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Detection of elements responsible for stage- and tissue-specific expression of mouse *Sry* using an in vitro Cre/*loxP* system

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Abstract

We have successfully specified essential sequences of the 5′ upstream region for the stage- and tissue-specific expression of mouse *Sry* by using an in vitro Cre/*loxP* system. Sry/Cre plasmids carrying *Sry* 5′ sequences of various sizes were transfected into the primary cultured cells from different tissues of CAG/loxP/CAT/loxP/LacZ transgenic fetuses on 11.5-day post coitus (dpc) or 13.5-dpc. Stage- and tissue-specific regulation of *Sry* expression was disrupted by the deletion of positions 7549–7660 (from −0.4 to −0.5 kb region). In vitro transcription assay also suggested that the region contains element(s) responsible for stage- and tissue-specific expression of mouse *Sry*. *SRY* promoter of Shiba goat (Capra hircus var Shiba), a native Japanese miniature goat, showed the tissue-specific activity in the cells from urogenital ridges of the male mouse, but not in the cells from female mice, indicating a possibly different mechanism among species in the regulation of *Sry* expression.

Keywords: Transgenic mice; Cre/*loxP*; Primary culture; *Sry*; Shiba goat

The *SRY*/*Sry* gene, an intronless transcription factor, initiates male sex determination in eutherian mammals [1,2]. *Sry* expression occurs in developing genital ridges in many species, including humans [3–5], mice [6–9], cows [10], pigs [11], and sheep [12]. In mouse fetal gonads, *Sry* transcripts can be detected on 10.5-day postcoitus (dpc) and show a peak on 11.5-dpc. After 12.5-dpc, *Sry* transcripts are not detected, and testicular cords can be seen in the male gonads. Thus, the temporal expression pattern of *Sry* suggests that *Sry* is part of a pathway of genes regulating sex determination in male gonads. A common regulatory mechanism of *Sry* expression among species may not be postulated for incredibly variable sequence among different groups of mammals despite the high sequence homology of the putative *Sry* regulatory region within each taxonomic group of species; primates, bovines, and rodents [13]. We have previously determined the stage-specific regulatory element of mouse *Sry* in the 5′ upstream region by means of a gel shift assay [14]. However, despite the pivotal role of *Sry* in sex determination, its promoter has been poorly characterized because no available *Sry*-expressing cell line has been established, and because of its temporal and extremely low expression in developing gonads.

The bacteriophage P1 Cre recombinase, which is a 38-kDa protein, recognizes specific 34-bp sequences called *loxP* sites [15–18]. The Cre/*loxP* system has provided us with a useful tool for manipulating gene expression in mice such as stage- and tissue-specific activation or inactivation

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of genes [19,20], and targeting of cell type-specific genes [21–23]. We have previously established an available in vitro Cre/*loxP* system using the primary cultured cells for investigating the regulatory upstream region of sex determination-related genes, including *Sry* [24].

In this study, we have successfully further narrowed the sequences of *Sry* 5′ region that are important to its stage-specific expression and have clarified the role of the 5′ region of mouse *Sry* by an in vitro Cre/*loxP* system. We also investigated using the goat *SRY* promoter whether or not this common regulatory mechanism of *SRY* expression is present among different species.

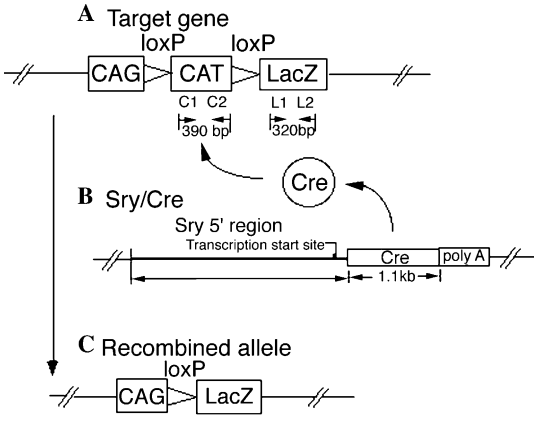

Fig. 1. The structure of the target gene and Cre-mediated recombination. The arrows under the gene construct in (A) indicate the positions and directions of the primers used for polymerase chain reaction (PCR) to detect the CAT and LacZ transgenes in CAG/loxP/CAT/loxP/LacZ transgenic mice. The number under the gene construct in (B) indicates the size of the DNA fragments. Before recombination, the loxP-flanked CAT gene is expressed under the control of the CAG promoter. After Cre-mediated recombination (B), the lacZ gene is expressed, followed by deletion of the CAT gene (C).

