A male-specific role for SOX9 in vertebrate sex determination

Jill Kent1,*†, Susan C. Wheatley1,* Jane E. Andrews2, Andrew H. Sinclair2 and Peter Koopman1,3

1Centre for Molecular and Cellular Biology and 3Department of Anatomical Sciences, The University of Queensland, Brisbane, Queensland 4072, Australia
2Department of Paediatrics and Centre for Hormone Research, The University of Melbourne, Royal Children’s Hospital, Parkville, Melbourne, Victoria 3052, Australia
*The first two authors contributed equally to this work
†Author for correspondence (e-mail: J.Kent@mailbox.uq.oz.au)

SUMMARY

Mutation analyses of patients with campomelic dysplasia, a bone dysmorphology and XY sex reversal syndrome, indicate that the SRY-related gene SOX9 is involved in both skeletal development and sex determination. To clarify the role SOX9 plays in vertebrate sex determination, we have investigated its expression during gonad development in mouse and chicken embryos. In the mouse, high levels of Sox9 mRNA were found in male (XY) but not female (XX) genital ridges, and were localised to the sex cords of the developing testis. Purified fetal germ cells lacked Sox9 expression, indicating that Sox9 expression is specific to the Sertoli cell lineage. Sex specificity of SOX9 protein expression was confirmed using a polyclonal antiserum. The timing and cell-type specificity of Sox9 expression suggests that Sox9 may be directly regulated by SRY. Male-specific expression of Sox9 mRNA during the sex determination period was also observed in chicken genital ridges. The conservation of sexually dimorphic expression in two vertebrate classes which have significant differences in their sex determination mechanisms, points to a fundamental role for SOX9 in testis determination in vertebrates.

Sox9 expression was maintained in the mouse testis during fetal and adult life, but no expression was seen at any stage by in situ hybridisation in the developing ovary. Male-specific expression was also observed in the cells surrounding the Müllerian ducts and in the epididymis, and expression in both sexes was detected in the developing collecting ducts of the metanephric kidney. These results suggest that SOX9 may have a wider role in the development of the genitourinary system.

Key words: SOX9, sex determination, evolution, mouse, chick

INTRODUCTION

In recent years a number of genes have been isolated that have roles in mammalian sex determination and gonadogenesis. In spite of this, we are far from understanding the molecular mechanisms underlying these processes (reviewed by Goodfellow and Lovell-Badge, 1993). Several different sex determination mechanisms are known which are genetic or environmental, dominant or dosage-sensitive, reversible or irreversible. These mechanisms are likely to have evolved separately, but are thought to be associated with a more conserved gonadogenesis pathway common to all vertebrate species (Marshall Graves, 1995).

Sex in mammals depends on the inheritance of a heteromorphic pair of sex chromosomes, X and Y. In the male (XY), the Y chromosome is dominant and leads to testis formation (Ford et al., 1959; Jacobs and Strong, 1959; Welshons and Russell, 1959). The testes produce sex hormones which induce the formation of secondary male sexual characteristics (Jost, 1947). However, this mechanism of sex determination is not typical of all vertebrate classes. For example, in most avian species the female is the heterogametic sex (ZW) and the male is homogametic (ZZ; Ohno, 1961). Evidence suggests that the W chromosome is required for the development of ovarian tissue, but that dosage of Z chromosomes appears to influence testicular development (Thorne and Sheldon, 1993). Further, it is the ovary that produces sex hormones required for the formation of the majority of female secondary sexual characteristics in birds (Wolff and Wolff, 1951).

Genetic analysis of sex-reversed individuals resulted in the isolation of the testis-determining gene on the mammalian Y chromosome, termed SRY (Gubbay et al., 1990; Sinclair et al., 1990). This gene is expressed in the testis during the critical period of sex determination (Koopman et al., 1990) and is able to direct male development in an XX transgenic mouse (Koopman et al., 1991). This demonstrates that SRY is the major switch gene in mammalian sex determination. SRY encodes a protein containing a DNA binding domain known as the HMG box (Nasrin et al., 1991). The HMG box is the only part of SRY conserved between species, and point mutations in the corresponding region of the SRY gene cause sex reversal in humans (Berta et al., 1990; Jäger et al., 1990; 2813 Development 122, 2813-2822 (1996) Printed in Great Britain © The Company of Biologists Limited 1996 DEV3508
Harley et al., 1992; Pontiggia et al., 1994). These features suggest that the sex-determining function of SRY depends on its ability to bind to DNA sequences involved in the regulation of genes downstream in the sex-determining cascade.

