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Role of Steroidogenic Factor 1 and Aromatase in Temperature-Dependent Sex Determination in the Red-Eared Slider Turtle

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ABSTRACT Red-eared slider turtles are genetically bipotential for sex determination. In this species, as in many other reptiles, incubation temperature of the egg determines gonadal sex. At higher incubation temperatures females are produced and increasing temperature appears to increase estrogen production in the embryonic brain. Treatment of eggs incubating at a male-producing temperature with exogenous estrogen causes ovaries to form. At a female-biased incubation temperature, prevention of estrogen biosynthesis or administration of nonaromatizable androgens results in the development of testes. In mammals, steroidogenic factor 1 (SF-1) regulates most genes required for estrogen biosynthesis, including aromatase. In both mammals and red-eared sliders, SF-1 is differentially expressed in males and females during gonadogenesis. We have examined both SF-1 gene expression and aromatase activity in embryos incubating at different temperatures and after manipulation to change the course of gonadal development. Our findings indicate a central role for SF-1 in enacting the effect of estrogen. Estrogen treatment directly or indirectly downregulates SF-1 and, ultimately, causes development of females. The inhibition of estrogen results in upregulation of SF-1 and male hatchlings. Thus, SF-1 may lie at the center of one molecular crossroad in male versus female differentiation of the red-eared slider. *J. Exp. Zool. 290:597–606, 2001.*

Sex steroid hormones appear to play a role in the differentiation of the gonad in vertebrates. In species with genetic sex determination, modulators of sex hormones can override genetic influences to redirect gonadal differentiation. When exposed to aromatase inhibitor early in development, genotypic female chickens develop testes rather than ovaries (Elbrecht and Smith, '92; Vaillant et al., 2001; Smith and Sinclair, 2001). Administration of exogenous estrogen during development in marsupial mammals will cause genetic males to develop ovaries (Burns, '61; Shaw et al., '88; Pask and Renfree, 2001). In female mice with a targeted disruption of the estrogen receptors α and β, the ovary in adulthood transforms into a testicular-like structure containing seminiferous tubules and Sertoli-like cells as well as expression of Müllerian inhibiting substance and Sox9 (Couse et al., '99). Transplantation of XX genital ridge tissue into adult male rats will result in the differentiated tissue having all cell types characteristic of a testis (Taketo et al., '84). Administration of exogenous steroids to vertebrates lacking sex chromosomes can cause complete and functional sex reversal in many fish, amphibians (Hayes, '98), and reptiles (Crews et al., '88; Govoroun et al., 2001; Chang et al., 2001).

It is evident that the downstream events in the differentiation of the gonad are similar. With the exception of SRY, genes thus far implicated in the primary sex development of mammals have now also been identified in other vertebrates. Steroidogenic factor 1 (SF-1, a.k.a. Ad4BP) is a transactivator of most enzymes involved in the biosynthesis of steroid hormones, including sex steroids (Morohashi and Omura, '96). Analysis indicates that SF-1 is expressed at the earliest stages of urogenital ridge development; disruption

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of the gene encoding SF-1 results in newborns that lack adrenal glands and gonads (Lala et al., '92; Morohashi et al., '92; Ikeda et al., '94; Luo et al., '94a; Shen et al., '94). Both male and female embryos express SF-1; shortly after differentiation of the Sertoli cells and formation of testicular cords, SF-1 expression persists in males but diminishes in females (Luo et al., '94b). In addition to being critical to sex steroid biosynthesis, SF-1, along with SOX9, upregulates expression of Müllerian inhibiting substance (MIS) in Sertoli cells of developing testes (Arango et al., '99) and the MIS receptor in testes and Müllerian ducts (de Santa Barbara et al., '98). MIS in turn appears to downregulate aromatase gene expression (DiClemente et al., '92; Rouiller-Fabre et al., '98). SF-1 is encoded by the Ftz-F1 gene, a homolog of the *Drosophila* Ftz-F1 gene, and study of mice with a targeted disruption of Ftz-F1 reveals abnormal function of pituitary gonadotropes (Morohashi, '99) as well as a malformed ventromedial hypothalamus (Luo et al., '94a; Shinoda et al., '95). A brain-specific transcript of aromatase has been detected in rats, and the gene encoding it contains a consensus SF-1 binding site (Honda et al., '93). In reptiles and birds, it is thought that SF-1 might regulate aromatase, hence synthesis of ovary-determining estrogen, in developing ovaries (Fleming et al., '99; Smith et al., '99a; Western et al., 2000).

