Develop. Growth Differ. (1998) **40**, 243–254

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

Judith M. Bergeron,[1]* Manfred Gahr,[2] Karen Horan,[3] Thane Wibbels[4] and David Crews[1†]

[1]*Institute of Reproductive Biology, Department of Zoology, University of Texas at Austin, Austin, Texas 78712, USA,* [2]*Max Planck Institut für Verhaltensphysiologie, D8130 Seewiesen Post, Starnberg, Germany,* [3]*University of Chicago Hospitals, Department of Obstetrics and Gynecology, MC2050, Chicago, Illinois 60637 and* [4]*Department of Biology, University of Alabama at Birmingham, Birmingham, Alabama 35294, USA.*

Many reptiles exhibit temperature-dependent sex determination where the incubation temperature of the egg determines the gonadal sex of the individual. If exogenous estrogen is administered during the temperature-sensitive period to embryos incubating at a male-producing temperature, the temperature effects can be overridden and females will be produced. Inhibiting production of endogenous estrogens at female-biased incubation temperatures results in embryos developing as males rather than females. Thus, estrogen–estrogen receptor-dependent mechanisms appear to play a key role in female sex determination. The present study characterized the expression of the estrogen receptor during the critical period of temperature sensitivity in the red-eared slider turtle, *Trachemys scripta*. Polymerase chain reaction was used to amplify estrogen receptor cDNA. A portion of the estrogen receptor cDNA was used to produce probes for *in situ* hybridization analyses to localize and quantitate levels of estrogen receptor mRNA at different stages of development in embryos from different incubation temperatures. Estrogen receptor mRNA is expressed in the gonadal tissues of both putative males and putative females even before the gonads begin to resolve as ovaries or testes. There is a greater abundance of estrogen receptor mRNA in putative females at the beginning of the temperature-sensitive period as compared to putative males. In embryos from a female-producing incubation temperature, levels of estrogen receptor mRNA are higher in the beginning of the temperature-sensitive window compared to levels after the ovary is differentiated. These results support the hypothesis that estrogen–estrogen receptor dependent processes are important during sex determination and gonadal differentiation in temperature-dependent sex determination.

Key words: estrogen, estrogen receptor, gonads, temperature-dependent sex determination.

Introduction

In reptiles with temperature-dependent sex determination (TSD), embryonic incubation temperature determines whether the individual will be male or female. In the red-eared slider turtle (*Trachemys scripta*), a TSD species, constant temperatures below 28.6°C induce all male hatchlings while temperatures above 29.6°C produce all female hatchlings. Increasing incubation temperature within the narrow 28.6–29.6°C range between these two groups results in increasing female : male ratios (reviewed in Crews *et al.* 1994). Shifting eggs from low to high temperatures (and vice versa) established a window of sensitivity in which the effects of the initial incubation temperature could be reversed (Wibbels *et al.* 1991).

Extensive studies using steroid hormone treatments or inhibitors of steroid production suggest that temperature may be acting by regulating the steroid environment that directs gonadal differentiation (see Crews 1994 or Crews *et al.* 1994 for review). Application of exogenous sex steroids to the egg is also capable of reversing sex: estrogens and the aromatizable androgens testosterone (T) and androstenedione (AE) produce females at male-biased incubation temperatures. Cytochrome P450 aromatase

*Author to whom all correspondence should be addressed.
†Author to whom reprint requests should be addressed.
Received 3 June 1997; revised 11 November 1997; accepted 26 November 1997.

is the enzyme that converts T to estradiol (E2) and AE to estrone (E1). Administration of the non-steroidal, specific aromatase inhibitors CGS 16949A and CGS 20267 (Ciba-Geigy, Summit, NJ, USA) to eggs incubating at a female-producing temperature will reverse the temperature effects on sex determination, producing a significant number of male hatchlings (Crews & Bergeron 1994; Dorizzi *et al.* 1994; Rhen & Lang 1994; Wibbels & Crews 1994). At a female-biased temperature (which normally results in ≈ 80% female : 20% male), only males were produced after aromatase inhibitors were administered. Stopping the production of estrogens therefore was enough to stop the production of females.

Using autoradiographic procedures, Gahr *et al.* (1992) demonstrated that ^{3}H-E2 is concentrated in the liver, the adrenal-kidney (mesonephros)-gonad (AKG) complex, and in some bony structures in the developing embryo. This pattern occurred in embryos from both male- and female-producing incubation temperatures, at stages in the beginning of the temperature-sensitive period, as well as during and after gonadal differentiation. That estrogen concentrating tissues are found during the critical period of sex determination supports the hypothesis that estrogen-ER dependent mechanisms may have a role in female sex determination.

Co-localization studies combining autoradiography with immunocytochemistry led to the discovery that the antibodies to human ER (H222Spy and H226Spy), which are immunoreactive in a wide variety of species representing every vertebrate class, including lizards, snakes, and alligators, consistently fail to recognize turtle ER (M. Gahr & D. Crews, unpubl. data). If there are significant differences in the amino acid sequence between mammalian and turtle ER, such differences may be responsible for the failure of these mammalian antibodies to bind the turtle ER. It is important then to identify the estrogen receptor (ER) in the embryo, and to determine which cells express these key proteins relative to the period of sex determination and gonadal differentiation.

The present study sought to determine the location of cells expressing ER mRNA in the developing reproductive system of the red-eared slider. Through a quantitative analysis, the abundance of ER mRNA was examined and compared across developmental stages and across different incubation temperatures. Reverse-transcription followed by PCR (polymerase chain reaction; RT-PCR) was used to clone portions of cDNA of the estrogen receptor (ER) in the red-eared slider. These clones were used as probes in the *in situ* hybridization assays to detect cell populations expressing ER in the developing gonad.