Attempts to identify other sex-determining genes on the basis of the DNA-binding properties of SRY have not been successful. An alternative approach is to identify genes which map to loci associated with sex reversal. This approach has implicated a recently identified gene, \textit{SOX9}, in mammalian sex determination. \textit{SOX9} is a member of a family of genes related to \textit{SRY} by the HMG box (Denny et al., 1992b; Wright et al., 1993). \textit{SOX} genes encode proteins with domains characteristic of transcription factors and are expressed in a variety of developing tissues (Stevanovic et al., 1993; van de Wetering et al., 1993; Uwanogho et al., 1994; Connor et al., 1995; Hosking et al., 1995). \textit{Sox9} expression has been shown to occur at sites of chondrogenesis in mouse embryos (Wright et al., 1995) and defects in \textit{SOX9} have been associated with the bone dysmorphology syndrome campomelic dysplasia (CD; Foster et al., 1994; Wagner et al., 1994). A large proportion of XY CD patients exhibit partial or complete sex reversal as well as skeletal abnormalities. In these cases, genital morphologies range from minor variants such as hypospadias and cleft scrotum to female genitalia with streak-like gonadal rudiments (Houston et al., 1983). These phenotypes demonstrate a role for \textit{SOX9} not only in skeletal development but also in sex determination.

Whilst the sex reversal seen in CD patients clearly establishes a role for \textit{SOX9} in mammalian sex determination, it is not yet known how \textit{SOX9} contributes to the molecular genetic pathway of testis development. Several models can be envisaged. First, \textit{SOX9} could act as a transcription factor required for expression of \textit{SRY}. In this model, expression of \textit{Sox9} would be expected to occur in the genital ridges prior to the onset of Sry expression at 10.5 dpc (Koopman et al., 1990; Hacker et al., 1995; Jeske et al., 1996), and would be seen in both sexes since \textit{Sry} is the only Y-linked component of the male sex-determining pathway. Second, \textit{SOX9} protein might interact with \textit{SRY} to form a complex that initiates testis development. In this model, expression of \textit{Sox9} would be expected in both sexes in the period covering the onset of \textit{Sry} expression and the start of overt testis differentiation at 12.5 dpc. A third possibility is that \textit{SOX9} might be directly or indirectly responsive to \textit{SRY}. \textit{Sox9} expression in this model would be restricted to the developing male gonads, beginning shortly after the onset of \textit{Sry} expression. Fourth, \textit{SOX9} might play a relatively late role in the differentiation rather than the determination of testes, perhaps acting as a transcription factor for genes involved in synthesis of steroid hormones or genes important for the architecture of the testis. In this fourth model, a relatively late onset of transcription might be expected in the fetal testes and associated structures.

To distinguish between these models, we have investigated the localisation of \textit{Sox9} mRNA and protein in the developing mouse gonad. A profile of expression was observed which is consistent with a role in male but not female sex determination, and with the possibility that \textit{Sox9} may respond directly to activation by \textit{SRY}. The observation of a similar expression pattern in a phylogenetically distant species, the chicken, confirms a fundamental function for \textit{SOX9} in vertebrate testis determination.

**MATERIALS AND METHODS**

**Collection and sexing of tissue samples**

CD1 mouse embryos were staged by fore- and hindlimb morphology (Rugh, 1968). For embryos younger than 13.5 days postcoitum (dpc), chromosomal sex was determined by toluidine blue staining of amniotic cells and scoring the presence or absence of Barr bodies (Palmer and Burgoyne, 1991).

