Expression of *Sry*-related genes in the developing genital ridge/mesonephros of the chick embryo

D. McBride, H. Sang and M. Clinton*

Division of Development and Reproduction, Roslin Institute (Edinburgh), Roslin, Midlothian
EH25 9PS, UK

A reverse transcription-polymerase chain reaction approach was used in an attempt to identify a chick homologue to *Sry*. Gene transcripts from the genital ridge were analysed at the stage of development at which the expression of such a gene might be expected. A number of *Sry*-related genes expressed in the developing genital ridge of the chick were identified.

Introduction

During embryonic development, the actions of regulatory genes commit the undifferentiated cells of the genital ridge along the testicular or ovarian pathway. The gene responsible for testis determination in mammals has been identified (Gubbay *et al.*, 1990; Sinclair *et al.*, 1990; Koopman *et al.*, 1991). In mice, this gene is designated *Sry* for Sex determining region, Y-chromosome. The *Sry* protein contains a DNA-binding motif known as the HMG-box, found in a wide variety of proteins that bind to DNA with different degrees of sequence specificity (Nasrin *et al.*, 1991; Harley *et al.*, 1992; Rimini *et al.*, 1995). This region is highly conserved in all mammalian *Sry* genes sequenced to date, while sequences outside the HMG-box are very poorly conserved between mammalian species (Laudet *et al.*, 1993; Payen and Cotinot, 1993; Tucker and Lundrigan, 1993; Whitfield *et al.*, 1993; Tucker and Lundrigan, 1995). Homologues of the *Sry* gene are present in all mammalian species so far examined but none have yet been identified in a non-mammalian species.

In birds, the female is heterogametic with regard to sex chromosomes, with one Z chromosome and one W chromosome. It would therefore seem unlikely that an identical sex-determining system could operate in birds and mammals. However, it is possible that an avian homologue to *Sry* could act as a testis-determining gene if its expression was triggered by a different mechanism from that seen in mammals. The aim of this study was to identify an avian homologue to *Sry*. Other workers have attempted such studies using approaches based on the polymerase chain reaction (PCR). These studies involve PCR amplification using genomic DNA as template, and, while unsuccessful at identifying an avian homologue of *Sry*, a number of avian sequences of *Sry*-related (*Sox*) genes have been reported (Griffiths, 1991; Coriat *et al.*, 1993). In contrast, a reverse transcription-PCR approach was used to examine gene transcripts in the chick genital ridge at the time of gonadal differentiation, that is, at a time and location that an avian

Fig. 1. Schematic representation of mouse *Sry* (a) and human SRY (b) demonstrating relative positions of PCR primers, R5, O5 and R3 (—). ■ HMG-box region of protein.

homologue of *Sry* might be expected to be expressed (Koopman *et al.*, 1990). Combinations of degenerate PCR primers based on the sequences of a number of mammalian *Sry* genes were used.

The aim of this study was to identify a number of sequences of *Sry*-related genes, some of which correspond to avian sequences reported in earlier studies. One of these sequences corresponded to the avian homologue of SOX-9, a gene recently implicated in sex reversal and chondrogenesis in mammals (Foster *et al.*, 1994; Wagner *et al.*, 1994; Wright *et al.*, 1995). This sequence has been used to isolate a genomic clone for chicken SOX-9.

Materials and Methods

Materials

Degenerate PCR primers were designed based on the published sequences of mammalian *Sry* genes. Primers were R5-GTG/CAAGCGACCCATGAATTCA/TTTT/CATC/ GGTG/ATGGT, R3-ATCTTCGAATTCCGAC/TGAGGTT/ CGA/GTAT/CTTG/ATA and O5-AGA/GTGGCT/CCT/AA/ GG/CAGAATTCCAG/AA/CATG. Primers correspond to nucleotides at positions 7–40(R5), 208–239(R3) and 59–84(O5) of the HMG Box of the mouse *Sry* gene (Fig. 1). For ease of subcloning, primers were designed to include an EcoRI restriction enzyme site.

*Correspondence.

Received 7 June 1996.