Materials and Methods

Animals

Freshly laid eggs from the red-eared slider turtle, *Trachemys scripta*, were obtained commercially (Robert Kliebert, Hammond, LA, USA). After transport to the laboratory, they were held at room temperature until we established embryo viability by candling of eggs. The eggs were randomized to eliminate any clutch effect, then placed in groups of 30 in covered trays containing moistened vermiculite (vermiculite: water, 1:1). Egg trays were placed in reach-in incubators (Precision, Chicago, IL, USA) programed to maintain a constant temperature of 26.0, 29.2 or 31.0°C. Temperature fluctuations were monitored daily by checks on incubator programing, a computerized data logger (HOBO temperature logger, Onset Computer Corporation, Pocasset, MA, USA), and a calibrated incubator thermometer. During the entire period of incubation, egg trays were rotated daily from shelf to shelf to avoid any effects of a temperature gradient within the incubator. Egg development was monitored by candling and dissecting representative eggs to verify developmental characteristics specific to a particular stage (Yntema 1968).

Tissue collection

Three developmental stages were sampled: stage 15, in the beginning of the temperature-sensitive period (TSP); stage 19, during the TSP; and stage 23, well after the TSP. When the embryos reached one of these developmental stages, they were anesthetized in ice, killed with rapid decapitation and dissected under a dissecting microscope. The AKG complex was removed and immediately frozen in liquid nitrogen, all within 1–2 min of decapitation to maintain RNA integrity. Frozen AKG samples were stored at −80°C until use for *in situ* hybridization.

Cloning and sequencing

Computerized sequence analysis software (Microgenie, Beckman, Fullerton, CA, USA) was used to compare published sequences of the ER of human (Green *et al.* 1986; Greene *et al.* 1986), rat (Koike *et al.* 1987), chicken (Krust *et al.* 1986; Maxwell *et al.* 1987), frog (Weiler *et al.* 1987), and trout (Pakdel *et al.* 1989) that show highly conserved amino acid sequences. Because of such conservation in sequence in other vertebrates, DNA primers designed from the DNA-binding domain and the steroid-binding domain were selected to generate turtle cDNA clones of ER using RT-PCR. The primers used were: primer G: 5′-GG(ATG)CA(TC)AA(CT)GA(TC)TA(TC)ATGTG-3′ from the DNA binding domain and primer U:

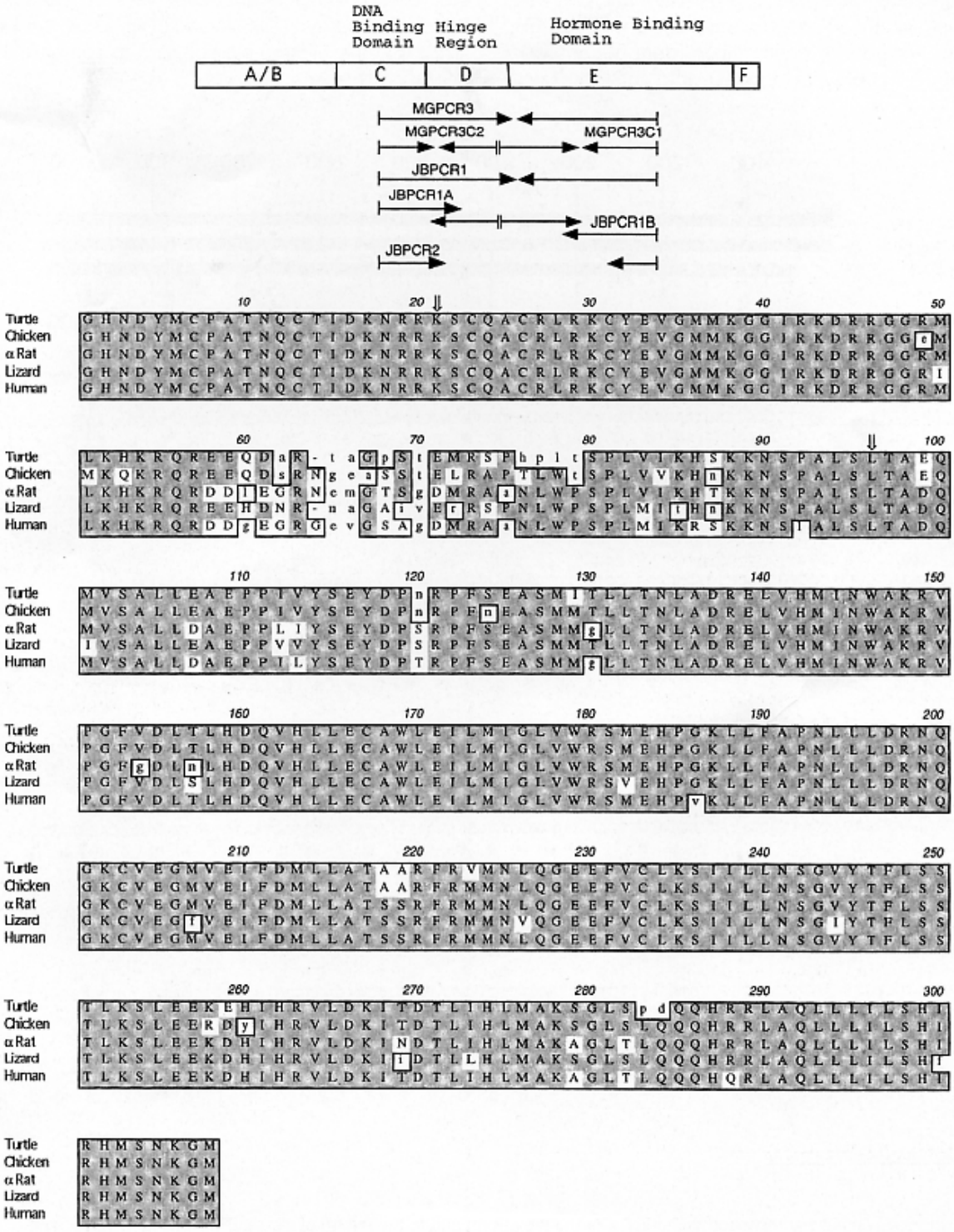
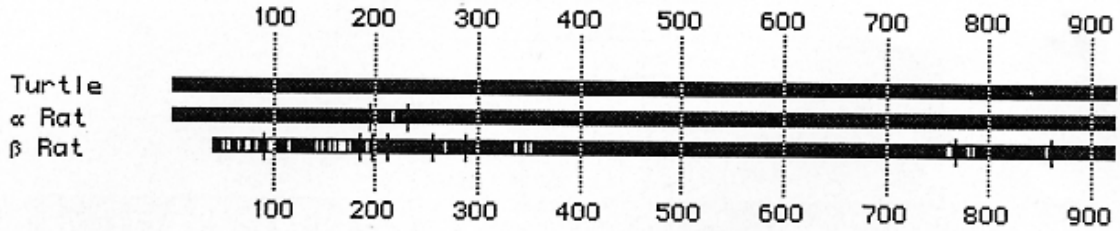


