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Estradiol and Incubation Temperature Modulate Regulation of Steroidogenic Factor 1 in the Developing Gonad of the Red-Eared Slider Turtle*

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ABSTRACT

Red-eared slider turtles are genetically bipotential for sex determination, with incubation temperature of the egg determining gonadal sex. At higher incubation temperatures, females are produced, possibly due to increased biosynthesis of estrogen. Exogenous estrogen causes the formation of ovaries, and prevention of estrogen biosynthesis results in the development of testes. In mammals, steroidogenic factor 1 (SF-1) regulates most genes required for estrogen biosynthesis from cholesterol. In mammals as well as red-eared slider turtles, SF-1 is differentially expressed in males and females during gonadogenesis. To examine a possible role for SF-1 in temperature-dependent sex determination, we assayed its expression in red-eared slider turtles after treatments that alter sex development during gonadogenesis of the wild-type organism.

We examined gonadal SF-1 expression in embryos 1) incubating at three different temperatures, 2) after treating eggs with estrogen at a male-producing temperature, and 3) after inhibition of estrogen biosynthesis at a female-producing temperature. Our findings suggest that both temperature and estrogen lie upstream of SF-1 in a sex-determining regulatory hierarchy in red-eared slider turtles and that estrogen directly or indirectly modulates the regulation of SF-1 expression. (*Endocrinology* **142:** 1403–1411, 2001)

AMONG AMNIOTES (reptiles, birds, and mammals), sex can be determined genetically by the inheritance of sex chromosomes or environmentally by the incubation temperature of eggs during embryogenesis (1, 2). Genotypic sex determination (GSD) occurs in mammals where males are XY heterogametic, and the SRY protein triggers male development. Sex steroid hormones, including estrogen, are not thought to be involved in primary sex determination of mammals (3, 4), although female to male gonadal sex reversal occurs during adulthood in mice lacking α/β estrogen receptors (5), and exogenous estrogen feminizes the gonad in genetic male marsupials (6). GSD also occurs in birds where females are ZW heterogametic. In birds, SRY is apparently not present in either sex, and an ovary-determining mechanism is as yet unknown, but estrogen does appear essential for female sex determination. In chickens, for example, inhibiting aromatase, the enzyme that converts testosterone to estrogen, causes genotypic females to develop as phenotypic males (7), and exogenous estrogen causes transient feminization of genotypic males (8).

Temperature-dependent sex determination (TSD) occurs in all crocodilians, most turtles, and some lizards (9). These reptiles are genetically bipotential (10) and no functional SRY has been identified (11, 12). Estrogen is implicated in female sex determination of reptiles and specifically in conveying the effect of temperature in TSD reptiles (1, 2, 13, 14). In the red-eared slider turtle (*Trachemys scripta* elegans), a TSD species, application of 17β-estradiol (E_2) on eggs incubating at an all male-producing temperature results in 100% female hatchlings (9). This effect of E_2 treatment is both time- and dose-dependent. The period of E_2 sensitivity overlaps the temperature-sensitive period (TSP) and is synergistic with the effect of temperature. Hatchlings that result after E_2 treatment at an all male-producing temperature are morphologically and histologically equivalent to those incubated at a temperature that produces only females. Application of aromatase inhibitor (AI) to eggs incubating at a female-biased temperature results in 100% male hatchlings. Higher doses of AI are required to produce this effect at increasingly female-biased temperatures. Male hatchlings from this treatment group are morphologically and histologically comparable to normal, temperature-derived males. Throughout the TSP, estrogen receptor is expressed in gonads of both putative males and females (15). Despite much effort, however, the initial endogenous source of estrogen in TSD reptiles remains unclear (13). Furthermore, little is known about molecular mechanisms underlying endogenous or manipulated sex determination in TSD species. Despite the apparent lability of triggers that initiate sex determination among amniotes, subsequent gonadogenesis and the underlying molecular components identified to date are largely conserved.

Steroidogenic factor 1 (SF-1) (16), also named Ad4BP (17), is known in mammals to regulate many genes in the reproductive axis. In steroidogenic tissues, SF-1 regulates the expression of most enzymes required to synthesize estrogen from cholesterol: cytochrome P450 steroid hydroxylases, including aromatase, and 3βHSD (18, 19). In addition, SF-1 is preferentially expressed in developing testes compared with ovaries. SF-1 is, therefore, of clear interest in the study of sex determination in birds and reptiles. It has been cloned and its expression examined in chickens (20, 21), alligators (22), and red-eared slider tur-

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tles (14, 23). Regulation of SF-1 itself has not yet been fully characterized in any organism.

In this study we asked whether manipulating sex determination by shifting the incubation temperature, by treatment with E_2, or by treatment with AI to inhibit estrogen biosynthesis would affect the expression of SF-1 in red-eared slider turtles. Adrenal-kidney-gonad complexes in red-eared slider turtles cannot be separated until late in gonadogenesis, and SF-1 is strongly expressed in adrenal as well as gonad (23). To quantify SF-1 message in gonad alone we therefore used *in situ* hybridization analysis. Our findings support the involvement of SF-1 in the regulatory hierarchy of TSD in red-eared slider turtles and show that incubation temperature and estrogen directly or indirectly modulate the regulation of SF-1 expression.

Materials and Methods

Tissue collection

Freshly laid red-eared slider turtle eggs were purchased from Kleibert Turtle Farms (Hammond, LA) and kept at room temperature until viability was established by candling. Viable eggs were randomized to eliminate clutch effects and were placed in covered trays containing moistened vermiculite. The trays were secured in plastic bags and placed in incubators (Precision, Chicago, IL) at 26, 29.4, or 31 C. Incubator temperatures were continuously monitored with HOBO data loggers (Onset Computer Corp., Bourne, MA), and verified daily with calibrated incubator thermometers. Temperatures fluctuated less than 0.1 C. Egg boxes were rotated within the incubators each day. Developmental progression was monitored regularly by comparing external characteristics of a sampling of embryos against staging guidelines established by Yntema (24).

Probe preparation

Cowan and Wibbels (14) provided a 457-bp clone of red-eared slider turtle SF-1 that we subcloned into the pCRII vector (Invitrogen, San Diego, CA) to eliminate most sequences in common with an alternate transcript, ELP1, as defined in mouse gene structure (25). Before performing *in situ* hybridizations, a Southern blot analysis was conducted to determine the binding specificity of probes derived from this 330-bp subclone (Fig. 1). Genomic DNA was isolated according to the method of Sambrook *et al.* (26) from multiple red-eared slider turtle embryos and digested with *Bam*HI, *Eco*RI, or *Hin*dIII (New England Biolabs, Inc., Beverly, MA). Three sets of the three digests were electrophoresed (10 μg/lane) simultaneously on a 0.7% agarose gel and transferred to a BrightStar Plus nylon membrane (Ambion, Inc., Austin, TX). The membrane was baked for 30 min at 80 C, then cut into thirds. Each of the resulting equivalent blots was hybridized to one of three DNA probes (Fig. 1A). Probes were synthesized by linear amplification using PCR Strip EZ (Ambion, Inc.) and [α-^{32}P]deoxy-ATP (3000 Ci/mmol, 10 mCi/ml; NEN Life Science Products-DuPont, Boston, MA) to a specific activity of more than 10^9 cpm/μg and were used at a concentration of 10^6 cpm/ml UltraHyb hybridization solution (Ambion, Inc.). Blots were hybridized overnight at 43 C, then washed for 40 min (with one solution change) at each of three stringencies: 0.2 × SSC (standard saline citrate)/0.1% SDS at room temperature, 0.1 × SSC/0.1% SDS at 43 C, and 0.1 × SSC/0.1% SDS at 68 C (26) (Ambion, Inc.). Bound probe was visualized by PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, CA; Fig. 1B) at each level of stringency (final wash shown). Southern blot findings combined with previous Northern blot analyses (23) indicate that the 330-nucleotide probe binds a single copy of SF-1 only.

