THE JOURNAL OF EXPERIMENTAL ZOOLOGY 281:409–416 (1998)

Temperature-Dependent Sex Determination in the Red-Eared Slider Turtle, *Trachemys scripta*

THANE WIBBELS,[1*] JON COWAN,[1] AND ROBERT LEBOEUF[2]
[1]*Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294-1170*
[2]*Department of Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, Alabama 35294-1170*

ABSTRACT Temperature-dependent sex determination (TSD) in the red-eared slider turtle, *Trachemys scripta*, has been the subject of a variety of past studies. Incubation temperature appears to affect sex determination in a dose-dependent fashion. This suggests that temperature could be affecting a dosage-sensitive element in the sex-determination cascade. Sex determination in *T. scripta* is sensitive to estrogen, and data from many studies support the hypothesis that endogenous estrogen production may be involved in female sex determination. However, this hypothesis has not yet been evaluated through aromatase expression studies in this species. Several recent studies have cloned cDNAs for genes that could be involved in sex determination and/or sex differentiation. The cDNAs for SF-1 and MIS have been cloned in *T. scripta*, indicating that these may represent conserved elements in the sex-determination/sex-differentiation cascade of reptiles. The SOX9 cDNA also has been cloned in *T. scripta* (Spotila et al., '98), and it shows a sex-specific expression pattern. Future studies targeted at aromatase expression as well as the expression of factors such as SOX9, SF-1, and MIS will begin to provide a more comprehensive picture of the events involved in TSD in *T. scripta*. Further, such studies could help pinpoint the temperature-sensitive element(s). *J. Exp. Zool. 281:409–416, 1998.*

Temperature-dependent sex determination (TSD) has been the subject of numerous studies over the past several decades in a variety of reptiles. The most detailed investigations of the physiology of TSD have focused on several species of turtles and on the American alligator (see reviews by Raynaud and Pieau, '85; Lance and Bogart, '94; Lang and Andrews, '94; Wibbels et al., '94; Jeyasuria et al., '94; Pieau, '96). The red-eared slider turtle, *Trachemys scripta*, has been the subject of many of these physiologic studies for several reasons. First, there are several logistical advantages to using *T. scripta*. Eggs from this turtle can be obtained commercially in large numbers during April through July of each year (Robert Kliebert, Hammond, LA). These eggs have a relatively thin shell, which allows for the accurate staging of eggs (via candling) during experiments. The thin egg shell also facilitates decreased mortality during steroid hormone treatment studies, since the hormones can be applied topically to the egg shell rather than injected. Second, temperature sensitivity and gonadal differentiation have been well described in this turtle, thus providing a basis for optimizing the experimental design of physiologic studies. The purpose of this article is to attempt to provide a concise summary of what is currently known on the physiology of sex determination in *T. scripta*.

DOSAGE EFFECT OF TEMPERATURE

An early study by Bull et al. ('82) indicated that sex determination in *T. scripta* was sensitive to incubation temperature, with relatively warm temperatures producing females and cooler temperatures producing males. The thermosensitive period was shown to approximate the middle third of incubation (Wibbels et al., '91a), which is consistent with that shown for several other reptiles with TSD (Yntema, '79; Bull and Vogt, '81; Pieau and Dorizzi, '81; Yntema and Mrosovsky, '82; Ferguson and Joanen, '83; Bull, '87; Webb et al., '87; Deeming and Ferguson, '88; Lang and Andrews, '94). In *T. scripta*, this thermosensitive period precedes and then overlaps the initial stages

Grant sponsor: Mississippi-Alabama Sea Grant Consortium; Grant number: NA56RG0129; Grant sponsor: University of Alabama at Birmingham.
*Correspondence to: Thane Wibbels, Department of Biology, University of Alabama at Birmingham, Birmingham, AL 35294-1170. E-mail: biof009@uabdpo.dpo.uab.edu
Received 17 February 1998; Accepted 18 February 1998

of sexual differentiation of the gonads (Wibbels et al., '91a). Typically, *T. scripta* has a *pivotal temperature* (temperature producing a 1:1 sex ratio) of approximately 29.0 to 29.5°C (Bull et al., '82; Crews et al., '91; Etchberger et al., '91; Wibbels et al., '91a,b; Wibbels and Crews, '95).

More detailed studies of *T. scripta* have indicated that temperature appears to exert a "dosage effect" on sex determination. The dosage effect depends on the "potency" of the temperature (i.e., the warmer or cooler the temperature, the more potent it is in producing females or males, respectively) and the duration of the temperature (Wibbels et al., '91a). Similar findings have been shown in the alligator (Lang and Andrews, '94) and in the map turtle (Bull et al., '90). These findings suggest that incubation temperature may be regulating the production of a factor(s) in the sex determination cascade, which, if produced in sufficient dosage, initiates a cascade toward a specific sex. But what element(s) in the sex-determination cascade is temperature sensitive?

