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A male-specific role for *SOX9* in vertebrate sex determination

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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 *cSOX9* 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;

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, *SOX9*, in mammalian sex determination. *SOX9* is a member of a family of genes related to *SRY* by the HMG box (Denny et al., 1992b; Wright et al., 1993). *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). *Sox9* expression has been shown to occur at sites of chondrogenesis in mouse embryos (Wright et al., 1995) and defects in *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 *SOX9* not only in skeletal development but also in sex determination.

Whilst the sex reversal seen in CD patients clearly establishes a role for *SOX9* in mammalian sex determination, it is not yet known how *SOX9* contributes to the molecular genetic pathway of testis development. Several models can be envisaged. First, SOX9 could act as a transcription factor required for expression of *SRY*. In this model, expression of *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 *Sry* is the only Y-linked component of the male sex-determining pathway. Second, SOX9 protein might interact with SRY to form a complex that initiates testis development. In this model, expression of *Sox9* would be expected in both sexes in the period covering the onset of *Sry* expression and the start of overt testis differentiation at 12.5 dpc. A third possibility is that *SOX9* might be directly or indirectly responsive to SRY. *Sox9* expression in this model would be restricted to the developing male gonads, beginning shortly after the onset of *Sry* expression. Fourth, *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 *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 *Sox9* may respond directly to activation by SRY. The observation of a similar expression pattern in a phylogenetically distant species, the chicken, confirms a fundamental function for *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 toludine blue staining of amniotic cells and scoring the presence or absence of Barr bodies (Palmer and Burgoyne, 1991).

Chicken (*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 W-specific *Xho*I repeat from a genomic DNA template. Amplification of cytoplasmic *β-actin* mRNA was performed as a control. Multiplex PCR reactions were performed with 100 ng each of W-specific primers *Xho*1 (AACTACCACTTTTCTCACGG) and *Xho*2 (TTCAGAGTGATAACGCATGG) (Kodama et al., 1987) and 100 ng each of Actin 1 (TGGATGATGATATTGCTGC) and Actin 2 (ATCTTCTCCATATCATCCCA) (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 *Xho*I repeat product was present, and ZZ (male) if only the *β-actin* product was present.

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

Whole-mount in situ hybridisation

Expression of mouse *Sox9* was detected using the probe Sox9.5a (Wright et al., 1995). A 381 bp chicken *SOX9* (*cSOX9*) fragment was generated by PCR amplification of cDNA from urogenital systems of 5.5-7.5 d male and female embryos. Primers c*SOX9*.1 (CCCCAACGCCATCTTCAA) and *cSOX9*.2 (CTGCTGATGCCGTAGGTA) were designed using the *cSOX9* cDNA sequence (accession no. U12533). The *cSOX9* 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 MZ8 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 *Bam*HI, *Eco*RI or *Hin*dIII. This was hybridised overnight at 65°C with an [α-^{32}P]dCTP-labelled *Pst*I fragment generated from the plasmid containing the 381 bp *cSOX9* PCR product. The filter was washed to a stringency of 0.1× SSC, 0.2% SDS at 65°C and exposed to film overnight. The intensity of the *cSOX9* 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 *Sox9* cDNA (Wright et al., 1995) or pGAL0 (Kato et al., 1990) containing the *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 (CPQTHSPQDWEQPVYTQVTR) was synthesised and conjugated to diptheria toxin (QIMR Peptide Unit) and used to immunise a Dutch rabbit. Test bleeds confirmed the presence of antibodies reacting with a protein of approximately 65×10^3 M_r 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 age and sex. 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 95ºC 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 horseradish 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 (GAACAGACTCACATCTCT) 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% CO_2 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 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*

