsp-Sry*/XX genital ridges of plate A show the planes of the sectioning images in plate C. Transgenic expression is found widely in the gonadal area of the genital ridges both at 9 and 13 ts. In the 12.5-dpc (30 ts) testis, the transgene is expressed intensely in Sertoli cells in the newly formed testicular cord both at the posterior (left, a smaller broken arrow in A) and middle (right, a large broken arrow in A) portions. Embryos at approximately 11.0, 11.5 and 12.5 dpc show 12, 18 and 30 ts, respectively. ce, coelomic epithelium of the gonadal area; ms, mesonephros; tc, testicular cord. Scale bars in the sectioning images indicate 1 mm in B and 10 μm in C.

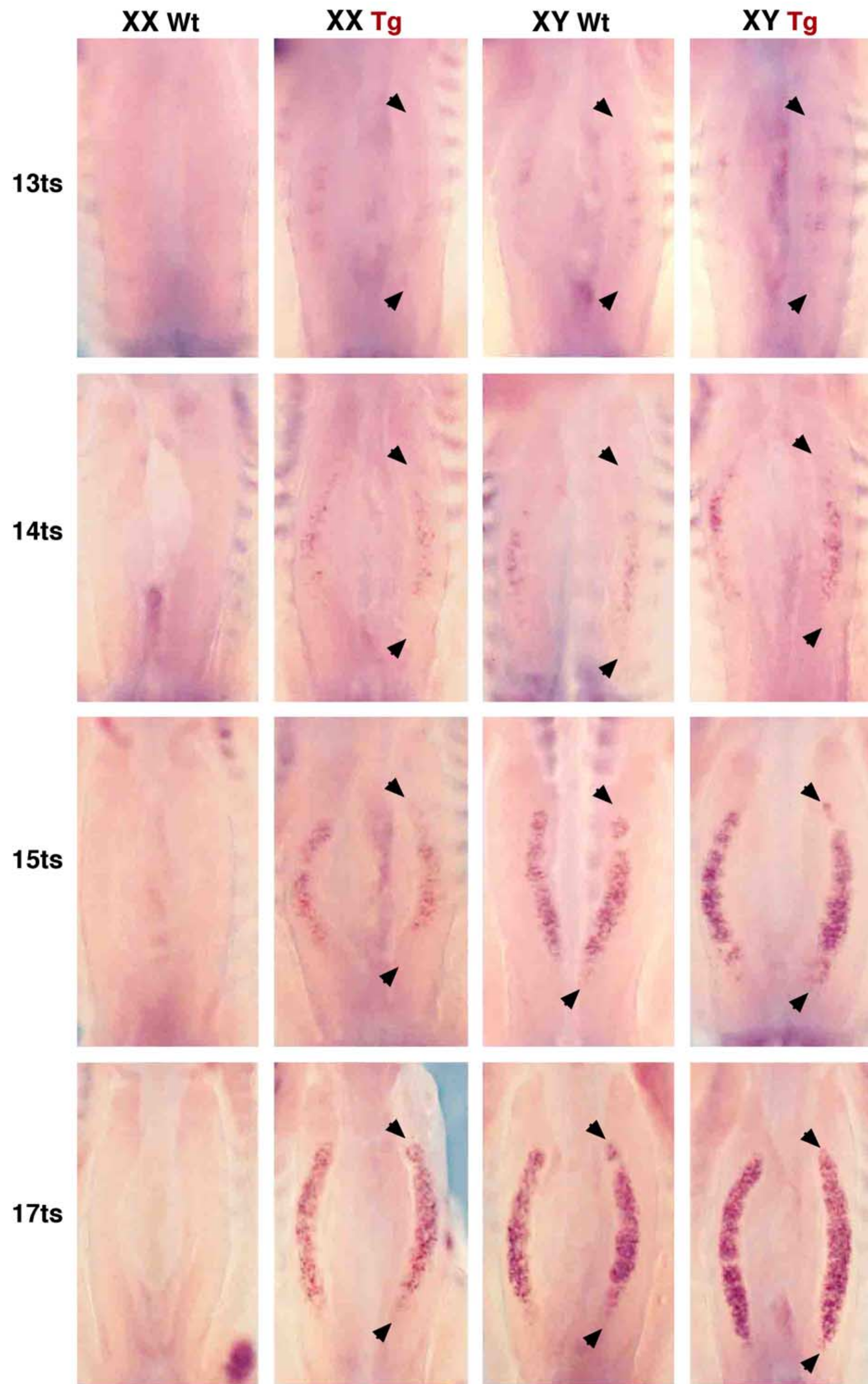


Fig. 5. Whole-mount in situ hybridization analyses showing spatiotemporal patterns of *Sox9* expression in the XX and XY genital ridges of *Hsp-Sry* (Tg) and wild-type (Wt) embryos. *Sox9* expression is first detected in the central region of gonads at 13–14 ts and, subsequently, expands to both anterior and posterior ends until 17 ts in both XX and XY *Hsp-Sry* embryos. This is quite similar to the spatiotemporal pattern of *Sox9* expression in XY wild-type gonads. In XX wild-type embryos, no signal was detected in the gonadal area in any of the developmental stages examined. In all embryos, positive signals were also observed in the chondrocytes of the body. Anterior is shown on the top in each plate, while anterior and posterior edges of the gonadal area are indicated by arrowheads. Embryos at approximately 11.0 and 11.5 dpc show 12 and 18 ts, respectively.

8A). This was in contrast to the close association between SOX9-positive cells and germ cells in wild-type/XY and *Hsp-Sry*/XX gonads. At 18 ts, however, no appreciable difference in the distribution of these cells was detected between *Hsp-Sry*/XY and wild-type/XY gonads, where SOX9-positive cells were aggregated around germ cells to form cord-like structures. Moreover, a comparative analysis of anti-SF1/Ad4Bp and anti-SOX9 staining in the *Hsp-Sry*/