1. CCCAGGCGGC TCGAAGGAAG CTGGCTGACC AGTACCCGCA TCTGCACAA
GCCGAGCTCA GCAAGACGCT GGGCAAGCTG TGGAGGCTGC TGAATGAGAG
CGAGAAGCGT CCCTTCGTGG AGGAGGCCGA GCGGCTGCGG GTGCAGCACA
AGAAGGACCA CCCCAC
2. TCTAAGATCG AGAGGAGAAA AATCATGGAG CAGTCGCCGG ACATGCACAA
CGCGGAGATC TCCAAGCGCC TGGGCAAGCG GTGGAAGCTG CTGAAGGACA
GCGAGAAGAT CCCCTCCATC CGGGAGGCGG AGAGGCTGCG GCTCAAGCAC
ATGGCCGATT ACCCCGAC
3. TCCCAGGGGCC AGCGCGGCAA GATGGCACAG GAGAACCCCA AAATGCACAA
CTCGGAAATC AGCAAGCGGC TGGGCGCGGA GTGGAAGCTG CTCTCCGAGG
CGGAGAAGCG GCCCTACATC GACGAGGCCA AGCGGCTGCG GGCGCAACAC
ATGAAGGAAC ACCCCGAC
4. TCCCAGGGGCC AGCGGCGGAA GATGGCCCAG GAGAATCCCA AGATGCACAA
CTCGGAGATC AGCAAGCGCC TCGGGGCCGA TTGGAAGCTG CTGAGCGATG
CCGAGAAGCG GCCCTTCATC GACGAGGCCA AGAGGCTGCG GGCCGTGCAC
ATGAAGGAGT ACCCCGAT
5. TCGCAGCACG AGCGCCGCAA GATCATGGAC CAGTGGCCCG ACATGCACAA
CGCCGAGATC TCCAAGCGCC TGGGTCCGCG CTGGCAGCTC CTGCACGACT
CCGAGAAGAT CCCCTTTGTG AAGGAGGCCG AGCGCCTGCG CGTCAAGCAC
ATGGCCGACT ACCCCGAC
6. TCGCAGATCG AACGGCGGAG GATCATGGAG CAGTCGCCCG ACATGCACAA
CGCCGAGATC TCCAAGCGGC TGGGCAAGCG CTGGAAGCTG CTCAAGGACA
GCGACAAGAT CCCCTTCATC CGGGAGGCGG AGCGGCTGCG CCCTCAAGCA
CATGGCGGAC TACCCCGAC

Fig. 2. Nucleotide sequences of representative clones from the six groups of PCR-derived clones (PCR-primer sequences are not shown).

cDNA synthesis

ISA-Brown eggs from commercial hens were incubated, on the day of lay at 38°C ± 0.5°C. After 5, 6 and 7 days of incubation embryos (50 per day) were isolated and placed in sterile Petri dishes for dissection of genital ridges/mesonephros. RNA extracted from dissected tissues, from both male and female embryos, was combined and cDNA was synthesized using a commercially available first strand cDNA synthesis kit (Pharmacia Biotech, St Albans).

PCR

PCR reactions were performed on cDNA templates using primer combinations R5 with R3, and O5 with R3.

PCR reactions were performed in 50 µl of 10 mmol Tris-HCl l⁻¹, 1.5 mmol MgCl₂ l⁻¹, 50 mmol KCl l⁻¹, 0.1 mg ml⁻¹ gelatine, pH 8.3, containing 200 µmol dNTPs l⁻¹, 1 µmol primers l⁻¹ and 1 unit of Taq polymerase (Boehringer

Table 1. Number of individual clones in each group of *Sry*-related sequences

Sequence group	Number of individual clones
1	21
2	19
3	11
4	1
5	3
6	4

Mannheim UK, Lewes). After an initial denaturation step of 94°C for 2 min, DNA was denatured at 94°C for 1 min, annealed at 60°C for 2 min and extended at 72°C for 2 min. Reactions were subjected to 30 cycles of amplification and

Fig. 3. Predicted amino acid sequences of chicken *Sox* transcripts compared with protein sequences of mammalian homologues. Blanks indicate non-conservative substitutions; +, conservative substitutions; and ; identical amino acids.

