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Research Article

Regulation of *Amh* during sex determination in chickens: *Sox* gene expression in male and female gonads

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Abstract. During mammalian sexual development, the SOX9 transcription factor up-regulates expression of the gene encoding anti-Müllerian hormone (AMH), but in chickens, *Sox9* gene expression reportedly occurs after the onset of Amh expression. Here, we examined expression of the related gene *Sox8* in chicken embryonic gonads during the sex-determining period. We found that *cSox8* is expressed at similar levels in both sexes at embryonic day 6 and 7, and only at the anterior tip of the gonad, suggesting that SOX8 is not responsible for the sex-specific increase in *cAmh* gene expression at these stages. We also found that several other chicken *Sox* genes (*cSox3*, *cSox4* and *cSox11*) are expressed in embryonic gonads, but at similar levels in both sexes. Our data suggest that the molecular mechanisms involved in the regulation of *Amh* genes of mouse and chicken are not conserved, despite similar patterns of *Amh* expression in both species.

Key words. Sex determination; Amh; chicken; testis.

Even though molecular mechanisms of patterning and morphogenesis are surprisingly well conserved during metazoan evolution, mechanisms governing sex determination and gonadal development are diverse, even among vertebrates. In mammals, the heterogametic pairing of sex chromosomes (XY) results in male development. The mouse and human sex-determining gene, *Sry*/*SRY* (sex-determining region on Y chromosome), has been identified [1, 2] and is known to cause the bipotential gonad to differentiate into a testis [3]. However, *Sry* is not a conserved sex-determining gene as it exists only in mammals [4–7]. In contrast to mammals, in birds males are homogametic (ZZ) and females are heterogametic for the sex chromosomes (ZW). Whether avian sex is determined by a master female-determining gene on the W chromosome or by Z chromosome gene dosage is still unclear [8].

There are reports for the mouse that several genes are expressed predominantly in the developing testis but not in the ovary and, therefore, are likely to be important for male sex determination and differentiation. Some of these genes, such as *Amh* (anti-Müllerian hormone) and *Sox9* [9–12] are expressed similarly in mouse and chicken gonads, suggesting that there could be some degree of similarity between the molecular pathways and sexual development in chicken and mouse. However, some differences are evident. For example, *Sf1* (steroidogenic factor 1) and *Gata4* are predominantly expressed in the male gonad in mice [13, 14], while in chicken expression levels of both genes are similar between male and female gonads [12, 15].

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Sox genes represent a family related by the *Sry*-type high-mobility group (HMG) box, and function as transcription factors in various developmental processes through binding to a conserved core DNA sequence [16]. Twenty *Sox* genes have been identified in mouse and human, and are classified by their HMG box sequences into subgroups A–H [17, 18]. The expression pattern of each gene tends to be conserved in mouse and chicken. Among them, *Sox9* (group E) is known to act as a sex-determining gene. Mutations of human *SOX9* cause campomelic dysplasia, a severe skeletal malformation syndrome associated in most cases with XY sex reversal [19, 20], and ectopic expression of *Sox9* in XX mouse fetal gonads induces testis formation [21].

AMH plays an important role in inducing the regression of the Müllerian ducts in males, which normally give rise to the uterus, oviducts, upper vagina and fallopian tubes in females [22]. Analysis of mouse and human *Amh* gene regulation has uncovered several factors important for modulating *Amh* expression. For example, SF1 up-regulates *Amh* expression by cooperative interaction with WT1 [23], GATA-4 [24], SOX9 [25] and SOX8 [26]. Mice mutant for the SOX-binding site or the SF1-binding site in the *Amh* promoter revealed that SOX proteins are essential for *Amh* expression, while SF1 enhances the final expression level [27]. Oréal et al. [28] were the first to describe the chicken *Amh* promoter and showed that it has little overall homology with the mouse *Amh* promoter, but contains two putative SOX-binding sites and one SF1-binding site, suggesting that the mouse and chicken *Amh* promoters are similarly regulated. However, chicken *Sox9* is expressed too late to be a *cAmh* regulator [28, 29], but another SOX protein might substitute for SOX9 and, together with SF1, regulate *cAmh* expression.