Materials and methods

Mice. Two transgenic mouse lines were used: the transgenic mouse line [25] carrying a CAG (CMV enhancer, chicken β-actin promoter, and fusion intron of chicken β-actin and rabbit β-globin)/loxP/CAT/loxP/LacZ (denoted as a CAG/loxP/LacZ) fusion gene (Fig. 1A), which directs LacZ expression upon Cre-mediated excision of the *loxP*-flanked CAT gene located between the CAG promoter and the LacZ gene (Fig. 1C); and the transgenic mouse line expressing GFP on the Y chromosome under the control of the CAG promoter, produced by Ichida et al. [26]. The CAG/loxP/LacZ transgene was identified by PCR using two different primer sets (Fig. 1A). For the LacZ gene, forward primer 5′-GCG-TTA-CCC-AAC-TTA-ATC-G-3′(L1) and reverse primer 5′-TGT-GAG-CGA-GTA-ACA-ACC-3′(L2) were used, and for the CAT gene, 5′-CAG-TCA-GTT-GCT-CAA-TGT-ACC-3′(C1) and 5′-ACT-GGT-GAA-ACT-CAC-CCA-3′(C2) were used. The GFP transgene was identified by observation of fluorescence on the body under a hand-type illuminator with a UV filter. Mice were kept under regulated temperature (22–25 °C), a humidity of 40–60%, and an illumination cycle (14 h light, 10 h dark), and were provided with food and water ad libitum. The animal experiments were conducted according to the Guide for the Care and Use of Laboratory Animals, College of Agriculture, University of Tokyo.

Construction of the Sry/Cre fusion gene. The pUCSry plasmid containing 14.6-kb mouse genomic *Sry* was kindly provided by Dr. R. Lovell-Badge (National Institute of Medical Research, UK). The 9.9 kb *Sry* (positions 3895–13775, denoted by Dolci et al. [27]) previously shown to induce testis development in XX transgenic mice [28] was excised once from the pUCSry with *Stu*I and then subcloned into pBluscriptSK$^-$ (Stratagene, La Jolla, CA) at the *Eco*RV site, which is denoted as a pSry9.9 plasmid. The 1.1 kb Cre coding region was excised from a pxAwNCre plasmid (kindly provided by Dr. I. Saito, University of Tokyo, Japan) with *Xba*I. The 468 bp Sry coding region of the pSry9.9 plasmid was excised with *Eco*RV and replaced with the 1.1 kb Cre coding region to replace Sry expression with Cre expression, and is denoted as a 4.1Sry/Cre plasmid (Fig. 2A). We carried out the cloning of all DNA, using the bacterial strain DL652 kindly provided by Dr. P. Koopman (Queensland