ESTROGENS AND SEX DETERMINATION

Several studies examining turtle species other than *T. scripta* have provided direct support for the hypothesis that female incubation temperature is correlated with increased estrogen production and aromatase activity in the developing gonads (Desvages and Pieau, '91; '92a,b; Dorizzi et al., '91; Desvages et al., '93; Pieau, '96; Jeyasuria et al., '94; Jeyasuria and Place, '98). A more recent study has shown increased levels of aromatase mRNA in adrenal-kidney-gonad complexes at female incubation temperatures in the diamondback terrapin, *Malaclemys terrapin* (Jeyasuria and Place, '98). In *T. scripta*, there is a lack of data regarding gonadal estrogen levels, and although portions of the aromatase gene have been cloned (D. Crews, personal communication), studies on aromatase expression are still in progress. There have been previous studies that examined steroid production by adrenal-kidney-gonad complexes and steroid levels from whole-body homogenates and pooled serum (White and Thomas, '92a,b), but higher levels of estrogen were not detected at female temperatures.

While data on endogenous levels of gonadal aromatase activity and estrogen levels are lacking in *T. scripta*, there is a large amount of indirect evidence suggesting that estrogen could be involved in the sex-determination cascade. Previous studies in *T. scripta* indicate that treatment of eggs incubated at male temperature with estrogens or estrogen-related compounds results in the production of females (Crews et al., '91; Wibbels and Crews, '92). These findings are consistent with data from other reptiles with TSD (Pieau, '69, '70, '74; Gutzke and Bull, '86; Bull et al., '88; Lance and Bogart, '91, '92; Lang and Andrews, '94). The time period during which sex determination is sensitive to exogenous estrogen in *T. scripta* is similar to the thermosensitive period (Wibbels et al., '91b). Further, estrogen and incubation temperature appear to have a synergistic effect on sex determination (e.g., estrogen has a more potent feminizing effect near the pivotal temperature) (Wibbels et al., '91b; Wibbels and Crews, '95). In fact, dosages as low as 1.0 ng applied to the egg shell have been effective in feminizing embryos incubated near the pivotal temperature (Wibbels and Crews, '95). Recently, estrogen receptor expression has been examined during TSD in *T. scripta* (Bergeron et al., in press). The results of that study indicate that estrogen receptor mRNA is present in gonads prior to sex determination at both male and female temperatures. Thus the sex-determination system of *T. scripta* definitely appears sensitive to estrogen.

While there is a paucity of data on aromatase activity during sex determination in *T. scripta*, there have been several studies that have attempted to block aromatase activity. Of particular interest, the nonsteroidal aromatase inhibitors fadrozole and letrozole have been shown to induce male sex determination (Crews and Bergeron, '94; Wibbels and Crews, '94). Thus aromatase activity and estrogen production during sex determination are clearly areas of interest for future studies of *T. scripta*. Based on recent aromatase data from the saltwater terrapin (Jeyasuria and Place, '98), studies of *T. scripta* will most certainly benefit from high-resolution/sensitivity techniques for examining aromatase expression. In light of recent studies on aromatase activity in the brain during TSD (Merchant-Larios, '98; Jeyasuria and Place, '98), it will be of considerable interest to also compare expression between the brain and gonads. If aromatase activity and estrogen production are shown to be higher at female temperatures in *T. scripta*, the question still remains: What is the temperature-sensitive element that regulates this system?

ANDROGENS AND SEX DETERMINATION?

While estrogens and aromatase have been the focus of most studies on the physiology of TSD, there have been several studies in *T. scripta* suggesting that nonaromatizable androgens could

have a role in the male sex-determination cascade. Several studies have indicated that the nonaromatizable androgen 5αDHT can induce male sex determination (Wibbels et al., '92; Wibbels and Crews, '95; Crews et al., '96), but only when using incubation regimes that result in mixed sex ratios in the control groups (e.g., near the pivotal temperature). These studies also required high dosages of 5αDHT (relative to estrogen dosage necessary to feminize embryos), and they could not predictably masculinize all embryos within treatment groups regardless of dosage. There are also data indicating that the 5α-reductase inhibitors 4MA and MK906 can feminize embryos, but again, only when using near-pivotal incubation temperatures (Crews and Bergeron, '94). Thus the data on the possible involvement of nonaromatizable androgens and 5α-reductase in *T. scripta* are far from conclusive. Further, the production of males in response to 5αDHT has not been reported in any other reptiles with TSD, and therefore, this issue remains controversial (Rhen and Lang, '94). It should be noted, however, that the great majority of previous studies have not used near-pivotal incubation temperatures. Nevertheless, the effects of 5αDHT are definitely not as distinct and predictable as those of estrogen, and this fact has prompted some researchers to exclude nonaromatizable androgens from having a major role in sex determination (Pieau, '96).

