Expression of *Sox9*, *Mis*, and *Dmrt1* in the Gonad of a Species With Temperature-Dependent Sex Determination

Christina Shoemaker, Mary Ramsey, Joanna Queen, and David Crews*

Sex determination in vertebrates, the process of forming an ovary or testis from a bipotential gonad, can be initiated by genetic or environmental factors. Elements of the downstream molecular pathways underlying these different sex-determining mechanisms have been evolutionarily conserved. We find the first evidence that *Sox9* expression is preferentially organized in the testis early in the temperature-sensitive period in a species with temperature-dependent sex determination (*Trachemys scripta*). This pattern occurs before sexually dimorphic *Mis* expression and in a temporal hierarchy that is similar to mammals. Furthermore, we extend previous findings that *Dmrt1* expression at early stages of sex determination has a dimorphic pattern consistent with a possible upstream role in determining the fate of the bipotential gonad. *Developmental Dynamics 236:1055–1063, 2007.* © 2007 Wiley-Liss, Inc.

Key words: temperature-dependent sex determination; reptile; *Sox9*; *Mis*; *Dmrt1*; testis differentiation

Accepted 10 January 2007

INTRODUCTION

In vertebrates, the bipotential gonad first forms as a thickening of ventromedial mesonephric tissue. These genital ridges have the potential to be directed toward either ovarian or testicular fates and go through a second phase of development wherein sex is determined. In the third phase of gonadal development, sexual fate is committed and differentiation into a testis or an ovary occurs. In organisms exhibiting genotypic sex determination (GSD), including mammals, birds, and some reptiles, genetic factors determine the sexual fate of the initially bipotential gonad. In other vertebrates, environmental factors direct sexual development, such as in temperature-dependent sex determination (TSD) found in all crocodilians and many turtles. While the initial upstream factor determining gonadal sex differs radically between TSD and GSD, many of the same genes are involved in the downstream process of gonad differentiation.

In organisms with TSD, genes involved in early phases of sex-determining of the bipotential gonad are expected to be expressed in a dimorphic manner before or early in the temperature-sensitive period (TSP), whereas genes more integral to downstream testis or ovary differentiation should be expressed dimorphically after the TSP. To clarify their location in the temperature-dependent sex-determining molecular network, we examined the expression of *Sox9*, *Mis*, and

ABBREVIATIONS MPT male-producing temperature **FPT** female-producing temperature **TSP** temperature-sensitive period **TSD** temperature-dependent sex determination **GSD** genetic sex determination **Mis** Müllerian-inhibiting substance **Dmrt1** doublesex mab3-related transcription factor 1 **Sox9** SRY-like HMG-box 9 **Mis-R2** Mis receptor type II **PP1** protein phosphatase type I **RT-PCR** reverse-transcriptase polymerase chain reaction **qPCR** quantitative real-time PCR **ISH** in situ hybridization

Department of Integrative Biology, University of Texas at Austin, Austin, Texas
Grant sponsor: National Science Foundation; Grant number: IBN 200001269.
***Correspondence to: David Crews, Department of Integrative Biology, University of Texas at Austin, Austin, TX 78712. E-mail: crews@mail.utexas.edu**

DOI 10.1002/dvdy.21096
Published online 26 February 2007 in Wiley InterScience (www.interscience.wiley.com).

© 2007 Wiley-Liss, Inc.

Dmrt1 in the red-eared slider turtle, *Trachemys scripta*. In this species, the gonad is sensitive to the effect of temperature during the middle third of embryonic development, and after this window closes, the gonad becomes committed to an ovarian or testicular fate (Bull et al., 1990; Wibbels et al., 1991). The timing of this window in *T. scripta* lasts from approximately stage 14 (Greenbaum staging series) through stage 18 at a female-producing temperature (FPT) and through stage 19 at a male-producing temperature (MPT; Wibbels et al., 1991; Greenbaum, 2002). Cooler incubation temperatures (25–27°C) produce all male hatchlings and warmer temperatures (31–35°C) result in all female hatchlings, with varying sex ratios produced by temperatures in between (Wibbels et al., 1991). Shifting eggs during the TSP from one end of the temperature spectrum to the other redirects gonadal development, resulting in 100% sex reversal (Crews et al., 1994).

Analysis of human patients with campomelic dysplasia, roughly two thirds of whom develop as XY females, revealed the importance of the *Sry*-related gene *Sox9* (*SRY-like HMG-box 9*) in the molecular sex-determining cascade (Foster et al., 1994). Extensive studies in the mouse have since shown *Sox9* to be both necessary and sufficient to cause the determination and differentiation of a testis (Vidal et al., 2001). In both humans and mice, SOX9 interacts directly with SF1 (*Steroidogenic factor 1*) to up-regulate the expression of *Müllerian-inhibiting substance* (*Mis* or *anti-Müllerian hormone*, *Amh*; de Santa Barbara et al., 1998; Arango et al., 1999). *Mis*, a member of the transforming growth factor-β (TGF-β) superfamily, is the first factor secreted by differentiated Sertoli cells in the testis, and causes the regression of the Müllerian ducts, anlagen which otherwise develop into the uterus, cervix, and fallopian tubes in females (Behringer et al., 1990).

The regulatory relationship between *Sox9* and *Mis* in mammals has not been strictly conserved across phyla or mechanisms of sex determination. In the chicken, another vertebrate with GSD, sexually dimorphic expression of *Mis* in the gonad precedes *Sox9*, and the relationship between the two has yet to be fully characterized (Oreal et al., 1998; Smith et al., 1999b). Expression patterns in species with TSD have thus far appeared similar to those in chicken. In the American alligator, *Alligator mississippiensis*, dimorphic expression of *Mis* occurs in the middle of the TSP, preceding the onset of testis-specific *Sox9* (Western et al., 1999). Similarly, expression of *Sox9* does not become testis-specific until the end of the TSP in both the leopard gecko and the Olive Ridley sea turtle, *Lepidochelys olivacea* (Moreno-Mendoza et al., 1999; Valleley et al., 2001), although *Mis* has not yet been characterized in these species. In the red-eared slider turtle, *Mis* expression has also been shown to be male-specific by the middle of the TSP (Takada et al., 2004). Furthermore, *Sox9* expression appears in one study to be testis-specific after the TSP (Spotila et al., 1998), while in another study, reverse-transcriptase polymerase chain reaction (RT-PCR) showed comparable levels of expression in both sexes at all stages examined (Kettlewell et al., 2000).