Previously, we hypothesized that SOX8 might be a candidate transcription factor for regulating the chicken *Amh* gene [26, 30]. Mouse *Sox8* is expressed male specifically during gonad development. Its expression starts prior to the onset of *Amh* gene expression, and encodes a protein product that can up-regulate mouse *Amh* together with SF1 in vitro [26]. Group E Sox genes, consisting of Sox8, Sox9 and Sox10, show moderate levels of amino acid similarity and have similar genomic organization, suggesting that group E *Sox* genes may originate from one ancestral gene [31]. Although expression patterns of *Sox9* and *Sox10* overlap to a limited extent [32, 33], expression of *Sox8* overlaps substantially with expression of *Sox9* [31, 32] and to a lesser extent, *Sox10* [33, 31]. This fact suggests that there is some functional redundancy between SOX8 and SOX9, similar to that published for SOX1, SOX2 and SOX3 in lens formation [34], L-SOX5 and SOX6 in cartilage formation [35] and SOX7, SOX17 and SOX18 in vasculogenesis [36–38].

In this study, we analyzed the expression patterns of chicken *Sox8* in developing gonads during the sex-determining window. If *cSox8* contributes to *cAmh* gene expression, one would expect to find *cSox8* predominantly expressed in the embryonic testis and prior to the onset of *cAmh*. We found this not to be the case, suggesting that SOX8 is not responsible for sex-specific expression of *cAmh* in chicken. We also tested the expression patterns of several other *cSox* genes which are expressed in embryonic testis, and similarly found that they were not expressed male specifically.

Materials and methods

Chicken embryos. Fertilized chicken eggs were obtained from local suppliers (Ingham, Brisbane, Australia and Saitama Experimental Animal Supply, Saitama, Japan) and maintained at 18 °C until use. Eggs were transferred to an incubator at 37.5 °C and allowed to develop for 5, 6 or 7 days. Staging was confirmed at dissection according to Hamburger and Hamilton [39]. The entire urogenital ridge of each embryo was explanted for whole-mount *in situ* hybridization. Sexing was performed by PCR as described elsewhere [40] using genomic DNA purified from a hind limb of each embryo.

Amplification of HMG box sequences. Total RNA was isolated from left and right gonads of day 6 male embryos by standard methods [41]. RNA (0.5 μg) was then treated with DNaseI and first-strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen) according to the manufacturer's protocol. For amplification of the HMG box, the PCR reaction was carried out in a solution containing 1 x NH_4 buffer (Bioline), 100 M $MgCl_2$, 100 μM dNTPs, 0.4 μM primers and 0.5 unit Biotaq DNA polymerase (Bioline) with 4.5 min denaturation at 95 °C followed by 40 cycles of amplification at 95 °C for 30 s and 57 °C for 30 s. The PCR product was cloned into pGEM-T Easy (Promega). Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and M13 reverse primer, and electrophoresis was carried out by the Australian Genome Research Facility, Brisbane, Australia. Primer sequences used were as follows; G7A1: 5'-AGC G(A/G)C CCA TGA ACG C(A/C/G/T)T T-3' and G7B1: 5'-CGC (C/T)GG TA(C/T) TT(A/G) TA(A/G) TC(A/C) GGG T-3'. The PCR reaction was also carried out using genomic DNA as a template.

RT-PCR of *Sox* genes, *Amh* and *Actin*. The left and right gonads of staged, sexed embryos were pooled (ten and five embryos of each sex for day 6 and day 7, respectively) to isolate total RNA using the RNeasy Mini kit (Qiagen) with the optional on-column DNase digestion with the RNase-free DNase set. The first-strand cDNA was synthesized from 1 μg of total RNA using Power-

