

Structural and functional characterization of the mouse *Sox9* promoter: implications for campomelic dysplasia

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Received December 24, 1998; Accepted January 8, 1999

DDBJ/EMBL/GenBank accession nos AB022193–AB022195

Mutations in *SOX9* cause campomelic dysplasia (CD), a dominant skeletal dysmorphology and XY sex reversal syndrome. The CD phenotype is sensitive to dosage and expression levels of *SOX9*. *Sox9* is expressed during chondrocyte differentiation and is up-regulated in male and down-regulated in female genital ridges during sex differentiation. In order to study the sex- and tissue-specific regulation of *Sox9*, we have defined the transcription start site and characterized the mouse *Sox9* promoter region. The *Sox9* proximal promoter shows moderately high nucleotide similarity between mouse and human. Transient transfection experiments using various deletion constructs of the 6.8 kb upstream region of mouse *Sox9* fused to a luciferase reporter showed that the interval between 193 and 73 bp from the transcription start site is essential for maximal promoter activity in cell lines and in primary male and female gonadal somatic cells and liver cells isolated from 13.5 d.p.c. mouse embryos. This minimal promoter region was shown by DNase I hypersensitive site assay to be in an 'open' state of chromatin structure in gonads of both sexes, but not in the liver. Promoter activity was higher in testis than in ovary and liver, but deletion of the region from –193 to –73 bp abolished this difference. We conclude that the proximal promoter region is in part responsible for the sex- and tissue-specific expression of the *Sox9* gene and that more distal positive and negative elements contribute to its regulation *in vivo*, consistent with the observation that translocations upstream from *SOX9* can result in campomelic dysplasia.

INTRODUCTION

The *Sox9* gene encodes a transcription factor that is critical for chondrogenesis and testis determination in vertebrates. Mutations

in human *SOX9* cause campomelic dysplasia (CD) (1,2), a disorder characterized primarily by defects of the skeleton, but also affecting the development of the testes, kidneys, heart and brain. *Sox9* is expressed in mesenchymal condensations prior to and during chondrogenesis (3) and has been shown to activate *Col2a1*, the gene encoding type II collagen, the major component of the cartilage matrix (4,5). More recently, *SOX9* has been shown to regulate the genes encoding aggrecan (6) and type XI collagen (7) and may also regulate other structural and/or patterning genes involved in cartilage and bone development.

Male to female sex reversal is found in ~75% of XY CD patients, indicating an additional role for *Sox9* in the testis-determining pathway. *Sox9* is more highly expressed in male than female genital ridges in mouse, chicken and turtle embryos, suggesting that *Sox9* is a fundamental sex-determining gene common to all vertebrates (8–10). The timing and sexually dimorphic expression indicates that *Sox9* is downstream from *Sry* in mammals, but expression in chick and turtle gonads must be independent of *Sry*, which is lacking in these species. Furthermore, the expression of *Sox9* in chondrogenic condensations and in the central nervous system, notochord and kidney in mouse embryos (3,8) implicates multiple positive or negative pathways in *Sox9* regulation during embryogenesis.

Campomelic dysplasia with sex reversal is caused by mutations in, or translocations around, one copy of *SOX9*. It is clear that threshold levels of *SOX9* activity are critical for normal function. An understanding of the sex- and tissue-specific regulation of *Sox9* is therefore central to a molecular analysis of the sex-determining and chondrogenic pathways and will provide a basis for searching for regulatory mutations in non-translocation cases of CD for which a structural mutation cannot be found.

As a first step towards understanding the molecular mechanisms of *Sox9* regulation, we have characterized the mouse *Sox9* promoter and flanking genomic regions in mouse fetal tissues and cultured cells. We find that a 120 bp region close to the transcription start site is associated with maximal promoter activity. This region contributes to, but does not fully account for, the sex- and tissue-specific expression of *Sox9* seen *in vivo*. These findings implicate the involvement of more distal regulatory elements in addition to those in the proximal promoter.