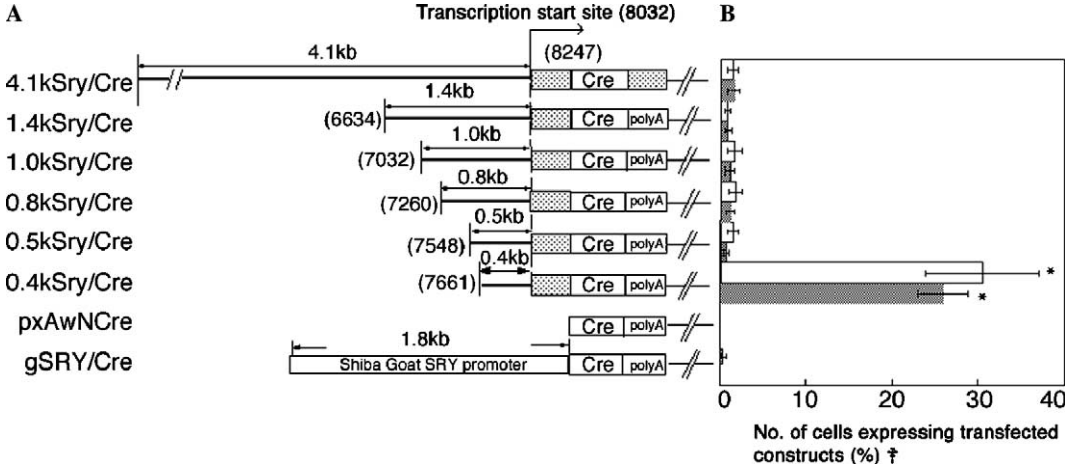

Fig. 2. Schematic structures of the Cre genes with sequentially deleted 5′ flanking regions of mouse *Sry* and with the goat *SRY* promoter region (A) and their promoter activities (B). pxAwNCre was used as a negative control. Results are expressed as percentages of the X-gal-positive cells in the primary cultured cells that were prepared from CAG/loxP/CAT/loxP/LacZ transgenic fetuses and transfected with each of the constructs. Transfection efficiency was expected by comparing the cells transfected with CAG/Cre constructs as described in Table 1. The numbers in parentheses indicate the positions at the 5′ region denoted by Dolci et al. [27]. Dotted squares in the gene constructs indicate the 5′ untranslated sequences of mouse *Sry*. The data are means ± SE of five independent experiments. Open bars represent the results obtained when constructs were transfected to the cells from the male urogenital ridges of 11.5-dpc, and the filled bars represent the results obtained when transfected to the female urogenital ridges of 11.5-dpc fetuses. *The mean value was significantly higher than other constructs; †X-gal-positive cells/total cells examined.

University, Australia) as host cells for the plasmid. The 1.4-kb 5′ region of mouse *Sry* was excised with *Hpa*I and *Eco*RV, and was inserted into the *Sma*I site of the pxAwNCre plasmid; the resulting plasmid was denoted as a 1.4kSry/Cre. 1.4kSry/Cre was digested with *Xho*I and then digested partially with Eco T22I to obtain three kinds of Sry/Cre fragments of different sizes. The fragments were inserted into pBluescriptSK$^-$ (Stratagene, La Jolla, CA) that had been digested with *Xho*I and *Pst*I; the resulting plasmids were denoted as 1.0kSry/Cre, 0.8kSry/Cre, and 0.5kSry/Cre. A 0.4kSry/Cre was generated by digesting the 1.4kSry/Cre plasmid with *Acc*I at the original site of pxAwNCre and at position 7661 of *Sry* to eliminate an *Sry* 5′ region of approximately 1.0 kb.