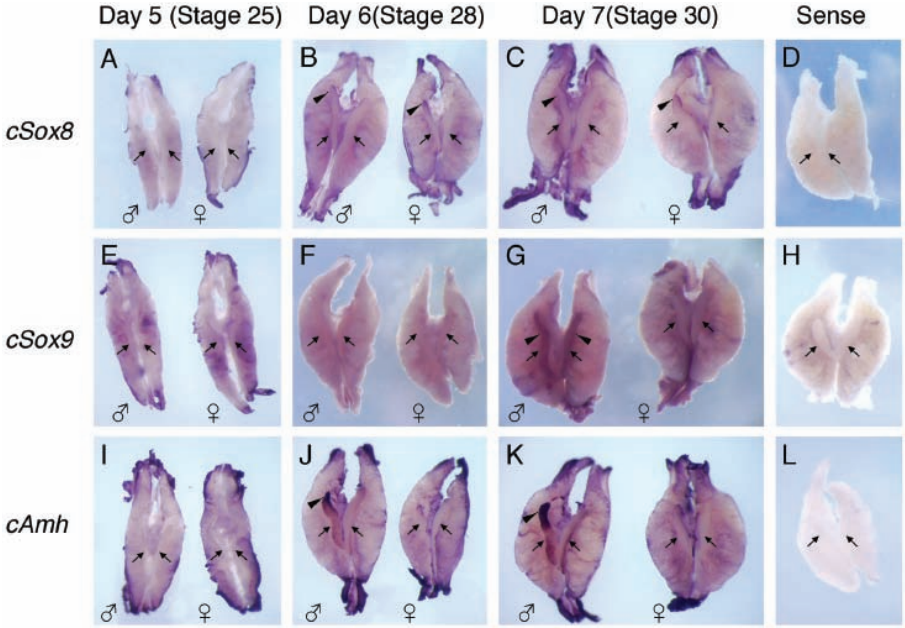

Figure 1. Whole-mount *in situ* hybridization of *cSox8*, *cSox9* and *cAmh* in the chicken embryonic gonad/mesonephros. In each panel, male (♂) and female (♀) gonad/mesonephros are left and right, respectively. Probes used are shown at the left of each panel. The samples in A–C, E–G and I–K were hybridized with antisense probe. In D and L a day 6 sample hybridized with sense probe. In H a day 7 sample was hybridized with sense probe. Developmental stages (days and Hamburger-Hamilton stage) of gonad/mesonephros are labeled above. Arrow shows the position of gonad. Arrowheads point to regions of positive staining.

Script reverse transcriptase (Clontech) and oligodT. PCR amplification was carried out using the QuantiTect SYBR Green PCR kit (Qiagen) with uracil-N-glycosylase and the 7900HT Sequence Detection System (Applied Biosystems). Samples were incubated at 50 °C for 2 min, then 95 °C for 15 min, followed by 40 cycles of amplification at 94 °C for 30 s, 54.2 °C for 1 min and 72 °C for 1 min. For the amplification of *cSox8* and *cSox9*, 85.4 °C for 15sec was added after each 72 °C step of each amplification cycle. Primer sequences used were as follows; cSox3-1: 5'-GCACCAGCACTACCAGAG-3' and cSox3-2: 5'-CGA ATG CGG ACA CGA ACC) for *cSox3* [29], cSox4F: 5'-TCG GGG GAT TGG CTG GAG TC-3' and cSox4R: 5'- CTC AGC CGA TCC TCG TTT CC-3' for *cSox4*, cSox8RTF : 5'-CTA CAA GGC TGA CAG CGG GC-3' and cSox8RTR: 5′-AGG CCG GGC TCT TGT GAG TC-3' for *cSox8*, cSox9F: 5'-CCC CAA CGC CAT CTT CAA-3' and cSox9R: 5'-CTG CTG ATG CCG TAG GTA-3' for *cSox9*, cSox11F2: 5'-AAG CAG GAG GCG GAC GAC GA-3' and cSox11R2: 5'-CGC CCC GCA CCT CCT CGT AG-3' for *cSox11*, cAmh-1: 5'-GTG GAT GTG GCT CCC TAC CC-3' and cAmh-2: 5'-GCA GCA CCG AGG GCT CCT CC-3' for *cAmh* [29] and Actin-1: 5'-TGG ATG ATG ATA TTG CTG C-3' and Actin-2: 5'-ATC TTC TCC ATA TCA TCC C-3' [29] for *Actin*. To calculate the relative amount of gene expression levels, the $\Delta\Delta C_T$ method was used. Three independent pooled samples were analyzed. Maximum average values were set as 100%.