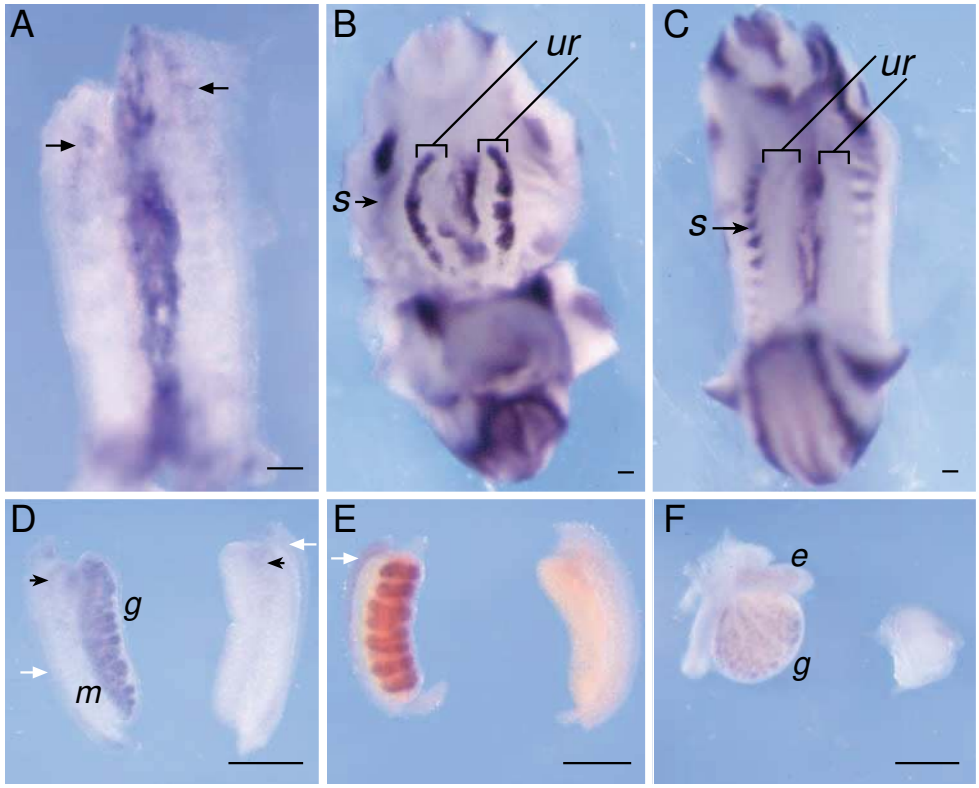

Fig. 1. Expression of *Sox9* in the murine fetal gonads. Ventral views after in situ hybridisation with an antisense *Sox9* riboprobe. (A) A pair of 10.5 dpc male genital ridges showing very faint staining for *Sox9* (arrow) as compared to the tissue between the ridges which is strongly expressing *Sox9*. Partially dissected 11.5 dpc male (B) and female (C) embryos. Staining is present in the developing skeletal system (s indicates the developing ribs) of both sexes and in the male but not the female genital ridges. The anterior ends of the genital ridges overlying the mesonephroi (urogenital ridges, ur) are indicated with square brackets. (D-F) Comparison of male and female gonads (g) and attached mesonephros (m) at 12.5 (D), 13.5 (E) and 17.5 (F) dpc (male left; female right). Staining is present in the male gonad, specifically in the sex cords and subsequently in the seminiferous tubules. Additional sites of expression are (D) the Müllerian duct (white arrow) and the cranial end of the mesonephros (black arrow) in both sexes, (E) the cells surrounding the male Müllerian duct (arrow) and (F) the epididymis (e). Scale bar, 0.1 mm (A-C); 0.5 mm (D-F).

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).

In the metanephros (the definitive kidney) of both sexes at 12.5 dpc, *Sox9* expression was detected in the branching ureteric bud, but not in the surrounding metanephric blastema (Fig. 6A). The cells surrounding the presumptive ureter were also stained at this time. In the chicken at 6.5 and 7.5 d, the cells of the branching ureteric bud were also found to express *cSOX9* (Fig. 6B).