Chicken (\textit{Gallus domesticus}) eggs were incubated at 37.8°C in a humidified incubator. Embryo age was measured in days of incubation (d). Sex of embryos was determined by PCR amplification of a \textit{W}-specific \textit{XhoI} repeat from a genomic DNA template. Amplification of cytoplasmic \textit{\beta}-actin mRNA was performed as a control. Multiplex PCR reactions were performed with 100 ng each of \textit{W}-specific primers \textit{XhoI} (AACATCCACTTTCTCACGG) and \textit{Xho2} (TTCA-GAGTGATAACGCATGG) (Kodama et al., 1987) and 100 ng each of Actin 1 (TGGATGATGATTTGCTG) and Actin 2 (ATCTTTCATATCATTCCA) (Kost et al., 1983) in 20 μl reactions. PCR reactions were denatured at 95°C for 5 minutes, subjected to 35 cycles of 95°C 30 seconds, 56°C 30 seconds, 72°C 30 seconds and then 5 minutes at 72°C. PCR reactions were performed on a Perkin Elmer thermal cycler. Products of the reactions were analysed on a 1% agarose gel. Chromosomal sex was determined to be ZW (female) if the \textit{XhoI} repeat product was present, and ZZ (male) if only the \textit{\beta}-actin product was present.

Embryos were either partially dissected to remove the head, tail, limb buds and viscera overlying the urogenital ridges (gonads and mesonephros), or the urogenital ridges were explanted.

**Whole-mount in situ hybridisation**

Expression of mouse \textit{Sox9} was detected using the probe \textit{Sox9.5a} (Wright et al., 1995). A 381 bp chicken \textit{Sox9} (c\textit{Sox9}) fragment was generated by PCR amplification of cDNA from urogenital systems of 5.5-7.5 d male and female embryos. Primers c\textit{Sox9.1} (CCCCAACGCCATCTTCAA) and c\textit{Sox9.2} (CTTGCGATGCGCGTAGTGA) were designed using the c\textit{Sox9} cDNA sequence (accession no. U12533). The c\textit{Sox9} PCR product was cloned into pCR-script (Stratagene) and the identity of the product verified by sequencing. Antisense and sense riboprobes were prepared and hybridised as described by Christiansen et al. (1995). Photographs were taken on a Leitz M28 stereomicroscope using Kodak Ektachrome film.

**Southern blot analysis**

A Southern blot was prepared containing 15 μg of either male or female chicken genomic DNA digested with either \textit{BamHI}, \textit{EcoRI} or \textit{HindIII}. This was hybridised overnight at 65°C with an [ε-\textit{32P}]dCTP-labelled \textit{PstI} fragment generated from the plasmid containing the 381 bp c\textit{Sox9} PCR product. The filter was washed to a stringency of 0.1x SSC, 0.2% SDS at 65°C and exposed to film overnight. The intensity of the c\textit{Sox9} bands were quantified using a Molecular Dynamics phosphorimager and ImageQuant software. Densitometry was also performed on an image of the ethidium bromide-stained gel and this confirmed that equal amounts of male and female DNA had been loaded.

**Cell transfection**

COS-1 cells, at a density of 5×10^5-10^6 per 6 cm plate, were transfected with 10 μg of either pSG5 (Stratagene) containing a 2.2 kb \textit{Sox9} cDNA (Wright et al., 1995) or pGALO (Kato et al., 1990) containing the \textit{Sox9} coding sequence using Lipofectamine reagent in
serum-free OptiMem medium according to the manufacturer’s instructions (Gibco BRL). The transfection mix was left on overnight, then removed and the cells fed with complete medium. After 48 hours the cells were harvested and samples prepared for immunoblotting.

**Generation of SOX9 polyclonal antiserum**

A peptide representing the carboxy terminus of murine SOX9 (CPQTHSPQDWEQPVYTQVR) was synthesised and conjugated to diphtheria toxin (QIMR Peptide Unit) and used to immunise a Dutch rabbit. Test bleeds confirmed the presence of antibodies reacting with a protein of approximately 65kD, expressed from Sox9 cDNA transfected into COS-1 cells. Preimmune serum did not react with this protein. Anti-SOX9 antibodies were affinity purified using the same peptide conjugated to SulfoLink Coupling Gel according to the manufacturer’s instructions (Pierce).