Fig. 1. Upper: Schematic diagram of functional domains of the estrogen receptor cDNA (adapted from Evans 1988) with a depiction of our clones and subclones generated to characterize the turtle ER. Lower: Comparison of the deduced protein sequence of *Trachemys scripta* estrogen receptor to published sequences of ER of other species (see text for references). Outlined regions are highly conserved, with shading indicating identical amino acids. The hinge region covers amino acids 21–96 and is indicated by arrows above the actual sequence.

5'TCCAT(TG)CC(CT)TT(AG)TT(AG)CTCAT-3' from the steroid-binding domain; bases in parentheses indicate degenerate positions. Southern blot analysis was performed on the PCR products, using chicken ER cDNA as a probe for confirmation (data not shown). Resulting cDNA were cloned into pGEM 7zf+ (Promega, Madison, WI, USA) resulting in three independent clones spanning 0.9 kb. The clones were sequenced using Sequenase, version 2.0 (United States Biochemical, Cleveland, OH, USA) and analyzed with the Microgenie software to compare to ER genes of other species.




```
        10         20         30         40         50         60         70         80         90        100
GGGCACAATGATTACATGTGTCCCGCTACTAACCAGTGCACCATCGACAAGAACAGGAGAAAGAGCTGTCAGGCCTGCCGGCTGCGAAAGTGCTATGAAG
  G  H  N  D  Y  M  C  P  A  T  N  Q  C  T  I  D  K  N  R  R  K  S  C  Q  A  C  R  L  R  K  C  Y  E

       110        120        130        140        150        160        170        180        190        200
TGGGAATGATGAAAGGTGGGATCCGAAAAGACCGCAGAGGCGGACGTATGCTGAAACACAAACGTCAGAGAGAGGAACAGGATGCCAGGACTGCAGGGCC
V  G  M  M  K  G  G  I  R  K  D  R  R  G  G  R  M  L  K  H  K  R  Q  R  E  E  Q  D  A  R  T  A  G  P

       210        220        230        240        250        260        270        280        290        300
TTCTACTGAGATGAGAAGCCCCCACCCTCTGACAAGTCCTCTTGTGATTAAGCATAGCAAGAAGAACAGTCCAGCTTTGTCCCTTACAGCAGAGCAGATG
  S  T  E  M  R  S  P  H  P  L  T  S  P  L  V  I  K  H  S  K  K  N  S  P  A  L  S  L  T  A  E  Q  M

       310        320        330        340        350        360        370        380        390        400
GTCAGTGCCTTACTGGAAGCTGAACCACCCATAGTCTATTCAGAGTATGATCCTAACAGACCTTTCAGTGAAGCCTCCATGATCACCCTGTTGACCAACC
  V  S  A  L  L  E  A  E  P  P  I  V  Y  S  E  Y  D  P  N  R  P  F  S  E  A  S  M  I  T  L  L  T  N

       410        420        430        440        450        460        470        480        490        500
TTGCAGACAGGGAACTGGTGCACATGATCAACTGGGCAAAAAGGGTCCCAGGGTTTGTGGATTTAACACTCCATGATCAGGTACATCTACTGGAATGTGC
L  A  D  R  E  L  V  H  M  I  N  W  A  K  R  V  P  G  F  V  D  L  T  L  H  D  Q  V  H  L  L  E  C  A

       510        520        530        540        550        560        570        580        590        600
CTGGTTAGAGATACTGATGATTGGCTTAGTCTGGCGTTCAATGGAACATCCGGGAAAACTCTTATTTGCACCTAATCTATTACTGGACAGGAATCAAGGG
  W  L  E  I  L  M  I  G  L  V  W  R  S  M  E  H  P  G  K  L  L  F  A  P  N  L  L  L  D  R  N  Q  G

       610        620        630        640        650        660        670        680        690        700
AAGTGCGTAGAGGGCATGGTGGAGATCTTTGACATGTTGCTGGCCACTGCTGCTCGGTTCCGTGTGATGAATCTCCAGGGGGAGGAATTTGTGTGCCTTA
  K  C  V  E  G  M  V  E  I  F  D  M  L  L  A  T  A  A  R  F  R  V  M  N  L  Q  G  E  E  F  V  C  L

       710        720        730        740        750        760        770        780        790        800
AGTCAATCATCCTGCTCAATTCAGGTGTGTATACATTTCTTTCCAGCACCTTGAAATCTTTGGAAGAGAAGGAGCATATTCATCGTGTTCTGGACAAAAT
K  S  I  I  L  L  N  S  G  V  Y  T  F  L  S  S  T  L  K  S  L  E  E  K  E  H  I  H  R  V  L  D  K  I

       810        820        830        840        850        860        870        880        890        900
CACAGATACATTGATTCATTTAATGGCCAAGTCGGGTCTCTCTCCAGATCAGCAACACAGACGACTGGCTCAGCTCCTCCTCATACTCTCGCACATCAGG
  T  D  T  L  I  H  L  M  A  K  S  G  L  S  P  D  Q  Q  H  R  R  L  A  Q  L  L  L  I  L  S  H  I  R

       910        920
CACATGAGCAACAAAGGAATGGA
  H  M  S  N  K  G  M  X
```

Fig. 2. Upper: Schematic comparison of the ER cDNA to the α-form and to the β-form of the rat ER. The alignment begins with the nucleotide (nt) of the rat ER that corresponds to the first nt of our tER clones. The horizontal black bars represent the cDNA sequences for each gene, with the numbers on the top and bottom line indicating the nt. Small vertical lines perpendicular to the rat sequence bars indicate extra nt that are not present in the tER cDNA; white lines in the rat sequence bars indicate non-conserved nt regions when compared to tER. The rat α-form is clearly more similar to the tER than is the β-form. Lower: Nucleotide sequence of the portion of the turtle ER (tER) cDNA characterized in the present study. PCR primers used to generate the clones are indicated with underlining.