STEROIDOGENIC FACTOR-1

In light of the aromatase and estrogen data reviewed above, the nuclear orphan receptor steroidogenic factor 1 (i.e., SF-1) represents a plausible candidate for involvement in the sex-determination cascade of reptiles with TSD. SF-1 (also called Ad4BP) originally was identified in mouse and bovine (Lala et al., '92; Morohashi et al., '92) and is a member of the nuclear hormone receptor superfamily of transcription factors (Honda et al., '93). SF-1 also appears to be the mammalian homologue of the *Drosophila* transcription factor *Fushi tarazu* factor 1, or FTZ-F1 (Lala et al., '92; Morohashi et al., '92). In mammals, SF-1 acts as a global mediator of steroidogenesis by regulating steroidogenic enzyme expression (Caron et al., '97). Of particular interest, SF-1 has been shown to regulate aromatase gene expression in mammalian gonadal tissue (Lynch et al., '93; Carlone and Richards, '97), and it exhibits sexually dimorphic expression in the differentiating gonads (Ikeda et al., '94). It is also expressed by embryos in other regions of the reproductive endocrine axis: the anterior pituitary and the ventromedial hypothalamic nucleus (Ingraham et al., '94). Additionally, SF-1 has been shown to regulate the expression of mullerian inhibiting substance during testicular differentiation (Shen et al., '94).

We have recently used a PCR-based approach to clone a putative cDNA for SF-1 in *T. scripta*. Adrenal-kidney-gonad complexes from male embryos (stage 23; Yntema, '68) were used to isolate total RNA for producing cDNA template. Degenerate primers based on mammalian SF-1 sequences were used in the PCRs. 5′ and 3′ rapid amplifications of cDNA ends (RACE) were used to clone the 5′ and 3′ noncoding regions of the cDNA. Sequencing was performed on a Perkin Elmer Applied Biosystems 377 automated sequencer. We are currently in the process of isolating and sequencing multiple clones to independently verify the sequence of our original clone.

The predicted sequence of the putative *T. scripta* SF-1 cDNA clone shows high homology to mammalian SF-1 (Fig. 1). The clone includes the highly conserved regions I, II, and III and FTZ-F1 box typical of mammalian SF-1. The predicted amino acid sequence is as high as 100% in the FTZ-F1 box and in region III (see Fig. 1). These data indicate that a homologue to SF-1 exists in reptiles, thus suggesting the conservation of this gene in amniotic vertebrates. We are currently in the process of using probes based on this *T. scripta* SF-1 to examine the expression of SF-1 during TSD.

MULLERIAN INHIBITING SUBSTANCE

Expression of mullerian inhibiting substance (MIS) has been shown to be an early indicator of testicular differentiation in mammals. MIS expression occurs shortly after the onset of SRY expression, at a time when the gonads begin to morphologically differentiate as testes (Hacker et al., '95). Thus MIS expression represents a molecular marker for testicular differentiation in sex-determination studies. Unfortunately, it has not been used in any past studies of reptiles with TSD due to the lack of any homologous probes for reptilian MIS.

The primary physiologic role of MIS is to stimulate the regression of the mullerian ducts during male sexual differentiation (Lee and Donahoe, '93; Behringer, '95). Because of the "freemartin effect" (Lillie, '17), MIS also has been suggested to be involved in testicular differentiation in mammals (Vigier et al., '87); however, more recent studies indicate that MIS-deficient mice develop testes