Continued evidence of molecular conservation between sex-determining mechanisms across phyla came with the discovery of a mammalian gene, *Dmrt1*, that possesses functional similarity to sex-determining genes in both nematodes (*mab-3*) and flies (*doublesex*; Shen and Hodgkin, 1988; Burtis and Baker, 1989). Loss of *Dmrt1* is thought to be responsible for the male-to-female sex reversal seen in XY humans with chromosome 9 deletions (Flejter et al., 1998), and *Dmrt1* has since been studied in a variety of vertebrates. Although it seems to play a downstream role in testis differentiation in mammals (Raymond et al., 1999), it has been proposed to be a master sex-determining gene in both chicken and medaka (Nanda et al., 1999; Matsuda et al., 2002). In organisms with TSD, testis-specific expression of *Dmrt1* early in the TSP has been demonstrated in the Olive Ridley sea turtle (Torres-Maldonaldo et al., 2002). In the American alligator, gonadal expression of *Dmrt1* before and during the TSP is also greater in males, but increases through development in both sexes, raising questions about its function (Smith et al., 1999a). In *T. scripta*, dimorphic expression during the TSP has been reported, beginning at either stage 15 (Kettlewell et al., 2000) or stage 17 (Murdock and Wibbels, 2003).

To clarify the nature of the gene regulatory network underlying testis development in organisms with TSD, we analyzed the expression patterns of *Sox9*, *Mis*, and *Dmrt1* by whole-mount in situ hybridization (ISH) and quantitative real-time polymerase chain reaction (qPCR) during the period of sex determination and differentiation in *T. scripta*. Whereas the expression of all three of these genes has been reported as male-specific, the localization of expression patterns and the temporal hierarchy of expression in relation to each other have not been detailed. We visualize and compare localized expression patterns of each gene at the earliest stage of bipotential gonad formation (stage 15), in the middle and at the end of the TSP (stages 17 and 19), as well as at two stages of gonad differentiation (stages 21 and 23). We find the first evidence in an organism with TSD that *Sox9* is expressed in a testis-specific manner early in the TSP, before the onset of testis-specific *Mis* expression. Furthermore, we support previous findings and confirm in *T. scripta* that *Dmrt1* shows sexually dimorphic expression at the beginning of the TSP, in a pattern that is consistent with a possible upstream role in testis determination.

RESULTS AND DISCUSSION

Sox9 Expression Is Dimorphic Near the Beginning of the TSP

Previous reports have shown that levels of *Sox9* expression in *T. scripta* are similar at MPT and FPT during the TSP, and only become higher in the testis during differentiation (Spotila et al., 1998). Our data are consistent with this report, and we also find evidence of an earlier dimorphism in the localized organization of this transcription factor. Early in the TSP, *Sox9* expression at MPT occurs in clusters of cells surrounding other nonexpressing cells. We suggest that these clustered cells are perhaps presumptive Sertoli cells of the develop-