Probe preparation

In situ hybridization probes were prepared from JBPCR1A (392 bp, see Figs 1,2). Sense and antisense riboprobes were synthesized by transcription with Sp6 and T7 polymerase, incorporating ^{35}S-CTP (NEN Du Pont, Boston, MA, USA) at a specific activity of 10^8–10^9 d.p.m./µg probe. 'Cold' antisense competitor probes were prepared by incorporating ^{3}H-UTP (NEN Du Pont) of low specific activity (10^3–10^4 d.p.m./µg) to facilitate quantitation. After degrading the cDNA transcription template, the probes were then purified by two rounds of ethanol precipitation with ammonium acetate. The yield of the synthesized probe was calculated from counting the final product on a liquid scintillation counter (Beckman) and dividing the counts by the specific activity of the probe.

Tissue preparation and hybridization

The AKG was cross-sectioned at 20 µm on a refrigerated cryostat (2800 Frigocut, Reichert-Jung, Nussloch, Germany). The sections were melted onto RNase-free poly L-lysine-coated microscope slides and allowed to dry at room temperature prior to storage at −80°C with desiccant. At the time of the assay, tissue sections were fixed in 4% paraformaldehyde, followed by rinses with phosphate-buffered saline (PBS). Sections were then treated with freshly prepared triethanolamine and acetic anhydride and rinsed in 2 × SSC, followed by dehydration in ethanol and delipidation in chloroform. The air-dried samples were then prehybridized overnight at 45°C in a hybridization solution containing 50% formamide, 10% dextran sulfate, 0.3 mol/L NaCl, 10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA, 1 × Denhardt's solution, 10 mmol/L dithiothreitol, and 2.5 mg/mL tRNA. Hybridization was carried out under the same conditions, except that tRNA was reduced to 0.5 mg/mL and an ER-specific ^{35}S-labeled probe was added at a final concentration of 0.3 µg probe × length (kb)/mL. Both prehybridization and hybridization were carried out in airtight containers lined with wet paper towels to keep the chamber humid. Following hybridization, sections were washed in