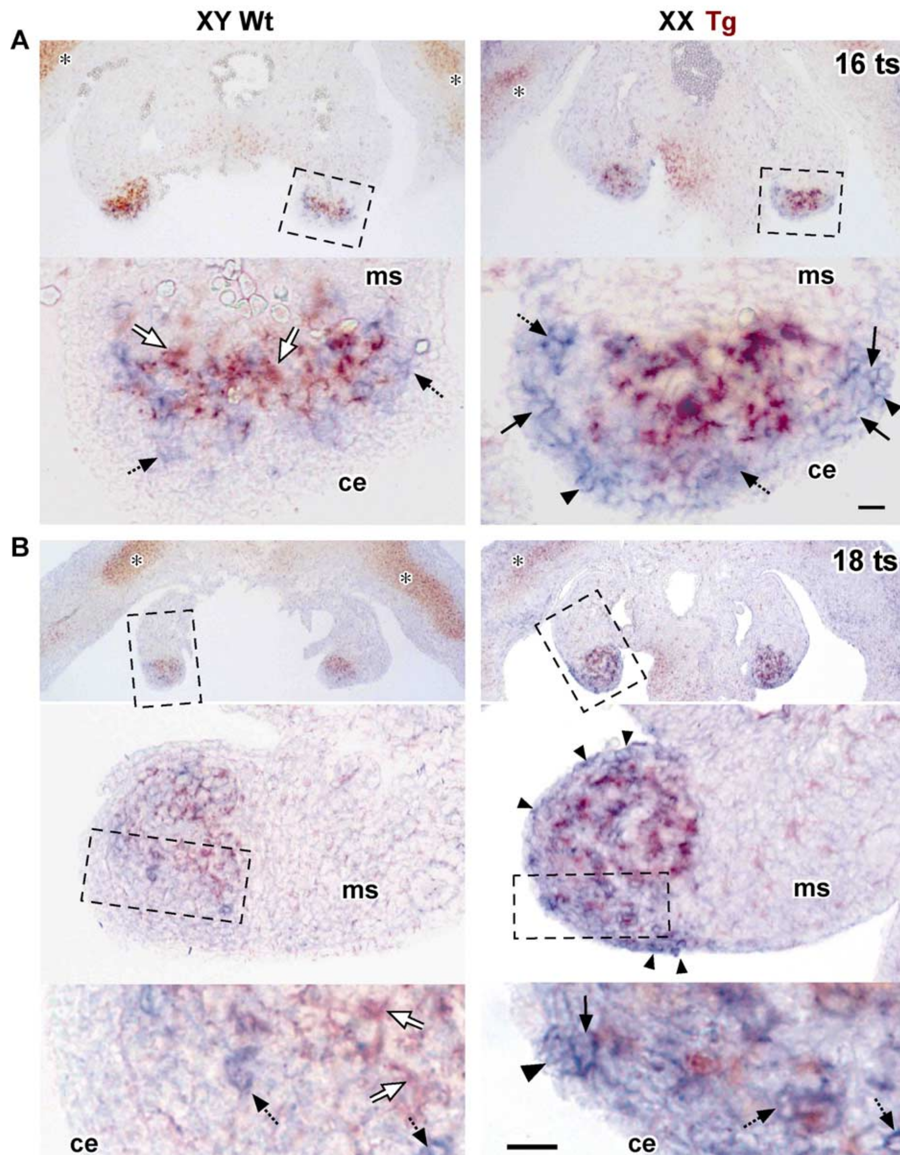


Fig. 6. Double-labeling in situ hybridization analysis showing a direct comparison between the expression domains of *Sry* and *Sox9* in wild-type/XY and *Hsp-Sry*/XX genital ridges at 16 ts (A) and 18 ts (B). The embryos were hybridized with FITC-labeled *Sox9* (brown) and DIG-labeled *Sry* (blue) probes. Transverse sections were prepared in the middle position of the gonads of the stained embryos. In the wild-type/XY gonads at 16 ts, *Sox9* expression domain is observed in the inner area of the *Sry*-positive domain (left in A). At 18 ts, *Sry* expression was reduced in parts of *Sox9*-positive cells, and several *Sry*-positive/Sox9-negative cells were found outside of *Sox9*-positive domain (left in B). In the *Hsp-Sry*/XX gonads at 16–18 ts (right in A and B), *Sox9* expression domains clearly exhibit similar to those in wild-type/XY gonads at the same stages, despite having a wider distribution of *Sry*-positive cells in the entire gonadal area including the coelomic epithelium and the several cell layers just beneath it. Moreover, all *Sry*-positive cells located within (arrowheads) and just beneath (solid arrows) the coelomic epithelium show no *Sox9* expression, even though some of them highly express the transgenic *Sry* transcripts. Broken or open arrows indicate the cells expressing only *Sry* or *Sox9* transcripts, respectively. Each higher magnification image is indicated by the broken rectangle in the upper panel. Asterisk, developing cartilage; ce, coelomic epithelium of the gonadal area; ms, mesonephros. Scale bars indicate 100 μm.

XY gonads at 15–16 ts revealed that all SOX9-positive cells including the cells located in such expanded area likely express SF1/Ad4Bp (Fig. 8B). No SF1/Ad4Bp-negative/SOX9-positive cells were detected in the *Hsp-Sry* gonads, suggesting that *Sox9* up-regulation occurs properly in supporting cell type. Moreover, at all stages which we examined, we could not detect any SOX9-positive reactions in the cells within and just beneath coelomic epithelium where some of them highly express *Sry* transcripts in *Hsp-Sry* genital ridges from earlier stages. In addition, anti-SOX9 reactions were observed predominantly in the nucleus at all stages. In some SOX9-positive cells, its reactions were likely observed in both their nucleus and cytoplasm, but the cells with only cytoplasmic SOX9 were not found even at 14–15 ts.

Quantitative analysis of SOX9-positive cells per gonadal area revealed no significant differences among the three male genital ridges at 14 ts, but in the *Hsp-Sry*/XY gonads at 15 and 16 ts, their numbers were significantly ($P < 0.01$) increased as compared with those in wild-type/XY and *Hsp-*