RED-EARED SLIDER TURTLE

It is not possible in mammalian in vivo preparations to modulate the differential expression of SF-1 and aromatase and hence determine their function in gonadal differentiation. Here the utility of species lacking sex chromosomes is evident. There are at least three advantages of an animal model system having temperature-dependent sex determination (TSD) for the study of sex determination in vertebrates. First, the developmental timing of sex determining genes has been identified with simple temperature-shift experiments. Second, merely changing the incubation temperature can vary the strength of the sex-determining signal. And third, because these reptiles lay eggs, the embryo can be manipulated in ways not feasible in viviparous species.

In the red-eared slider turtle (*Trachemys scripta elegans*), gonadal sex is determined by the temperature experienced during embryogenesis, a process known as TSD. The pattern of TSD in the red-eared slider is one of low temperatures (e.g., 26°C) producing males and higher temperatures (e.g., 31°C) producing females. Mid-range temperatures (28.6–29.4°C) produce mixed-sex ratios, with increasing temperature increasing the fraction of females. Incubation temperature exerts its effect only during the mid-trimester of development. Thus, small changes in temperature acting during a narrow window completely switch the path of sex determination.

In the red-eared slider, the physical stimulus of temperature is transduced into an endocrine signal that directs gonadal and sexual development; specifically, estrogens and their aromatizable precursors stimulate female development in the absence of an activated male developmental pathway, whereas nonaromatizable androgens stimulate the male-determining cascade as the female cascade remains inactive (Crews et al., '94; Crews, '96). A synergy exists between incubation temperature and estrogen (Bull et al., '90; Wibbels et al., '91). A retrospective analysis of the published data on the effects of varying doses of estradiol-17β (E2) at different incubation temperatures in directing female sex determination reveals that the Michaelis-Menten equation fit all dose-response data, suggesting that both incubation temperature and estrogen interact with a single protein (estrogen receptor, or ER) driving a reversible process (Sheehan et al., '99). Treatment with inhibitors of aromatase or reductase will cause males and females to develop at otherwise female- and male-producing temperatures, respectively. It is important to note that this is an "all-or-none" effect and hermaphrodites are not produced (Wibbels et al., '91; Crews and Bergeron, '94). Finally, simultaneous administration of dihydrotestosterone and E2 to eggs incubating at an intermediate temperature results in ovotestes (Crews et al., '94), a condition never found in nature. Taken together, these results suggest that the proximate trigger for gonadal differentiation involves the differential gene activation pathways for sex steroid hormones.

Many of the genes identified in the pathway leading to gonadogenesis in mammals have also been found in TSD reptiles. For example, DMRT1 has been identified in mice, chickens, and the American alligator (*Alligator mississippiensis*), a species with TSD. In all of these species, DMRT1 exhibits sex-dependent expression whereby it is significantly higher in the developing testes of all three species at a crucial timepoint in differentiation (Smith et al., '99a). An additional link between mammals and TSD reptiles is SOX9 (Moreno-Mendoza et al., 2001; Torres-Maldonado et al., 2001) and SF-1.

ROLE OF SF-1 IN SEX DETERMINATION

In mammals, SF-1 expression becomes differential in developing gonads just as testes and ovaries become distinguishable (Ikeda et al., '93; Hatano et al., '94). At embryonic day 12.5 in mice, expression is high in males and very low in females. This difference continues, coincident with rapid testicular differentiation, until late in gonadal development (Ikeda et al., '94). The pattern of SF-1 expression in chicken and alligator following histological distinction of gonadal sex differs from that in mammals. SF-1 levels become less abundant in the genetically- or temperature-determined male than female as gonadal sex becomes distinct in chicken (Smith et al., '99b) and alligator (Western et al., 2000), respectively. In chicken, SF-1 message expression falls to an almost negligible level in males while remaining high in females, correlating with the pattern of aromatase expression in chickens (Andrews et al., '97; Smith et al., '97). Aromatase is upregulated by SF-1 in mammalian granulosa cells (Carlone and Richards, '97), where it converts testosterone to estrogen.

Expression of SF-1 mRNA in the red-eared slider is equivalent at male- and female-producing temperatures in the early (stage 14) gonadal ridge. At this stage, gonads from the two incubation temperatures are bipotential and histologically indistinguishable. As the gonads becomes histologically distinct, the pattern of SF-1 expression changes, continuing to increase at the male-producing incubation temperature but declining at the female-producing incubation temperature (Fig. 1). SF-1 mRNA is also evident in all stages examined in both adrenal and brain, and is expressed equally at both male- and female-producing incubation temperatures. The distribution of SF-1 within the gonad also indicates a sex-based difference developing over time (see Fig. 4). In stages 14–16, SF-1 appears to be distributed evenly throughout the bipotential gonad. During the middle and end of the TSP (stages 17–19), SF-1 signal becomes striated in presumptive testes. By stage 23, well after the TSP, the difference in SF-1 mRNA distribution is dramatic. Signal in testes is markedly striated and the most abundant signal lies inside the developing sex cords (see Fig. 4). A lower level of expression is seen in surrounding interstitial space. By contrast, signal in ovaries is close to background (as measured in kidney) and largely vacuolated.