For the RT-PCR amplification of *cSox12* and *cSox14*, the same cDNA for the real-time RT-PCR was used. The PCR reaction was carried out in the same solution as HMG box amplification with 4.5 min denaturation at 95 °C followed by 40 cycles of amplification at 95 °C for 30 s and 50 °C for 30 s. Primer sequences used were as follows; cSox12F: 5'-AGA TCT CCA AGC GCC TGG GTC G-3' and cSox12R: 5'-GGT AGT CGG CCA TGT GCT TG-3' for *cSox12*, cSox14F: 5'-GAG GTT CCT CAC ACC TTG GC-3' and cSox14R: 5'-ACA CGG AGG AAT CCC AGT CC-3' for *cSox14*.

Probes. The previously reported *cAmh* probe [9] was obtained by RT-PCR amplification of an 821-bp fragment, using primers cAMHRTF (5'-ACG GTG CGC GCC CAC TGG CAG G-3') and cAMHRTR (5'-ACG TCG TGA CCT GCA AGC CCT C-3') and RNA prepared from 5.5-day-old whole embryo. The *cSox8* probe was cloned by PCR using primers chSox8C2F (5'-CTG CAG AGC TCC AAC TAC TAC A-3') and chSox8C2R (5'-GAG CTC TGT CCT TTT GGA GAG T-3') and chicken genomic DNA as the template. The fragment corresponds to nucleotides 1228–1589 of the *cSox8* mRNA sequence (accession No. AF228664). PCR products of *cAmh* and *cSox8* were cloned into the pGEM-T Easy vector. The *cSox9* probe was reported previously by Kent et al. [11]. The *cSox11* fragment was cloned by SacI digestion of *cSox11* cDNA and subsequent insertion into pBluescriptII KS vector. The fragment corresponds to nucleotides 667–967 of the *cSox11* mRNA sequence (accession No. AB012237). The *cSox4* probe was obtained by KspI digestion of *cSox4* cDNA and subsequent insertion into pBluescriptII KS vector. The fragment corresponds to nucleotides 576–965 of the chicken *Sox4* mRNA sequence (accession No. AY493693).

***In situ* hybridization.** Sense and antisense digoxygenin-labeled RNA probes were generated by *in vitro* transcrip-

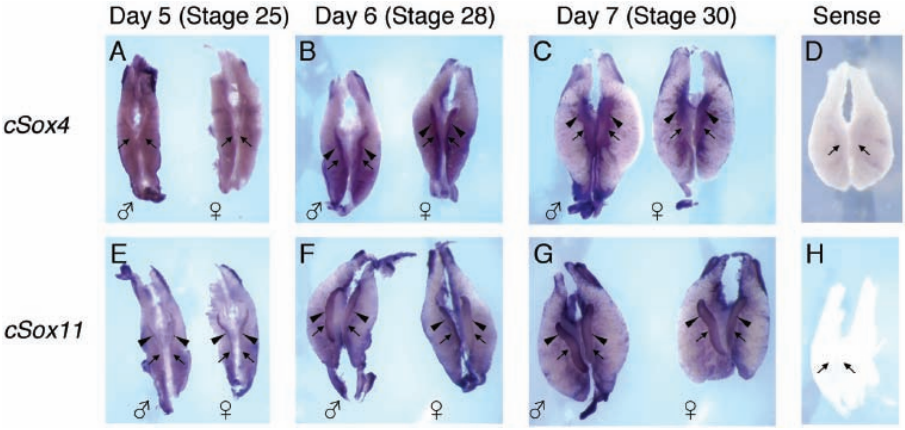

Figure 2. Whole-mount *in situ* hybridization of *cSox4* and *cSox11* in the chicken embryonic gonad/mesonephros. In each panel, male (♂) and female (♀) gonad/mesonephros are left and right, respectively. Probes used are shown at the left of each panel. A–C and E–G were hybridized with antisense probe. D and H show day 6 samples hybridized with sense probe. Developmental stages (days and Hamburger-Hamilton stage) of gonad/mesonephros are labeled above. Arrow shows the position of gonad. Arrowheads point to regions of staining.

tion. Whole mount *in situ* hybridization was performed as described using the maleic acid buffer (MABT) method [42]. Experiments were carried out at least twice for each probe, with similar results.