Construction of the 5′ region of the goat SRY/Cre fusion gene. Genomic DNA Library was prepared using genomic DNA extracted from a male fetus of Shiba goat (Capra hircus var Shiba), a native Japanese miniature goat bred in Experimental Station for Bio-Animal Science, The University of Tokyo, Iwama-machi, Ibaraki, Japan. The genomic DNA Library was used to screen for the *SRY* containing DNA fragment. Genomic DNA extracted was subjected to digestion with *Sau*3AI. Two terminal bases (4 bases were removed from a single strand by *Sau*3AI) were filled with G and A by using a Lambda GEM-12-XhoI Half Site Arm kit (Promega, Madison, WI) Packaging was done by using a Gigapack II Packaging Extract (Stratagene, La Jolla, CA). Shiba Goat male DNA was PCR-amplified, using primer-F (5′-CCT-TCA-TTG-TGT-GGT-CTC-GTG-AAC-3′) and Primer-R (5′-AGC-CCG-GGT-ATT-TGT-CTC-GGT-GTA-TAG-3′), which were designed using the published sequence of HMG box of the caprine SRY [29]. Condition employed for PCR was as follows; 1 cycle of 2 min at 94 °C, 3 min at 65 °C, 3 min at 72 °C, and 25 cycles of 2 min at 94 °C, 1 min at 65 °C, 1 min at 72 °C, and finally the reaction mixture was incubated at 72 °C for 10 min. The resulting DNA fragments were retrieved from the agarose gel after electrophoresis, and ^{32}P-labeled by means of a random labeking kit (Amersham, Buckinghamshire, UK). Plaque hybridization was performed and the DNA was extracted from the positive plaque. The isolated phage DNA was digested with *Eco*RI and ligated to a plasmid vector pUC18 for sequencing (GenBank Accession No. D82963). Based on the cloned Shiba Goat SRY sequence one forward primer and one reverse primer were designed: Shiba SRY F1(5′-AGC-GCT-TAG-GTA-CAT-TCA-AAT-GT-3′) and Shiba SRY R(5′-AGC-GCT-GTT-CTT-ACC-AGA-GAC-TG-3′). All of these primers were designed to generate a restriction site for Aor51HI. The PCR products were cloned into pGEM-T easy vector and sequenced. The Shiba Goat SRY 5′ region was excised with Aor51HI and inserted into the *Sma*I site of pxAwNCre plasmid.

CAG/Cre fusion gene. The Cre-expressing plasmid, pCAG/Ncre [25], containing the bacteriophage P1 Cre gene and driven by the CAG promoter [30] and the polyadenylation signal were the kind gifts of Dr. I. Saito, Institute of Medical Sciences, University of Tokyo, Japan.

Preparation of primary cultured cells from the tissues. Transgenic fetuses were prepared by mating the CAG/loxP/LacZ transgenic females to a double transgenic male carrying the CAG/loxP/LacZ and also expressing the CAG/GFP on Y chromosome. Immediately after the pregnant transgenic mice had been sacrificed by cervical dislocation, the urogenital ridges of 11.5-dpc female and male fetuses were individually collected under a fluorescence stereomicroscope (M2 FL II, Leica, Germany), as previously reported [24]. The 13.5-dpc female and male gonads, respectively, were collected from the fetuses produced by mating the CAG/loxP/LacZ transgenic male to the females from the same transgenic mouse line. The brains and livers were used as control tissues. The tissues were minced and treated with 0.1% trypsin, 0.01% EDTA in phosphate-buffered saline (PBS, pH 7.4) at 37 °C for 10 min, and then dissociated by repeated pipetting. After the viability of the cells had been checked by trypan blue staining, approximately 5×10^4 cells/well were poured into noncoated 24-well dishes containing F12/DME medium (Gibco-BRL, New Yolk, NY) and 10% FBS.

Transfection of plasmids. Transfection of plasmids was performed immediately (0 h), at 3 h, or at 10 h, respectively, after the cell suspension was poured into culture dishes. Plasmids were transfected using Effectene Transfection Reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions, and the transfected cells were cultured at 37 °C under 5% CO_2 for 3 days.

Detection of β-galactosidase activity in the cells. At 3 days after transfection, the cells were washed three times with PBS (pH 7.4), fixed with 0.25% glutaraldehyde, and washed with PBS. The β-galactosidase activity was revealed by staining for approximately 14 h at 37 °C in PBS (pH 7.4) containing 2 mM $MgCl_2$, 4 mM $K_3Fe(CN)_6$, 4 mM $K_4Fe(CN)_6$, 0.01% Tween 20, and 0.4 mg/ml 5-bromo-4-chloro-3-indoly-β-D-galacto-pyranoside (X-gal).