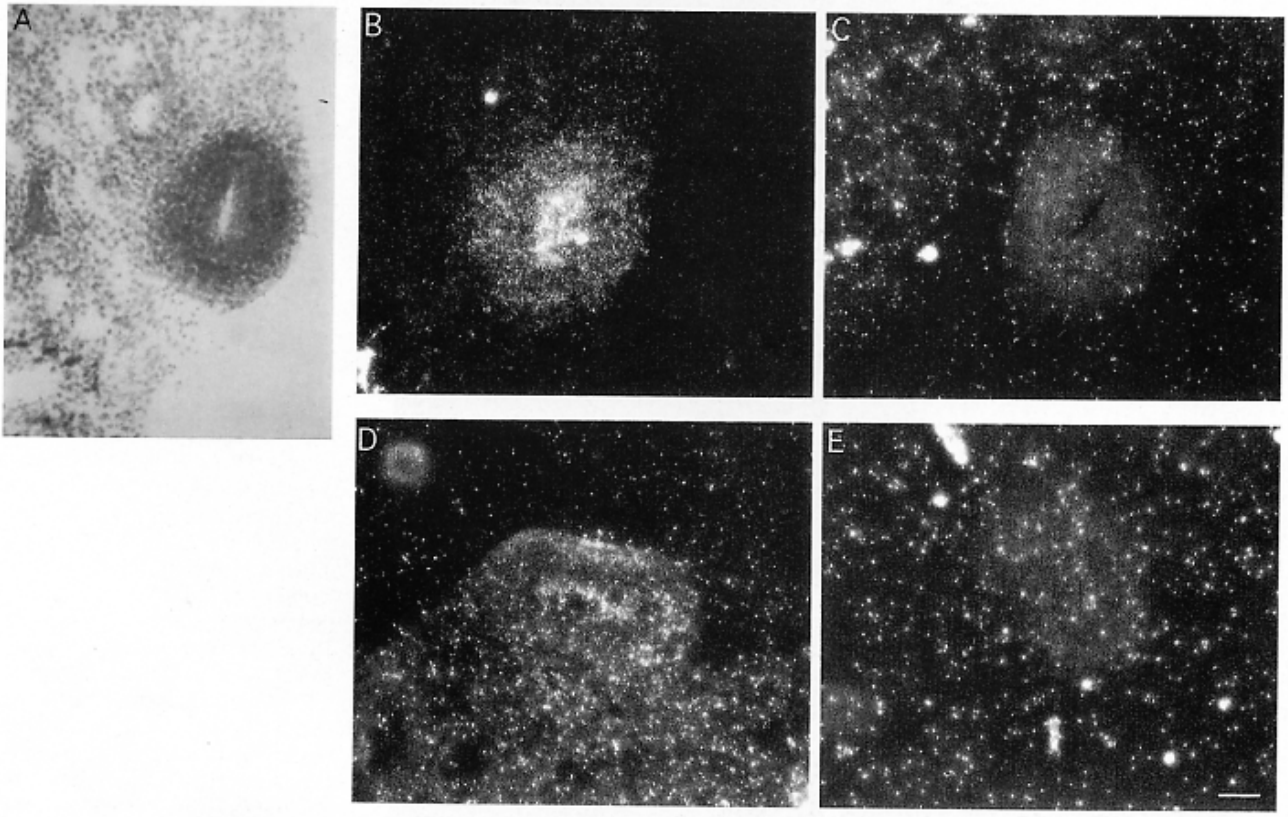


Fig. 3. Histological and *in situ* hybridization sections of oviduct from a stage 23 embryo at a female-producing temperature showing localization of tER. (A) Light-field microscopy. (B) Dark-field microscopy of the same section following *in situ* hybridization with the ^{35}S-labeled tER antisense probe. (C) Subsequent section of oviduct from the same animal following *in situ* hybridization with ^{35}S-labeled tER sense probe. (D) Subsequent section of oviduct from the same animal following *in situ* hybridization with ^{35}S-labeled tER antisense probe plus 100-fold excess non-radioactive antisense probe. (E) Subsequent section of oviduct from the same animal following *in situ* hybridization with ^{35}S-labeled tER antisense probe after the sections were degraded with RNase. Bar, 100 µm.

increased stringency washes to remove non-specific signal and dehydrated in alcohol, then air dried. The slides were then dipped in Kodak NTB-2 autoradiographic emulsion, dried, and exposed at 4°C. After 2 weeks, slides were developed in D-19 developer (Eastman Kodak, Rochester, NY, USA), fixed, and rinsed in water. Sections were immediately stained using hematoxylin-eosin staining, then coverslipped using Permount (Sigma Chemical Co., St Louis, MO, USA).

Analysis

Final results were analyzed using dark field microscopy. Specific labeling is defined as clusters of silver grains lying over stained AKG cells at a density of three times that of background. Quantitation of specific tissue areas and cells was carried out using the University of Washington Grain Counting program, version 1.102. For each individual AKG, the imaging system was programed to select the five highest labeled cell clusters within the gonadal tissues, then count the silver grains per cell. For background controls within each AKG cross-section, the system then repeated the selection and counting process for cell clusters in the kidney. The grains per cell for the less-labeled kidney section was subtracted from the grains per cell for the attached gonadal section to give a measure of mRNA abundance (grains per cell) for each individual animal. A preliminary assay was conducted in 1995 ($n=5$ or 6 per group) with a larger assay following collection of more tissue samples ($n=$ 8–12 per group) in 1996. The background (kidney) grains per cell between the two assays were com-

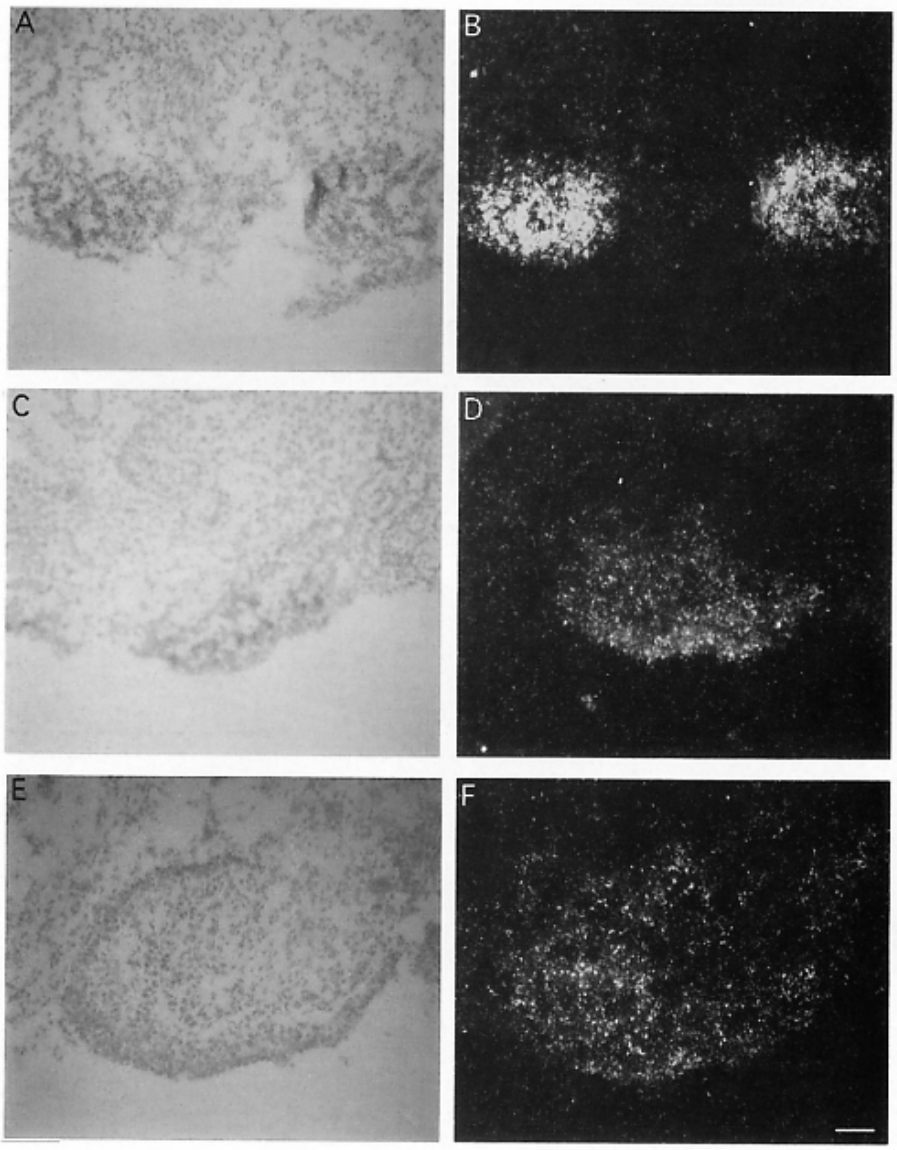


Fig. 4. Histological and *in situ* hybridization sections of turtle gonadal ridges from embryos at a female-producing incubation temperature (31°C), indicating ER mRNA expression. These are photographs of individual embryos to demonstrate specific localization. They may not, therefore, be representative of the mean values of tER mRNA expression for each group. The top row (A,B) are from an embryo in the beginning of the temperature sensitive period, or TSP, (stage 15). The second row (C,D) are from an embryo during the TSP (stage 19). The bottom row (E,F) are from an embryo after the TSP (stage 23), when the gonad has differentiated into an ovary. Bar, 100 μm.