Results and discussion

To compare the temporospatial expression of *cSox8* and *cAmh* in embryonic gonads, we employed whole-mount *in situ* hybridization analysis using female and male gonad/mesonephros complexes isolated from day 5, 6 and 7 chicken embryos (Hamburger and Hamilton stages 25, 28 and 30, respectively [39]). These stages cover the temporal window at which sexual dimorphism in the gonad first becomes apparent [43]. In addition to providing spatial information relating to gene expression, whole-mount *in situ* hybridization is commonly used as a semiquantitative guide to gene expression levels between different tissues hybridized with the same probe and incubated under the same conditions, as in the experiments described below.

As expected, *cAmh* was expressed at higher levels in male than in female gonads at day 6 and 7 (fig. 1J, K). Expression levels in the right male gonads were higher than in the left, possibly reflecting the asymmetric development of avian genital ridges [43]. We did not observe expression of *cAmh* at day 5 (fig. 1I), even though Oréal et al. [28] reported that *cAmh* is expressed weakly and at similar levels in male and female gonads at day 5 by section *in situ* hybridization. This may reflect lower sensitivity of our whole mount *in situ* hybridization method.

Previous workers have reported that male-specific high-level expression of *cSox9* is preceded by expression of *cAmh* in the chick [28, 29]. We analyzed the temporal expression of *cSox9* in chicken embryos. No signal was observed in male or female gonads at day 5 or day 6 (fig. 1E, F). High levels of *cSox9* expression were observed in day 7 male gonads, while no signal was observed in the day 7 female (fig. 1G). This compares to the results of Oréal et al. [28] who described very faint expression of *cSox9* in day 6 gonads by *in situ* hybridization using sections. Our data demonstrate high levels of *cAmh* expression in day 6 male gonad, preceding the high levels of *cSox9* expression first detected in day 7 male gonad. They suggest that SOX9 is not responsible for the male-specific up-regulation of *cAmh*, but may play a role in the maintenance and/or amplification of *cAmh* expression in the male gonads once transcription is initiated. Our results support the previous observation that the male-specific high levels of *cAmh* expression precede testicular *cSox9* expression [28, 29].

We next examined expression of *Sox8*. At day 5, no *cSox8* expression was observed (fig. 1A). At day 6 and 7, *cSox8* expression was observed at the anterior tip of both male and female right gonads at similar levels (fig. 1B, C). This expression profile is different from that of *cAmh*, both in spatial distribution of transcripts and degree of sex specificity, suggesting that SOX8 could not be responsible for sex-specific up-regulation of *cAmh* in chicken.

The expression patterns of mouse and chicken *Sox8* imply that the functions of SOX8 are conserved in most but not all tissues between the two species. For example, *Sox8* is expressed in brain, skeletal muscle, eye, somite, dermomyotome, limb, digits, gut, spinal cord and dorsal root ganglia in both species [30, 31, 44]. However, some differences are evident in embryonic heart and gonad: in chicken, *cSox8* is expressed in the embryonic heart, testis and ovary, whereas in mouse, *Sox8* expression occurs predominantly in the embryonic testis, but not in the ovary or heart [31, 44]. Given these observations, SOX8 may contribute to the male-specific activation of *Amh* expression during gonadgenesis in mice but not chicken.

In mouse, only two SOX proteins, SOX8 and SOX9, have been identified as regulators of *Amh* expression in the embryonic gonad [26]. Based on our data, and previous studies [28, 29], neither SOX8 nor SOX9 can be responsible for the onset of high levels of *cAmh* expression