Chick sequence	Nucleotide (%)	Amino acid (%)
cSox-9	90	98
cSox-11	90	98
cSox-14	86	98
cSox-3	86	96
cSox-12	88	92
cSox-4	93	100

Transformant clones were picked and cultured overnight in LB medium containing ampicillin.

Plasmid DNA was prepared using commercially available reagents (Qiagen Ltd, Dorking) and sequenced by the dideoxy chain termination method using a Sequenase kit (Amersham International plc, Slough). Sequences were compared against international nucleotide and protein databases using the Blast package of analysis programmes (Altschul *et al.*, 1990).

A chick genomic library in lambda EMBL-3 was plated on *E. coli* strain NM621 and screened using the protocol of Maniatis *et al.* (1982). Purified insert from PCR clone pBS-cSOX9 was labelled using the Ready-To-Go kit (Pharmacia Biotech) for use as probe. DNA from positive EMBL-3 clones was used as template in PCR reactions using primers R5 and R3. The resulting PCR product was purified on a gel, subcloned into Bluescript KS M13 and sequenced.

Reverse transcription-PCR was used to examine *Sry*-related transcripts in the genital ridge/mesonephros of developing chick embryos. The two primer combinations were used and PCR products of the expected sizes, 233 bp and 181 bp, were obtained. PCR products were digested with *Eco*R1, gel purified and subcloned into Bluescript KS M13. A total of 60 clones was analysed and the sequences fell into six groups of identical clones (Table 1). Nucleotide sequences of representative clones from each group are shown (Fig. 2). The sequence of each group of clones was compared with that of mammalian *Sry* and *Sox* genes. Although overall, *Sry* genes are poorly conserved between species, the HMG-box regions of individual *Sox* genes, including *Sry*, show a high degree of conservation (Griffiths, 1991; Coriat *et al.*, 1993; Laudet *et al.*, 1993; Payen and Cotinot, 1993; Tucker and Lundrigan, 1993; Whitfield *et al.*, 1993). On this basis, the sequences obtained in

```

↓ CCCAGGCGGC TCGAAGGAAG CTGGCTGACC AGTACCCGCA TCTGCACAAC
51  GCCGAGCTCA GCAAGACGCT GGGCAAGCTG TGGAGGTGAG CGGGAGGTGG
101 GAGCGATGCC TGGGGTGGGC GGGGGGACGC AGCCTGCAGA GTTTGCTCCG
151 GCTGTGACGC GCGAGGAGGA GAGAGGAAAG GGAGGGAGAG GATGGTTGGA
201 AGCCTGGAAA AGTTGCCGCA AAGTTTTGCA TCGCTGGCA AAGTTGTTCCG
251 GCGGAATAGG AGGGGGAGAG CAGGGCAAGG AGAGCCTGGG CTGCCCTCTG
301 CTCCTGCAA AGGTTTTATG CTGCTTGAGC CGTGCCCTGT GTGTGCTGCA
351 GCCGGGGGAG CGCTTGGTGA TTGTGCAGGG GAAGCGATGG GCCTAGATTG
401 CATCAGCTCT GTGCAGAGCG AGGGGAGGGG GCAAAAGCTG CCAACCCCCG
451 GGTGGGTGCC CAGGGGCGGC TGGGTGCCCT CCGCCTTCCA AAATAAGGCA
501 GGGAGGGGTG AAAATCAGGA GGCAAGTGAG TCACCTGGCT GCCAATGAGT
551 GTGACTGCAG AAAGGAGGGG CGGAGGGGAA TCCTAAATCC ACAGCGGGAG
601 GAGAAAGTGG TGAGCAACTC TGGTCTGGC AGCCGGCGCG AGAGCCGTCA
651 CCCACAGTGC CCGGGGATGG GGGAGCTGAG GAGCACTGTG CTCTCACACC
701 TCTCGTTTGG TCATTGAAAC GCGGGGTGTC AACCAGCATG TGCCCCAAAG
751 TGAGGGTTCA GTGCTGGAAG GGGGGAGGTC GCATCCCTGT TTGTGGGGAC
801 TGGGAGAGGT TTGGGGCTGA TGTCAATGGC AGAGCGGAGG GAAACACTTG
851 CGCCTCTCCC CCGTGAGCTG CAGCTGACCC CTCACCGTGT TTTCCCCCCC
901 CTTCCCCATC CTCCTCAGGC TGCTGAATGA GAGCGAGAAG CGTCCCTTCG
951 TGGAGGAGGC CGAGCGGCTG CGGGTGCAGC ACAAGAAGGA CCACCCCGAC
1001 TACAA