The probe used in *in situ* hybridizations was made by run-off transcription (26) from the 330-bp template using enzymes from New England Biolabs, Inc. and [α-^{35}S]CTP from NEN Life Science Products-DuPont. Riboprobe was synthesized to a specific activity of 8×10^8 cpm/μg and used at a concentration of 0.3 μg probe × length (kb)/ml hybridization solution.

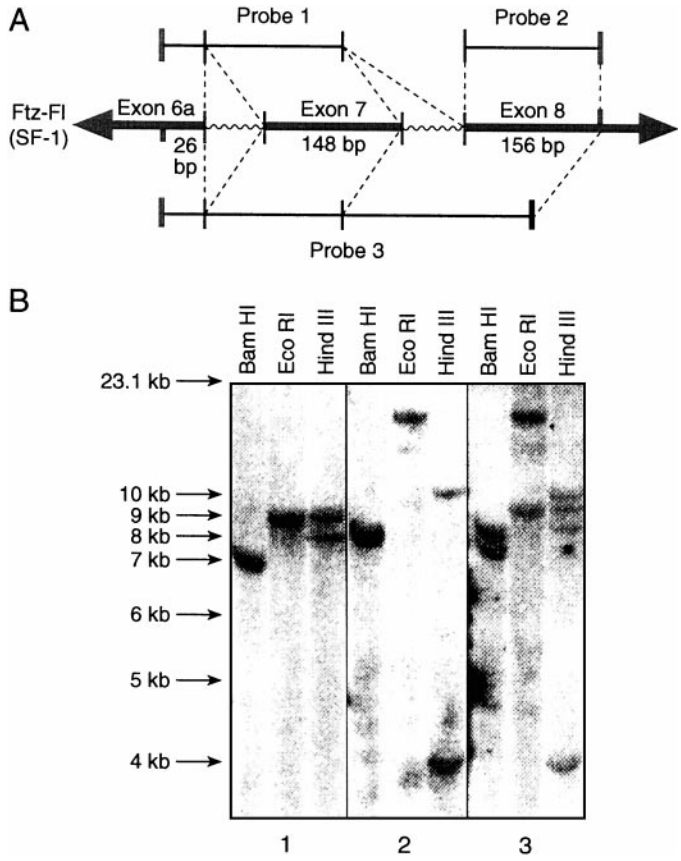

FIG. 1. Binding specificity of the SF-1 probe is validated by Southern blot analysis. Schematic of the 3′-region of Ftz-F1 (25) (A), the gene that encodes SF-1, with the locations of three probe sequences indicated. Probe 3 was subsequently used in *in situ* hybridizations. Probes 1, 2, and 3 were hybridized to equivalent genomic DNA blots 1, 2, and 3, respectively (B). In lanes 1 and 2, blot 3 has two bands, which correspond to single bands in blots 1 and 2. This suggests that *Bam*HI and *Eco*RI each cut the genomic DNA in the intron between exons 7 and 8. In lane 3, blot 3 has four bands, which correspond to two bands each in blots 1 and 2. Although the two bands in blot 1 suggest *Hin*dIII cut in the intron between exons 6a and 7, the two bands in blot 2 may indicate multiple tandem repeat sequences among individuals pooled to prepare the blots. Alternatively, in red-eared slider turtles there may be more than one exon in the sequence corresponding to exon 8 in mouse SF-1. Overall, these data indicate that probe 3 bound a single copy of Ftz-F1 only, supporting its continued use in hybridization protocols.

In situ hybridization

All eggs were initially incubated at 26, 29.4, or 31 C. At stage 17 they were randomly divided into treatment and control groups. Eggs in the continuous temperature groups, which also served as controls for manipulations below, were retained at their starting temperatures. At stages 15, 17–19, 21, and 23, a subset of these eggs was taken from each temperature, and their embryos were quickly frozen on dry ice, then stored at −80 C for later analysis.

Four other groups were treated at stage 17. A group of eggs incubating at the all male-producing temperature of 26 C was shifted to the all female-producing temperature of 31 C. Another group was shifted from 31 to 26 C. A group of eggs incubating at 26 C was treated with 5 μg E_2 (Sigma, St. Louis, MO) in 5 μl 95% ethanol, as previously described (9). Carrier controls were treated with ethanol only at 26 C. Finally, a group of eggs incubating at 29.4 C was treated with 100

Table 1. *In situ* hybridization sample size in each treatment by stage, and hatchling sex ratios from each treatment

Treatment	Embryonic stage						♂:♀ sex ratio
	15	17	18	19	21	23	
26 C	7	10	13	14	8	7	12:0
31 C	7	12	12	12	8	8	0:12
26→31 C			8	8	8	6	2:93
31→26 C			15	15	15	15	39:8
E_2@26 C			6	8	8	8	0:65
29.4 C	17	15	16	21	15	15	15:71
AI@29.4 C			15	14	14	14	24:0

Minimum sample numbers were determined by power analysis. Actual numbers assayed exceeded those minimums in most treatment/stage groups. The *line* demarcates two assays. Hatchling sex was identified morphologically for each individual and corroborated histologically in a sampling of individuals. Ethanol carrier control embryos are not shown. E_2, 17β-Estradiol; AI, aromatase inhibitor.

μg (in 5 μl 95% ethanol) of the aromatase inhibitor fadrozole (CGS16949A, Ciba-Geigy, Summit, NJ). Carrier controls were spotted with ethanol only at 29.4 C. Subsets of eggs from each treatment group were taken at stages 18, 19, 21, and 23, and the embryos were frozen at −80 C until use. A subset of individuals from all groups above were maintained until hatching, then sexed (Table 1). Two *in situ* hybridizations were performed to accommodate the large number of individuals assayed. To determine whether statistical comparisons could be made between the two assays, consecutive slides of 10 29.4 C untreated individuals from 3 different stages were assayed in each experiment. Using the JMP program (version 3.2.2, SAS Institute, Inc., Chicago, IL), a multivariate ANOVA was performed on resulting SF-1 expression values. No significant difference was found between assays ($F = 0.71$; $P = 0.42$).