It is significant that in embryos continuously incubated at an intermediate, female-biased temperature (29.4°C), SF-1 expression is higher in individuals with histologically identifiable testes compared to ovaries, similar to expression patterns found in 26°C males compared to 31°C females, respectively. It is important to remind the reader that at these intermediate temperatures, hermaphrodites are not produced. Further, in those individuals in which gonadal sex was still histologically indistinguishable late in, and even after, the TSP (stages 21 and 23, respectively), the level of SF-1 was intermediate— neither male nor female (Fig. 2). One explanation for these characteristics is a threshold effect at the molecular level as initiated by temperature. These data indicate a close temporal association between differential SF-1 expression and gonadal dimor-

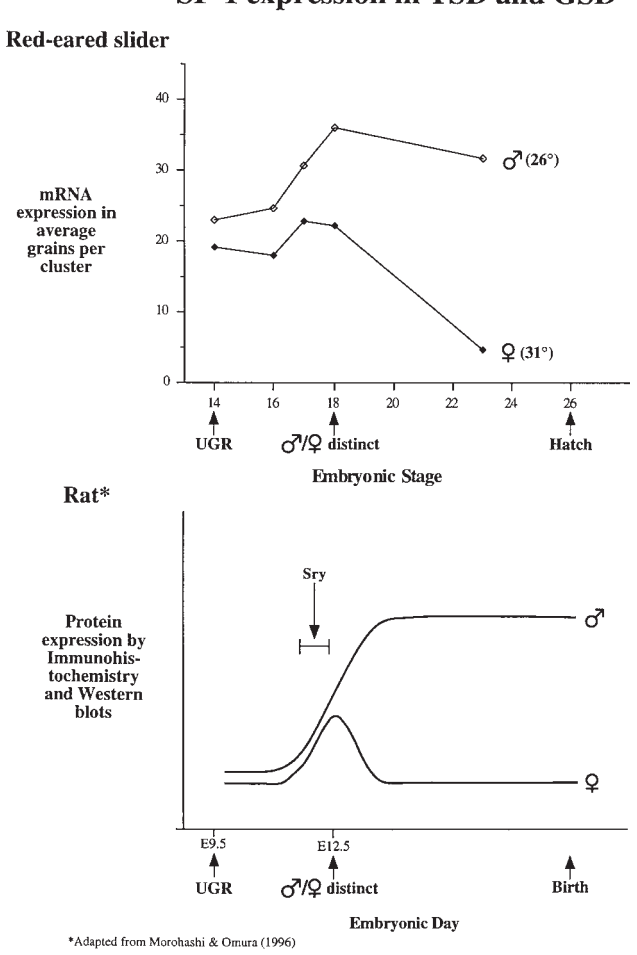

Fig. 1. Comparison of the expression of steroidogenic factor 1 (SF-1) mRNA throughout embryogenesis in the red-eared slider turtle (*Trachemys scripta elegans*) at male-producing (26°C) and female-producing (31°C) incubation temperatures (after Fleming et al., '99) and amount of SF-1 protein during gonadogenesis in the rat (after Morohashi and Omura, '96). The approximate timing of the developmental distinction between ovary and testis defined by gray area.

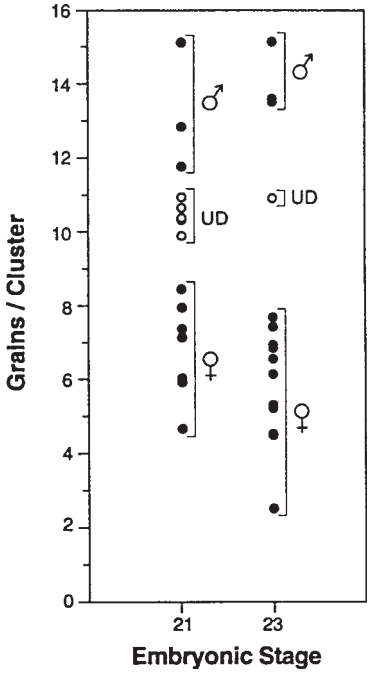

Fig. 2. In the red-eared slider turtle (*Trachemys scripta elegans*), incubation at an intermediate temperature (29.4°C) that produces a mixed sex ratio results in variation in SF-1 mRNA. This suggests a dosage effect of SF-1. Data points represent SF-1 expression values for individuals from stages 21 and 23 (late and post temperature-sensitive period). Redrawn from Fleming and Crews, 2001.