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RESULTS

Identification of the *Sox9* transcription start site

Sox9 is strongly expressed in mouse fetal testes and limb bud chondrocytes (3,8,9). In order to delimit the promoter of mouse *Sox9*, we mapped genomic clones in the *Sox9* region (Fig. 1A) and determined the transcription start site in both tissues. Primer extension experiments using 11.5 d.p.c. limb bud RNA indicated transcription start sites 363–360 bp upstream from the first ATG, with the strongest band at –361 bp (Fig. 1B). This position is similar to that in the human *SOX9* gene reported previously (Fig. 2; 2). RNase protection assays using a probe spanning the *Sox9* promoter confirmed this result. Protected fragments of equal size were detected in both 11.5 d.p.c. limb bud and 13.5 d.p.c. testis RNA (Fig. 1C). In the ovarian sample, protected bands could be detected at the same position by longer exposure of the autoradiograph (data not shown). Since no intron splicing acceptor consensus sequences are found around this region, we conclude that the gonad- and chondrocyte-specific expression of *Sox9* utilizes the same transcription start site and is regulated via the same promoter.

Conserved sequence motifs in the proximal upstream region

Previous studies have shown that the sex- and tissue-specific expression of *Sox9* during embryogenesis is conserved among human, mouse and chick (2,8,9). Some evolutionary conservation of important regulatory sequences is therefore expected.

In order to compare the nucleotide sequences upstream of the transcription start site, we isolated human and chick *Sox9* genomic DNAs by library screening or PCR. This region was moderately conserved between human and mouse (~70%; Fig. 2). Comparison with the chick upstream sequence revealed three conserved elements (two CCAAT boxes and a TATA box), the positions and orientation of which were conserved, suggesting that these elements are important for *Sox9* transcription (Fig. 2). The mouse *Sox9* promoter also contains putative GATA and CREB transcription factor binding sites, some of which are conserved (Fig. 2). We also checked for possible binding sites for SRY, DAX1, SF-1 and WT-1, which function as key regulators in the male or female differentiation pathway of mammalian sex determination (11–15). Of these, only one putative SRY/SOX9 binding site (CACAAAT) was found in mouse and human (Fig. 2). The mouse and human upstream sequences also contain a (CA)_n repeat, an additional motif to which SRY protein reportedly binds (16). These observations suggest that DAX-1, SF-1 and WT-1 are not directly involved in *Sox9* transcriptional regulation via the proximal promoter and are compatible with the hypotheses that SRY may regulate *Sox9* transcription and *Sox9* may be transcriptionally autoregulatory.

Open chromatin structure immediately upstream of the *Sox9* promoter

It is generally accepted that local chromatin structure affects gene transcription (17). We used DNase I hypersensitivity assays to reveal regions of open chromatin around the *Sox9* gene that could interact with positive or negative regulatory factors (Fig. 3). Assay of genomic fragments between –4 and +1.4 kb from the transcription start site revealed a single DNase I hypersensitive