pared using a Student's *t*-test. Mean grains per cell were determined for each group (defined by developmental stage and incubation temperature). Three-way analysis of variance (ANOVA) was used to compare means of all the groups, followed by hierarchical two-way ANOVA to determine if significance came from differences in the ER mRNA levels of different stages, different incubation temperatures, or from each assay. All statistics were performed using Systat 5.2.1.

Results

Cloning and sequencing

The amino acid sequence comparison of the turtle ER (tER) cDNA to other species showed that tER had the characteristic conservation of amino acid sequence in the DNA (100%) and steroid-binding (>90%) domains, but had greater difference in the less conserved hinge region (Fig. 1, lower). This degree of homology would characterize the present clone as ER. Comparison of tER cDNA to the two forms (α and β) of ER that have been identified (Kuiper *et al.* 1996) shows that we characterized the putative α form (Fig. 2, upper). The fragment used for *in situ* hybridization probes is the subclone JBPCR1A from nt1–392 (Fig. 2, lower).

In situ *validation*

Sections hybridized to the ^{35}S-labeled ER antisense probe showed intense specific localization of silver

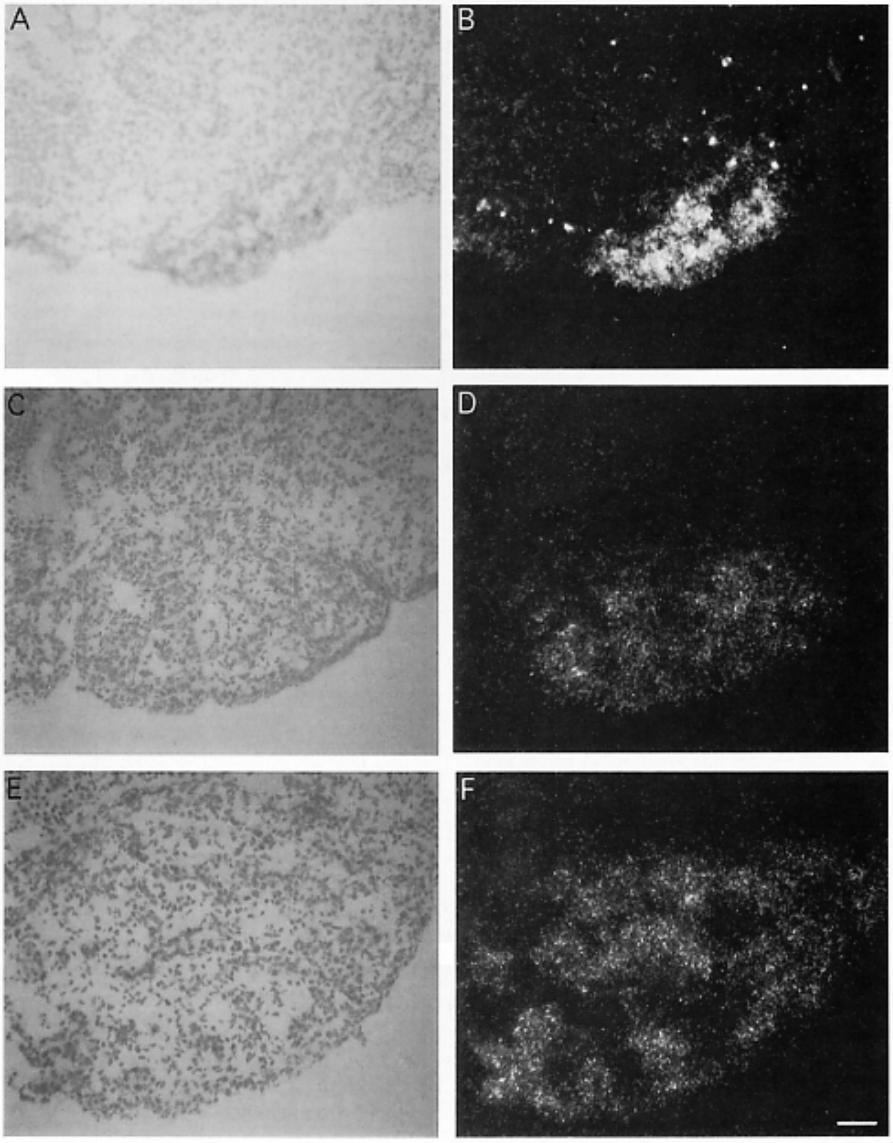


Fig. 5. Histological and *in situ* hybridization sections of turtle gonadal ridges from embryos at a male-producing incubation temperature (26°C), indicating ER mRNA expression. These are photographs of individual embryos to demonstrate specific localization. They may not, therefore, be representative of the mean values of tER mRNA expression for each group. The top row (A,B) are from an embryo at the beginning of the temperature sensitive period, or TSP, (stage 15). The second row (C,D) are from an embryo during the TSP (stage 19). The bottom row (E,F) are from an embryo after the TSP (stage 23), when the gonad has differentiated into a testis. Bar, 100 μm.

grains over the cells in the gonadal ridges and in the differentiated gonads, but not in the adrenal or kidney cells. There was no specific signal over the background level in the adjacent tissue section that had been hybridized to the ^{35}S-labeled ER sense probe. The specific labeling of the antisense probe was eliminated by the addition of 100-fold excess 'cold' antisense competitor probe and by RNase pre-treatment (Fig. 3).