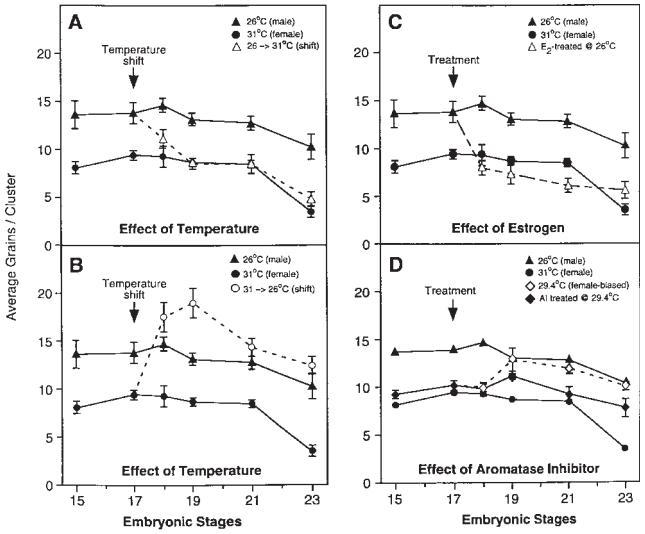

Fig. 3. Modulation of the pattern of expression of SF-1 mRNA throughout embryogenesis in the red-eared slider turtle (*Trachemys scripta elegans*). SF-1 expression is significantly higher in gonads of embryos incubating at a temperature that produces all males (26°C) compared to one that produces all females (31°C). Shifting eggs from the male- to the female-producing incubation temperature in the middle of the TSP causes downregulation of SF-1 to the female level. (**A**) Shifting eggs from the female- to the male-producing incubation temperature results in upregulation of SF-1 expression to the male pattern. (**B**) Treatment with estradiol (E2) treatment of eggs incubating continuously at a male-producing temperature results in downregulation of SF-1 to the female level. (**C**) Continuous incubation at the female-biased temperature of 29.4°C causes an SF-1 expression curve that lies between the male- and female-producing incubation temperatures and contains, at least at late developmental stages, mixed effects of males and females. (**D**) In eggs incubating at the female-biased temperature, inhibition of estrogen biosynthesis by treatment with aromatase inhibitor (AI) causes upregulation of SF-1 expression to the level of 26°C males.

phism, and suggest that a threshold of SF-1 may be important to continued differentiation.

To further examine the effect of temperature on gonadal expression of SF-1 in the red-eared slider, eggs were shifted from an all-male (26°C) to an all-female (31°C) producing incubation temperature—or vice versa—at stage 17, the middle of the TSP. In the male-to-female shift, SF-1 message levels were downregulated to a female level (Figs. 3,4). The pattern of this change suggests either a drop to basal expression or active repression of SF-l expression following the temperature cue. In the female-to-male shift, SF-1 expression levels went up rapidly, initially surpassing even the 26°C male controls before falling back to that level (Figs. 3,4). This change in regulation could involve at least two steps: initial upregulation followed by a secondary, balancing downregulation. This data suggests that SF-1 is in the TSD molecular pathway.

Gonadal sex of red-eared slider turtle can also be manipulated during the TSP by treating eggs incubating at a male-producing temperature with E2, or by treating eggs incubating at a female-producing temperature with an aromatase inhibitor (AI), which inhibits estrogen biosynthesis (Figs. 3,4). Such manipulations produce female and male offspring, respectively. Following E2 treatment of eggs incubating at a male-producing temperature, gonadal SF-1 expression was downregulated and became statistically and histologically indistinguishable from temperature-derived females. Treatment of eggs incubating at a female-biased temperature with AI had the opposite effect, but this effect was delayed compared to temperature shift and E2 treatments (Figs. 3,4). It is possible that existing endogenous estrogen may remain active for some time, thereby accounting for this delay. The modulation of SF-1 expression by E2 and AI treatments provides molecular support for a critical, endogenous effect of estrogen in TSD, and suggests that SF-1 is at least one of the genes regulated (directly or indirectly) by estrogen in this TSD species.