Frozen whole red-eared slider turtle torsos were embedded in OCT compound (Tissue-Tek, Miles, Elkhart, IN) and sectioned on a cryostat (2800 Frigocut, Reichert-Jung, Nussloch, Germany) at 20 μm. Sections were placed serially on sets of five poly-L-lysine-treated slides, air-dried, and stored at −80 C. One slide from each individual was hybridized to SF-1 antisense probe. For one individual per each treatment stage, a consecutive slide was hybridized to SF-1 sense probe. Three additional consecutive slides from each of three individuals were used for further probe validation: ribonuclease treatment of the mounted tissue before hybridization with labeled antisense probe, and 100- and 500-fold cold antisense probe competition. The *in situ* hybridization protocol used has been previously described (27). The emulsion exposure time was 9 days (4 C).

Darkfield quantification of silver grains in specifically labeled cells, defined as having a density of silver grains at least three times that of background, has been previously described (15, 28). Briefly, slides were computer coded and randomized. The Grains Counting Program (University of Washington) then selected 45 of the most densely labeled clusters, each cluster approximating the size of a single cell, from the middle of a gonad in each individual (5 clusters, from 3 representative fields in each of 3 sections) and counted the silver grains in each cluster. Average grains per cluster were corrected for background in each individual by subtracting average grains per cluster measured in the adjacent kidney tissue. Using the JMP program, the corrected individual measures were used to calculate means and SEs and to perform two-way ANOVAs (Figs. 2-5). *Post-hoc* comparisons were made using the Dunn-Sidak method to provide a significance level of $\alpha' = 1 - (1 - 0.05)^{1/k} = 0.0012$, where k is the number of individual comparisons for an experimentwise $\alpha = 0.05$ (29).

Results

SF-1 expression differs at male- and female-producing incubation temperatures

The development of red-eared slider turtles has been divided into 26 stages (with hatching at stage 26) based on external features of the embryo (24). At 26 and 31 C incubation, the TSP begins at approximately stage 15, and bipotential gonads are present at that time. Developing testes can first be distinguished from ovaries in some individuals at about stage 18/19. Commitment to gonadal sex begins at stage 17 (seen in 3% of males and 20% of females), and sex is committed in all individuals by stage 21 at these temperatures (30). Continuous incubation of eggs at 26 C produces all male hatchlings, whereas incubation at 31 C produces all female hatchlings (Table 1) (30, 31). Using semiquantitative *in situ* hybridization, we compared SF-1 gonadal expression in embryos incubating at these temperatures from stages spanning the TSP and found distinct male and female patterns. SF-1 expression was significantly higher at the male- than the female-producing temperature in all stages examined (Fig. 2A). These data expand our earlier descriptive evidence for differential expression of SF-1 (23).

At stages 15 and 17, SF-1 messenger RNA (mRNA) appeared evenly dispersed in the bipotential gonads of embryos at both male- and female-producing temperatures. From stages 18–23, as early sex tubules proliferate in putative testes (30), message became localized in cells of the tubules and, to a lesser extent, the interstitial space (Fig. 2B). In putative ovaries during the same period, when immature sex cords regress and the cortical region proliferates, SF-1 expression became faint and uniform throughout (Fig. 2C). In some ovaries there appeared to be a slightly greater concentration of message in the cortical than in the medullary region. Results from sense probe hybridizations and other validation controls were negative. There was no apparent difference in SF-1 expression in adrenal glands at male- compared with female-producing temperatures or as a result of the treatments described below.

Gonadal SF-1 expression at an intermediate incubation temperature

In red-eared slider turtles, incubation between 28.6–29.6 C results in varying ratios of male to female hatchlings with no intersexes. As temperature increases within this range, the male/female sex ratio decreases. We incubated eggs at 29.4 C, a temperature that produces an approximately 20:80 female-biased hatchling ratio (Table 1) (9), and examined SF-1 expression at stages spanning the TSP. The resulting curve lies intermediate to the all male (26 C)- and all female (31 C)-producing temperature curves (Fig. 3A). We then sexed, by histological analysis, all stage 21 and 23 individuals from