**Preparation of protein extracts**

Male and female urogenital ridges from 11.5, 12.5 and 13.5 dpc mouse embryos were pooled according to sex and age. Soluble fractions were prepared from these, and adult gonads and limb buds from 11.5 dpc embryos as follows. Tissues were homogenised and then spun at 5000 rpm for 5 minutes and the supernatant removed to a fresh tube. This was spun at 13000 rpm to isolate the soluble fraction, and sample buffer (Laemmli, 1970) was added. Untransfected and COS-1 cells transfected with Sox9 cDNA were harvested into sample buffer. All samples were sonicated using a Vibracell Ultrasonic Processor (Sonics and Materials, Connecticut, USA) at 40% duty with the output control set at 4 for 20 seconds with no pulse using a microtip. The protein content of all samples was determined using a Pierce BCA protein assay kit and β-mercaptoethanol was added to 5%. Samples were heated to 95°C for 5 minutes and resolved on a 10% SDS-polyacrylamide gel.

**Immunoblotting**

Immunoblotting was performed as described by Wheatley et al. (1993). Affinity purified anti-SOX9 antiserum was diluted to approximately 30 μg/ml. Goat anti-rabbit horse- radish peroxidase (HRP)-conjugated Ig (Vector Laboratories) was diluted to 0.1 μg/ml.

**RT-PCR analysis**

RNA extraction, DNase I treatment and RT-PCR analysis were performed as described previously (Koopman, 1993). Multiplex or parallel PCR amplifications of each cDNA sample were performed using 25 ng each of Hprt primers Hprt1A and Hprt1B (Koopman et al., 1990) and/or 250 ng each of the Sox9 cDNA specific primers 9.5b (GTGGCAAGTATTGGTCAA) and 9.5c (GAACAGACTCA-CATCTCT) in 25 μl (parallel) or 50 μl (multiplex) reactions. Samples were subjected to 26-36 cycles of 94°C 30 seconds, 55°C 1 minute, 72°C 1 minute on a Perkin Elmer Cetus thermal cycler. Products from the parallel reactions were combined and analysed on a 2.2% agarose gel.

Care was taken to arrest the amplification reactions in the linear phase. To achieve this, reactions were periodically sampled to determine a point when the product became detectable on an agarose gel. Amplifications were then allowed to proceed for 6 additional cycles. The amount of Hprt product therefore indicated the amount of starting template, and comparison between the intensity of the Hprt and Sox9 bands (352 bp and 319 bp respectively) allowed the relative level of Sox9 expression in each sample to be assessed. Multiplex PCR of c-kit and Hprt from germ cells employed c-kit primers as described by Rossi et al. (1993) which produce a 385 bp band. Amplification conditions were as for Sox9 and Hprt except that an annealing temperature of 60°C was used.

**Preparation of germ cell samples**

Mouse germ cells were isolated by puncture of 13.5 dpc gonads as described by Hogan et al. (1994). An additional level of selection was employed to increase the purity of the sample. Briefly, cells were plated on tissue culture dishes in DMEM containing 10% fetal calf serum and incubated at 37°C in 5% CO2 for 4 hours (Rossi et al., 1993). Non-adherent germ cells were collected and total RNA was extracted from the germ cells and from the gonads from which they had been removed. The identity of the germ cells was verified by PCR amplification of the germ cell marker c-kit (Rossi et al., 1993).

**RESULTS**

**Dimorphic SOX9 expression in mouse and chicken gonads**

In both mouse and chicken the gonads form as a thickening (the genital ridge) on the ventral medial side of the mesonephros. In mouse the genital ridge is visible at 10 days postcoitum (dpc) and in chicken at about 4 days of incubation (d). Male and female gonads are morphologically indistinguishable until 11.5 dpc in mouse and 5.5 d in chicken. SOX9 expression was visualised during the sex determination period in mouse (10.5-13.5 dpc) and chicken (5.5-7.5 d) by whole mount in situ hybridisation of explanted gonads or partially dissected embryos. Expression of Sox9 in chondrogenic tissues (Wright et al., 1995) provided a positive control for expression in urogenital structures and a basis for comparison of expression levels between embryos.