```

Fig. 4. Sequence of genomic cSox9 clone, pBS 71101. Splice donor and acceptor sequences are underlined (PCR-primer sequences are not shown).

this study were compared with nucleotide databases and assigned as homologues of the most closely matching mammalian sequences. None of the amplified sequences corresponded to a chick homologue of *Sry*, but all sequences represented members of the *Sox* gene family (Fig. 3).

The predicted amino acid sequences of the six groups of clones were compared with the amino acid sequences of mammalian *Sox* genes. This enabled identification of the probable mammalian homologues: Group 1 sequences differ by one amino acid from mouse *Sox 9*; Group 2 sequences differ by one amino acid from human SOX11; Group 3 sequences differ by one amino acid from mouse *Sox14*; Group 4 sequences differ by two amino acids from human SOX3; Group 5 sequences

show four amino acid differences from mouse *Sox12*; and Group 6 sequences are identical to human SOX4.

To avoid confusion, the nomenclature of Wright *et al.* (1995) was adopted and these clones were designated cSox9, cSox11, cSox14, cSox3, cSox12 and cSox4. A summary of comparisons of chick sequences with mammalian homologues is shown (Table 2).

One of these sequences (Group 1) represents the chick homologue of *Sox-9*, a gene recently implicated in sex reversal in mammals. This clone was used to screen a chick genomic library. Positive clones were used as a template in PCR reactions with the primers R5 and R3. The resulting PCR product, designated pBS 71101, contained 1066 bp rather than

the expected 233 bp. The 1066 bp fragment was subcloned and sequenced. Analysis of this sequence was found to comprise the HMG box of cSox9 with 833 of intronic sequence flanked by consensus splice sites. The nucleotide sequence of this genomic clone is shown (Fig. 4).

Discussion

Reverse transcription-PCR was used to examine the *Sry*-related transcripts present in the genital ridge/mesonephros of chick embryos between day 5 and day 7 of development. In terms of gonadal development, days 5–7 of embryogenesis in chicks correspond to days 10–12 in mice, and represent the period during which *Sry* is expressed. It could reasonably be assumed that should a *Sry* homologue function in the same manner in the chick, the gene would be expressed at this time in the developing gonad. The failure to detect expression of an *Sry* homologue in the differentiating genital ridge coupled with previous unsuccessful attempts to identify chick *Sry* suggest that the mechanism of sex determination in chicks does not depend on this developmental switch.

Expression of a number of *Sry*-related *Sox* genes was detected, although practical difficulties in isolating genital ridge tissues free from contaminating mesonephric tissue made it impossible to be certain that this expression occurred in the genital ridge. A mutation in one of these *Sox* genes has been implicated in sex-reversal in mammals (Foster *et al.*, 1994; Wagner *et al.*, 1994). A chick genomic library was screened using the PCR clone cSox9 as a probe to study this gene in chickens. DNA extracted from positive clones was used as a template in PCR reactions containing the HMG box primers. The PCR product was larger than predicted, suggesting the presence of intronic sequences, which was subsequently confirmed by DNA sequence analysis. The position of the intron within the DNA binding motif of cSox9 corresponds exactly to that recently published for mouse *Sox9* (Wright *et al.*, 1995).

In conclusion, these results suggest that the sex-determination mechanism in chicks is not dependent on a homologue of the mammalian testis determining gene. However, expression of a *Sry*-related gene also thought to be involved in mammalian gonadal development was demonstrated in the genital ridge/mesonephros at a time co-incident with chick gonadal differentiation.