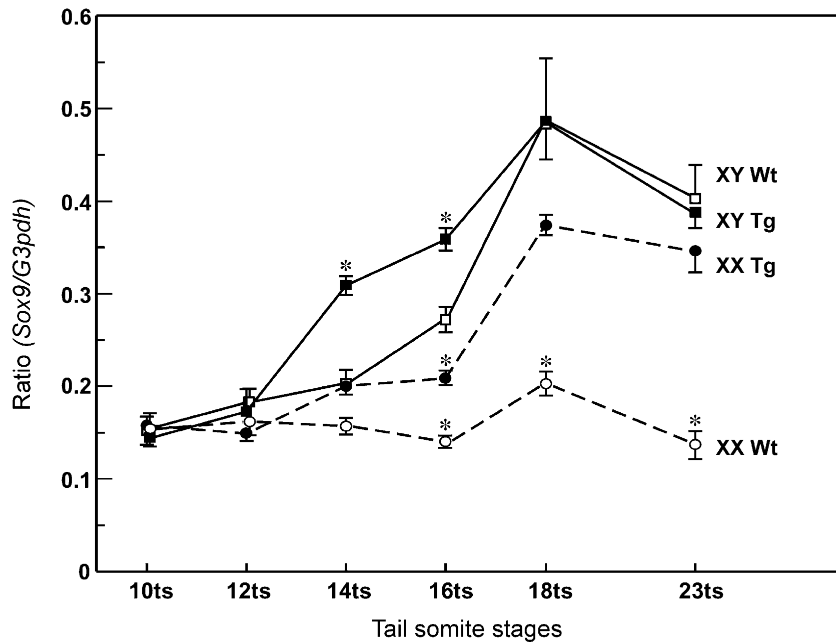


Fig. 7. Real-time RT-PCR analysis showing changes in *Sox9* transcript levels during early phases of testis differentiation in XX (broken lines and circle) and XY (solid lines and rectangle) genital ridges of the *Hsp-Sry* (Tg; closed mark) and wild-type (Wt; open mark) embryos. Vertical axis represents *Sox9*/*G3pdh* amplicon ratio, while horizontal axis represents the developmental stage of tail somite (ts) numbers. The closed (Tg) or open (Wt) circles and rectangles represent the mean values ± standard error. Each asterisk on error bar indicates a significant difference ($P < 0.01$) as compared with the data in XY wild-type genital ridges at the same stage. The seven independent experiments were performed at each stage. The expression level of *Sox9* transcripts was significantly ($P < 0.01$) higher in the *Hsp-Sry*/XY genital ridges at 14 and 16 ts, as compared with those of wild-type/XY and *Hsp-Sry*/XX genital ridges at the same stages. Its expression level in the *Hsp-Sry*/XX genital ridges at 16 ts is significantly ($P < 0.01$) lower than that of wild-type/XY genital ridges.