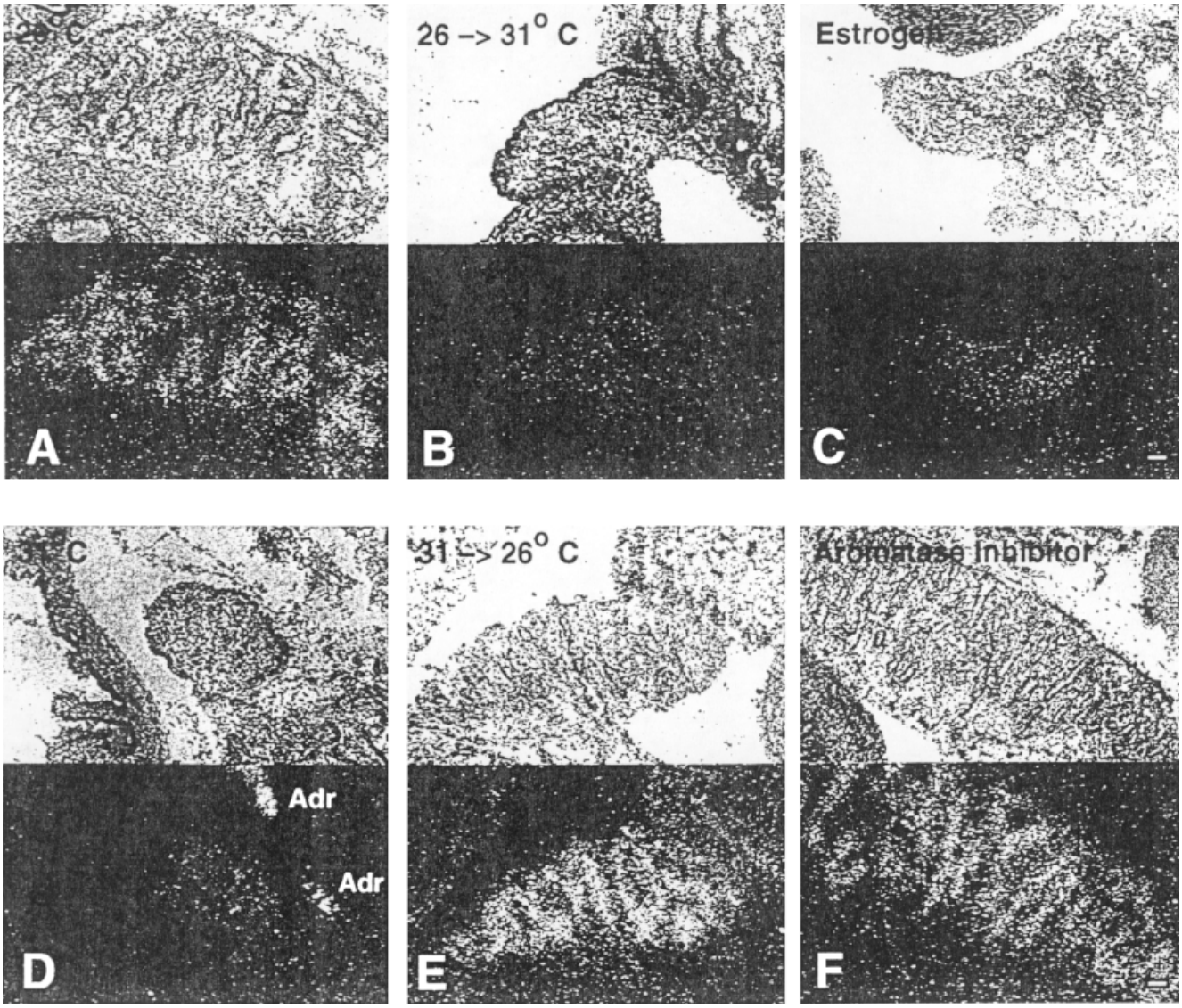

Fig. 4. Localization and abundance of SF-1 mRNA in the embryonic gonad of manipulated red-eared slider turtles (*Trachemys scripta elegans*). Presented are the results of the sex effect of temperature and estrogen SF-1expression patterns. Lightfield and darkfield images are shown for each treatment. All individuals shown here are at embryonic stage 23, after the temperature-sensitive period (TSP). The scale bar is 100 μm. (**A**) Testis from embryo incubated at 26°C (male-producing temperature). (**B**) Ovary from an individual incubated at 26°C until stage 17 (mid-TSP), then shifted to 31°C (female-producing temperature). (**C**) Ovary from embryo incubated at 26°C, and treated with estradiol (E2) at stage 17. (**D**) Ovary from embryo incubated at 31°C. (**E**) Testis from embryo incubated at 31°C until stage 17, then shifted to 26°C. (**F**) Testis from embryo incubated at 29.4°C (female-biased temperature) and treated with aromatase inhibitor (AI) at stage 17.