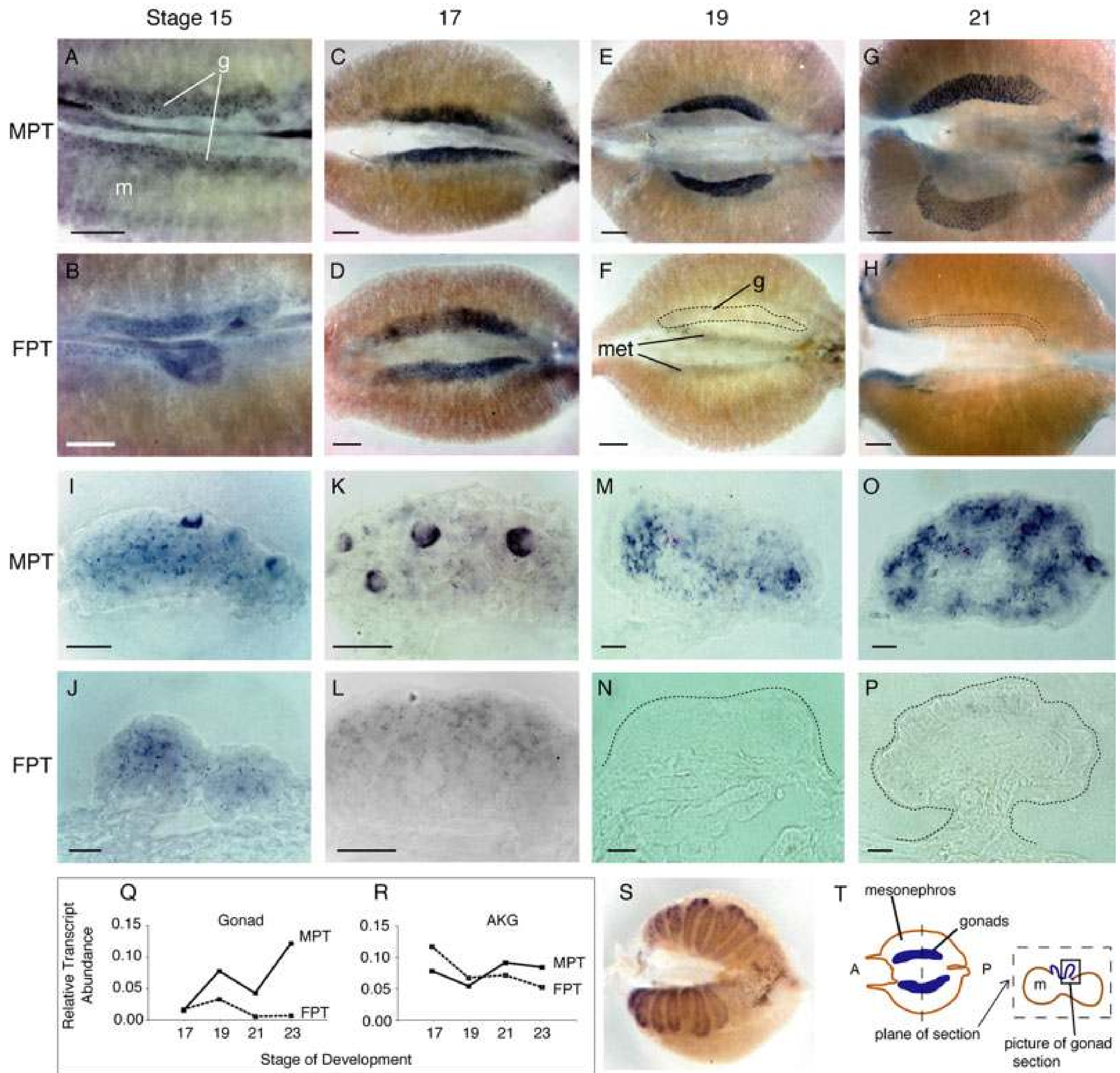

Fig. 1. Developmental expression of *Sox9* in embryonic gonads of the red-eared slider turtle measured by in situ hybridization and quantitative real-time polymerase chain reaction (qPCR). **A–H:** Whole-mount in situ hybridization (ISH) was used to examine *Sox9* expression patterns in turtle gonads dissected from embryos at indicated stages (n>8). **A–H:** Ventral view of representative adrenal–kidney–gonad (AKG) complexes are shown; see cartoon for morphology (T). **I–P:** Tissues are then sectioned to 20 μm thickness, producing transverse sections. **F:** Metanephric staining at female-producing temperature (FPT) is visible through the mesonephros. **S:** This is further illustrated by a dorsal view of a stage 21 AKG. **Q,R:** qPCR measured as relative transcript abundance normalized to the constitutively expressed gene *PP1* shows a dramatically different pattern in the gonad (Q) vs. the AKG (R), reflecting inclusion of strong metanephric expression. Dashed lines indicate external edge of gonad. g, gonad; m, mesonephros; met, metanephros; MPT, male-producing temperature. Scale bar = 300 μm in A–H, 20 μm in I–P.

ing seminiferous tubules. While levels of *Sox9* expression at FPT are similar, transcript localization is diffuse and appears unorganized throughout the gonad. It is not unlikely that this differential localization corresponds to a functional dimorphism early in the sex-determining period. Thus, we find the first evidence of *Sox9* expression in an organism with TSD that suggests similarity to the pattern seen in mammals. These findings are consistent with a possible upstream role for *Sox9* in TSD, although this was not investigated in the current study and warrants future attention.

At the beginning of the TSP (stage 15), *Sox9* is expressed at comparable levels in gonads at both male-producing (n = 12/15; Fig. 1A) and female-producing (n = 9/12; Fig. 1B) temperatures. However, sections of gonads reveal that, at MPT, *Sox9* transcripts are concentrated in cells surrounding nonexpressing cells (Fig. 1I), whereas expression at FPT remains dispersed throughout the gonad (Fig. 1J). At this

stage at MPT, *Sox9* is also expressed in dorsal metanephric tissue in half of the embryos examined (n = 7/15; data not shown).

As the sex-determining period progresses and temperature exerts its effect (stage 17), organized *Sox9* expression persists at MPT (n = 15/16; Fig. 1C,K). There is strong staining in dorsal metanephric tissue and the posterior tip of the mesonephros, although it is unclear if posterior mesonephros is actually a contributing source of gonadal cells (Fig. 1C). In addition, approximately half of MPT embryos show staining in developing Wolffian ducts (n = 7/16; data not shown). Although *Sox9* expression at this stage is retained in gonads at FPT in comparable levels to gonads at MPT (n = 8/11; Fig. 1C,D), it still occurs in a diffuse pattern spread throughout the gonad (Fig. 1L). Metanephric expression is also detected in most embryos at FPT (n = 7/11), but Wolffian duct expression is not. In situ data are confirmed by qPCR studies in which total RNA was extracted from one sample per sex/stage. Each sample was composed of either pooled gonads or pooled adrenal–kidney–gonad (AKG) dissected from at least 20 embryos. This procedure was technically possible beginning at stage 17, and *Sox9* expression is seen at comparable levels at both MPT and FPT at this stage (Fig. 1Q).

At a stage when sexual fate is committed at an MPT but remains somewhat reversible at an FPT (stage 19), *Sox9* expression increases in virtually all gonads developing at MPT (n = 7/8; Fig. 1E,Q) in a pattern that is suggestive of Sertoli cells organizing into sex cords (Fig. 1M). Expression is down-regulated in all FPT gonads examined by ISH (n = 0/12; Fig. 1F,N), but is still detectable by qPCR, a discrepancy we are investigating (Fig. 1Q). Expression is maintained in dorsal metanephric tissue in some embryos at both MPT (n = 4/8) and FPT (n = 7/12), while Wolffian duct expression disappears from males.

During testis differentiation (stages 21 and 23), *Sox9* is strongly expressed in all gonads examined developing at MPT (n = 13/13 and 12/12, respectively; Fig. 1G,Q). In these gonads, expression continues in an increasingly reticulated pattern, probably corresponding to the expansion of primitive sex cords and their subsequent transformation into seminiferous tubules (Fig. 1O). In contrast, only a few cells weakly expressing *Sox9* were observed in several differentiating ovaries at stages 21 and 23 (n = 3/12 and 5/12, respectively; Fig. 1H,P,Q). In both sexes, metanephric expression remains strong in the beginning of gonad differentiation (stage 21, n = 9/13 at MPT, n = 11/12 at FPT; Fig. 1S) and then diminishes to a faint level (stage 23, n = 9/12 at MPT, n = 3/12 at FPT).