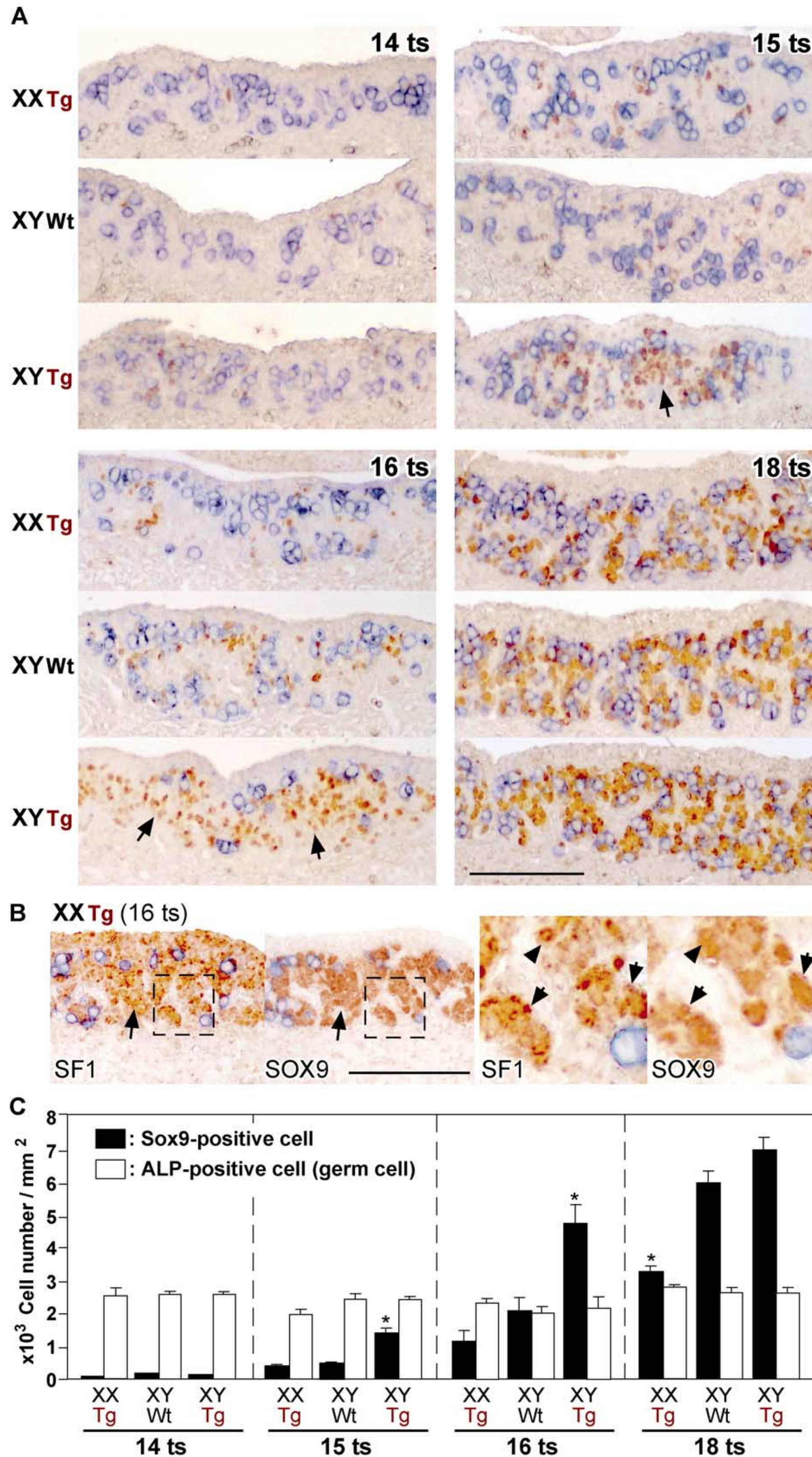
Sry/XX (solid bars in Fig. 8B). At 18 ts, the number of SOX9-positive cells in *Hsp-Sry*/XY gonads was similar to that in wild-type/XY gonads but was significantly ($P <$ 0.01) lower in *Hsp-Sry*/XX gonads than in wild-type/XY gonads. Therefore, these results clearly suggest that the rate of increase of SOX9-positive cells between 14 and 16 ts is significantly higher in *Hsp-Sry*/XY gonads than that in wild-type/XY gonads, which is consistent with the quantitative RT-PCR data showing a significantly high level of expression of *Sox9* transcripts in *Hsp-Sry*/XY gonads at similar stages (Fig. 6). The number of germ cells showed no significant differences among the three male gonads at any stage (open bars in Fig. 8B).

Discussion

In the present study, we established an XX sex-reversed mouse where the transgene was activated along the entire gonadal region across a broad span of developmental stages in order to examine the effects of misexpression of *Sry* transcripts on endogenous *Sox9* expression. In this *HSP-Sry* line, the proper testis development was observed in most of *HSP-Sry*/XX embryos isolated at 12.5–13.5 dpc, suggesting the sufficient levels of the transgene activation in the supporting cell lineage. The present RNA analysis of XX gonads of this *Hsp-Sry* line showed that the transgene-derived *Sry* transcripts were expressed from at least 9 to 30 ts along the entire gonadal area. This is in contrast to the transient center-to-pole wave of endogenous *Sry* expression in normal XY gonads during 12 to 24 ts. Since *Hsp-Sry*/XY gonads express both endogenous *Sry* gene and *Hsp-Sry* transgene (data not shown), this transgenic line allows us to study the influence of misexpression and overexpression of *Sry* transcripts on male-specific *Sox9* activation in developing gonads.