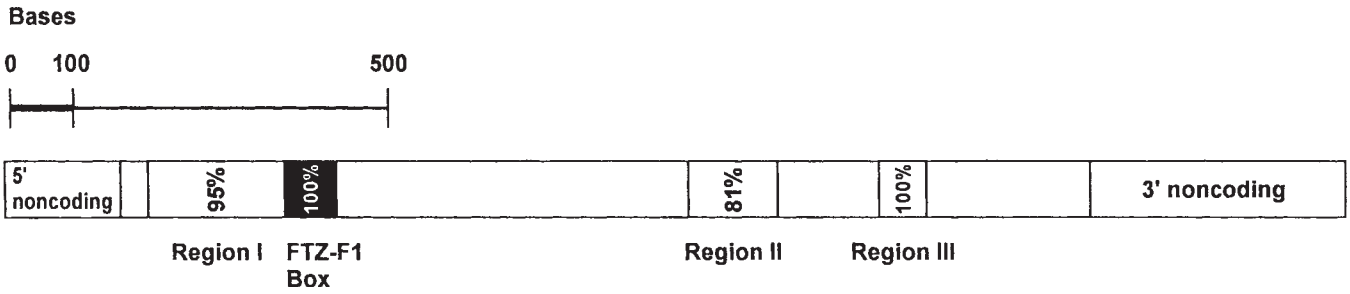

Fig. 1. Putative steroidogenic factor 1 (SF-1) cDNA isolated from *T. scripta*. The clone includes the highly conserved regions I, II, and III and FTZ-F1 box typical of mammalian SF-1. Percentages refer to homologies between *T. scripta* and mammalian SF-1.

and viable sperm (Behringer, '95). It also has been shown that MIS can stimulate "endocrine sex reversal" in embryonic ovaries (Vigier et al., '89). While this latter finding may not seem relevant to normal mammalian sex determination (since steroid hormone production appears to be a downstream event), it may be relevant to TSD in reptiles because aromatase activity appears to be a pivotal event. It has been proposed that MIS expression in mammals is regulated by SF-1 (Shen et al., '94; Giuili et al., '97), with the sexually dimorphic expression of SF-1 closely coinciding with the sexually dimorphic expression of MIS.

We have recently used PCR-based strategy to isolate a putative MIS clone in *T. scripta*. Degenerate primers based on the structure of chicken and mammalian MIS were used in the PCRs. RNA was isolated from embryonic testes (stage 23; Yntema, '68) for the production of cDNA template. 5′ and 3′ RACEs were used to isolate the 5′ and 3′ noncoding regions of the cDNA. Sequencing was performed on a Perkin Elmer Applied Biosystems 377 automated sequencer. We are currently in the process of isolating and sequencing multiple clones in order to independently verify the sequence.

The putative *T. scripta* MIS clone is shown in Fig. 2. Regions 1 through 5 refer to exons 1 through 5 identified in the chicken and mammalian MIS genes (Cate et al., '86; Munsterberg and Lovell-Badge, '91; Haqq et al., '92; Eusebe et al., '96; Neeper et al., '96). The *T. scripta* clone is similar in size and GC content to chicken and mammalian MIS cDNAs. Regions of high homology between chicken and mammalian MIS also show similar homology in the *T. scripta* clone. The sequence data indicate that this clone is MIS rather than other TGF-β family members. As with the chicken and mammalian sequences, the region of the *T. scripta* clone corresponding to the C-terminal domain of chicken MIS shows the highest ho-

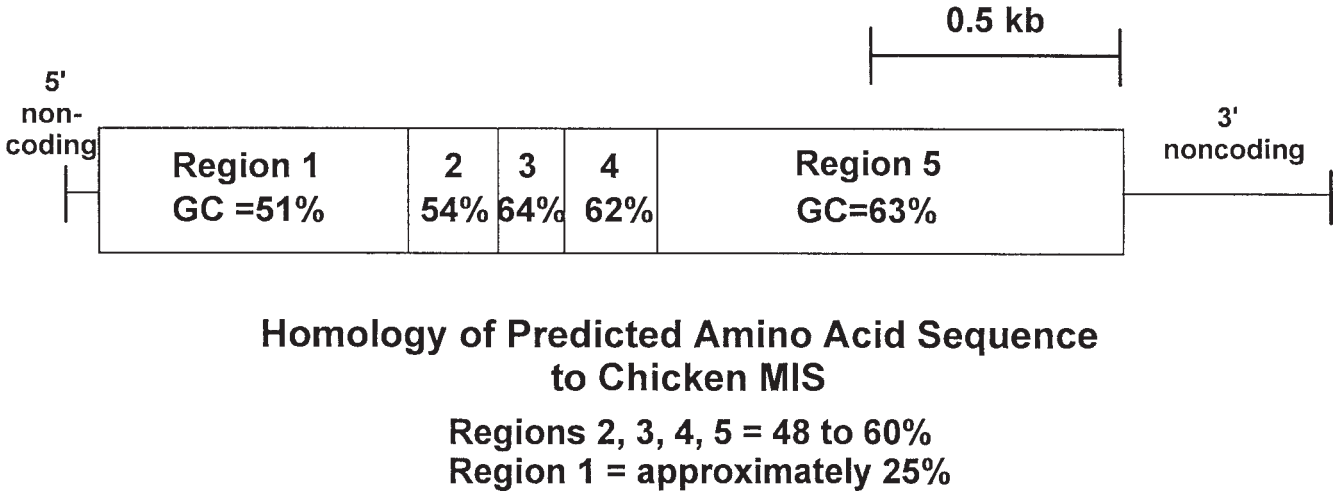

Fig. 2. Putative mullerian inhibiting substance cDNA isolated from *Trachemys scripta*. Regions 1 through 5 refer to exons 1 through 5 identified in chicken and mammalian MIS (see Cate et al., '86; Eusebe et al., '96; Neeper et al., '96). GC content is shown for each region.