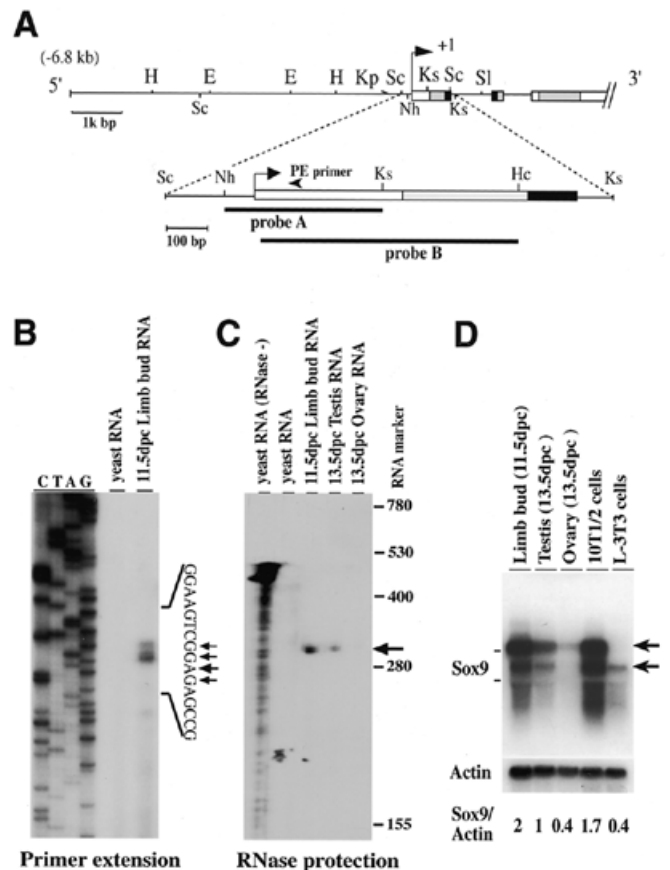


Figure 1. Identification of the *Sox9* transcription start site. (A) Schematic representation of the 5'-region of mouse *Sox9*. The box indicates the *Sox9* gene (shaded box, ORF; black box, HMG box) and the arrow indicates the position of the transcription start site. Recognition sites for various restriction endonucleases are shown (Sl, *SalI*; E, *EcoRI*; H, *HindIII*; Kp, *KpnI*; Sc, *SacI*; Nh, *NheI*; Ks, *KspI*; Hc, *HincII*). Solid lines represent probes used in RNase protection and northern blot experiments. The primer position used in the primer extension experiments is indicated by the arrowhead (PE primer). (B) Primer extension experiments. The left four lanes show the sequence ladder as a marker. Arrows indicate the four bases in the *Sox9* upstream sequence (right) implicated as transcription start sites, the largest arrow corresponding to the most commonly used start site. (C) RNase protection assay performed using a 452 base *Sox9* probe (probe A). The expected 310 base protected fragments (arrow) were detected in the limb bud and testis RNAs. (D) Northern blot analysis (probe B) showing *Sox9* mRNA expression in mouse fibroblast cell lines and embryonic tissues. The lower panel shows the same blot hybridized with a β -actin probe and the numbers at the bottom (*Sox9*/Actin) indicate the *Sox9* expression level relative to that of actin (the relative *Sox9* mRNA amount in the 13.5 d.p.c. testis was set as 1).

site located in the region immediately upstream of the *Sox9* promoter, around the *SacI* and *NheI* sites at –193 and –73 bp, respectively (Fig. 3). The same hypersensitive site was detected in male and female gonadal somatic cells and in 10T1/2 and 3T3 cells which express *Sox9*, but not in liver cells. These findings implicate the region around the *SacI* and *NheI* sites in the *Sox9* promoter in accessibility to regulatory factors.

A more extensive search in the regions from –8.6 to –4 kb and from +1.4 to +10 kb revealed no additional DNase I hypersensitive sites (data not shown). We conclude that DNase I hypersensitive sites in the range from –8.6 to +10 kb of the *Sox9* gene are restricted to the proximal promoter region.