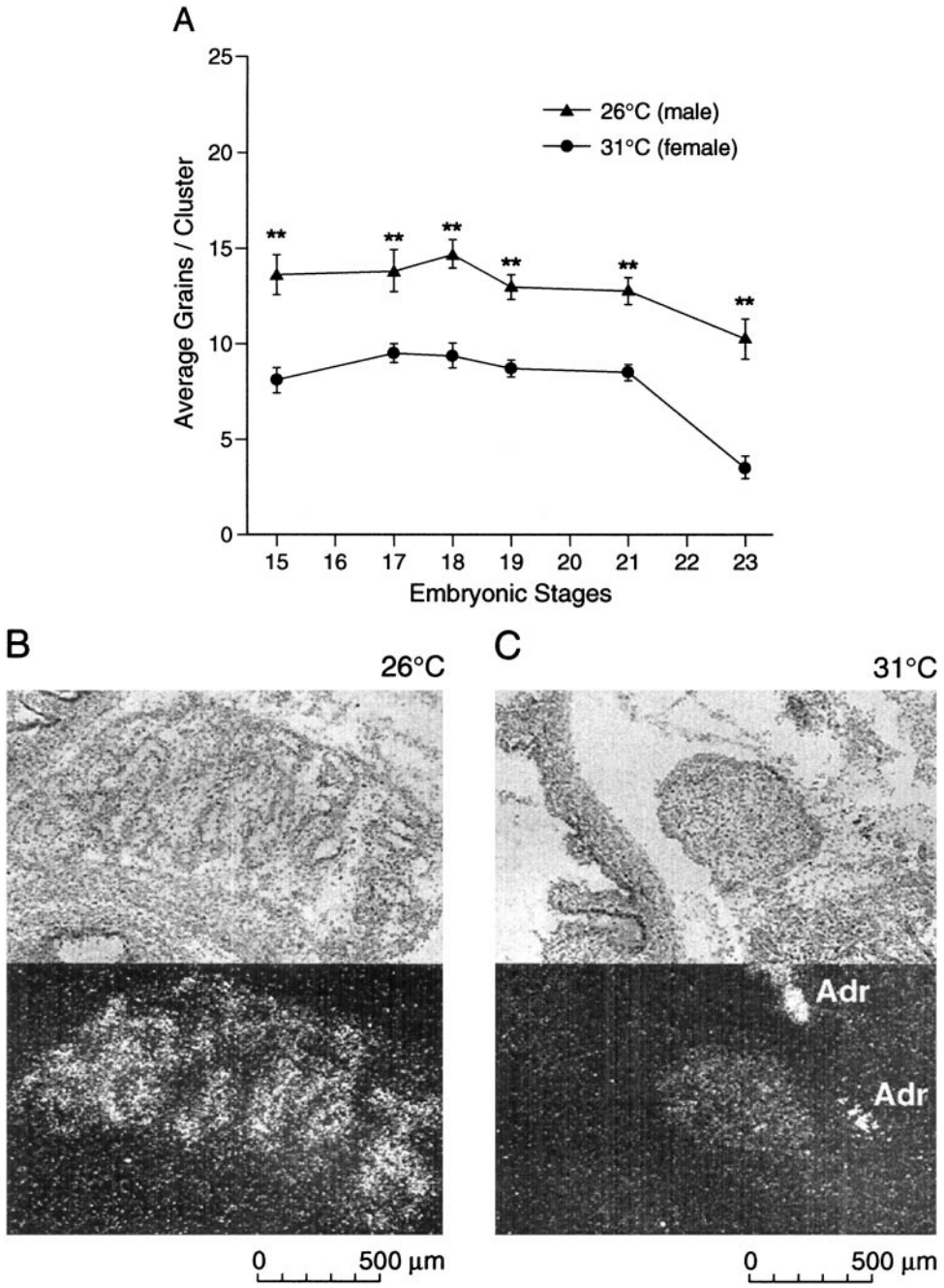

FIG. 2. SF-1 mRNA expression is higher in developing red-eared slider turtle gonads at male (26 C)- compared with female (31 C)-producing incubation temperatures. Quantitation of gonadal SF-1 expression (A) shows a significant difference in SF-1 expression between incubation temperatures at each developmental stage assayed. Stages span the temperature-sensitive period. Mean values and SEs are given; sample sizes are listed in Table 1. *, $P < 0.05$; **, $P < 0.0012$ (by α' test). Light-field and darkfield images of a post-TSP testis (B) at the male-producing temperature (note the predominant localization of SF-1 signal in the sex cords) and ovary (C) at the female-producing temperature. Adr, Adrenal tissue.

the 29.4 C incubation temperature and found three groups of individuals: those with developing testes or ovaries, and a third group that still appeared histologically bipotential. Of the sex-identified individuals, approximately 80% were female, and 20% were male, as hatching data would predict. High or low levels of SF-1 message corresponded to identified males or females, respectively (Fig. 3B). This pattern along with the corresponding distribution of message agree with the profiles of 26 C males *vs.* 31 C females described above. Intermediate levels of SF-1 message were associated with the histologically bipotential individuals at both stages 21 and 23. At the younger stages assayed, gonadal sex could not be distinguished histologically in most individuals (there is no established marker for gonadal sex in red-eared slider turtles), but effects of both sexes may be present in the SF-1 expression data of these stages as well. The intermediate position of the 29.4 C expression curve is, therefore, the result of pooled male and female effects and does not represent an intermediate, graded response by all individuals to the intermediate temperature.

Switching incubation temperatures during the temperature-sensitive period alters SF-1 expression

Although sensitivity to temperature begins at approximately stage 15 in red-eared slider turtles, gonadal sex is

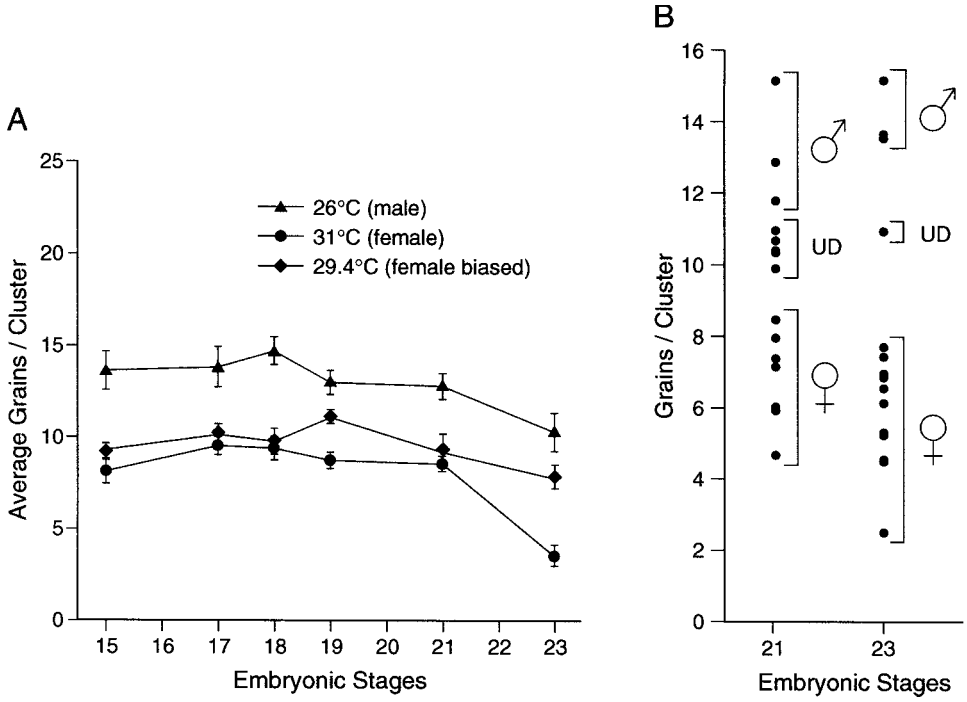

FIG. 3. Developmental SF-1 mRNA expression in red-eared slider turtle gonads at an intermediate, female-biased temperature (29.4 C). The curve for average SF-1 expression at 29.4 C (A) lies intermediate to curves produced at all male- and all female-producing temperatures. Mean values and SEs are given; sample sizes are listed in Table 1. The gonadal SF-1 expression value for each late stage 29.4 C individual (B) was plotted then correlated with its histologically identified sex, revealing that 29.4 C data are comprised of both male and female effects. ♂, testes; ♀, ovaries; UD, histologically bipotential.

not committed until later, and changing the incubation temperature midway through the TSP will still alter the hatchling sex ratio (30). To determine whether temperature shifts that change sex outcome also affect SF-1 expression, we performed two opposing shifts and analyzed SF-1 expression levels at four subsequent stages. When red-eared slider turtle eggs are shifted from a male (26 C)- to a female (31 C)-producing incubation temperature at stage 17, approximately 100% female hatchlings result (Table 1) (30). After such a shift, we found that gonadal SF-1 expression correspondingly changed from a male to a female pattern (Fig. 4A). One stage after treatment, gonadal SF-1 expression was already significantly lower in the shifted embryos than in 26 C male controls. There was no significant difference between shifted embryos and 31 C female controls at any stage assayed. Distribution of SF-1 mRNA within gonads also followed the female pattern (Fig. 4B compared with Fig. 2C).

Eggs shifted from a female (31 C)- to a male (26 C)-producing incubation temperature at stage 17 result in approximately 80% male hatchlings (Table 1), as commitment to female gonadal sex is already seen in approximately 20% of individuals by this stage (30). With this shift, average gonadal SF-1 expression was significantly higher in shifted embryos than in 31 C female controls by one stage after treatment (Fig. 4A). Shifted values initially surpassed those of 26 C male controls as well, but by stages 21 and 23 they had declined and were statistically the same as the male controls. All stage 21 and 23 individuals assayed were sexed histologically, and an approximately 80:20 male-biased ratio was found, as predicted by hatching data. SF-1 expression was high in identified males and low in identified females (data not shown).