The authors thank P. Koopman for assistance in assigning clones as homologues to mammalian sequences. This work was supported by a commission from the Ministry of Agriculture, Fisheries and Foods.

References

- Altschul SF, Gish W, Miller W, Meyers EW and Lipman DJ (1990) Basic local alignment search tool *Journal of Molecular Biology* **215** 403–410
- Coriat A-M, Muller U, Harry JL, Uwanogho D and Sharpe PT (1993) PCR amplification of SRY-related gene sequences reveals evolutionary conservation of the SRY-box motif *PCR Methods and Applications* **2** 218–222
- Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, Weissenbach J, Mansour S, Young ID, Goodfellow PN, Brook JD and Schafer AJ (1994) Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene *Nature* **372** 525–530
- Griffiths R (1991) The isolation of conserved DNA sequences related to the human sex-determining region Y gene from the lesser black-backed gull (*Larus fuscus*) *Proceedings of the Royal Society of London* **224** 123–128
- Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Munsterberg A, Vivian N, Goodfellow P and Lovell-Badge R (1990) A gene mapping to the sex determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes *Nature* **346** 245–250
- Harley VR, Jackson DJ, Hextall PJ, Hawkins JR, Berkovitz GD, Sockanathan S, Lovell-Badge R and Goodfellow PN (1992) DNA binding activity of recombinant SRY from normal males and XY females *Science* **255** 453–456
- Koopman P, Munsterberg A, Capel B, Vivian N and Lovell-Badge R (1990) Expression of a candidate sex-determining gene during mouse testis differentiation *Nature* **348** 450–452
- Koopman P, Gubbay J, Vivian N, Goodfellow PN and Lovell-Badge R (1991) Male development of chromosomally female mice transgenic for *Sry*. *Nature* **351** 117–121
- Laudet V, Stehelin D and Clevers H (1993) Ancestry and diversity of the HMG box superfamily *Nucleic Acids Research* **21** 2493–2501
- Maniatis T, Fritsch EF and Sambrook J (eds) (1982) *Molecular Cloning: a Laboratory Manual* Cold Spring Harbour Laboratory, New York
- Nasrin N, Buggs C, Kong XF, Carnazza J, Goebl M and Alexander-Bridges M (1991) DNA-binding properties of the product of the testis-determining gene and a related protein *Nature* **354** 317–320
- Payen EJ and Cotinot CY (1993) Comparative HMG-box sequences of the *Sry* gene between sheep, cattle and goats *Nucleic Acids Research* **21** 2772
- Rimini R, Pontiggia A, Spada F, Ferrari S, Harley VR, Goodfellow PN and Bianchi ME (1995) Interaction of normal and mutant SRY proteins with DNA *Philosophical Transactions of the Royal Society of London* **350** 215–220
- Sinclair AH, Berta P, Palmer MS, Hawkins JR, Griffiths BL, Smith MJ, Foster JW, Frischauf AM, Lovell-Badge R and Goodfellow PN (1990) A gene from the human sex-determining region encodes a protein with homology to a conserved DNA-binding motif *Nature* **346** 240–244
- Tucker PK and Lundrigan BL (1993) Rapid evolution of the sex determining locus in Old World mice and rats *Nature* **364** 715–717
- Tucker PK and Lundrigan BL (1995) The nature of gene evolution on the mammalian Y chromosome: lessons from *Sry*. *Philosophical Transactions of the Royal Society of London* **350** 221–227
- Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, Pasantes J, Bricarelli FD, Keutel J, Hustert E, Wolf U, Tommerup M, Scempp W and Scherer G (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9 *Cell* **79** 1111–1120
- Whitfield LS, Lovell-Badge R and Goodfellow PN (1993) Rapid sequence evolution of the mammalian sex-determining gene SRY *Nature* **364** 713–715
- Wright E, Hargrave MR, Christiansen J, Cooper L, Kun J, Evans T, Gangadharan U, Greenfield A and Koopman P (1995) The *Sry*-related gene *Sox9* is expressed during chondrogenesis in mouse embryos *Nature Genetics* **9** 15–20