Dimorphic *Mis* Expression Occurs Midway Through the TSP

It has been shown in *T. scripta* that *Mis* expression is undetectable in the bipotential gonad, is up-regulated in developing testes during the TSP and becomes increasingly stronger as sex determination occurs and differentiation begins (Takada et al., 2004). Our data are consistent with these findings as well, and further reveal that, in contrast to *Sox9*, both the level of *Mis* expression as well as its cellular organization appear similar in MPT and FPT gonads early in development. At the start of the TSP, *Mis* transcripts are organized in similar clusters of cells in both sexes. It is not until after the sex-determining period, and after *Sox9* patterns are dimorphic, that *Mis* is up-regulated in males and down-regulated in females. These results are consistent with a role for *Sox9* upstream of *Mis*, although a regulatory relationship between them in this species remains to be shown.

Mis expression is virtually equivalent at both male- and female-producing temperatures at the earliest stage of gonad formation (stage 15; n = 10/12 at both MPT and FPT; Fig. 2A,B,I,J). As the sex-determining period progresses (stage 17), *Mis* expression is up-regulated in the gonads of embryos incubating at MPT (n = 16/16; Fig. 2C,K) and persists but is down-regulated at FPT (n = 11/12; Fig. 2D,L,Qb). As sexual fate is committed and gonad differentiation occurs (stages 19, 21, and 23), developing testes strongly express *Mis* in a pattern similar to *Sox9*, possibly in the preSertoli cells of nascent seminiferous tubules (Fig. 2E,M,G,O,Q). Expression is also seen at MPT in dorsal metanephric tissue, as well as progressively in a cranial to caudal wave in the Müllerian ducts (Fig. 2S). In contrast, expression at FPT at stage 19 is undetectable in half of the gonads examined and faint in the rest (n = 7/14; Fig. 2F,N,Q). In later stages of ovarian differentiation (stages 21 and 23), expression at FPT falls to undetectable levels (n = 0/12 and 0/12, respectively; Fig. 2H,P,Q). Although qPCR levels at FPT appear close to zero in all stages examined, this finding is an artifact produced by the extremely high expression levels seen later at MPT (see Fig. 2Qa,Qb).

Dmrt1 Expression Is Consistent With a Possible Upstream Role in *T. scripta*

We used whole-mount ISH to confirm previous reports that *Dmrt1* expression is dimorphic before sex is determined. At the beginning of the TSP (stage 15), all embryos developing at MPT show punctate *Dmrt1* expression in the gonad (n = 12/12; Fig. 3A,I), while half of FPT embryos show weaker, diffuse gonadal expression (n = 6/12; Fig. 3B,J). At stage 17, expression of *Dmrt1* continues in gonads at MPT (n = 14/14; Fig. 3C,K) and is detected in only a very few cells in gonads at FPT (n = 2/9; Fig. 3D,L). Up-regulation of expression at MPT is localized in clusters of cells surrounding nonexpressing cells (Fig. 3K). qPCR confirms a difference in *Dmrt1* transcript levels at this stage, and although expression in both sexes is low overall, relative transcript abundance is three times higher at MPT than at FPT (Fig. 3Q).

By stage 19, *Dmrt1* expression is upregulated further at MPT (n = 7/7; Fig. 3E,Q). Expression at FPT declines in all ISH gonads examined (n = 0/10; Fig. 3F,N), but is still detectable by the more sensitive method of qPCR (Fig. 3Q). Sectioned gonads reveal continued *Dmrt1* expression in cells organized in what are possibly developing sex cords of the testis (Fig. 3M). Finally, during differentiation (stages 21 and 23), expression at MPT continues to increase (n = 12/12 and 8/8, respectively; Fig. 3G,O,Q) and is