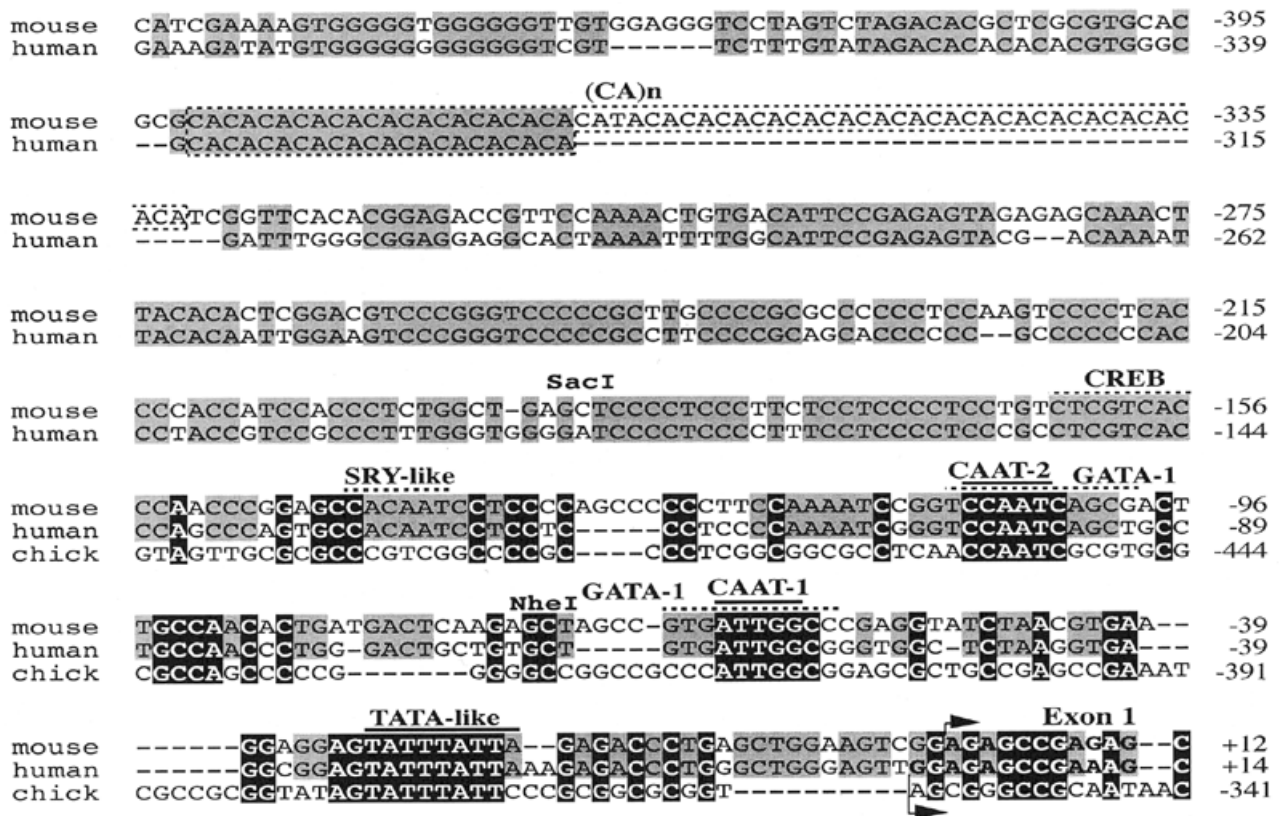


Figure 2. Nucleotide sequences and putative regulatory elements of the putative *Sox9* proximal promoter regions in mouse, human and chicken. The shaded and black boxes indicate mouse/human and mouse/human/chick homology, respectively. No appreciable similarity to the chick sequence was found outside the region shown. The three elements conserved among the three species (two CCAAT boxes and a TATA-like sequence) are indicated by solid lines. Other putative *cis* elements are indicated by broken lines. Arrows show the transcription start sites of the mouse (this study) and human (2) genes. Mouse and human sequences are numbered relative to their major transcription start site; chick numbering is relative to the first methionine codon (ATG) of the ORF.

Expression levels of *Sox9* mRNA in fetal tissues and cell lines

As a basis for interpreting transfection studies using *Sox9* reporter constructs, we next examined the expression level of *Sox9* mRNA in cells and tissues used in this study. Northern blotting detected two major *Sox9* transcripts of 5.5 and 2.3 kb (Fig. 1D). *Sox9* mRNA was found to be expressed at high levels in the limb bud and 10T1/2 cells, which can be induced to chondrogenesis under appropriate culture conditions (S. Wheatley and P. Koopman, unpublished data). *Sox9* expression in the male gonad was 2.5-fold higher than that in the ovary, in agreement with published data (9). L-3T3 cells showed a low level of *Sox9* expression similar to that in the ovary, while no expression was detectable in liver, even by RT-PCR analysis (data not shown).

Localization of the minimal promoter containing tissue-specific regulatory elements

In order to localize regulatory sequences in the 5' flanking region of mouse *Sox9*, transient transfections were performed using various lengths of *Sox9* upstream DNA (starting at -6.8 kb to +251 bp and ending at +315 bp relative to the transcription start site) fused to a luciferase reporter. These constructs were co-transfected with a pEF-LacZ reporter. Results were standard-

ized to *LacZ* expression levels, to control for plasmid copy number in each well, and to luciferase expression levels obtained using a β -actin-luciferase construct, to control for differences in transfection efficiency between cell types.