At 10.5 dpc, before overt sexual differentiation, Sox9 expression in the mouse urogenital ridge was limited to a faint, diffuse band on the lateral side of the genital ridge in both sexes (Fig. 1A). At 11.5 dpc, Sox9 expression differed strikingly between males and females, with strong staining seen in male, but not in female, genital ridges (Fig. 1B,C). Sox9 expression was not present in the mesonephroi of either sex at this stage, but staining was observed in the tissue lying between the mesonephroi (Fig. 1A-C), which surrounds the dorsal aorta. At 12.5-13.5 dpc, Sox9 expression became localised to the sex cords in the testis, which at this stage consist of Sertoli and germ cells (Fig. 1D,E). Sox9 expression was maintained in the sex cords as they differentiated into the seminiferous tubules (Fig. 1F). No Sox9 mRNA was detectable by in situ hybridisation in the female genital ridge after 10.5 dpc (Fig. 1C-F), although expression was detected by the more sensitive technique of reverse transcriptase polymerase chain reaction (RT-PCR; data not shown).

In the chicken, cSOX9 expression was not detected in the genital ridge of either males or females at 5.5 d, but was prominent in the tissue lying between the mesonephroi (Fig. 2A,B). At 6.5 and 7.5 d, cSOX9 expression was observed in the male genital ridge, but remained absent in the female genital ridge (Fig. 2C-F). At these stages, staining in the right testis was often greater than staining in the left testis (Fig. 2C), possibly reflecting the asymmetric development of avian genital ridges. cSOX9 expression was not apparent in the mesonephroi of either sex at the stages examined. The
expression pattern of cSOX9 in the chicken skeletal system was very similar to that observed in mouse (data not shown), confirming that the orthologous gene was being detected. The higher level of cSOX9 expression in the male gonad could stem from a dosage difference between males (ZZ) and females (ZW), if the cSOX9 gene is located on the Z chromosome. To establish whether cSOX9 is Z-linked, a chicken cSOX9 probe was hybridized to male and female chicken genomic DNA. Comparison of the signal intensity between the sexes revealed no significant differences, indicating that cSOX9 is not located on either of the sex chromosomes (Fig. 3).

SOX9 protein expression in the mouse gonad

An anti-SOX9 polyclonal antiserum was produced in order to examine the expression of SOX9 protein during gonad development. A number of steps were taken to ensure the specificity of the antibody. The antiserum was raised against a C-terminal peptide outside the HMG box of SOX9 in order to exclude potential cross-reactivity with other SOX proteins, including SRY. BLAST and FASTA database searches revealed no significant match between the immunogenic peptide and any known murine proteins. Immunoblot analysis was performed on COS-1 cells transfected with Sox9 constructs. Using Sox9 cDNA, a protein band of approximately 65×10^3 M_r was detected in transfected but not in untransfected cells (Fig. 4A), whilst a construct containing the Sox9 open reading frame linked to a portion of the GAL4 gene generated a protein of approximately 80×10^3 M_r. Significantly, only one band was observed by immunoblotting of adult testis despite the known expression of several Sox genes in the testes (Koopman et al., 1990; Denny et al., 1992a; Connor et al., 1995; Kanai et al., 1996), and the translational complexity of this tissue. This band has the same M_r as the SOX9 band seen in COS-1 cells transfected with Sox9 cDNA. The same sized protein was also detected at high levels in mouse limb buds (Fig. 4B), a tissue which has been shown by in situ hybridisation to express high levels of Sox9 mRNA (Wright et al., 1995). These experiments show that the antiserum specifically detects SOX9.

Immunoblot analysis of SOX9 protein in developing mouse urogenital ridges confirmed the differential expression between males and females seen in in situ hybridisation experiments. A band of approximately 65×10^3 M_r representing SOX9 protein was detected in male, but not in female, urogenital ridges at 11.5, 12.5 and 13.5 dpc (Fig. 4B), stages at which high levels of Sox9 mRNA are confined to the male gonads.

Localisation of Sox9 mRNA to the Sertoli cell population

Sex cords in fetal testes at 13.5 dpc are composed of two cell types, the somatic Sertoli cells and the germ cells. It is known that germ cells are not involved in sex determination (McLaren, 1985). In order to determine in which cell type Sox9 is expressed, germ cells were purified from 13.5 dpc male gonads. Total RNA was extracted from these cells and from the gonads from which they had been isolated, and semi-quantitative PCR performed to analyse the expression of Sox9 relative to the ubiquitously expressed gene Hprt. Sox9 mRNA was detected in the male gonad, but not the male germ cells or the ovary (Fig. 5A), indicating that Sox9...
Sex-specific expression of SOX9 is expressed in the somatic cells. Over-amplification of these PCR reactions revealed low levels of Sox9 expression in the germ cells (not shown). Together with the in situ hybridisation results, these experiments indicate that Sox9 is expressed at high levels in the Sertoli cells, and at lower levels in the germ cells.