Receptor distribution

In all stages, at all temperatures tested, ER mRNA was expressed in the urogenital tissues, but not in the adrenal or kidney (Figs 4,5). Even before the gonads began to differentiate as ovaries or testes, presumptive gonads showed intense hybridization to antisense ER mRNA. This signal remained specific to the gonadal cells during gonadal development, at all temperatures tested. Gonadal differentiation was accompanied by differences in the localization of ER mRNA. Ovaries showed general distribution throughout both the cortex and the medulla (Fig. 4), whereas the testes showed specific labeling over the testicular cords (Fig. 5).

Receptor quantitation

Three-way ANOVA comparing temperature, developmental stage, and assay effects showed significant effects ($F = 4.26$; d.f. = 4; $P < 0.01$). At extreme temperatures, in the beginning of the TSP (stage 15) when the urogenital ridge was undifferentiated, ER mRNA levels were greater in embryos from 31°C than in embryos from 26°C ($F = 10.81$; d.f. = 1; $P < 0.01$; Fig. 6). This relationship was reversed at the stages during gonadal differentiation, where the 26°C gonads showed higher ER mRNA levels than the 31°C animals ($F = 6.28$; d.f. = 1; $P < 0.02$). After the TSP had ended and the gonads were differentiated (stage 23), the ER mRNA levels were not different across temperatures ($F = 2.77$; d.f. = 1; $P < 0.11$). In comparing stages within temperatures, the 31°C embryos showed a decrease in the gonadal expression of ER mRNA as they went from beginning stages to the differentiated stage ($F = 4.66$; d.f. = 2; $P < 0.02$). These stage-related differences were not seen in the embryos from 26°C ($F = 2.40$; d.f. = 2; $P < 0.11$).

Within the intermediate temperature (29.2°C), there was no difference in the abundance of ER mRNA among the three embryonic stages sampled ($F = 1.15$;

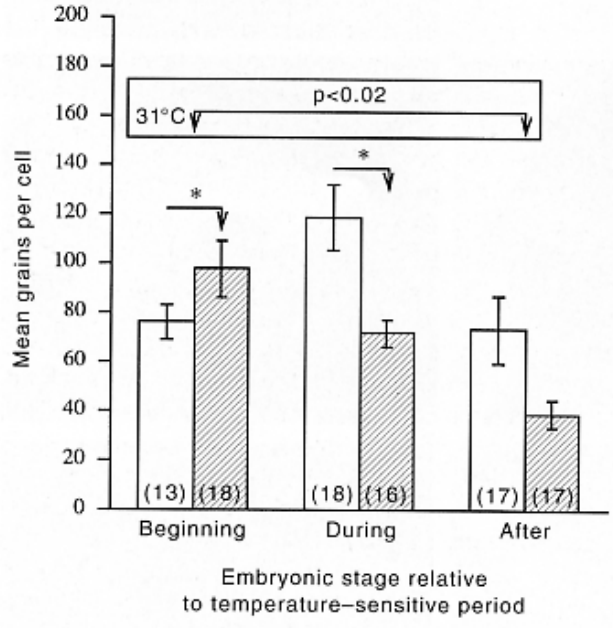


Fig. 6. Mean grains per cell at embryonic stages relative to the temperature-sensitive period. Shown are the values for the extreme temperatures (□, 26°C, male-producing; ▨, 31°C, female-producing). Significant differences comparing the temperature within stages are indicated by arrows above the bars. The box at the top indicates the significant differences when comparing stages within a temperature.

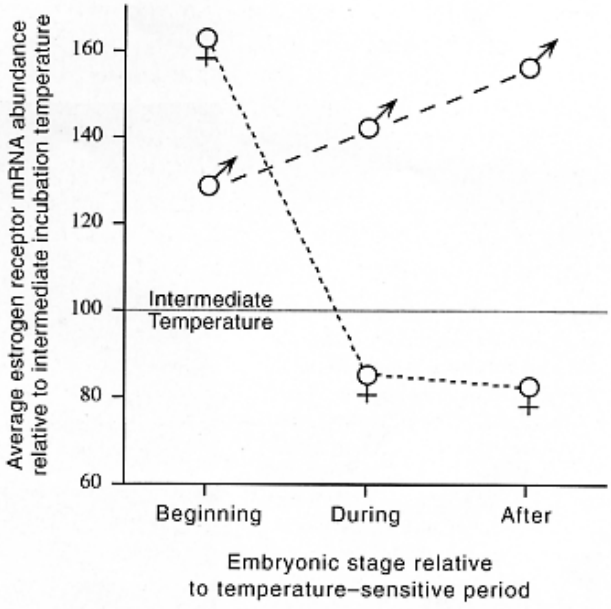


Fig. 7. Abundance of gonadal ER mRNA across stages at extreme temperatures (26°C, male; 31°C, female) relative to the threshold temperature (29.2°C). The tER mRNA levels at threshold temperatures were set to equal 100. The tER mRNA levels of the extreme temperature groups were then adjusted as a ratio of the corresponding stage from the threshold temperatures. The threshold temperatures in the 2 years that produced the subjects of the present study resulted in an approximately 60 : 40 female : male ratio.

d.f. = 2; $P < 0.33$). In the beginning stage, gonadal ER mRNA abundance in the embryos from 29.2°C was slightly lower than both 26 and 31°C, but this difference was only significant compared to the putative females ($F = 27.34$; d.f. = 1; $P < 0.01$). During and after the TSP (stages 19 and 23, respectively), embryos from the intermediate temperature fell between those of the 26 and 31°C embryos, but these values were not significantly different from either of the two extreme temperatures. At 29.2°C, the stages before visible gonadal differentiation contained both male and female gonads in unknown proportions. If there are already sex-specific genetic factors that cannot be accounted for by macroscopic characteristics, this may confound analysis. To examine the intermediate temperature effects, the means for the intermediate temperature groups were set at 100 and the extreme temperature means were adjusted proportional to the adjusted intermediates (Fig. 7).