High levels of luciferase reporter activity were observed in both 10T1/2 and L-3T3 cells using the full-length (-6.8 kb) construct pGL6.8-Luc and progressive deletions to the *SacI* site in the proximal promoter at -193 bp (pSc-Luc; Fig. 4A). However, deletion of the region from -193 bp to the *NheI* site at -73 bp (pNh-Luc) almost completely abolished expression of the reporter construct. A further deletion to the *SmaI* site at +251 bp in the 5'-untranslated region (5'-UTR) (pSm-Luc) abolished the remaining weak luciferase activity to a level similar to that of the negative control pGL-b-Luc.

Similar results were obtained by transfection of these constructs into male and female gonadal somatic cells and liver cells obtained directly from mouse fetal tissues (Fig. 4B). These data indicate that the region from -193 to -73 bp is essential for maximum *Sox9* promoter activity in a range of cell types. This region corresponds well with the DNase I hypersensitive site determined in these cells.

Promoter activities in 10T1/2 cells and testis were consistently higher than those in L-3T3 cells, liver and ovary (Fig. 4). Deletion of the *SacI*-*NheI* (-193 to -73 bp) region abolished this differential. These data suggest that some of the sex and tissue

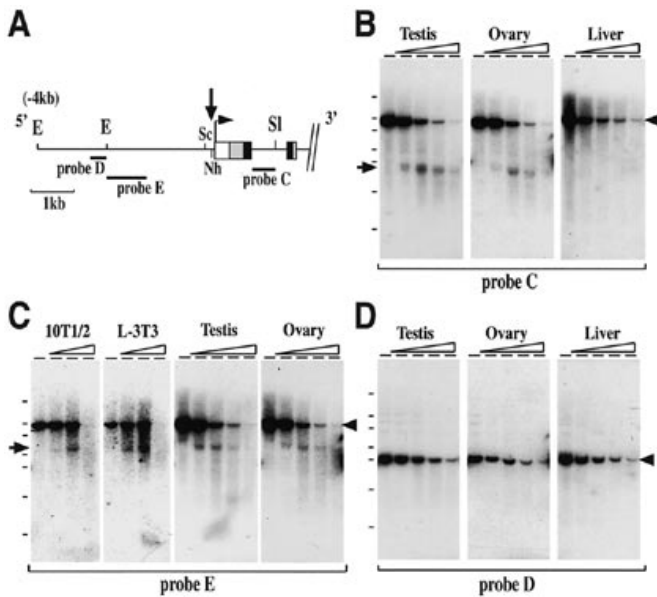


Figure 3. DNase I hypersensitive site analysis. (A) Schematic diagram showing the *Sox9* promoter region and fragments used as probes. (B–D) Nuclei from the various cells (10T1/2, L-3T3 and testis, ovary and liver cells collected from 13.5 d.p.c. embryos) were treated with increasing amounts of DNase I (left to right, shown by triangles) or no DNase I (left lane in each panel). After DNA extraction and digestion with *EcoRI* and *SalI*, they were hybridized with probe C, D or E. Arrowheads indicate the original *Sox9* band [3.8 kb *EcoRI*–*SalI* fragment, (B) and (C); 1.6 kb *EcoRI* fragment, (D)]; arrows show novel bands due to DNase I cleavage [1.4 kb, (B); 2.2 kb, (C); none, (D)]. The sizes of these bands indicate a single DNase I hypersensitive site at the position indicated by an arrow in (A). DNA markers are shown as bars at the left of (B), (C) and (D) (12.0, 4.0, 3.0, 2.0, 1.5, 1.0 and 0.5 kb).

specificity of *Sox9* expression is due to *cis* elements in the proximal promoter, between –193 and –73 bp from the transcription start site.

DISCUSSION

Sox9 is expressed in a complex sex- and tissue-specific manner during embryogenesis (3,8,9). Further, heterozygous mutations in human *SOX9*, including translocations affecting expression levels, lead to XY sex reversal and skeletal dysmorphism in campomelic dysplasia patients (1,2). These observations indicate that the regulation of *Sox9* expression is complex and critical for normal development.