mology, and the region corresponding to exon 1 shows the lowest homology. We have expressed a segment of the *T. scripta* MIS (corresponding to regions 4 and 5) in a pET vector system. The expressed protein cross-reacts with antisera raised against a region of exon 5 of chicken MIS.

We are now in the process of making probes based on the *T. scripta* MIS clone for examining the expression of MIS relative to incubation temperature and SF-1 expression during TSD.

SOX9

While previous studies have failed to isolate an SRY homologue in reptiles (Spotila et al., '94), the cDNA from a closely related gene (i.e., SOX9) recently has been cloned in *T. scripta* (Spotila et al., '98). In mammals, mutations of SOX9 are associated with abnormal testis development and male-to-female sex reversal (Schafer et al., '95). This suggests that SOX9 may have a role in the male sex-determination cascade. Further, it has been hypothesized that SRY actually may regulate SOX9 during sex determination (Graves, '95, '98); however, direct evidence is currently lacking.

In contrast to SRY, SOX9 may represent a conserved element in the sex determination of vertebrates because it also has been identified in birds and reptiles (Clinton, '98; Smith et al., '98; Spotila et al., '98; Western et al., '98). The recent cloning of SOX9 in *T. scripta* provides a new avenue for investigating the sex-determination cascade (Spotila et al., '98). Data from that study are consistent with the hypothesis that SOX9 may be involved in male sex determination because it is expressed at higher levels in adrenal-kidney-gonad complexes at male temperatures.

DAX1

DAX1 is another gene that has been implicated in sex determination. This gene has been identified in mammals and is located on the short arm of the X chromosome (Zanaria et al., '94; Guo et al., '96). DAX1 is a member of the nuclear hormone receptor superfamily of transcription factors (Burris et al., '96). Mutations of DAX1 are associated with adrenal hyperplasia and hypogonadotropin hypogonadism (Muscatelli et al., '94; Swain et al., '96). It appears that abnormally high expression of this gene (due to gene duplication) results in male-to-female sex reversal (Bardoni et al., '94; Swain et al., '96). The expression pattern of DAX1 in embryonic gonads suggests that it may be important in ovarian differentiation (Swain et al., '96). Additionally, DAX1 is expressed in the adrenal, hypothalamus, and pituitary gland, suggesting that it could play a role in steroidogenesis (Burris et al., '96). The expression pattern of DAX1 is similar to that of SF-1, suggesting that these two transcription factors could act as coregulators in the sex-determination cascade (Burris et al., '96).

The cloning of DAX1 has not been reported in any reptile, but unpublished data (Lance, in press) indicates that a DAX1-like gene may be expressed by the alligator ovary. Considering the proposed roles of DAX1 in ovarian differentiation and steroidogenesis, it would be of significant interest to clone DAX1 in *T. scripta* and examine its expression pattern during TSD.

FUTURE STUDIES

Considering the data reviewed above, estrogen production and aromatase activity remain a priority for future studies of TSD in *T. scripta*. However, it is clear that there are several new areas that invite further investigation. With regard to aromatase activity, there is an obvious need to verify aromatase expression levels in *T. scripta* using high-sensitivity techniques. The recent cloning of SOX9, SF-1, and MIS will provide new avenues for probing the molecular biology of TSD in *T. scripta*. Data from such studies should begin answering basic questions and thus provide a more comprehensive picture of the events involved in TSD. As examples: Is SF-1 involved in the regulation of aromatase and MIS? Is SOX9 involved in testicular differentiation? Does DAX1 exist in *T. scripta*, and if it does, is it involved in ovarian differentiation? Answers to such questions will require not only expression studies but also examination of gene structure to identify regulatory elements. Further, these studies will no doubt uncover other factors in the sex-determination cascade. And finally, we get back to the basic question: What is the temperature-sensitive element of TSD in *T. scripta*? At this point, it is still unknown, but in comparison with just a few years ago, we have several more factors that may help provide the answer.

ACKNOWLEDGMENTS

Alex Elbrecht of Merck, Sharp, and Dohme graciously furnished the chicken antisera for MIS.

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