Postnatal expression of Sox9

In a mature testis, all the stages of spermatogenesis can be found simultaneously. During the first 35 days postnatum (dpn) however, spermatogenesis occurs in a co-ordinated manner throughout the testis, so that a given time point corresponds to a single stage of spermatogenesis. RT-PCR analysis of total RNA isolated from 7, 14, 21, and 28 dpn testes was employed to determine levels of Sox9 mRNA at these stages, as compared with an Hprt control. Similar levels of Sox9 mRNA were detected at the three latter stages, but at 7 dpn lower levels of Sox9 were observed (Fig. 5B). RT-PCR analysis demonstrated that expression of Sox9 is maintained in mature adult testis, but not in the adult ovary, and this was confirmed at the level of protein expression by immunoblot analysis (Figs 4A, 5C). Sox9 mRNA was also detected in testes from adult XXSxr a and XXSxr b sex reversed mice (Fig. 5C) which lack germ cells (McLaren et al., 1984), demonstrating that Sox9 is expressed in the somatic cells of the adult testis.

Sox9 expression in the genitourinary system

Sox9 expression in the murine genitourinary system was not restricted to the gonads. At 12.5 dpc both the Müllerian duct (Fig. 1D) and the mesonephric duct (not shown) expressed Sox9 in the male and the female. By 13.5 dpc, this staining was retained only in the male and extended to the mesenchymal cells surrounding the Müllerian duct (Fig. 1E). In males the Müllerian duct fails to differentiate further and disappears by birth. At 15.5 dpc staining was still detected (not shown) but was not observed at 17.5 dpc, when the duct has completely regressed (Fig. 1F). The cranial end of the mesonephros was weakly positive for Sox9 in both sexes at 12.5 dpc, but staining was restricted to the mesonephric tubules of the male at 17.5 dpc during differentiation into the epididymis (Fig. 1F).

DISCUSSION

SOX9 is in an unusual group of developmental genes whose pivotal roles in the embryo were revealed by studies of human genetic diseases. Mutations in SOX9 result in partial or complete sex reversal in approximately 75% of XY CD patients (Houston et al., 1983; Tommerup et al., 1993; Foster et al., 1994; Wagner et al., 1994). In order to distinguish between several potential modes of action of SOX9 in the molecular genetic pathway of male sexual development, we have examined the expression of SOX9 in developing mouse and chicken gonads. In the mouse, we have established that

---

Fig. 2. Expression of cSOX9 in the embryonic chicken genital system. Ventral view of male (A,C,E) and female (B,D,F) genital ridges (g) and mesonephroi (m) after whole-mount in situ hybridisation with an antisense cSOX9 riboprobe. (A,B) 5.5 d; (C,D) 6.5 d; (E,F) 7.5 d. Genital ridges are indicated by arrows. In A, B and D the tissue expressing cSOX9 between the urogenital ridges is tissue surrounding the dorsal aorta. Scale bar, 0.5 mm.
cSOX9 probe. Intensity of male markers are in kb. difference of the BamHI, partial digestion products were apparent. The size or Z (2:1) chromosomes. When female DNA was digested using with an autosomal ratio (1:1) rather than a location on the W (0:1) ratio: . Size markers are in kb.

Fig. 3. cSOX9 is autosomal in chickens. Genomic DNA was digested with either BamHI, EcoRI or HindIII and probed with a cSOX9 probe. Intensity of male cSOX9 bands were compared against the corresponding female band. The ratio of intensity (male:female) is shown at the base of the tracks and is consistent with an autosomal ratio (1:1) rather than a location on the W (0:1) or Z (2:1) chromosomes. When female DNA was digested using BamHI, partial digestion products were apparent. The size difference of the Sox9 band in this track, compared with its male counterpart, may be due to an insensitive female BamHI site. Size markers are in kb.