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

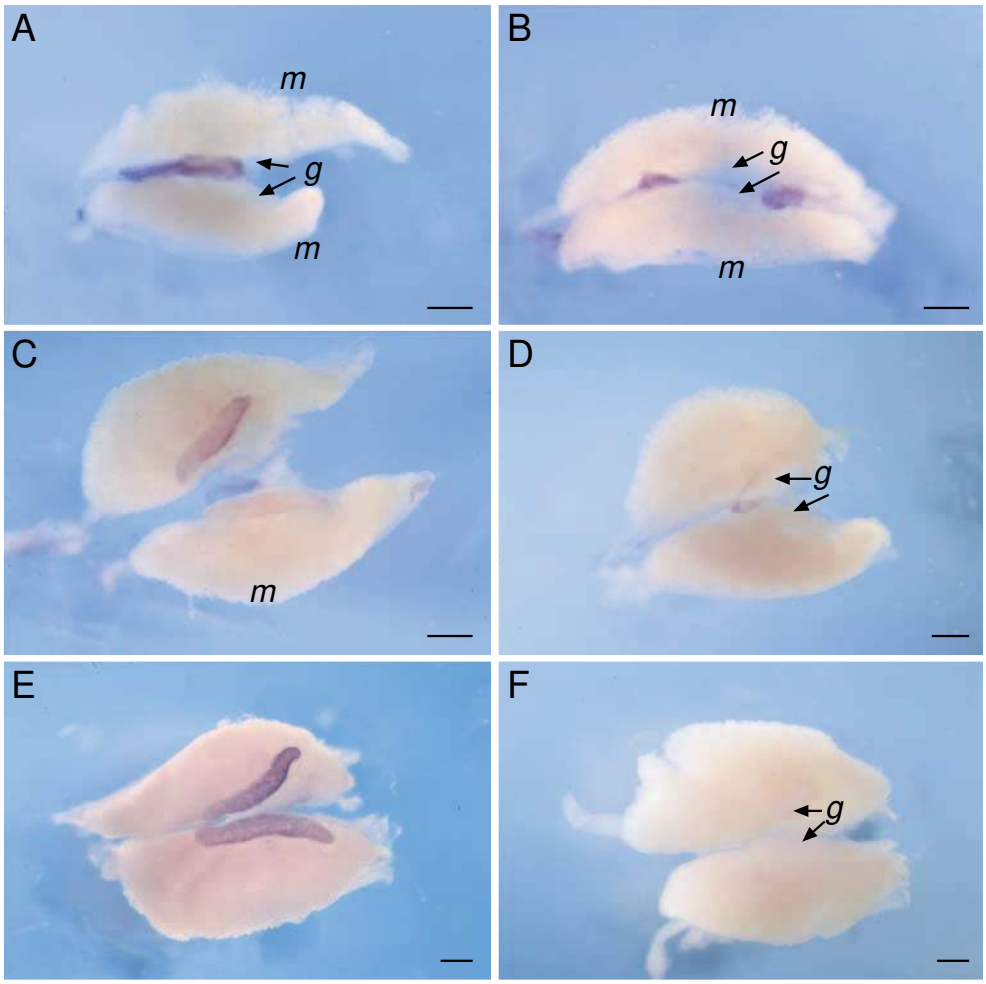

Fig. 2. Expression of *cSOX9* in the embryonic chicken genital system. Ventral view of male (A,C,E) and female (B,D,F) genital ridges (g) and mesonephroi (m) after whole-mount in situ hybridisation with an antisense *cSOX9* riboprobe. (A,B) 5.5 d; (C,D) 6.5 d; (E,F) 7.5 d. Genital ridges are indicated 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.

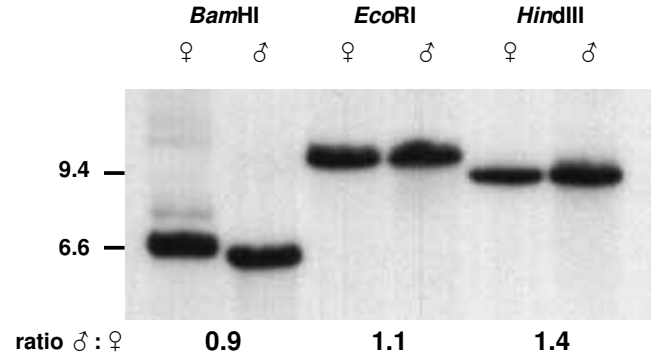

Fig. 3. *cSOX9* is autosomal in chickens. Genomic DNA was digested with either *Bam*HI, *Eco*RI or *Hin*dIII and probed with a *cSOX9* probe. Intensity of male *cSOX9* bands were compared against the corresponding female band. The ratio of intensity (male:female) is shown at the base of the tracks and 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 *Bam*HI, 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 *Bam*HI site. Size markers are in kb.