Estrogen treatment down-regulates SF-1 expression to the female pattern

When red-eared slider turtle eggs incubating continuously at an all male-producing temperature are treated with E_2 during the TSP, the sex-determining effect of temperature is overridden, and 100% female hatchlings result (Table 1) (9). To determine whether this treatment alters SF-1 expression as well, we spotted eggs incubating at 26 C with E_2 at stage 17 and assayed four subsequent developmental stages for SF-1 message. One stage after treatment and in all other stages examined, the level of gonadal SF-1 expression was significantly lower in E_2-treated embryos compared with 26 C male controls (Fig. 5A). The effect of treatment between E_2-treated embryos and 31 C female controls was not significant, and distribution of message was equivalent in these two groups (Fig. 5B compared with Fig. 2C). No significant effect of vehicle alone was found on SF-1 expression or hatchling sex ratios (data not shown).

Treatment with aromatase inhibitor up-regulates SF-1 expression to the male pattern

When the biosynthesis of estrogen is blocked in red-eared slider turtles by treatment with AI during the TSP, the female-biased sex-determining effects of an intermediate incubation temperature are overridden, and 100% male hatchlings result (Table 1) (9). To determine whether this treatment also alters SF-1 expression, at stage 17 we spotted eggs incubating at 29.4 C with a nonsteroidal AI at the minimum dosage required to produce all males at this temperature. We found that inhibition of estrogen biosynthesis directly or indirectly caused an up-regulation of SF-1 expression (Fig. 5A) and the male-like pattern of distribution (Fig. 5C compared with Fig. 2B). A significant increase over 29.4 C con-

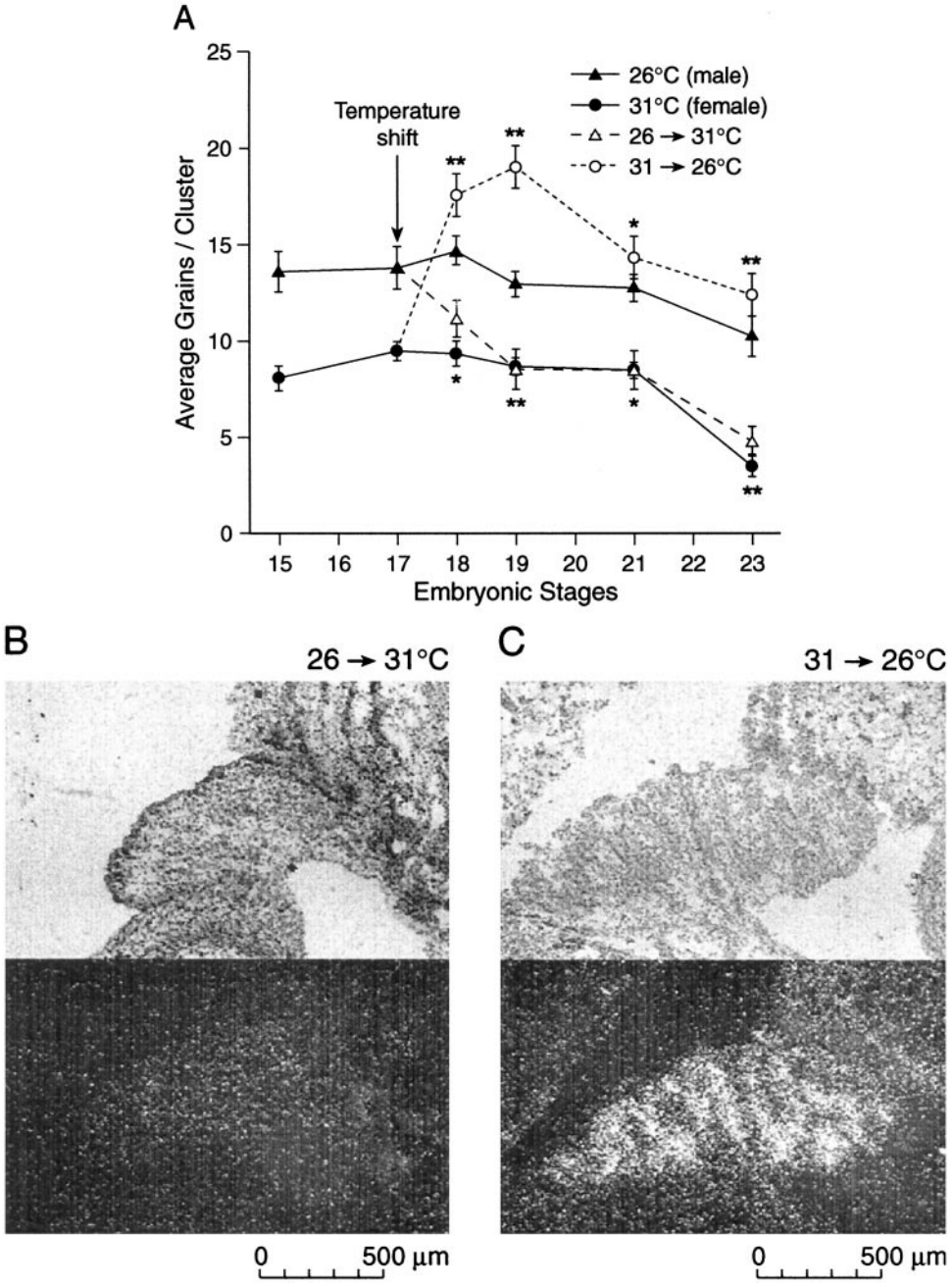

FIG. 4. SF-1 mRNA expression in developing red-eared slider turtle gonads is altered by shifting incubation temperatures during the TSP. Quantitation of gonadal SF-1 expression (A) for each temperature shift compared with controls that were continuously incubated at male- or female-producing temperatures. Mean values and SEs are given; sample sizes are listed in Table 1. *, $P < 0.05$; **, $P < 0.0012$ (by α' test). Light-field and darkfield images of post-TSP gonads after a male to female temperature shift (B) and a female to male temperature shift (C).

trols occurred, but not until three stages after treatment. At stages 19, 21, and 23, SF-1 gonadal expression in AI-treated males was statistically the same as that in 26 C males and showed no similarity to that in 31 C females. No significant effect of carrier alone was found on SF-1 expression or hatchling sex ratios (data not shown).

Discussion

SF-1 mRNA expression was examined here in developing gonads of a vertebrate species with TSD, the red-eared slider turtle. SF-1 message was assayed in embryos continuously incubated at one of three temperatures to establish naturally occurring expression patterns and to suggest transcriptional functions of SF-1 in a TSD model. SF-1 expression was then examined in embryos that had been manipulated by temperature or estrogen. Using the model system in this way allowed us to establish SF-1 expression relative to temperature and estrogen cues, which are both apparently involved in the TSD regulatory hierarchy (1), in a wild-type organism and begin understanding modulation of SF-1 expression in TSD.