Fig. 4. Immunoblot analysis of male and female urogenital ridges and adult gonads. (A) Mock and Sox9 transfected COS-1 cell and adult gonad samples. (B) Male and female urogenital ridge at 11.5, 12.5 and 13.5 dpc, testis, ovary and limb bud protein samples. 10 µg total protein (cell line samples) or soluble protein (all other samples) were loaded. Blots were exposed to X-ray film for 10 minutes (11.5 dpc samples) or 30 minutes (all other samples). Arrowheads indicate SOX9 as a protein of approximately 65×10^3 Mr, and the GALSOX9 fusion as a protein of approximately 80×10^3 Mr. Size markers are Mr×10^-3.

high level expression of Sox9 throughout the male genital ridge commences between 10.5 and 11.5 dpc. Further, we found that Sox9 is expressed in the Sertoli cell lineage, the development of which is thought to be one of the major events in testis determination (Burgoyne et al., 1988). Sox9 expression thus appears to follow that of Sry, the gene which is generally assumed to be responsible for triggering Sertoli cell development in mammals (Goodfellow and Lovell-Badge, 1993). Sry expression in the mouse fetus is first observed at 10.5 dpc, peaks around 11.5 dpc and is absent by 13.5 dpc (Hacker et al., 1995; Jeske et al., 1996). The timing, location and male-specificity of expression of both genes is consistent with SRY protein regulating the expression of Sox9, either directly or indirectly. It will be necessary to establish whether the regulatory sequences involved in directing Sox9 expression to the genital ridge contain consensus binding sequences for SRY, and to investigate the possibility of direct interaction by biochemical approaches.

We also observed expression of cSOX9 in chicken testes during the sex determining period. The conservation of male-specific expression of SOX9 suggests that it is important in testis development not only in mammals but throughout the vertebrate subphylum, irrespective of the type of primary sex determining switch. Non-mammalian vertebrates lack a Y
Sequence analysis of the SOX9 gene reveals that it encodes a protein with the characteristics of a transcription factor. The ability of SOX9 to act as a modular transcription factor, with DNA-binding and transactivation domains, has been demonstrated using a GAL4 assay in mammalian cells (A. McCormack, J. Bowles and P. K., unpublished observations). Further, in common with several SOX proteins, SOX9 binds the SOX consensus motifs AACAAGG and AACAAT (S. W. and P. K. unpublished observations). Together these observations suggest that SOX9 activates genes involved in testis development. The high level of conservation (91.3% similarity) between the mouse and chicken SOX9 protein sequences suggests that SOX9 acts in a similar manner in both of these species.

Two genes are known to be upregulated in the testis during its development and are potential targets for SOX9. These are 

**Ftz-F1**, which encodes the orphan steroid receptor steroidogenic factor 1 (SF1; Luo et al., 1994), and the gene encoding **AMH** (Münsterberg and Lovell-Badge, 1991). AMH is responsible for the regression of the Müllerian duct in males and is the earliest known secreted product of the Sertoli cells (Cate et al., 1986). SF1 is expressed in both the steroidogenic and the supporting cell lineages of both sexes, but a testis-specific up-regulation in transcript levels has been reported (Shen et al., 1994). A 114 bp sequence in the **AMH** promoter has been identified which appears to control its expression in the urogenital ridge in an SRY dependent, but indirect, manner (Haqq et al., 1993, 1994). SF1 has been shown to bind this sequence and repress, not activate, transcription (Haqq et al., 1994; Shen et al., 1994). Deletion of the putative ligand binding domains enables SF1 to activate the **AMH** promoter (Shen et al., 1994) suggesting that in vivo SF1 can repress AMH expression in the absence of a co-factor or ligand. The existence of a co-factor has also been suggested because AMH expression is Sertoli cell specific, while SF1 is not. The expression pattern of SOX9 is consistent with a role in the activation of the AMH gene and possibly in the male-specific up-regulation of SF1. A search of the 5’ regulatory regions of these genes revealed that they contain sites similar to the SOX consensus binding sites. The preferred DNA binding specificity of SOX9 is yet to be determined, but a goal of future work will be to define this sequence and to investigate SOX9 binding to these sites in the AMH and Ftz-F1 promoters.