Preparation of nuclear extracts. All steps after tissue collection were performed at 0–4 °C. Each tissue was suspended in NHS-1 solution containing 0.3 M sucrose, 10 mM Hepes–KOH (pH 7.9), 10 mM iminodiacetic acid (IDA), 5 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 0.1% Nonidet P-40 (NP-40), 0.1 mM Pefablock (Stratagene, La Jolla, CA), and 1 μg/ml each of leupeptin and pepstatin A. The tissue pairs were then homogenized, and the nuclei were collected in 30-ml cortex tubes by centrifugation (at 10,000 rpm for 10 min). The pellets were resuspended in NHS-1, layered on NHS-2 solution (1.75 M sucrose cushion in NHS-1 without NP-40), and centrifuged at 27,000 rpm for 1 h. Nuclei fractions were resuspended in NLB solution (350 mM NaCl, 25 mM Hepes–KOH, pH 7.9, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 0.1 mM pefablock, 10 mM IDA, and 0.5 mM NaF) and placed at room temperature for 30 min. After centrifugation at 14,000 rpm for 30 min, 0.5 g/ml powdered ammonium sulfate was added to the supernatant and the resulting solution was mixed for 30 min. The precipitated proteins were collected by centrifugation (at 14,000 rpm for 30 min), dissolved with 5 mg/ml of the dialysis buffer (25 mM Hepes–KOH, pH 7.9, 10% glycerol, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 10 mM IDA), and dialyzed for 2 h. The nuclear extracts were divided into 20-μl aliquots and stored at −80 °C until use.

In vitro transcription studies. 1.4kSry/Cre was excised with *Sal*I, and *Bgl*II (digested at Sry position 8207) was used as a 1.4kSry template. 1.0kSry/Cre, 0.7kSry/Cre, and 0.5kSry/Cre plasmids were excised with *Bam*HI and *Bgl*II, and used as 1.0kSry, 0.7kSry, and 0.5kSry templates. The 0.5kSry/Cre plasmid was excised with *Acc*I at *Sry* position 7661 and *Bgl*II, and was used as a 0.4kSry template. Each 50 μg of the nuclear extracts was incubated with 1 μg of the template at room temperature for 15 min and added to 10 μl of appropriate buffer (14 mM Hepes–KOH, pH 7.9, 14% glycerol, 20 mM KCl, 6.5 mM $MgCl_2$, 1.2 mM ATP, 1.2 mM CTP, 1.2 mM GTP, and 0.05 mM UTP), 20 U RNase inhibitor (Takara, Shiga, Japan), and 185 kBq [α-^{32}P]UTP (Amersham Biosciences, Buckinghamshire, UK) on ice. The mixture was incubated at room temperature for 1 h and then electrophoresed on 5% polyacrylamide gels in 1× TBE buffer. The gels were dried and exposed to X-ray film on an intensifying screen at −80 °C.

Results

Promoter activities of various 5′ upstream regions

First, to specify the sequences regulating stage- and tissue-specific expression of *Sry*, we constructed a variety of Sry/Cre plasmids with *Sry* 5′ regions of different sizes: positions 6634–8247 (1.4kSry/Cre, transcription start site is position 8032), positions 7032–8247 (1.0kSry/Cre), positions 7260–8247 (0.8kSry/Cre), positions 7549–8247 (0.5kSry/Cre), and positions 7661–8247 (0.4kSry/Cre), respectively (A in Fig. 2). The 4.1Sry/Cre that has been previously reported to be expressed in a stage- and tissue-specific fashion [24] was used as a positive control. pxAwNCre plasmids containing the Cre coding region alone were used as a negative control gene. Table 1 shows the results obtained from the experiments in which each of the Sry/Cre plasmids or the CAG/Cre plasmid

Table 1
Summarized results of LacZ-expressing cells in the primary cultured cells prepared from various tissues of 11.5-dpc and 13.5-dpc