Gonadal SF-1 expression was first examined in red-eared slider turtle embryos that were continuously incubated at an all male-producing (26 C), an all female-producing (31 C), or an intermediate (29.4 C) temperature. SF-1 message was significantly higher in 26 C males than in 31 C females. This difference was observed from the earliest stage examined, stage 15, which is during the indifferent phase of gonadal development and

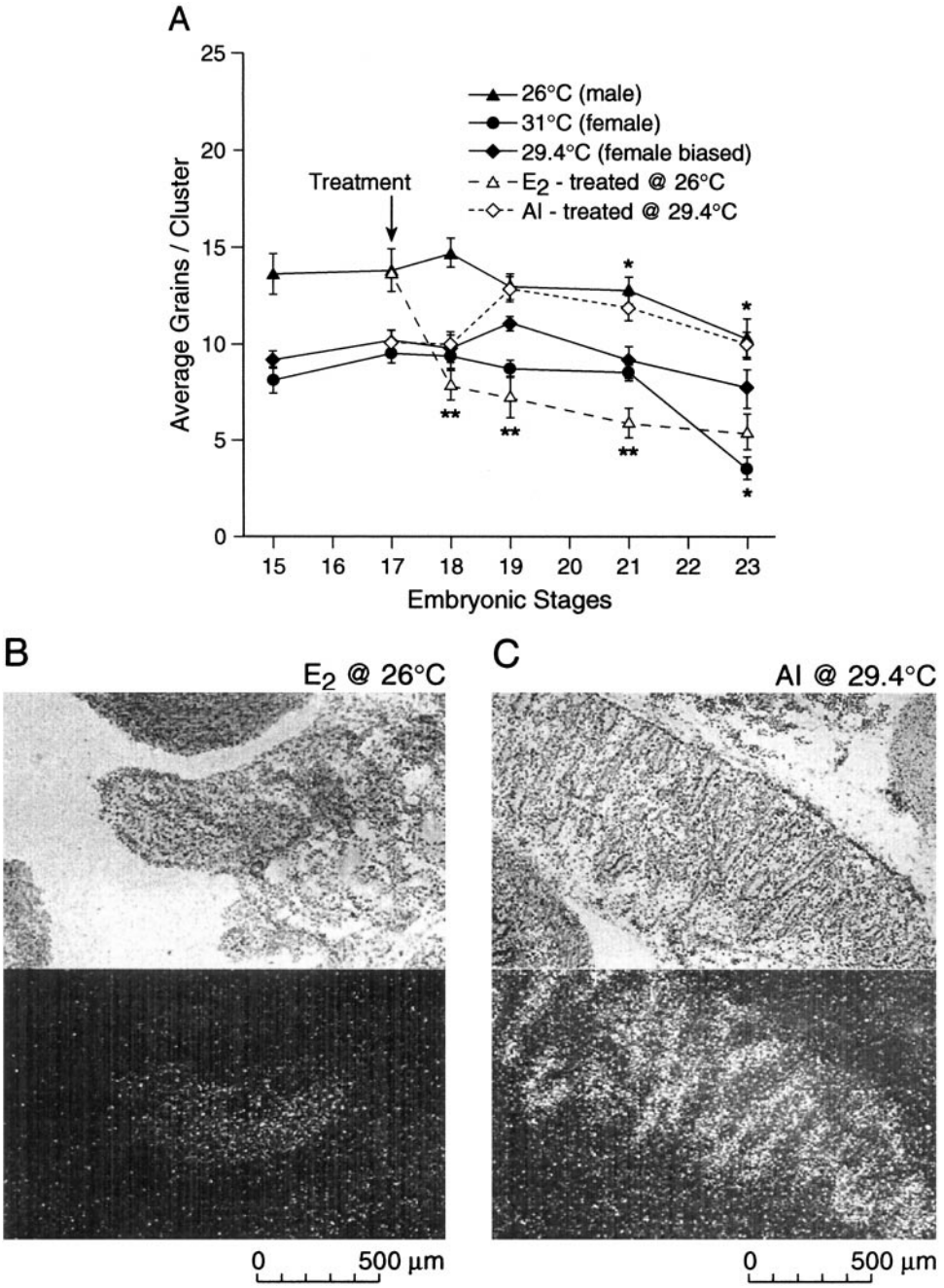

FIG. 5. SF-1 mRNA expression in developing red-eared slider turtle gonads is modulated after treatment during the TSP with E_2 at a male-producing temperature or with AI at a female-biased temperature. Quantitation of gonadal SF-1 expression (A) for each treatment compared with controls that were continuously incubated at the male-producing or female-biased temperature, respectively. The curve for SF-1 expression at a female-producing temperature is included for comparison. Mean values and SEs are given; sample sizes are listed in Table 1. *, $P < 0.05$; **, $P < 0.0012$ (by α' test). Lightfield and darkfield images of post-TSP gonads after treatment with E_2 at a male-producing incubation temperature (B) and with AI at a female-biased temperature (C).

marks the approximate beginning of the TSP (30). Morphological distinction of testes from ovaries cannot be detected in red-eared slider turtles until three or four developmental stages later (approximately 10.5–14 days at 26 C and 3–4 days at 31 C). In mammals, SF-1 is expressed without sex bias in males and females during the indifferent phase and may participate in the retention of primary steroidogenic tissue during this time (18). Although a similar function could be served by SF-1 in red-eared slider turtles, it is not clear why its expression appears sex biased at this time.

After the indifferent phase, SF-1 expression is higher in differentiating testes than ovaries in both mammals (18, 19) and red-eared slider turtles, which may indicate homologous SF-1 function. In developing mammalian testes, SF-1 functions include regulation of Mullerian inhibiting substance (MIS), which causes regression of the Mullerian ducts. This gene regulation is quantitative; increased levels of SF-1 cause increased MIS expression (32), although the high physiological level of MIS appears unnecessary to effect known functions in mammals. Interestingly, high levels of MIS, which can inhibit expression of aromatase (33), may be critical in developing TSD males, where exogenous estrogen causes female development at male-producing incubation temperatures (9). MIS has been cloned in red-eared slider turtles and is expressed developmentally in the adrenal-kidney-gonad complex of putative males at a time comparable to its expression in mammals (14); SF-1 message is predominantly localized in the sex cords, the site of MIS expression in vertebrates.

Transcriptional targets of SF-1 during differentiation of ovaries have not been identified in mammals. In at least some adult tissues, SF-1 regulates aromatase expression (18, 19), and there is evidence that a lower level of SF-1 may be sufficient to regulate aromatase compared with other P450 steroid hydroxylases (34). In TSD turtles, gonadal aromatase activity has been measured during the TSP (2), when it is thought to catalyze the synthesis of estrogens involved in female sex development. SF-1 expression in red-eared slider turtles could regulate aromatase expression during ovarian differentiation.