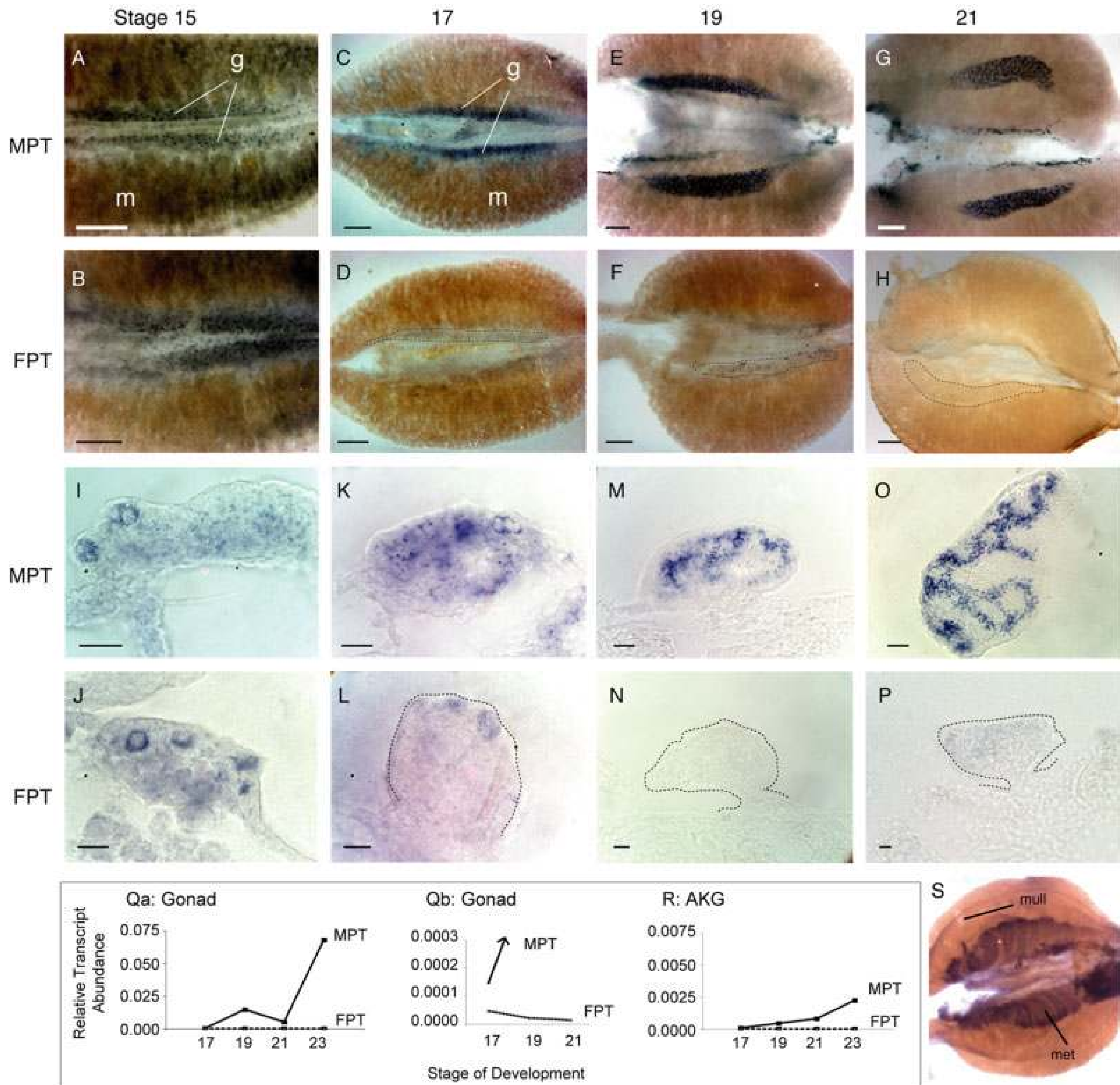

Fig. 2. Developmental expression of *Mis* in embryonic gonads of the red-eared slider turtle measured by in situ hybridization (ISH) and quantitative real-time polymerase chain reaction (qPCR). **A–H:** Whole-mount ISH was used to examine *Mis* expression patterns in turtle gonads dissected from embryos at the indicated stages (n>8). Ventral view of representative adrenal–kidney–gonad (AKG) complexes are shown. **I–P:** Tissues are then sectioned to 20 μm thickness, producing transverse sections. **S:** A dorsal view of a male-producing temperature (MPT) stage 19 AKG complex shows strong expression in metanephric tissue as well as in the anterior portion of the Müllerian ducts. **Qa,R:** qPCR is measured as relative transcript abundance normalized to the constitutively expressed gene *PP1*. Although metanephric expression is significant, relative levels of *Mis* expression measured from gonad tissues (Qa) are over 20-fold higher than in the AKG (R). **Qb:** A magnification of the scale of gonad expression reveals the dimorphism present at stage 17, as well as the decrease in expression in female-producing temperature (FPT) gonads through development. Dashed lines indicate represents external edge of gonad. g, gonad; m, mesonephros; mull, Müllerian duct; met, metanephros. Scale bar = 300 μm in A–H, 20 μm in I–P.

virtually undetectable in all gonads at FPT (n = 0/12 and 0/12, respectively; Fig. 3H,P,Q). Throughout development, *Dmrt1* expression is not seen in other tissues of the AKG complex, including the mesonephros, dorsal metanephros, or Müllerian and Wolffian ducts.

In conclusion, for a sex-determining gene to be directly downstream of temperature in an organism with TSD, its expression should be sexually dimorphic just before or early in the TSP. In *T. scripta*, *Sox9* expression patterns are dimorphic early enough to be consistent with a sensitivity to temperature, and in the simplest case, *Sox9* may directly up-regulate *Mis* as it does in mammals. Alternatively, an undetermined upstream factor may sense and respond to a male-producing temperature and up-regulate both *Sox9* and *Mis*. Following their initial up-regulation in this model, *Sox9* ex-