Constructs	11.5-dpc				13.5-dpc	
	Gonads		Other tissues of males		Gonads	
	Male	Female	Liver	Brain	Male	Female
4.1kSry/Cre	1.4 ± 0.6	1.6 ± 0.7	ND	ND	ND	ND
1.4kkSry/Cre	0.8 ± 0.3	0.9 ± 0.4	ND	ND	ND	ND
1.0kSry/Cre	1.7 ± 0.8	1.1 ± 0.5	ND	ND	ND	ND
0.8kSry/Cre	1.8 ± 0.8	1.1 ± 0.5	ND	ND	ND	ND
0.5kSry/Cre	1.4 ± 0.6	0.7 ± 0.3	ND	ND	ND	ND
0.4kSry/Cre	30.4 ± 6.6	25.8 ± 2.9	18.8 ± 10.9	11.1 ± 5.6	33.2 ± 11.7	37.1 ± 18.5
gSRY/Cre	0.3 ± 0.3	ND	ND	ND	2.2 ± 1.3	ND
pxAwNCre	ND	ND	ND	ND	ND	ND

The cells were prepared from urogenital ridges or gonads of CAG/loxP/CAT/loxP/LacZ transgenic fetuses and were then transfected with each of the constructs, as shown in Fig. 1. Values are percentages of X-gal-positive cells in the primary cultured cells that were transfected with each of the different constructs. Differences of transfection efficiency were normalized by comparing the number of X-gal-positive cells against those that were transfected with CAG/Cre plasmids. The number of X-gal-positive cells was calculated in three different microscopic views (100×) and then summed up. ND, not detected.

was transfected into the primary cultured cells that had been prepared from gonads, livers, and brains of male and female CAG/loxP/LacZ transgenic fetuses on 11.5-dpc and 13.5-dpc. The rates of X-gal-positive cells in the primary cultured cells transfected with various Sry/Cre constructs were graphically compared with those that were transfected with CAG/Cre plasmid (B in Fig. 2). When five constructs other than the 0.4kSry/Cre were transfected into the cells isolated from 11.5-dpc urogenital ridges, only a small number (0.7 ± 0.3–1.8 ± 0.8%) of X-gal-positive cells were detected in those cells. No X-gal-positive cells were detected in the cells from 11.5-dpc brains and livers or 13.5-dpc gonads (Table 1 and B in Fig. 2). In contrast, when 0.4kSry/Cre plasmids were transfected, the rate of X-gal-positive cells significantly increased in the cells from the livers, brains, and gonads of both 11.5-dpc and 13.5-dpc fetuses: the rates of X-gal-positive cells ranged from 11.1 ± 5.6% in the cells from 11.5-dpc brains to 37.1 ± 18.5% in the cells from 13.5-dpc female gonads (Table 1 and B in Fig. 2).

Next, to investigate whether or not the −0.4 to −0.5 kb region (positions 7549–7660) have inhibitory activity for transcription of ubiquitous genes, this region (*Acc*I/*Acc*I, 100 bp fragment) was fused with a CAG/EGFP gene at the 5′ upstream region (*Sal*I site which located 5′ upstream of CMV enhancer or *Sna*BI site which located between CMV enhancer and chicken β-actin promoter) and then transfected into primary cultured cells from 13.5-dpc male gonads. The results indicate that the construct was almost equally expressed in the transfected cells as well as a CAG/EGFP gene, ubiquitously expressing EGFP (data not shown).

The activity of Shiba goat SRY promoter in mouse gonad cells

We compared by database analysis the sequences of the −0.4 to −0.5 kb 5′ region of goat *SRY* with those of other species: mice, humans, chimpanzees, gorillas, pigs, bulls, and sheep. An alignment of these sequences is shown in Fig. 3. The sequences of the −0.4 to −0.5 kb region of goat *SRY* were highly conserved compared with bovine *SRY* (88.3%), but conservation was low compared to mouse *Sry* (33.3%). To examine the activity of the goat *SRY* promoter in the mouse cells, we constructed a fusion gene of the −1.8 kb 5′ region of goat *SRY* with the Cre coding region (denoted as a gSRY/Cre). The gSRY/Cre plasmids were transfected into the cells isolated from 11.5- and 13.5-dpc urogenital ridges and other tissues of CAG/loxP/LacZ transgenic male mice, and the rate of X-gal-positive cells in those cells was examined. A very small number of X-gal-positive cells were detected in the cells from 11.5- and 13.5-dpc male gonads, while none were observed in the cells from female gonads (Table 1 and B in Fig. 2). X-gal-positive cells were not observed in the cells from 11.5-dpc livers nor brains and in the cells from 11.5-dpc and 13.5-dpc female gonads (Table 1).