In order to study the regulation of *Sox9*, we have localized and characterized the promoter region using a variety of molecular strategies. Our data suggest that a 120 bp region located near the transcription start site is essential for maximal promoter activity. This region was found to drive higher levels of *Sox9* expression in testicular than in ovarian somatic cells and liver cells, implicating this interval in sex- and tissue-specific regulation of *Sox9*. These findings will serve as a basis for further studies aimed at identifying the *cis*-regulatory elements within this region and for investigating whether mutations in this interval underlie some cases of CD.

Despite the importance of the promoter region, it appears that promoter activity alone is not sufficient to account for tissue-specific differences in *Sox9* expression levels. Northern blot

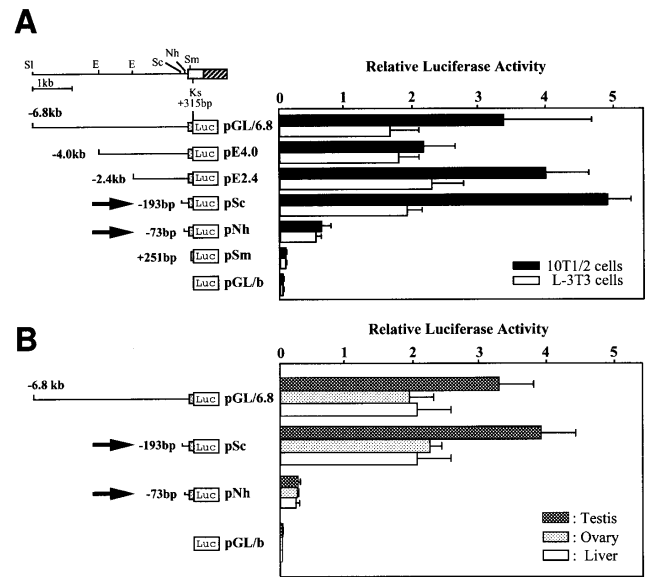


Figure 4. Promoter analysis using transient transfection of *Sox9* promoter constructs. (A) Transfection of 10T1/2 and L-3T3 cell lines; (B) transfection of gonadal somatic cells and liver cells isolated from the 13.5 d.p.c. embryos. Constructs contained various amounts of 5' flanking DNA (indicated at left) and terminated at a common 3'-end (*KspI* site, Ks, +315 bp). Each luciferase activity was measured, normalized to β -galactosidase values for the co-transfected construct pEF-LacZ (to control for copy number differences) and standardized to values obtained using p β A-Luc (to control for transfection efficiency; p β A-Luc = 1). Promoter activity was localized to the region of the *SacI* and *NheI* sites at –193 and –73 bp (arrows). Values obtained using pSc were significantly different from those obtained using pNh for 10T1/2, 3T3, fetal testis, fetal ovary and fetal liver cells ($P = 0.00002, 0.0004, 0.002, 0.0001, 0.02$, respectively, using a two-sample equal variance *t*-test). Cell type- and sex-specific differences between values obtained using pSc (10T1/2 versus 3T3, $P = 0.0002$; testis versus ovary, $P = 0.02$; testis versus liver, $P = 0.067$) were not seen using pNh (10T1/2 versus 3T3, $P = 0.3$; testis versus ovary, $P = 0.6$; testis versus liver, $P = 0.16$). Bars represent means and standard errors of measurements from four (A) or three (B) independent transfection trials.

analysis showed expression levels of *Sox9* 4-fold higher in 10T1/2 compared with L-3T3 cells and 2.5-fold higher in testis than ovary, whereas differences in mean luciferase reporter activities these cell and tissue types were substantially smaller. These findings suggest the involvement of some other regulatory mechanisms that could not be demonstrated by the present luciferase assay *in vitro*.

One possible mechanism is regulation at the level of chromatin organization, which we investigated using DNase I hypersensitivity assays. Although the activity of transfected *Sox9* promoter constructs was similar in liver cells to that seen in L-3T3 cells and ovary, the promoter region is in a closed chromatin conformation in liver tissue and therefore likely to be inaccessible to *trans*-acting factors, resulting in the inactivity of *Sox9* transcription in the liver. However, in gonads of both sexes and in 10T1/2 and L3T3 cells, this minimal promoter region was in an open state of chromatin structure. No sex- and cell-specific differences in DNase I hypersensitivity were detected. Therefore, it appears that an open chromatin structure is necessary for *Sox9* transcription,

but not sufficient for sex- and tissue-specific regulation of its transcription level.