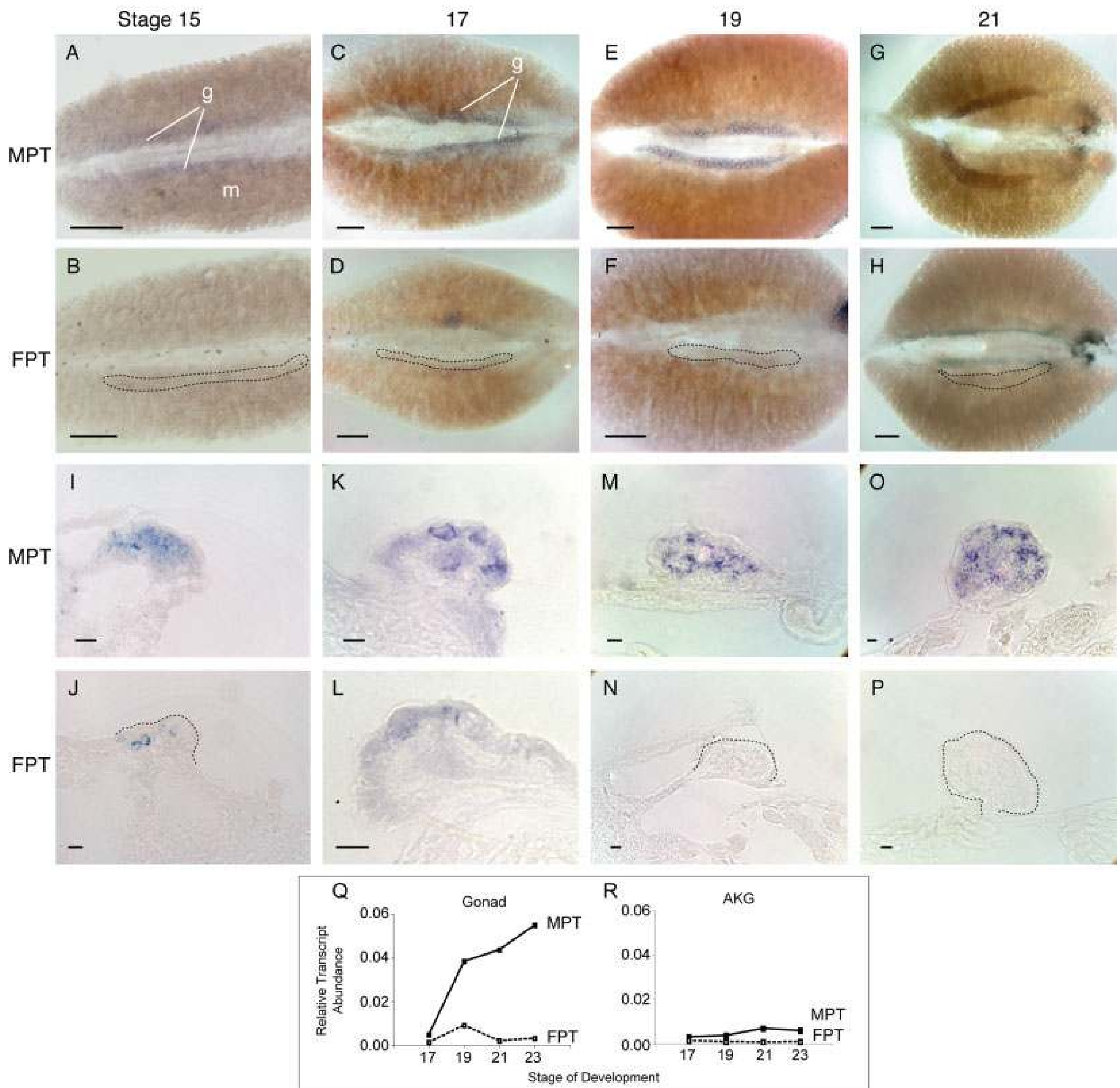

Fig. 3. Developmental expression of *Dmrt1* in embryonic gonads of the red-eared slider turtle measured by in situ hybridization (ISH) and quantitative real-time polymerase chain reaction (qPCR). **A–H:** Whole-mount ISH was used to examine *Dmrt1* expression patterns in turtle gonads dissected from embryos at the indicated stages (n>8). Ventral view of representative adrenal-kidney-gonad complexes are shown. **I–P:** Tissues are then sectioned to 20 μm thickness, producing transverse sections. **Q,R:** qPCR is measured as relative transcript abundance normalized to the constitutively expressed gene *PP1*. Dashed lines indicate external edge of gonad. g, gonad; m, mesonephros; MPT, male-producing temperature; FPT, female-producing temperature; AKG, adrenal–kidney–gonad complex. Scale bar = 300 μm in A–H; 20 μm in I–P.

pression in preSertoli cells may then direct their differentiation and organize and maintain expression of *Mis*. Furthermore, our findings of the spatial and temporal patterns of *Dmrt1* expression are also consistent with the possibility that *Dmrt1* could be a target of temperature, although this was not directly tested in the current study. To gain insight into these possibilities, and shed light on the hierarchy of genes in the regulatory network underlying TSD, we are currently investigating the response of these genes to sex-reversing shifts in temperature and elimination of transcript in the gonad in an *in vitro* organ culture system.

Tissue-Specific qPCR

The qPCR was performed on pooled AKG complexes as well as pooled gonads. Comparing relative expression levels between the two RNA sources reveals the problems associated with retaining extraneous tissue when examining sex-determining genes, as has

been common in previous RT-PCR studies. *Sox9* transcript levels in the two tissues reveal wholly different relative patterns of expression. In the gonad (Fig. 1Q), *Sox9* levels are comparable at MPT and FPT during the sex-determining period (stage 17), after which expression is consistently higher at MPT. In contrast, *Sox9* expression in the AKG as a whole (Fig. 1R) causes transcript abundance at FPT to rise above MPT at stages 17 and 19. During differentiation (stages 21 and 23), levels of expression in AKGs at MPT exceed FPT, but are much less dimorphic than in the isolated gonad. In contrast, expression of both *Mis* and *Dmrt1* show a similar temporal pattern in both the gonad (Figs. 2Q, 3Q) and the AKG (Figs. 2R, 3R): values at MPT are higher than at FPT and consistently increase through development. However, transcripts are found in much greater relative abundance in the gonad as compared with mesonephric and metanephric tissues. In the future, interpretation of data produced by qPCR will be more accurate when only the tissue of developmental interest is used.

Exploring Functional Roles of Genes in a Nonmodel System

Curiously, we find *Sox9* expression at female-producing temperatures both during and after the TSP, and no role for *Sox9* in females has yet been described. Expression is expected early in the TSP, when the gonad is still bipotential and temperature has yet to be translated into a molecular signal. If *Sox9* does not play a role in ovarian development, down-regulation of expression would be expected soon after. Although the current study investigated only transcript abundance, examining the subcellular localization of the SOX9 protein might explain its continued gene expression. As is the case in both mouse and human, SOX9 may be localized to the nucleus of preSertoli cells in the testis, exerting transcriptional control over downstream genes, whereas in ovarian cells remaining cytoplasmic and, thus, inactive as a transcription factor (de Santa Barbara et al., 2000; Lasala et al., 2004). Future work should investigate whether a similar nuclear-cytoplasmic shuttling system is important in TSD.

Similarly, *Mis* is expressed in gonads developing at both MPT and FPT, and it is possible that *Mis* hormone receptor expression is dimorphic and controls a critical window of sensitivity to the hormone itself. *Mis* is secreted from Sertoli cells in developing mammalian testes and in an autocrine/paracrine manner binds to its highly specific receptor, *Mis-R2*, found in the membranes of both Sertoli and Leydig cells (see Josso et al., 2001). The action of MIS to induce regression of the Müllerian duct is directed by a spatial dimorphism not of the hormone itself, but of *Mis-R2*, which is expressed in a cranial to caudal wave along the duct (Allard et al., 2000). Cloning the *Mis-R2* gene homolog in *T. scripta* and examining its expression could determine whether a similar mechanism may be involved. Thus, although the patterns of transcript expression of both *Sox9* and *Mis* appear more similar to mammals exhibiting genetic sex determination, the active gene products in *T. scripta* may in fact be evolutionarily conserved with other TSD species.