ROLE OF AROMATASE IN SEX DETERMINATION

The physiological equivalent of temperature appears to be a combination of the nature and abundance of steroidogenic enzymes, including aromatase and their products. Interestingly, in several turtle (Thomas et al., '92; White and Thomas, '92; Salame-Mendez et al., '98) and crocodilian (Smith and Joss, '94; Smith et al., '95) species, specific enzyme reaction products are not observed in the genital ridge at the beginning or during the TSP; the only activity detected in the gonad is observed after the temperature-sensitive window. This is not the case, however, in the European pond turtle (*Emys orbicularis*) (Pieau et al., '99). Radioimmunoassay of embryonic gonads from the pond turtle indicates that during the TSP, gonads from embryos at a female-producing incubation temperature have higher estrogen con-

tent than gonads at a male-producing temperature (Dorizzi et al., '91). Further, aromatase activity is very low in undifferentiated gonads and remains low in embryos at a male-producing temperature but increases exponentially in embryos at a female-producing temperature, an increase that begins several stages into the TSP. Shifting eggs from a male- to a female-producing incubation temperature results in an increase in aromatase activity, whereas the opposite manipulation decreases aromatase levels. In the pond turtle, these changes do not appear to be due to temperature modulation of aromatase activity, but rather to temperature-induced increases in expression of the aromatase gene (reviewed in Pieau et al., '99).

This information suggests that in some TSD species, including the red-eared slider turtle, extragonadal tissues, and not the gonad itself, produce steroid hormones during embryogenesis. There is increasing evidence that the brain may be both the temperature sensor and a site of steroid hormone synthesis. Not only does the brain contain temperature-sensitive neurons in the hypothalamus (Satinoff, '95), but in the Olive Ridley sea turtle (*Lepidochelys olivacea*), Merchant-Larios ('98) and colleagues (Salame-Mendez et al., '98) measured differences in estrogen content in the brain between incubation temperatures during the TSP, with concentration of E2 being much higher in the diencephalon of putative females than in putative males. Of equal significance is their failure to find differences in estradiol levels in the gonads of putative males and females at any developmental period examined. In the diamondback terrapin (*Malaclemys terrapin*), Place and coworkers (Jeyasuria et al., '94; Jeyasuria and Place, '97, '98; Blumberg et al., 2001) find aromatase transcripts in the brains of putative males and females prior to the TSP. Further, these transcripts are in greater abundance at the beginning of the TSP in females. Later in embryogenesis, during the TSP, transcript levels in the brains of putative males rise above those of females, while transcript levels in the gonads of putative females rise exponentially. Although these studies do not illustrate a mechanism by which neural differences are converted to gonadal differentiation, Merchant-Larios ('98) has raised the intriguing possibility that the different levels of steroid and enzyme activity before the onset of gonadal differentiation in the brain may play a role in establishing gonadal endocrine milieus specific to incubation temperature.

Using a tritiated water assay, we have compared the pattern of estradiol biosynthesis at male- and female-producing temperatures in the brain and adrenal-kidney-gonad (AKG) through development of the red-eared slider (Willingham et al., 2000). The pattern for both sexes in the AKG was one of increased activity after the TSP, but with no significant difference between sexes (Fig. 5, bottom panel). In the brain, however, putative females exhibit a significantly higher level of aromatase activity than putative males at the beginning of the TSP, after which activity in both male and