An alternative possibility is that SOX9 negatively regulates genes involved in ovarian development. **Dosage-sensitive sex reversal (DSS)** is a locus on the human X chromosome thought to be involved in ovary development. If the DSS region is duplicated in an XY individual, sex reversal occurs (Bardoni et al., 1994). A candidate gene, **DAX1**, has been cloned from this region (Muscatelli et al., 1994; Zanaria et al., 1994). Expression analysis in the mouse has demonstrated that Dax1 is present in both sexes during the early stages of gonadal differentiation, but that it is down-regulated only in the testis as overt sexual differentiation occurs (Swain et al., 1996). The observations that SOX9 is expressed during testis determination and, similarly to DSS, that levels of the gene product are critical for normal development may reflect the normal action of SOX9 as a repressor of DSS and ovary development. **Sox9** expression is not restricted to the sex determination

---

**Fig. 5.** RT-PCR analysis of Sox9 in the mouse gonad. (A) 13.5 dpc gonad, (B) postnatal testis at 7, 14, 21 and 28 dpn, (C) normal adult testes, and ovaries and testes from sex-reversed XXSxr a and XXSxr b gonad, (B) postnatal testis at 7, 14, 21 and 28 dpn, (C) normal adult testes, and ovaries and testes from sex-reversed XXSxr a and XXSxr b gonad. (A-C) Semi-quantitative PCR analysis was used to estimate the relative level of Sox9 in each sample as compared with an Hprt control. 36 (A,C) or 26 (B) cycles of PCR amplification were used. H2O sample was a negative control with no cDNA added. Amplification of a c-kit product in A confirmed the identity of the germ cell sample.

chromosome (Ohno, 1961) and do not appear to have an SRY gene (Griffiths, 1991; Coriat et al., 1993). Although cSOX9 is the earliest testis-specific gene so far detected in birds, it is unlikely that it acts as a substitute for SRY. The male phenotype represents the ‘default’ pathway in birds, excluding a dominant role for SRY in the absence of SOX9. Further, we found that cSOX9 is expressed after the first signs of sexual dimorphism are observed, inconsistent with a role as a switch in the sex-determining pathway. Finally, Southern analysis indicated that cSOX9 is not located on either of the sex chromosomes. In view of the similarity of the expression pattern of SOX9 between the two species, it will be interesting to identify genes involved in regulation of SOX9 in birds to elucidate how testis development occurs in the absence of SRY.

In mammals it is also possible for testicular differentiation to occur by an SRY-independent mechanism. Sertoli cell differentiation has been observed in the absence of all Y-derived sequences in some individuals including human XX males, XX true hermaphrodites (Abbas et al., 1990) and XX mice carrying an anti-Müllerian hormone (AMH) transgene (Behringer et al., 1990). It is not yet known whether SOX9 expression occurs in these cases or whether up-regulation of SOX9 transcription by a factor other than SRY is the cause of Sertoli cell development.
critical for the correct branching morphogenesis of the ureteric bud and the formation of a fully functional metanephric kidney. The expression of SOX9 is consistent with it having a major role in testis and genitourinary development in both mouse and chicken. The dimorphic expression pattern of cSOX9 in chickens also represents the first report of a testis-specific transcription factor in birds. The identification of factors with which SOX9 interacts in these two species will help to unravel both the conserved and divergent components of the vertebrate sex determining cascade.

We thank Edwina Wright, Jo Bowles, Murray Hargrave, Mike O’Neill, Michele Binder, Yvette Jeske and Shane McIntosh for help with this work. This work was funded by grants from the Australian Research Council, the National Health and Medical Research Council of Australia and the Wellcome Trust, UK (grant reference no. 043765).

REFERENCES


Nuclear receptor steriodogenic factor 1 regulates the Müllerian inhibiting substance gene: a link to the sex determination cascade. Cell 77, 651-661.


(Accepted 2 June 1996)

Note added in proof
Since submitting this data, Sudbeck and colleagues (P. Sudbeck, M. L. Schmitz, P. A. Baeuerle and G. Scherer) demonstrated transactivation by SOX9 of a reporter gene via the sequence AACAAAG.