EXPERIMENTAL PROCEDURES

Collection and Harvesting Embryos

Freshly laid red-eared slider eggs purchased from Clark Turtle Farms (Hammond, LA) were maintained as previously described (Wibbels et al., 1991). Briefly, viable eggs were randomized and placed in incubators (Precision, Chicago, IL) at 26.0°C or 31.0°C. Incubator temperatures were monitored daily with HOBO data loggers (Onset Computer Corp., Bourne, MA) and verified with calibrated incubator thermometers. Developmental progression was monitored by assessing external characteristics in a periodic sampling of embryos.

Cloning of Turtle Gene Homologs

Total RNA was extracted from pooled AKG tissues from a variety of sexes and stages and reverse-transcribed using oligo(dT) primers with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA). *Sox9* primers were designed to amplify a 558-bp subclone of the sequence reported by Spotila et al. (1998). Degenerate primers for *Mis* and *Dmrt1* were designed based on mouse, alligator, and chicken sequences. Amplified fragments were ligated into pCR4-TOPO vector (Invitrogen) and sequenced using M13F and M13R primers. The sequences of the *Mis* and *Dmrt1* clones were subsequently found to correspond to base pairs 140 to 339 of *T. scripta Mis* (accession no. AY235424) and base pairs 35 to 463 of *T. scripta Dmrt1* (accession no. AY316537).

Whole-Mount ISH

Riboprobes were reverse-transcribed in the presence of digoxigenin (DIG) -labeled UTP (Roche) using T3/T7 Megascript in vitro transcription kit (Ambion, Austin, TX) to produce antisense or sense DIG-labeled riboprobes. Turtle embryos were harvested from eggs at specific stages, and gonads were dissected and fixed overnight in 4% paraformaldehyde/phosphate buffered saline at 4°C. After fixation, whole-mount ISH was performed as described by Andrews et al. (1997) with modification. Briefly, tissues were dehydrated in an increasing methanol/PBTX series, rehydrated, and partially digested with 10 μg/ml proteinase K for 15–45 min, depending on tissue stage. After fixation in 0.2% glutaraldehyde, tissues were prehybridized overnight at 65°C. Hybridization of DIG riboprobe was carried out overnight at 65°C, followed by a series of increasing stringency washes in standard saline citrate. After anti–DIG-alkaline phosphatase Fab fragments (Roche) were preblocked in turtle embryo powder, tissues were incubated in 1:2,000 Ab dilution overnight at 4°C. Hybridization was visualized with BM purple (Roche) and whole tissues were photographed in 100% glycerol. AKGs were embedded in OCT medium (Fisher Scientific, Hampton, NH) and sectioned on a cryostat (2800 Reichert-Jung).

qPCR

Gonads from 20 individual turtles were pooled into one sample per stage/

sex for total RNA extractions using the RNAgents Total RNA Isolation Kit (Promega, Madison, WI). Total RNA was also extracted from pooled AKG complexes at each stage/sex for comparison. Total RNA was treated with Turbo DNA-free DNase I (Ambion, Austin, TX) and reverse-transcribed using the SuperScript First-Strand Synthesis for RT-PCR system (Invitrogen) with both oligo-(dT) and random hexamers. Relative gene expression levels were quantified using SYBR Green I dye (Invitrogen) and an ABI PRISM 7900HT real-time PCR cycler (ABI SDS 2.2.1 software). All samples were run in triplicate, and gene-specific PCR efficiencies were calculated from gene-specific standard curves. Relative transcript abundance corrected for PCR efficiency was normalized to expression of *PP1*, a constitutively expressed transcript across both stage and sex (Muller et al., 2002; Simon, 2003). Primers used to assay gene expression were designed across exon boundaries where possible using MacVector (Accelrys, San Diego, CA), and specificity was verified by agarose gel electrophoresis. Primers were as follows: *Mis* forward 5′-CGG CTA CTC CTC CCA CAC G-3′, reverse 5′-CCT GGC TGG AGT ATT TGA CGG-3′; *Dmrt1* forward 5′-CAA CTA CTC CCA ATA CCA GAT GGC-3′, reverse 5′-GGC TTC GCA GGC TGT TTT TC-3′; *Sox9* forward 5′-CCT GCC CTT CTG GTT CCG-3′, reverse 5′-TCC TCG TCC CTC TCT TTC TTC AG-3′; *PP1* forward 5′-CAG CAG ACC CTG AGA ACT TCT TCC TGC TG-3′, reverse 5′-GCG CCT CTT GCA CTC ATC AT-3′.

ACKNOWLEDGMENTS

We express our thanks to Raymond S. Porter, without whom this manuscript would not have been possible. We also thank three anonymous reviewers from whom we received invaluable comments. The work was assisted by Dr. James Skipper, Sarah Withycombe, and Sebastian Partesotti and was funded by a National Science Foundation grant awarded to D. Crews and Graduate Research Fellowships through The University of Texas at Austin awarded to C. Shoemaker.

REFERENCES

Allard S, Adin P, Gouedard L, di Clemente N, Josso N, Orgebin-Crist M, Picard JY, Xavier F. 2000. Molecular mechanisms of hormone-mediated Müllerian duct regression: involvement of beta-catenin. Development 127:3349–3360.

Andrews J, Smith C, Sinclair A. 1997. Sites of estrogen receptor and aromatas expression in the chicken embryo. Gen Comp Endo 108:182–190.

Arango NA, Lovell-Badge R, Behringer RR. 1999. Targeted mutagenesis of the endogenous mouse *Mis* gene promoter: in vivo definition of genetic pathways of vertebrate sexual development. Cell 99: 409–419.

Behringer RR, Cate RL, Froelick GJ, Palmiter RD, Brinster RL. 1990. Abnormal sexual development in transgenic mice chronically expressing Müllerian-inhibiting substance. Nature 345:167–170.

Bull JJ, Wibbels T, Crews D. 1990. Sex-determining potencies vary among female incubation temperatures in a turtle. J Exp Zool 256:339–341.