In red-eared slider turtle embryos that were continuously incubated at the intermediate temperature of 29.4 C, which typically produces about 20% male and 80% female hatchlings (9), gonadal SF-1 expression in most individuals became statistically comparable over developmental time to either 26 C males or 31 C females. Data from this temperature group indicated a close temporal association between differential SF-1 expression and gonadal dimorphism, suggesting a threshold effect of SF-1. An XY human has recently been identified with streak-like gonads containing immature tubules, with consequent male to female secondary sex reversal, attributed to decreased levels of functional SF-1 protein (35). Although the formation of sex tubules in mammalian testes is initiated by SRY (36), continued maturation may require a male threshold level of SF-1. In red-eared slider turtles, immature sex tubules are present during the middle of the TSP at both male- and female-producing incubation temperatures (30). A male threshold level of SF-1 in testes may be important in subsequent sex tubule maturation in this species as well.

Having established a baseline for SF-1 expression in red-eared slider turtles during incubation at continuous temperatures, we then examined whether altering the course of gonadal sex outcome during development altered SF-1 expression as well. Sex outcome was first manipulated by changing the incubation temperature in the middle of the TSP. When embryos are shifted from an all male (26 C)- to an all female (31 C)-producing incubation temperature, they become female (30). Here, such a shift resulted in down-regulation of gonadal SF-1 expression to a female level, and the pattern of change suggested either a drop to basal expression or active repression of SF-l expression. On the other hand, when embryos are treated by a female to male (31 to 26 C) temperature shift, approximately 80% become male (30). The remaining 20% appear already committed to develop as females despite the change in temperature cue. After this treatment, the mean level of SF-1 expression increased rapidly, initially surpassing even the 26 C male controls before falling back to that level, suggesting at least a two-phase regulation. Within this group, however, those embryos that could be histologically identified as female had equivalent SF-1 expression as 31 C female controls. Prior commitment to ovarian development appeared to disallow modulation of SF-1 expression regardless of the change in incubation temperature. Taken together, data from temperature treatments indicate that SF-1 expression is directly or indirectly modulated, at least before commitment, by the temperature trigger, that it lies in the TSD molecular path, and that its sex-specific levels are tightly controlled.

Sex outcome was next manipulated, and resulting SF-1 expression assayed, by altering the estrogen content of red-eared slider turtle eggs. When embryos incubating at an all male-producing temperature are treated with E_2, they develop as females (9). Here, after such a treatment, gonadal SF-1 expression was down-regulated, becoming statistically and histologically indistinguishable from that in 31 C female controls. On the other hand, when embryos incubating at a female-biased temperature are treated with AI to block biosynthesis of estrogen, they develop as males (9). After this treatment, SF-1 message increased to that of 26 C male controls, but this effect was delayed compared with temperature shift and E_2 treatments. Although AI inhibits new synthesis of estrogen, preexisting endogenous estrogen presumably remains active for some time and may account for this delay. Our experimental design does not allow conclusions about how direct the effect of estrogen is on SF-1 expression, as the first posttreatment measurement was taken one developmental stage (3 days) after application of E_2. Two lines of evidence suggest, however, that changes in SF-1 regulation were not simply an aftereffect of ovary formation. First, treatments were performed just as sex-specific gonadal development begins. Gonadal sex was still not clear in most individuals assayed one stage after treatment, but SF-1 expression had already changed significantly. Secondly, as noted above, a highly significant difference in SF-1 expression was found between 26 C males and 31 C females as early as three or four developmental stages before morphological or histological distinction of gonadal sex. Interestingly, treating pregnant rats with the synthetic estrogen diethylstilbestrol causes decreased levels of SF-1 in genotypic male embryos (37), although complete sex reversal has not been reported with this treatment in mammals.

Although direct measures of endogenous estrogen in TSD organisms have been conflicting or inconclusive, a large body of indirect evidence indicates that a critical estrogen effect lies downstream from the temperature cue in primary female TSD (1, 2, 13, 14). Our data provide support at a molecular level for this model. Estrogen and AI treatments that override temperature cues modulate SF-1 expression. A female-producing temperature and exogenous E_2 both result in low levels of SF-1 message; a male-producing temperature and decreased endogenous estrogen (AI treatment) both result in high levels of SF-1 expression. Finally, the level of SF-1 mRNA in developing red-eared slider turtle adrenal glands does not appear to be sex specific (23) and is unaffected by any of these treatments (Fleming, A., and D. Crews, unpublished data). Estrogen receptor is expressed in developing gonads, but not in adrenal tissue (15).

Taken together, the findings of this study suggest that at female-producing temperatures endogenous estrogen modulates the down-regulation of gonadal SF-1 early in the TSP. Yet a female-specific increase in gonadal aromatase activity has not been detected until midway through (2) or after (38) the TSP in any TSD species, indicating that estrogen biosynthesis may initially be extragonadal. Evidence for early estrogen synthesis has been found in the brain of the olive Ridley sea turtle and the diamondback terrapin (39, 40). Alternatively, the initial source of steroid hormones may be maternal and found in the yolk (41). Finally, it is possible that the differential endogenous expression of gonadal SF-1 is initiated by ligand-independent estrogen receptor or by

other factors altogether and is maintained by an estrogen-involved mechanism of feedback on SF-1 in the gonad.

Curiously, in alligators (which are also TSD reptiles) and chickens, the pattern of SF-1 gonadal expression is reversed during differentiation: expression is higher in females than in males (21, 22). The effect, if any, of exogenous E_2 on SF-1 expression has not been described in either group. E_2 treatment of genotypic male chicken embryos results in up-regulation of aromatase and down-regulation of MIS, whereas AI treatment of genotypic females has the opposite affect (42). We would predict similar results in red-eared slider turtles, with SF-1 at the center of this regulation, but it is unclear how reversed endogenous levels of SF-1 in red-eared slider turtles compared with alligators and chickens might result in similar regulation of both aromatase and MIS. Although considerable conservation of genes and their function in gonadal sex development exists among amniotes, a surprising number of evolutionary changes in regulation of these genes, as seen here in the regulation of SF-1, is becoming apparent.

The expression data presented here indicate that the functions of SF-1 during gonadogenesis may be largely conserved between red-eared slider turtles and mammals. Moreover, regulation of SF-1 in red-eared slider turtles is sex-specifically modulated by both the incubation temperature and the estrogen content of the developing egg. These data provide support at a molecular level for a critical endogenous effect of estrogen after a female temperature cue and indicate that SF-1, in turn, is one of the genes directly or indirectly affected by estrogen in female TSD. Finally, the relative amount of SF-1 mRNA expressed, high in developing testes compared with ovaries, appears pivotal within TSD and may indicate a threshold mechanism involving SF-1.