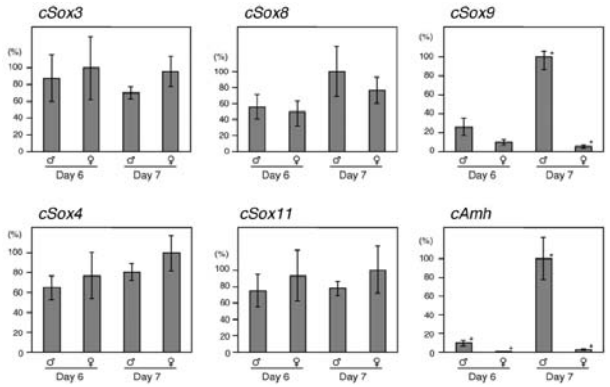

Figure 3. Quantitative, real-time RT-PCR analysis of *Sox* and *Amh* gene expression. Averages of three independent trials are shown as bars, with SEs shown as lines. Values marked with * were significantly different between males (♂) and females (♀) ($p = 0.0017$, 0.0047 and 0.012 for cSox9 at day 7, cAmh at day 6 and 7, respectively, using a two-sample equal variance t-test). Others were not significantly different between males and females ($p = 0.81$, 0.24, 0.74, 0.52, 0.17, 0.67, 0.38, 0.64, 0.49 for *cSox3* at day 6 and 7, *cSox8* at day 6 and 7, *cSox9* at day 6, *cSox4* at day 6 and 7, and *cSox11* at day 6 and 7, respectively).

in chicken, even though two SOX-binding sites are predicted in the *cAmh* promoter region. These observations prompted us to search for other *Sox* genes expressed in chicken male gonads that could be considered as candidate regulators of *Amh* expression. We conducted the analysis at day 6, since at this stage *cAmh* is expressed at high levels in male gonads while *cSox9* is either not expressed or expressed at a very low level.

We utilized degenerate RT-PCR on purified day 6 male gonad RNA using generic *Sry*-type HMG box primers to generate fragments for cloning into a plasmid vector. Twenty independent clones were sequenced, revealing that 12 clones were *cSox4* [45], 7 were *cSox11* [46] and 1 was *cSox9* [47].

One possible explanation for these results is that the degenerate primers used show a bias for amplification of *cSox4* and *cSox11* templates. To examine this possibility, we used the same degenerate primers to amplify *Sox* fragments from genomic DNA, in which all intronless *Sox* genes (Sox1, -2, -3, -4, -11, -12, -14, -21) capable of amplification by the primers are represented in equal proportions. Among ten clones amplified, none was *cSox4* or *cSox11*. Thus, primer bias does not explain our data relating to *cSox4* and *cSox11* expression in developing chicken gonads.

cSox4 and *cSox11* are expressed in male gonads at day 6, prompting us to examine the expression profiles of each in male and female gonads through the sex determination window. If both or either is expressed preferentially in male gonads, they could be considered a candidate for regulation of the *cAmh* gene. To evaluate the expression patterns of *cSox4* and *cSox11* in embryonic gonads, we employed whole-mount *in situ* hybridization analysis at the same stages previously used to profile *cSox8*, *cSox9* and *cAmh* expression. *cSox4* and *cSox11* signals were detected at similar levels in male and female gonads at all stages examined (fig. 2) suggesting that neither of them plays a role in sex-specific regulation of *Amh*.

The identification of *Sox* genes that are expressed in chicken embryonic gonads at day 5, 6 and 7 was previously attempted by McBride et al. [6]. Using RT-PCR amplification of the conserved *Sry*-type HMG box domain from RNA samples prepared from testes with mesonephroi attached, they found expression of *cSox3*, *cSox4*, *cSox9*, *cSox11*, *cSox12* and *cSox14*. Our data confirm that *cSox4, cSox9* and *cSox11* are indeed expressed, as is cSox3 (see below); however; we amplified the HMG box from day 6 gonad only, and this difference along with the differences in PCR primers, may explain the discrepancies in the data for *cSox12* and *cSox14*. Moreover, day 6 male gonad expresses *cSox4* and *cSox11* transcripts so abundantly that RT-PCR cloning is difficult for *Sox* genes expressed at low levels.

To examine the levels of gene expression quantitatively, we utilized RT-PCR and real-time RT-PCR analyses using RNAs isolated from pooled, sexed embryonic gonads at days 6 and 7 (fig. 3). As expected, *cAmh* and *cSox9* were expressed at different levels between males and females at day 7. At day 6, the expression levels of *cAmh* were statistically different between males and females ($p < 0.005$) while the expression levels of *cSox9* were not ($p > 0.1$). However, *cSox3*, *cSox4*, *cSox8* and *cSox11* were expressed at similar levels between males and females at days 6 and 7, suggesting that none of these *Sox* genes is responsible for the male-specific up-regulation of *cAmh* expression.