It seems likely that more distal enhancer/silencer elements are necessary for complete tissue-specific regulation of the *Sox9* gene. Since we could not detect any hypersensitive sites other than that in the proximal promoter region in the range from -8.6 to +10 kb of the *Sox9* locus, these regulatory elements may be present outside this interval. In some CD patients, translocation breakpoints map at considerable distances (up to 950 kb) from the *SOX9* gene, with no other gene sequences being found in this interval (18). Recent studies in transgenic mice bearing fragments of a human *SOX9* YAC indicate that regulatory elements driving expression of *SOX9* to various skeletal elements are scattered through a 350 kb region upstream of the *SOX9* locus in humans (19). Interestingly, the same study failed to locate sex- and tissue-specific elements in human *SOX9* that drive expression in the gonads of transgenic mice (19). If regulatory elements of the *Sox9* gene are similarly scattered in mice, their complete identification will present a formidable challenge.

MATERIALS AND METHODS

Genomic library screening and sequencing

Mouse *Sox9* genomic clones were isolated in our previous study (3). Chick *Sox9* genomic DNA clones were isolated from the Clontech (Palo Alto, CA) chick genomic libraries using a chick *Sox9* cDNA probe (8). Human *Sox9* 5'-flanking genomic DNA was identified by PCR using a promoter walker kit (Clontech) in combination with two human *SOX9* reverse primers: 5'-CGTCGCGCCGCTAC-CGCGGCGAGCACTTA-3' and 5'-GTGGCCAGTTCACAGCT-GCCCCGTCCAAGT-3' (2). All genomic DNA clones and PCR products were sequenced using the ABI Prism dye terminator cycle sequencing reaction kit. Nucleotide sequences were aligned by using the ClustalW multiple sequence alignment program (20). Putative transcription factor binding sites were defined by the TFSEARCH program (21) or from published papers.

RNA extraction and primer extension

Total RNA was extracted from various fetal tissues and 10T1/2 and L-3T3 cells by the guanidinium thiocyanate method (22). For primer extension, 20 µg of 11.5 d.p.c. limb bud total RNA or the control yeast RNA were separately annealed with 10 pmol of the ³²P-end-labeled primer (5'-GCCACTTGACCTCGTCTCTCTT-GCAAAGA-3', +80 to +109 bp from transcription start site) at 72°C for 40 min, and cDNA extension was carried out at 42°C for 40 min by the addition of AMV reverse transcriptase. The extension fragments were analysed on a denaturing 8% acrylamide-1× TBE sequencing gel. As a marker, sequencing samples of the mouse *Sox9* genomic DNA clone produced with the same primer were electrophoresed on the same gel. The sequencing gel was autoradiographed with X-ray film (Fuji Film, Tokyo, Japan).

RNase protection assay and northern blot analysis

The DNA fragments corresponding to probe A or B (Fig. 1A) were subcloned into pBluescript. Each clone was linearized with the appropriate restriction enzyme and antisense RNA probes were generated by *in vitro* transcription using [α -³²P]UTP with T3 or T7 RNA polymerase (Boehringer Mannheim, Mannheim, Germany). For RNase protection assay, after the full-length RNA

probe (probe A) was isolated from the acrylamide gel, the probe was hybridized with 10 µg of each total RNA sample or yeast RNA at 45°C for 12 h. After digestion with RNase A and T1 for 30 min at 37°C, the protected fragments were analysed on a denaturing 5% acrylamide-1× TBE sequence gel. For northern blot analysis, 20 µg of each extract were denatured in formamide-formaldehyde buffer, electrophoresed in 1% formaldehyde-agarose gels and transferred to a nylon membrane. Blots were hybridized with appropriate ³²P-labeled RNA probe at 75°C. Filters were finally washed with 0.1× SSC, 0.1% SDS at 75°C for 1 h and autoradiographed. The signal intensity was measured with a Bio-Rad (Hercules, CA) imaging densitometer (model GS-700).