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

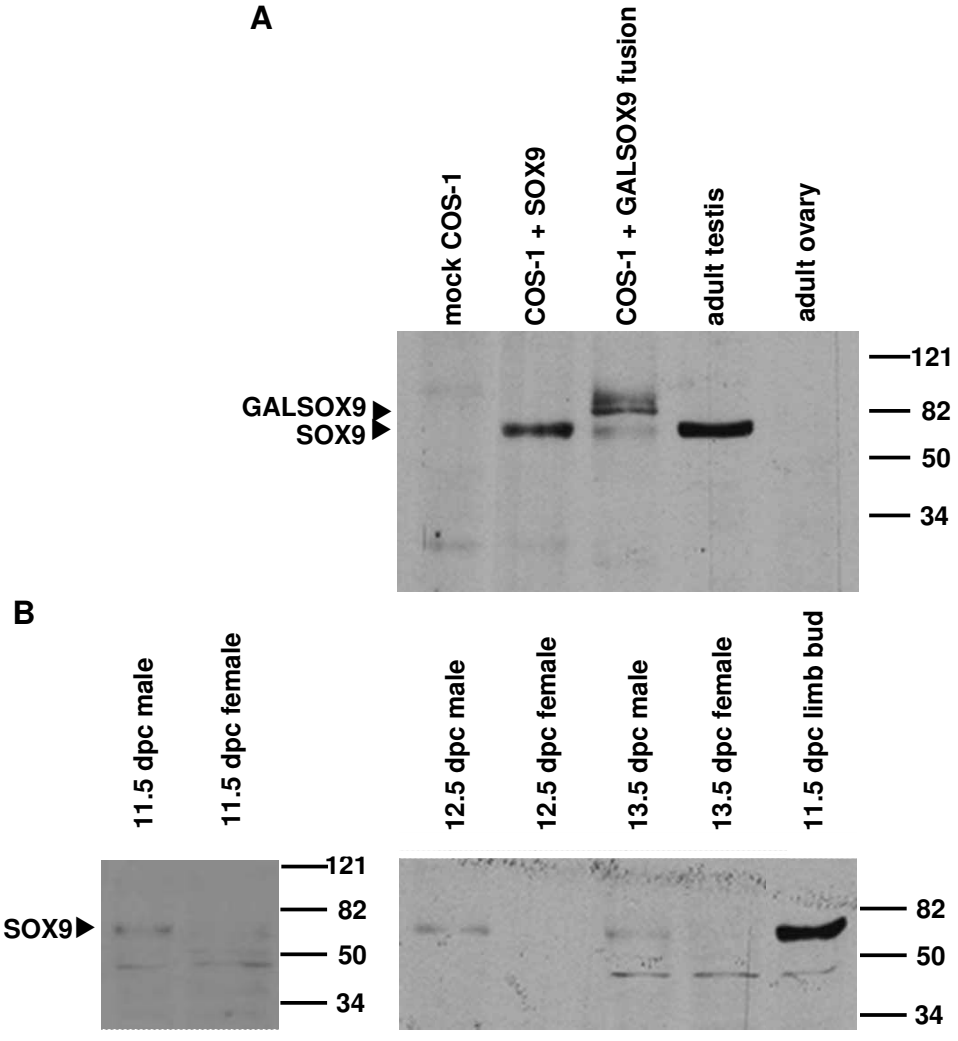

Fig. 4. Immunoblot analysis of male and female urogenital ridges and adult gonads. (A) Mock and *Sox9* transfected COS-1 cell and adult gonad samples. (B) Male and female urogenital ridge at 11.5, 12.5 and 13.5 dpc, testis, ovary and limb bud protein samples. 10 μg total protein (cell line samples) or 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 M_r and the GALSOX9 fusion as a protein of approximately 80×10^3 M_r. Size markers are $M_r \times10^{-3}$.

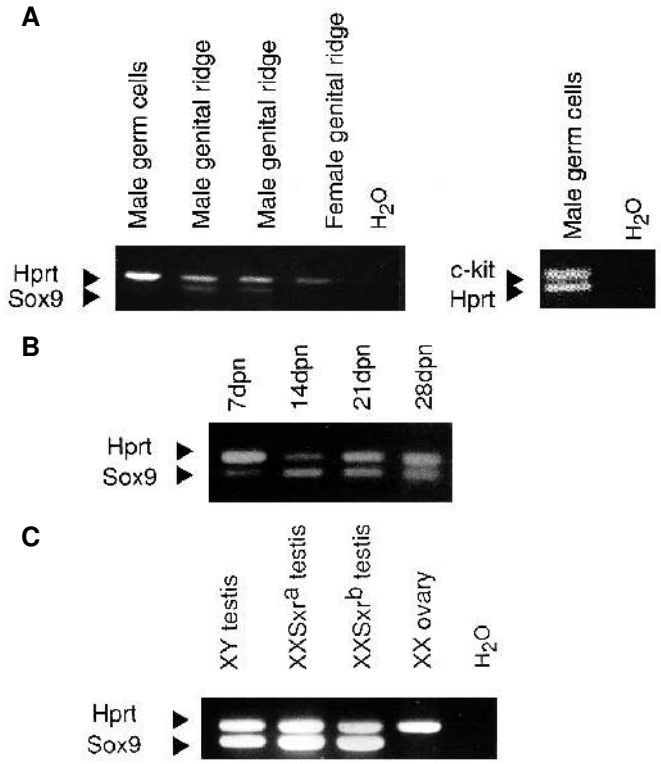

Fig. 5. RT-PCR analysis of *Sox9* in the mouse gonad. (A) 13.5 dpc gonad, (B) postnatal testis at 7, 14, 21 and 28 dpn, (C) normal adult testis, and ovaries and testes from sex-reversed XXSxr^a and XXSxr^b mice. (A-C) Semi-quantitative PCR analysis was used to estimate the relative level of *Sox9* in each sample as compared with an *Hprt* control. 36 (A,C) or 26 (B) cycles of PCR amplification were used. H_2O 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 is acts as a substitute for *SRY*. The male phenotype represents the 'default' pathway in birds, excluding a dominant role for *cSOX9* in male sex determination. 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.