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References

1. **Crews D** 1996 Temperature-dependent sex determination: the interplay of steroid hormones and temperature. Zool Sci 13:1–13
2. **Pieau C, Dorizzi M, Richard-Mercier N** 1999 Temperature-dependent sex determination and gonadal differentiation in reptiles. Cell Mol Life Sci 55:887–900
3. **Greco TL, Payne AH** 1994 Ontogeny of expression of the genes for steroidogenic enzymes P450 side-chain cleavage, 3β-hydroxysteroid dehydrogenase, P450 17α-hydroxylase/C_{17-20} lyase, and P450 aromatase in fetal mouse gonads. Endocrinology 135:262–268
4. **Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O** 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad Sci USA 90:11162–11166
5. **Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ, Korach KS** 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors α and β. Science 286:2328–2331
6. **Renfree M** 1994 Gonadal and genital differentiation. In: Short R, Balaban E (eds) The Differences between the Sexes. Cambridge University Press, New York, pp 222–225
7. **Elbrecht A, Smith RG** 1992 Aromatase enzyme activity and sex determination in chickens. Science 255:467–470
8. **Scheib D** 1983 Effects and role of estrogens in avian gonadal differentiation. Differentiation 23:S87–S92
9. **Crews D, Bergeron JM, Flores D, Bull JJ, Skipper JK, Tousignant A, Wibbels T** 1994 Temperature-dependent sex determination in reptiles: proximate mechanisms, ultimate outcomes, and practical applications. Dev Gen 15:297–312
10. **Bull JJ** 1980 Sex determination in reptiles. Q Rev Biol 55:3–21
11. **Coriat AM, Muller U, Harry J, Uwanogho D, Sharpe P** 1993 PCR amplification of SRY-related gene sequences reveals evolutionary conservation of the SRY-box motif. PCR Methods Appl 2:218–222
12. **Spotila JR, Spotila LD, Kaufer NF** 1994 Molecular mechanisms of TSD in reptiles: a search for the magic bullet. J Exp Zool 270:117–127
13. **Lance V** 1997 Sex determination in reptiles: an update. Am Zool 37:504–513
14. **Wibbels T, Cowan J, LeBoeuf R** 1998 Temperature-dependent sex determination in the red-eared slider turtle, *Trachemys scripta*. J Exp Zool 281:409–416
15. **Bergeron JM, Gahr M, Horan K, Wibbels T, Crews D** 1998 Cloning and *in situ* hybridization analysis of estrogen receptor in the developing gonad of the red-eared slider turtle, a species with temperature-dependent sex determination. Dev Growth Differ 40:243–254
16. **Lala DS, Rice DA, Parker KL** 1992 Steroidogenic factor 1, a key regulator of steroidogenic enzyme expression, is the mouse homolog of fushi tarazu-factor 1. Mol Endocrinol 6:1249–1258
17. **Morohashi K, Honda S, Inomata Y, Handa H, Omura T** 1992 A common *trans*-acting factor, Ad4-binding protein, to the promoters of steroidogenic P-450s. J Biol Chem 267:17913–17919
18. **Parker KL, Schimmer BP** 1997 Steroidogenic factor 1: a key determinant of endocrine development and function. Endocr Rev 18:361–377
19. **Morohashi K-i** 1999 Gonadal and extragonadal function of Ad4BP/SF-1: developmental aspects. Trends Endocrinol Metab 10:169–173
20. **Kudo T, Sutou S** 1997 Molecular cloning of chicken FTZ-F1-related orphan receptors. Gene 197:261–268
21. **Smith CA, Smith MJ, Sinclair AH** 1999 Expression of chicken steroidogenic factor-1 during gonadal sex differentiation. Gen Comp Endocrinol 113:187–196
22. **Western PS, Harry JL, Marshall Graves JA, Sinclair AH** 2000 Temperature-dependent sex determination in the American alligator: expression of SF-1, WT1 and DAX1 during gonadogenesis. Gene 241:223–232
23. **Fleming A, Wibbels T, Skipper JK, Crews D** 1999 Developmental expression of steroidogenic factor 1 in a turtle with temperature-dependent sex determination. Gen Comp Endocrinol 116:336–346
24. **Yntema CL** 1968 A series of stages in the embryonic development of *Chelydra serpentina*. J Morphol 125:219–252
25. **Ninomiya Y, Okada M, Kotomura N, Suzuki K, Tsukiyama T, Niwa O** 1995 Genomic organization and isoforms of the mouse ELP gene. J Biochem 118:380–389
26. **Sambrook J, Fritsch EF, Maniatis T** 1989 Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
27. **Young LJ, Lopreato GF, Horan K, Crews D** 1994 Cloning and *in situ* hybridization analysis of estrogen receptor, progesterone receptor, and androgen receptor expression in the brain of whiptail lizards *Cnemidophorus uniparens* and *C. inornatus*. J Comp Neurol 347:288–300
28. **Wennstrom KL, Crews D** 1998 Effect of long-term castration and long-term androgen treatment on sexually dimorphic estrogen-inducible progesterone receptor mRNA levels in the ventromedial hypothalamus of whiptail lizards. Horm Behav 34:11–16
29. **Sokal RR, Rohlf FJ** 1981 Biometry, Ed 2. Freeman, San Francisco
30. **Wibbels T, Bull JJ, Crews D** 1991 Chronology and morphology of temperature-dependent sex determination. J Exp Zool 260:371–381
31. **Bull JJ, Wibbels T, Crews D** 1990 Sex determining potencies vary among female incubation temperatures in a turtle. J Exp Zool 256:339–341
32. **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
33. **Rouiller-Fabre V, Carmona S, Merhi RA, Cate R, Habert R, Vigier B** 1998 Effect of anti-Müllerian hormone on Sertoli and Leydig cell functions in fetal and immature rats. Endocrinology 139:1213–1220
34. **Omura T, Morohashi K-i** 1995 Gene regulation of steroidogenesis. J Steroid Biochem Mol Biol 53:19–25
35. **Achermann JC, Ito M, Ito M, Hindmarsh PC, Jameson JL** 1999 A mutation in the gene encoding steroidogenic factor-1 causes XY sex reversal and adrenal failure in humans. Nat Genet 22:125–126
36. **Koopman P, Gubbay J, Vivian N, Goodfellow P, Lovell-Badge R** 1991 Male development of chromosomally female mice transgenic for Sry. Nature 351:117–121
37. **Majdic G, Sharpe RM, Saunders PTK** 1997 Maternal oestrogen/xenoestrogen exposure alters expression of steroidogenic factor-1 SF-1/Ad4BP in the fetal rat testis. Mol Cell Endocrinol 127:91–98
38. **Smith CA, Elf PK, Lang JW, Joss JMP** 1995 Aromatase enzyme activity during gonadal sex differentiation in alligator embryos. Differentiation 58:281–290
39. **Merchant-Larios H** 1998 Brain as a sensor of temperature during sex determination in the sea turtle *Lepidochelys olivacea*. J Exp Zool 281:510
40. **Jeyasuria P, Place A** 1998 Embryonic brain-gonadal axis in temperature-dependent sex determination of reptiles: a role for P450 aromatase CYP19. J Exp Zool 281:428–449
41. **Conley AJ, Elf P, Corbin CJ, Dubowsky S, Fivizzani A, Lang JW** 1997 Yolk steroids decline during sexual differentiation in the alligator. Gen Comp Endocrinol 107:191–200
42. **Nishikimi H, Kansaku N, Saito N, Usami M, Ohno Y, Shimada K** 2000 Mol Reprod Dev 55:20–30