In vitro transcription assay

To confirm the results shown in Table 1 and Fig. 2, we analyzed various 5′ upstream regions of *Sry* by an in vitro transcription assay. The constructs used for the in vitro transcription assay and the results obtained are shown in Fig. 4. When nuclear proteins from the livers of adult males were reacted with the templates containing various regions; 1.4-, 1.0-, 0.8-, 0.5-, and 0.4-kb fragments (Fig. 4B) transcripts were detected only in the reaction mixture with the template (Template 5) containing the −0.4-kb region from the *Sry* transcription start site, as shown in Fig. 4B. Transcripts were not detected in other templates (Template 1, Template 2, Template 3, and Template 4) that were larger than the −0.5-kb region from the transcription start site. The same negative results were obtained in the case when nuclear proteins from 11.5-dpc male urogenital ridges were allowed to react with each of the templates (data not shown).

Fig. 3. Comparison of the 5′ flanking sequences of Shiba goat *SRY* with those of other species. The sequences of the −0.4 to −0.5 kb (112 bp) region of goat *SRY* (equivalent to positions 7545–7665 of mouse *Sry*) were most highly conserved with bovine *SRY* (88.3%), and conservation was the lowest with mouse *Sry* (33.3%). The −1.8 kb upstream region of goat *SRY* showed an extremely low promoter activity in the mouse cells, as shown in Fig. 2. *Conserved nucleotides; the numbers indicate positions denoted by Dolci et al. [27]. GenBank Accession Numbers are D82963 (Shiba Goat), L08063 (human), AJ003068 (gorilla), AJ222687 (chimpanzee), U15569 (bull), AF026566 (sheep), U49860 (pig), and X67204 (mouse).

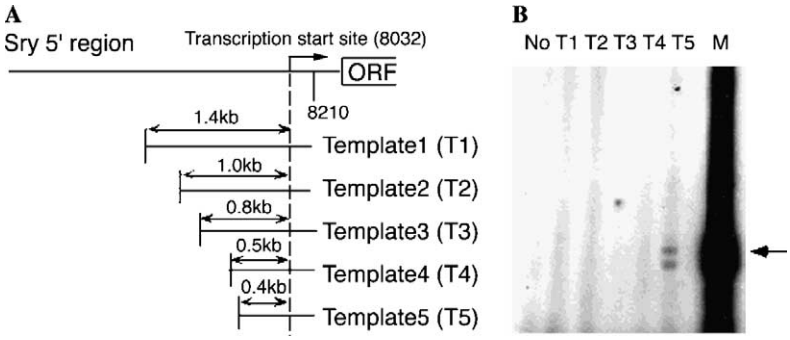

Fig. 4. In vitro transcription assays of the 5′ stepwise-deleted constructs of mouse *Sry*. (A) The differentially sized *Sry* promoter regions used as templates are schematically represented. The numbers in parentheses indicate the positions denoted by Dolci et al. [27]. (B) An in vitro transcription assay was performed in the solution containing [α-^{32}P]UTP and nuclear extracts from adult livers. No transcripts were observed when the *Sry* 5′ regions longer than 0.5 kb from the transcription start site were assayed as the templates in a reaction with nuclear extracts from adult liver, but they did appear when a −0.4-kb (T5) region was assayed. Arrows indicate the transcripts from the templates.