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 AACAAAG 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

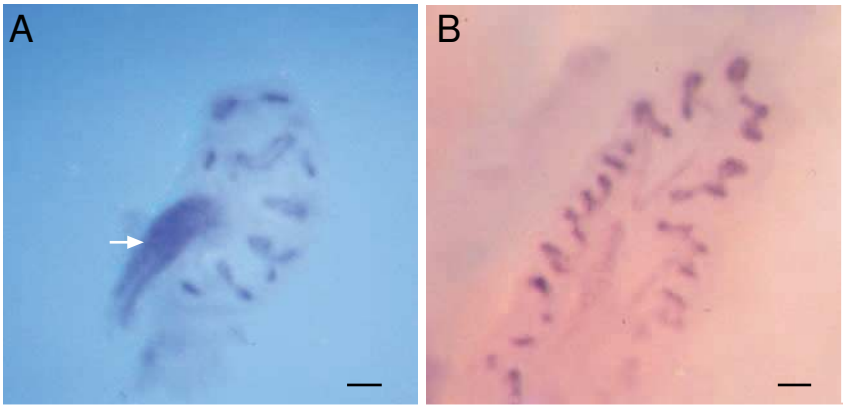

Fig. 6. Expression of *SOX9* in the metanephric kidney. (A) 12.5 dpc mouse kidney, (B) 7.5 d chicken kidney. Staining for *SOX9* is observed in the branching ureteric bud. The arrow indicates the ureter. Scale bar, 0.1 mm.

period. Prior to male-specific expression in the genital ridge, low levels of *Sox9* were detected in both male and female indifferent murine gonads. This low-level expression is not localised to the precursors of the Sertoli cells or their female equivalent, the granulosa cells, as these are scattered throughout the genital ridge (Magre and Jost, 1980). No gonadal abnormalities have been described in XX CD patients who have one mutated *SOX9* allele. This observation implies that one functional copy of *SOX9* is sufficient for normal gonadogenesis in females. *Sox9* expression was found to continue in the somatic portion of the testis throughout fetal, postnatal and adult life. These observations agree with human fetal and adult data and are consistent with a continuing role in the testis (Wagner et al., 1994). In the mouse, a transient down-regulation was consistently observed at 7 dpn, coinciding with the stage of spermatogenesis at which Sertoli cells proliferate (Bellvé, 1979). This observation suggests that *Sox9* is likely to be required for Sertoli cell differentiation, rather than proliferation.

Sox9 was also detected in tissues that are not part of the gonad, namely the cells surrounding the Müllerian duct and the mesonephros. *Sox9* is expressed by the cells surrounding the Müllerian duct from 13.5 dpc until the duct has regressed, a domain in which the AMH receptor is also expressed (Baarends et al., 1994). Expression in the mesonephros coincides with the redifferentiation of the mesonephric tubules to form the efferent ducts of the epididymis (Kent, 1987). These two morphogenetic events are controlled by the hormones AMH and testosterone, respectively (Byskov, 1986; Behringer et al., 1994). Therefore it is possible that *SOX9* may be involved in sex hormone-mediated differentiation. However, it is also possible that *SOX9* has a distinct role in the differentiation of the epididymis and Müllerian duct. No expression in these structures was detected in the chicken between 5.5 and 7.5 d. Investigation of *cSOX9* expression later in development will determine if expression is conserved during sex-specific differentiation of these tissues in chicken.

SOX9 expression in the genitourinary system of the mouse and chicken was also observed in the branches of the ureteric bud which differentiate to form the ureter and collecting duct system of the metanephric kidney. Interestingly, kidney abnormalities have been observed in one third of CD patients (Houston et al., 1983), suggesting that the level of SOX9 is 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.

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Note added in proof

Since submitting this data, Sudbeck and colleagues (P. Sudbeck, M. L. Schmitz, P. A. Baeuerle and G. Scherer (1996). Sex reversal by loss of the C-terminal transactivation domain of human SOX9. *Nature Genet.* **13**, 230-232) demonstrated transactivation by SOX9 of a reporter gene via the sequence AACAAAG.