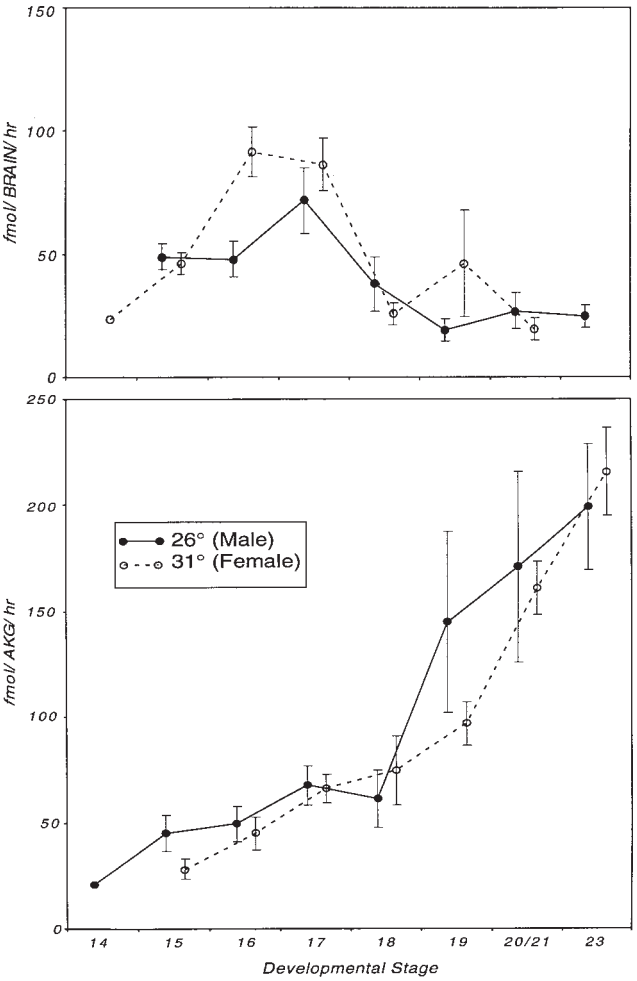

Fig. 5. Aromatase activity in the brain (top panel) and the urogenital system (bottom panel) of embryonic red-eared slider turtles (*Trachemys scripta elegans*) incubated at 26°C (male-producing temperature) and 31°C (female-producing temperature). At stage 16, activity is significantly higher in the brains of putative females than in putative males, implying a role for the brain and aromatase in sex determination in the red-eared slider turtle. Aromatase activity in the adrenal-kidney-gonad of embryos incubated at the male-producing temperature and the female-producing temperature does not change until the end of the temperature-sensitive period. Redrawn from Willingham et al., 2000.

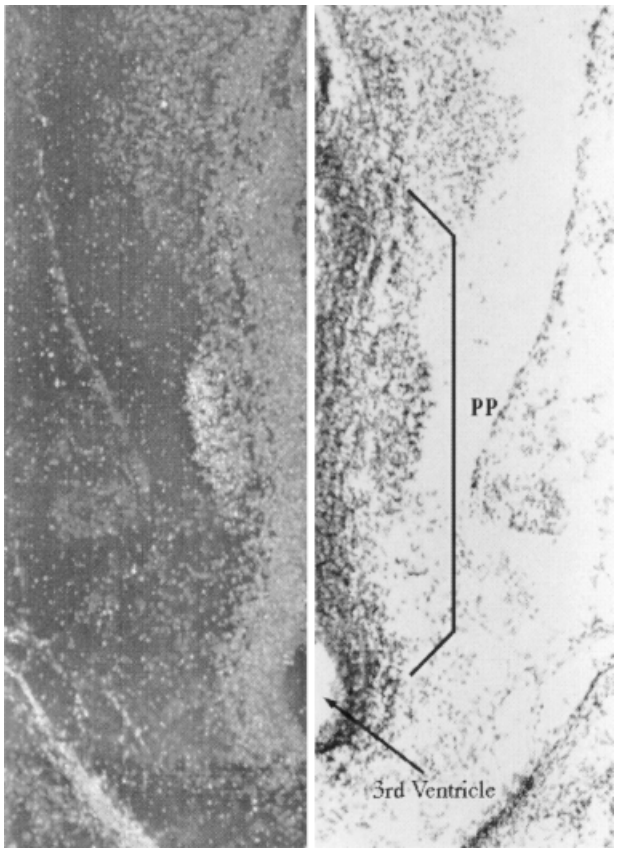

Fig. 6. Specific binding of radiolabeled anti-sense aromatase probe in the hypothalamus of an embryonic brain from 31°C (female-producing temperature) during the temperature sensitive period (stage 19) in the red-eared slider turtle (*Trachemys scripta elegans*). Left panel: Dark field. Right Panel: Light field is mirror image of dark field photomicrograph. Section is at the level of the periventricular region of the preoptic area-anterior hypothalamus (PP). Compare to distribution of SF-1 as illustrated in Fig. 5 of Fleming and Crews, 2000. Photograph manipulated using Adobe Photoshop 4.0; text labels added.