Burtis K, Baker B. 1989. Drosophila *doublesex* gene controls somatic sexual differentiation by producing alternatively spliced mRNAs encoding related sex-specific polypeptides. Cell 56:997–1010.

Crews D, Bergeron JM, Bull JJ, Flores D, Tousignant A, Skipper JK, Wibbels T. 1994. Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. Dev Genet 15:297–312.

de Santa Barbara P, Bonneaud N, Boizet B, Desclozeaux M, Moniot B, Sudbeck P, Scherer G, Poulat G, Berta P. 1998. Direct interaction of SRY-related protein SOX9 and steroidogenic factor 1 regulates transcription of the human anti-Müllerian hormone gene. Mol Cell Biol 18:6653–6665.

de Santa Barbara P, Moniot B, Poulat F, Berta P. 2000. Expression and subcellular localization of SF1, SOX9, WT1 and AMH proteins during early human testicular development. Dev Dyn 217:293–298.

Flejter W, Fergestad J, Gorski J, Varvill T, Chandrasekharappa S. 1998. A gene involved in XY sex reversal is located on chromosome 9, distal to marker D9S1779. Am J Hum Genet 63:794–802.

Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller P, Weissenbach J, Mansour S, Young I, Goodfellow PN, Brook J, Schafer A. 1994. Campomelic dysplasia and autosomal sex reversal caused by mutations in SRY-related gene. Nature 372:525–530.

Greenbaum E. 2002. A standardized series of embryonic stages for the emydid turtle *Trachemys scripta*. Can J Zool 80:1350–1370.

Josso N, di Clemente N, Gouedard L. 2001. Anti-Müllerian hormone and its receptors. Mol Cell Endocrinol 179:25–32.

Kettlewell JR, Raymond CS, Zarkower D. 2000. Temperature-dependent expression of turtle *Dmrt1* prior to sexual differentiation. Genesis 26:174–178.

Lasala C, Carre-Eusebe D, Picard J, Rey R. 2004. Subcellular and molecular mechanisms regulating anti-Müllerian hormone gene expression in mammalian and nonmammalian species. DNA Cell Biol 23:572–585.

Matsuda M, Nagahama Y, Shinomiya A, Sato T, Matsuda C, Kobayashi T, Morrey C, Shibata N, Asakawa S, Shimizu N, Hori H, Hamaguchi S, Sakaizumi M. 2002. *DMY* is a Y-specific DM-domain gene required for male development in the medaka fish. Nature 417:559–563.

Moreno-Mendoza N, Harley V, Merchant-Larios H. 1999. Differential expression of SOX9 in gonads of the sea turtle *Lepidochelys olivacea* at male- or female-promoting temperatures. J Exp Zool 284: 705–710.

Muller P, Janovjak H, Miserez A, Dobbie Z. 2002. Processing of gene expression data generated by quantitative real-time RT-PCR. Biotechniques 32:1372–1379.

Murdock C, Wibbels T. 2003. Expression of *Dmrt1* in a turtle with temperature-dependent sex determination. Cytogenet Genome Res 101:302–308.

Nanda I, Shan Z, Schartl M, Burt D, Koehler M, Nothwang H, Grutzner F, Paton I, Windsor D, Dunn I, Engel W, Staeheli P, Mizuno S, Haaf T, Schmid M. 1999. 300 million years of conserved synteny between chicken Z and human chromosome 9. Nat Genet 21:258–259.

Oreal E, Pieau C, Mattei M-G, Josso N, Picard J-Y, Carre-Eusebe D, Magre S. 1998. Early expression of AMH in chicken embryonic gonads precedes testicular SOX9 expression. Dev Dyn 212: 522–532.

Raymond CS, Parker E, Kettlewell JR, Brown LG, Page DC, Kusz K, Jaruzelska J, Reinberg Y, Flejter WL, Bardwell VJ, Hirsch B, Zarkower D. 1999. A region of the human chromosome 9p required for testis development contains two genes related to known sexual regulators. Hum Mol Genet 8:989–996.

Shen M, Hodgkin J. 1988. *mab-3*, a gene required for sex-specific yolk protein expression and a male-specific lineage in *C. elegans*. Cell 54:1019–1031.

Simon P. 2003. Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19:1439–1440.

Smith C, McClive PJ, Western PS, Reed KJ, Sinclair AH. 1999a. Conservation of a sex determining gene. Nature 402: 601–602.

Smith C, Smith M, Sinclair AH. 1999b. Gene expression during gonadogenesis in the chicken embryo. Gene 234:395–402.

Spotila LD, Spotila JR, Hall S. 1998. Sequence and expression analysis of *Wt1* and *Sox9* in the red-eared slider turtle, *Trachemys scripta*. J Exp Zool 281:417–427.

Takada S, DiNapoli L, Capel B, Koopman P. 2004. *Sox8* is expressed at similar levels in gonads of both sexes during the sex

determining period in turtles. Dev Dyn 231:387–395.

Torres-Maldonado L, Piedra AL, Moreno-Mendoza N, Valencia AM, Martinez AM, Merchant-Larios H. 2002. Expression profiles of *Dax1*, *Dmrt1*, and *Sox9* during temperature sex determination in gonads of the sea turtle *Lepidochelys olivacea*. Gen Comp Endocrinol 129:20–26.

Valleley E, Cartwright E, Croft N, Markham A, Coletta L. 2001. Characterisation and expression of *Sox9* in the leopard gecko, *Eublepharis macularius*. J Exp Zool 291:85–91.

Vidal VPI, Chaboissier M-C, de Rooij DG, Schedl A. 2001. *Sox9* induces testis development in XX transgenic mice. Nat Genet 28:216–217.

Western PS, Harry JL, Graves JAM, Sinclair AH. 1999. Temperature-dependent sex determination in the American alligator: *Amh* precedes *Sox9* expression. Dev Dyn 216:411–419.

Wibbels T, Bull JJ, Crews D. 1991. Chronology and morphology of temperature-dependent sex determination. J Exp Zool 260:371–381.