In the present study, to test whether misexpression or overexpression of *Sry* transcripts is capable of affecting male-specific up-regulation of the endogenous *Sox9* gene, we examined the spatiotemporal patterns of *Sox9* expression and the changes in its expression level and positive cell numbers in the developing male gonad of both *Hsp-Sry*/XX and *Hsp-Sry*/XY embryos. As a result, misexpression of *Sry* transcripts in the entire gonadal area from earlier stages promotes neither any advances in the timing nor ectopic up-regulation of *Sox9* expression in the pole positions at early phases of testis differentiation. Moreover, in the *Hsp-Sry* genital ridges, *Sox9* activation occurs in the SF1/Ad4Bp-positive somatic cells located in the inner gonadal area, but not in the cells within and just beneath the coelomic epithelium, despite a sufficiently high expression of *Sry* transcripts in some of these cells. Karl and Capel (1998) have demonstrated that both pre-Sertoli cells and interstitial cells have its origins within the coelomic epithelium of the genital ridge. Sekido et al. (2004) have postulated that the coelomic epithelial cells enter the gonads where they

differentiate into both supporting and interstitial cells probably by an asymmetric cell division. They have further suggested that *Sry* is activated only in the supporting cell type, but not in the coelomic epithelium and the presumptive bipotential precursors which are able to differentiate into both supporting and interstitial cell types (Sekido et al., 2004). With regards to the present findings showing no *Sox9* up-regulation in the *Sry*-positive cells within and just



beneath the coelomic epithelium in *Hsp-Sry* gonads, these reports suggest that *Sry* mRNA expression alone is not capable of inducing the male-specific *Sox9* up-regulation in the precursors before they have made the cell-fate decision to become a supporting cell type.

The present data showing no appreciable ectopic *Sox9* activation in the *Hsp-Sry* genital ridges have raised several possible regulations of *Sry* and *Sox9* at translational and post-translational levels. Phosphorylation by the cyclic AMP-dependent protein kinase has been previously described to modulate the DNA-binding ability of human SRY protein (Desclozeaux et al., 1998). Thevenet et al. (2004) demonstrated that human SRY interacts with the histone acetyl-transferase p300 and histone deacetylase-3 (HDAC3) in vitro. They also found the expression of p300 and HDAC3 in somatic cells of human and mouse genital ridges at the time of *Sry* expression, suggesting a possible regulation of SRY activity by acetylation and deacetylation during early phases of testis differentiation. Therefore, future studies are necessary to examine whether *Sry* transcripts are translated, modified and translocated properly in its ectopic expression sites of *Hsp-Sry*/XX genital ridges at early stages of testis differentiation. Another possible mechanism is that a male-specific *Sox9* up-regulation by SRY is required for co-factors that are expressed or activated in a center-to-pole pattern similar to the initial expression profile of *Sry*. SOX proteins are generally believed to require a partner protein to exert a regulatory function against their target genes (see reviews by Kamachi et al., 2000; Wilson and Koopman, 2002). The SOX-binding site in these naturally occurring enhancer sequences is not sufficient to exert a regulatory function without a second site nearby to bind a partner protein that cooperates with the SOX proteins. Therefore, these reports suggest that the partner proteins for SRY may be possibly required for male-specific *Sox9* up-regulation by SRY in the developing XY gonads. However, well-characterized gonadal transcriptional factors such as WT1 (Armstrong et al., 1992), SF1/Ad4Bp (Hatano et al., 1996; Ikeda et al., 1994; Morohashi, 1997), GATA4 (Tevosian et al., 2002), LHX9 (Birk et al., 2000; Mazaud et al., 2002) and EMX2 (Miyamoto et al., 1997) can probably be excluded as a candidate co-factor for SRY because all these factors are already expressed in the gonadal somatic cells at least from 10.5 dpc.