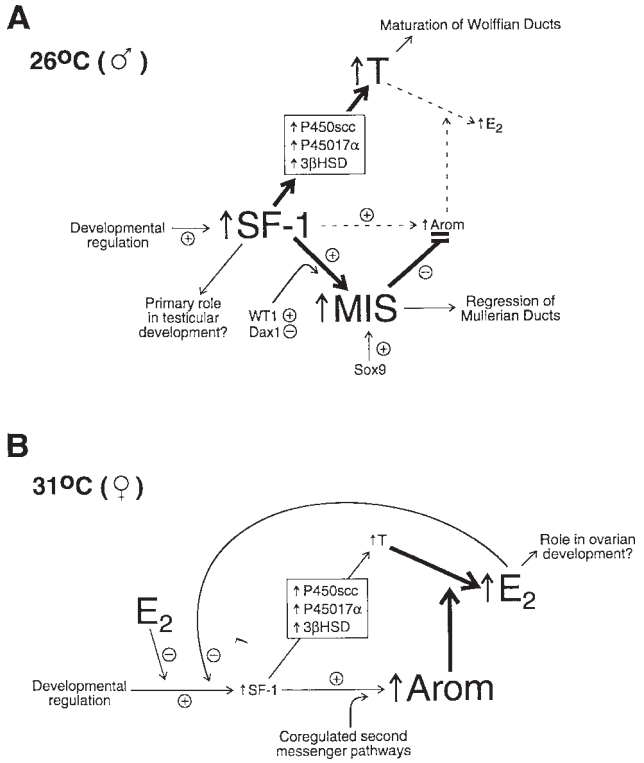

Fig. 7. Model suggesting how the level of SF-1—as affected by estrogen—could act as a central factor in the mutually restrictive regulation of MIS and aromatase (Arom) during the temperature sensitive period in red-eared slider turtle (*Trachemys scripta elegans*) gonads. Other genes are depicted as well as different steroidogenic enzymes. Direction of arrows indicate upregulation (↑) and downregulation (↓). Encircled positive and negative signs indicate stimulation and inhibition, respectively. **A**: Model at 26°C, or an all-male producing temperature. **B**: Model at 31°C or an all-female producing temperature.

female brains decrease, dropping below detection in females before hatch (Fig. 5, top panel). Further, preliminary evidence from in situ hybridization analysis suggests that aromatase mRNA is localized in the periventricular nucleus of the hypothalamus (Fig. 6). These results also point to the brain as a site of aromatase response to temperature in this species, and suggest further that the product of aromatase activity, estradiol, may induce alterations in the neuroendocrine axis controlling gonadal sex steroid hormone production.

MODEL FOR SEX DETERMINATION IN THE RED-EARED SLIDER

Given these new data, we have proposed a model that, while undoubtedly lying within a more complex network of regulatory mechanisms, incorporates the findings above for the red-eared slider (Fleming and Crews, 2001) (Fig. 7). A central feature in this model is regulation of multiple genes by SF-1. By manipulating gonadal sex outcomes in a variety of ways and, in all cases, observing two distinct levels of SF-1 message associated with the development of testes or ovaries suggests that SF-1 occupies a critical position in the sex determination process in the red-eared slider. In mammals, as in the red-eared slider, SF-1 gonadal expression is higher in differentiating testes than ovaries (Hatano et al., '94; Ikeda et al., '94; Fleming et al., '99). Thus, the relative amount of SF-1 message appears important in gonadogenesis of both mammals and turtles; this point is significant since mammals arose from turtle-like reptiles some 350 million years ago. In mammals, the number of SF-1 response elements

varies in promoters of genes it regulates. This variation affects, at least in part, the level of SF-1 required to attain full transcriptional activity of a given gene (Halvorson et al., '98; Naville et al., '99). Omura and Morohashi ('95) identified as many as five SF-1 response elements in promoters of steroidogenic P450 enzymes; yet, in the human, they found only one such site in the aromatase promoter. If there is an evolutionary conservation of function, the aromatase promoter in red-eared slider might also contain a single SF-1 response element, and a relatively low level of SF-1—as occurs at the female-producing temperature—which would be sufficient for its part in activating aromatase. In addition to SF-1, regulation of aromatase in mammals involves other transcription factors, coregulators, and second messenger pathways (Carlone and Richards, '97; Hammer et al., '99), and may be similarly complex in the red-eared slider.

CONCLUDING REMARKS

In vertebrates, genetic triggers either inherited from the parents or derived from the environment direct sex determination. In mammals and birds it is the genetic constitution established at the time of fertilization, whereas in many turtles it is the temperature experienced during the mid-trimester of embryogenesis that determines the type of gonad that develops. TSD is considered ancestral to genotypic sex determination and, indeed, except for the difference in the initial trigger, many of the same genes are involved in the process of gonadal differentiation. In the red-eared slider turtle, relatively low incubation temperatures produce all males whereas relatively high incubation temperatures produce all females. The transition from an all-male to an all-female sex ratio is abrupt, occurring over a 1°C range where mixed sex ratios, rather than intersex individuals, are produced. Sex steroid hormones appear to be the physiological equivalent of incubation temperature; administering exogenous steroid hormones or inhibiting their synthesis during incubation completely alters the normal temperature-induced sex determination outcome. Studies of the pattern and abundance of SF-1 gene expression and aromatase activity indicate a critical role for estrogen in TSD and a subsequent role in this path for SF-1. Further, estrogen directly or indirectly modulates regulation of SF-1 expression. The inherent ability of sex determination in species lacking sex chromosomes coupled with the rarity of intersexes and the likelihood that mammalian sex determination mechanisms are evolutionarily conserved makes the red-eared slider an excellent model for dissecting the molecular mechanisms underlying testicular and ovarian development during embryogenesis.

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