Detection of DNase I hypersensitive sites

Gonads and liver tissues were collected from 13.5 d.p.c. mouse embryos, treated with 0.05% trypsin, 200 IU/ml collagenase in phosphate-buffered saline (PBS)/EDTA at 37°C for 10 min and dissociated by repeated pipetting until a single cell suspension was achieved. After the viability (>95%) was checked by trypan blue staining, the cells were plated onto 10 cm dishes and incubated in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (FCS/DMEM) for 12 h. After washing with PBS several times for the removal of non-attached cells (i.e. blood cells in the liver and germ cells in the gonads), nuclei of the attached cells were isolated by homogenization of the cells in Nonidet P-40 (NP-40) lysis buffer (10 mM Tris-HCl pH 7.4, 15 mM NaCl, 60 mM KCl, 0.5 mM DTT, 1 mM EDTA, 1 mM EGTA, 0.5 mM spermidine, 0.5% NP-40, 10% sucrose) according to Wu's method (23). After addition of CaCl₂ and MgCl₂ (final concentrations 0.4 and 2 mM, respectively) into the nucleus samples, various concentration of DNase I (0–15 IU/ml) were incubated in the nuclear suspensions at 37°C for 15 min. An aliquot of 5 or 10 µg of each isolated DNA was digested with appropriate restriction enzyme, electrophoresed in 0.8% agarose gels and transferred to a nylon membrane. Blots were hybridized with each appropriate ³²P-labeled DNA probe at 65°C. Filters were washed with 0.2× SSC, 0.1% SDS at 65°C for 60 min and autoradiographed. About 30–40% of the male gonadal somatic cells attached on the dish were identified as Sertoli cells by immunofluorescence staining with anti-SOX9 antibody (8).

Construction of reporter plasmids, cell culture, transfection and luciferase activity assay

The *SalI* (in vector)-*KspI* fragment (from -6.8 kb to +315 bp) of the mouse *Sox9* genomic DNA was cloned upstream of the luciferase gene in pGL3-b-Luc (pGL6.8-Luc). Various 5'-deletion constructs of pGL6.8-Luc [pE4.0, pE2.4, pSc, pNh and pSm-Luc, possessing a common 3'-end (*KspI* site at +315 bp)] were made by digestion with each restriction enzyme and self-ligation (Fig. 1). The junction of the inserted DNA in each reporter plasmid was checked by sequencing. pEF(elongation factor promoter)-LacZ was co-transfected as an internal control for plasmid copy number and p β A(β -actin promoter)-Luc construct (a gift from Dr A. Ian Cassady, CMCB, University of Queensland) was used as a control to standardize for transfection efficiency. 10T1/2 and L-3T3 cells (0.5×10^5 cells/1 cm well) were seeded 1 day before transfection. Primary cultures of 13.5 d.p.c. gonadal somatic cells and liver cells were plated at a density of 2.0×10^5 cells/1 cm well as described above. After incubation

in FCS/DMEM for 12 h, the cells were washed with medium several times to discard the non-adherent cells. Transfection was performed using DOTAP or Fugene-6 reagent (Boehringer Mannheim). Luciferase reporter plasmid (3.3 mM, 1–2.5 µg) and pEF-LacZ (0.33 mM, 0.1 µg) were transfected into the cells on 1 cm dishes. After incubation for 48 h, luciferase and β-galactosidase activities were assayed as described previously (24). Each luciferase value was normalized to β-galactosidase as an internal control for copy number and the relative luciferase activity of pβA-Luc was set at 1 as standard promoter activity.

ACKNOWLEDGEMENTS

We wish to thank our colleagues Masami Kanai-Azuma, Josephine Bowles, Mats Nilsson, Paul Buxton, Susan Wheatley, Jill Kent, Andy Greenfield, Murray Hargrave, David Pennisi and Aaron Schindeler and Dr Hirokazu Fujimoto (Mitsubishi Kasei Institute of Life Sciences) for their advice and help with this work. We thank Jennifer Gardner and Jacqueline Emery for their help with isolation and sequencing of *Sox9* genomic DNAs, Shayama Wijedasa for secretarial and technical assistance and Laura Martin for help with statistical analyses. This work was supported by the Australian Research Council and the National Health and Medical Research Council of Australia.

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