One surprising finding from this study is that a *Sry* construct driven by a weak basal promoter of the *Hsp70.3* gene is capable of efficiently inducing a female-to-male sex reversal (three of four F0 transgenic mice), despite completely lacking 5′-upstream sequences that are capable of mimicking the spatiotemporal pattern of initial *Sry* expression (Albrecht and Eicher, 2001). This is clearly consistent with the previous report showing that the *Sry* construct with the deletion of 5′-sequences at 57 bp from the transcriptional start site is capable of efficiently inducing sex reversal (Koopman et al., 2001). Moreover, the present in situ hybridization data demonstrate that positive signals for the transgenic expression were stronger in the gonadal region than in mesonephric tissue in the developing genital ridges (Fig. 4). The intensity of the signals in Sertoli cells in the testis cords also appeared to be higher than that in interstitial somatic cells at 12.5 dpc (30 ts). This gonad-specific expression of the *Hsp-Sry* transgene may possibly be caused by the presence of a post-transcriptional mechanism to increase *Sry* message stability in the developing gonad, which has previously been suggested by Harley et al. (2003) and Koopman et al. (2001). More recently, post-transcriptional regulation of *Sox9* mRNA stability has also been implicated in mesenchymal differentiation into chondrocytes (Sitcheran et al., 2003). Therefore, with regard to the possible regulation of *Sry* message stability in developing gonads, this report suggests that a similar post-transcriptional regulation of *Sox9* mRNA stability may contribute to male-specific *Sox9* up-regulation during early phases of testis differentiation.

In conclusion, the present study is the first to demonstrate that ectopic expression of *Sry* transcripts in the entire gonadal area from earlier stages promotes neither any advances in the timing nor any appreciable ectopic activation of endogenous *Sox9* expression at early phases of testis differentiation. This suggests that *Sry* mRNA expression alone is not likely to provide timing or positional information needed for male-specific *Sox9* activation in the developing XY gonad. The present data also suggest that *Sry* is capable of up-regulating the *Sox9* expression level in pre-Sertoli cells dosage-dependently, although this *Sox9* up-regulation by *Sry* is limited to a very short period immediately after *Sox9* activation. This

Fig. 8. Immunohistochemical staining with anti-SOX9 Ab showing changes in SOX9-positive cell numbers in the wild-type/XY, *Hsp-Sry*/XX and *Hsp-Sry*/XY gonads during early phases of testis differentiation. (A) Sagittal sections showing SOX9-postive cells (brown) and ALP-positive germ cells (blue) in the middle portion of the male gonads of the *Hsp-Sry* (Tg) and wild-type (Wt) embryos during 14–18 ts. The positive reactions were first detected in all three male gonads at 14 ts. In *Hsp-Sry*/XY gonads isolated at 15 and 16 ts, the distribution of SOX9-positive cells expands into the gonadal area where few germ cells exist (arrows). Scale bar indicates 100 μm. (B) Sagittal sections showing anti-SF1/Ad4Bp (a marker for precursor cells of both Sertoli and Leydig cells) and anti-SOX9 staining of two consecutive sections of the *Hsp-Sry*/XY genital ridge at 16 ts (brown). Germ cells were also visualized by ALP staining (blue). All SOX9-positive cells including the cells located in the expanded area (arrows) are likely positive for anti-SF1/Ad4Bp staining. Higher magnification images (two panels at right) are indicated by the broken rectangle in the left two panels. Arrowheads indicate the corresponding cells expressing both SF1/Ad4Bp and SOX9. Scale bar indicates 100 μm. (C) Changes in the cell number per gonadal area of SOX9-positive cells (solid bar) and ALP-positive germ cells (open bar) during early phases of testis differentiation. Vertical axis represents the mean values of cell number per gonadal area (mm^2) ± standard error. Each asterisk on error bar indicate a significant difference ($P < 0.01$) as compared with the data in XY wild-type gonads at the same stage. No significant difference was detected among three male genital ridges at 14 ts, but in the gonads at 15 and 16 ts, their numbers were significantly ($P < 0.01$) increased as compared with those in wild-type/XY. At 18 ts, the number of SOX9-positive cells in *Hsp-Sry*/XY gonads showed a similar level to that in wild-type/XY gonads, although the number was significantly ($P < 0.01$) lower in *Hsp-Sry*/XX gonads than that in wild-type/XY gonads.

dosage-dependent *Sox9* up-regulation by *Sry* may positively support a direct interaction between *Sry* and *Sox9* at early stages of testis differentiation. However, evidence of a direct interaction would be required to identify and characterize the enhancer elements of the *Sox9* gene (Lovell-Badge et al., 2002).

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