We were unable to amplify *cSox12* and *cSox14* sequences by RT-PCR from chicken embryonic gonads. As a positive control, chicken genomic DNA was included as template. Signals were observed at expected size of 108 bp for *cSox12* and 331 bp for *cSox14* only from genome template, but not from gonad RNA samples, showing that neither gene is expressed in embryonic gonads at day 6 and day 7 (data not shown).

Previous studies have eliminated *cSox3* as a candidate for male-specific up-regulation of *Amh* expression because *cSox3* is expressed at similar levels in the male and female gonads at the sex-determining window [28, 29]. Our present data support this conclusion. We rule out *cSox8* because it is expressed in a different spatial pattern to *Amh*, and *cSox12* and *-14* because they are not expressed in gonads at sex-determining stages at all. We exclude *cSox4* and *cSox11* also, on the basis of equivalent expression levels between male and female. It is formally possible that *cSox4* and *cSox11* might be expressed in Sertoli cells in the male (the site of *Amh* expression) and in another cell lineage in females, in which *Amh* is

not expressed, but we consider this unlikely, especially considering that all genes found to be involved in sex-specific development of the gonads to date show a sexually dimorphic pattern of gene expression in fetal gonads when examined by whole-mount *in situ* hybridization. However, one still cannot exclude the possibility of SOX protein-mediated regulation of *cAmh* gene expression, and further extensive cloning of *cSox* genes may be necessary to discover a *Sox* gene expressed predominantly in chicken embryonic testis.

Alternatively, we have to consider that sex-specific *Amh* up-regulation is not mediated by SOX proteins in birds. Even though the putative SF1-binding site, like the two SOX-binding site in the *Amh* promoter, is conserved between mouse and chicken [28], and *cSf1* is co-expressed with *cAmh* at day 7 of chicken embryonic testis [15], the expression profiles of mouse and chicken *Sf1* show major differences. Before testis cord formation, *Sf1* is expressed at similar levels in males and females in both species**,** while subsequently, chicken *Sf1* expression is maintained in the testis, but is up-regulated in the ovary [12, 15, 29]. The opposite expression pattern is reported for mouse *Sf1* [reviewed in ref. 48]. This difference could be explained by the possibility that *SF1* functions in more steroidogenically active tissue (testis in mammals and ovary in birds), or that *Sf1* may not be associated with testis formation in birds [12]. Either way, the expression profile of *cSf1*, like that of *cSox9* [28] and *cSox8* (this study), suggests that it is not responsible for male-specific up-regulation of *cAmh* expression during chicken gonad genesis. Since both SF1 and SOX proteins are required for normal levels of *Amh* expression during sex determination in mouse [27], this may imply that there is a different mechanism of *cAmh* regulation in chicken compared with mouse, and that SOX protein is not a causative factor for sex-specific expression of *cAmh*.

Gonadal expression of *Sox8*, which has an evolutionarily conserved coding protein among vertebrates, has been studied in mouse, chicken and red-eared slider turtle [31, 49]. *Sox8* is expressed in the developing testes of all three species, implying a functional significance, but in chicken and turtle, *Sox8* is also expressed in the ovary. So far, up-regulation of mouse *Amh* is the only known molecular function for the SOX8 protein [26]. If this function is conserved among vertebrates, chicken SOX8 may have a protein partner which is expressed in males to activate or in females to suppress *cAmh*. Some genes are expressed sex specifically during gonadal differentiation in the mouse, including *Sf1* [13], *Wt1* [50], *Gata4* [14], *Lhx9* [51], *Wnt-4* [52] and *Dax1* [53]. However, chicken homologues of these genes are not candidates because they are expressed in both developing testis and ovary while *cAmh* is differentially expressed [15, 29]. *Dmrt1* is expressed only in developing testis in mouse, while chicken *Dmrt1* is expressed in both developing gonads with higher levels in testis, suggesting that it is not such a factor [15, 54]. The identification of a chicken SOX8-binding partner may clarify this possibility. Finally, further analysis of the *cAmh* promoter may reveal whether SOX proteins play a role in its up-regulation, and whether similarities exist in the mechanisms that regulate *Amh* expression in birds and mammals.

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