Discussion

We have previously shown that the in vitro Cre/*loxP* system (Fig. 1) using primary cultured cells from developing gonads of CAG/loxP/CAT/loxP/LacZ transgenic fetuses can be used for assay of the mouse *Sry* promoter, as low and transient expression of *Sry* is substituted for high and lasting expression of β-galactosidase in the transfected cells [24]. In addition, we have successfully determined the 5′ flanking sequences that contain important element(s) for stage- and tissue-specific expression of *Sry* [24]. In our present deletional assay, we have further narrowed the essential sequences of the mouse Sry 5′ flanking region for stage- and tissue-dependent expression down to the −0.4 to −0.5 kb (positions 7549–7660), as shown in Table 1 and Fig. 2. In the in vitro transcription assay the template (T5; A in Fig. 4) with the −0.4-kb region was found to have transcriptional activity in the cells from adult male livers, but the templates that were longer than this template did not have transcriptional activity in these cells. Many highly conserved potential elements (AP1, Barbie, GATA, Gfi1, cMyb, vMyb,NF1, Oct1, Sp1, and SRY) have been identified within the −0.2-kb upstream region of *Sry*/*SRY* by database analysis of 10 different mammalian species [13]. However, there is no experimental demonstration that has determined the true involvement of these factors in regulatory expression of *Sry*. The present results indicate that the −0.4-kb region does not contain any element responsible for the stage- and tissue-specific expression of the mouse *Sry*. Taken together with our previous results [24], it is certain that some trans-factor(s) regulating the stage- and tissue-specific activity of the *Sry* promoter may bind to positions 7549–7660. However, the fusion gene of the positions 7549–7660 to the ubiquitous gene

(CAG/EGFP) did not affect the level of EGFP expression (data not shown). The element could be a suppressing element for endogenous *Sry* promoter which is a very weak promoter but not strong to suppress the strong artificial promoter.

Furthermore, although the −0.5- to −1.4-kb region showed the stage- and tissue-specific activity in the deletional assay of *Sry* promoter, no transcripts were detected when the same regions were assayed as the templates in a reaction with nuclear extracts from 11.5-dpc urogenital ridges of male fetuses. This contradiction may be explained by the possibility that in the case of the in vitro transcription assay the factors derived from a large number of cells other than the *Sry*-expressing cells in the 11.5-dpc urogenital ridges may inhibit the stage- and tissue-specific activity of the −0.5- to −1.4-kb region of *Sry*. Although the mechanism by which the 5′ flanking region investigated in the present study regulates the stage- and tissue-specific expression of *Sry* is obscure, it is assumed that other 5′ upstream element(s) may correlate with these regions.

Yokouchi et al. [14] have reported that positions 5538–5639 (A4 fragment) contain a DNase I hypersensitive site and may be involved in stage-specific transcriptional regulation of the linear and/or circular molecule transcripts from the mouse *Sry* gene. As it is postulated that transcription factors bind to the A4 fragment in mouse fetal gonad cells at 11.5-dpc, we have attempted using an in vitro transcription assay to confirm the contribution of the A4 fragment to the stage- and tissue-specific activity of *Sry* promoter. The fusion of positions 5538–5639 endowed a stage- and tissue-specific promoter activity of *Sry* only in a very weak fashion. Moreover, the activity was not accelerated by the fusion of multiple A4 fragments (data not shown). It was because only less than 2% of the *Sry*-expressing cells in gonads was used to make the extracts based on the data of Fig. 2 and Table 1.

It is curious that, apart from the HMG box, the nucleotide sequences of *SRY* are poorly conserved among mammalian species [29,31], despite the primary role of *SRY* in sex determination. In addition, the positions 7549–7660 of mouse *Sry* are not conserved among species (Fig. 3). Introduction of human *SRY* did not induce sex reversal in XX type transgenic female mice [2]. Our present results indicate that the −1.8-kb 5′ upstream region of Shiba goat *SRY* is not fully active in the cells from male urogenital ridges and is not at all active in the female cells. By contrast, it has been reported that GFP is expressed in the male urogenital ridge of transgenic mice under the control of the porcine *SRY* promoter [32]. These findings suggest that there may be a different mechanism underlying the regulation of *SRY* expression among species.

In conclusion, in the present study we have identified an essential sequence of 5′ flanking regions for regulating stage- and tissue-specific expression of mouse *Sry*; positions 7549–7660. It will be of interest to investigate how known or unknown factors actually interact with the element in the sex-determining molecular pathway.

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