Discussion

The present study sought to determine when the genes of estrogen receptors are expressed during development, and further, if incubation temperature modulates the expression of genes encoding these receptors. We know estrogen production to be a principal element of female sex determination as a result of inhibition of aromatase, and therefore estrogen production, inducing males at female-producing incubation temperatures (Crews & Bergeron 1994; Dorizzi *et al.* 1994; Rhen & Lang 1994; Wibbels & Crews 1994). Gonadal ER expression, therefore, would also be expected during ovarian differentiation. Estrogen receptor expression though, cannot be induced by incubation temperature, as complete estrogen-induced sex reversal can occur at male-inducing incubation temperatures. Indeed, the present study has shown that ER is also expressed in cells of gonadal tissues of embryos from male-producing incubation temperatures (Fig. 5). In the mouse as in the turtle, ER is present in the testes prior to phenotypic differentiation of the gonad (Greco *et al.* 1992). In addition to its role in ovarian development, ER probably plays an important role in male sexual differentiation. Male mice born without the ER gene show reduced testis size and decreased fertility (Lubahn *et al.* 1993).

As shown in the present study, however, there are differences in the relative amounts of ER mRNA prior to differentiation of the urogenital ridge (Figs 6,7). Gonadal cells in putative females are expressing more ER mRNA than their putative male counterparts. The higher temperature may be enhancing ER expression during initiation of the ovary-determining cascade. The greater ER mRNA levels then drop as the gonad nears the end of the TSP and remains low after the gonad has differentiated into an ovary. Embryos from male-producing incubation temperatures express consistent ER mRNA levels in all three stages of gonadal development. In the rat, ER mRNA in the brain is known to decrease in response to increased E2 (Lauber *et al.* 1991). In this same study, sex differences in the basal levels of ER mRNA corresponded to sex differences in the ER protein. If temperature is acting by increasing embryonic estrogen production as has been hypothesized (Pieau *et al.* 1994; Salame-Mendez *et al.* 1997), the decrease in gonadal ER mRNA at a female-inducing incubation temperature would support that relationship. Further investigation into binding properties of the receptor may help elucidate the precise mechanisms involved.

The portion of tER cDNA used in the present study may also bind ER-β if it is expressed in the turtle as in the rat (Kuiper *et al.* 1996). Because we only have a form for the α form of tER, we were unable to distinguish ER expression as α or β. It is important to note that in rats, ER-β plays an important role in both male and female gonadal functions. The ER expression shown in the turtle gonads in the present study may reflect a similar pattern as in the rat.

The current study also provides new data regarding temperature differences in gene expression at different stages of embryonic development. At the beginning of the TSP, when the gonadal ridge is at its most plastic, there is a wide range of values for ER mRNA abundance (grains per cell) in embryos from all three incubation temperatures (26°C: range 30–121 grains per cell; 29.2°C: range 13–112 grains per cell; 31°C: range 31–206 grains per cell). During the TSP, however, the individual variability of the ER mRNA signal is reduced in those embryos incubating at 31°C (range 40–109 grains per cell), while the 26°C embryos still show considerable variability (range 28–252 grains per cell). The mRNA signal of the embryos from the intermediate temperature fall between the extremes (range 57–140 grains per cell). Although these animals are all at the same morphological stages as determined by external characteristics (Yntema 1968), underlying molecular events may indicate a difference in developmental stage. Genetic thermosensitivity relative to gonadal differentiation is being studied in the Olive Ridley sea turtle (*Lepidochelys olivacea*), indicating a similar dissociation of molecular and morphological timing (Merchant-Larios *et al.* 1997). These authors have proposed that the initiation of sex differences is an extragonadal signal and that gonadal differentiation results from this

signal. In the current study, the embryos from 31°C (female) are only about 2–3 days from being irreversible in terms of gonadal differentiation, whereas the 26°C (male) gonads are 7–10 days from being irreversible. The reduced variability of the 31°C embryos may result from being well into the ovary-determining cascade. If this ovary-determining pathway includes a feedback mechanism through the ER, it could explain the more coordinated expression observed among these embryos at 31°C.

Differential rates of sexual development may be important to TSD species. In nature, unlike a laboratory incubator, nest temperatures may undergo daily temperature fluctuations. This seemingly might interrupt the sex determining process if the embryos are switching between male- and female-inducing incubation temperatures. The outcome of sexual differentiation then, would depend on what temperature the embryo experiences during the TSP. In the loggerhead sea turtle (*Caretta caretta*), Georges *et al.* (1994) demonstrate mathematically that it is the proportion of development that occurs above or below the threshold temperature. The variability of ER mRNA levels in the present study may be a reflection of synchronicity that comes from being further along in development. This would support the hypothesis that temperature may interact with genetic factors in a positive feedback manner to determine gonadal sex. This may not be exclusive to TSD species as shown with mammalian sex determination and gonadal differentiation in which events early in gonadal differentiation involve support cells (Sertoli cells in testes, follicle cells in ovaries), which then direct gonadal differentiation (McLaren 1991).

As estrogen-ER dependent processes may be critical to the mechanisms of TSD, it is important to examine any estrogen-ER differences between male- and female-producing temperatures. As we begin to elucidate these differences, we can then look more closely at the temperature range that produces varying sex ratios for a better understanding of sex-determining mechanisms. In embryos from stages 15 and 19, the urogenital ridge cannot be distinguished as ovary or testes by examination of histological differences. There may, however, be essential differences in the molecular process that would enable us to determine at an earlier stage if the gonad is developing as a testis or an ovary. At stages 19 and 23, gonadal ER mRNA levels per cell in embryos from the threshold incubation temperature were between those of either the male- or female-producing incubation temperatures (Fig. 6). Thus, the gonads may already be committed, and the ER levels reflect the genetic processes that have been initiated.

Following differentiation of the gonads as ovaries or testes (stage 23), sex-specific ER mRNA levels are

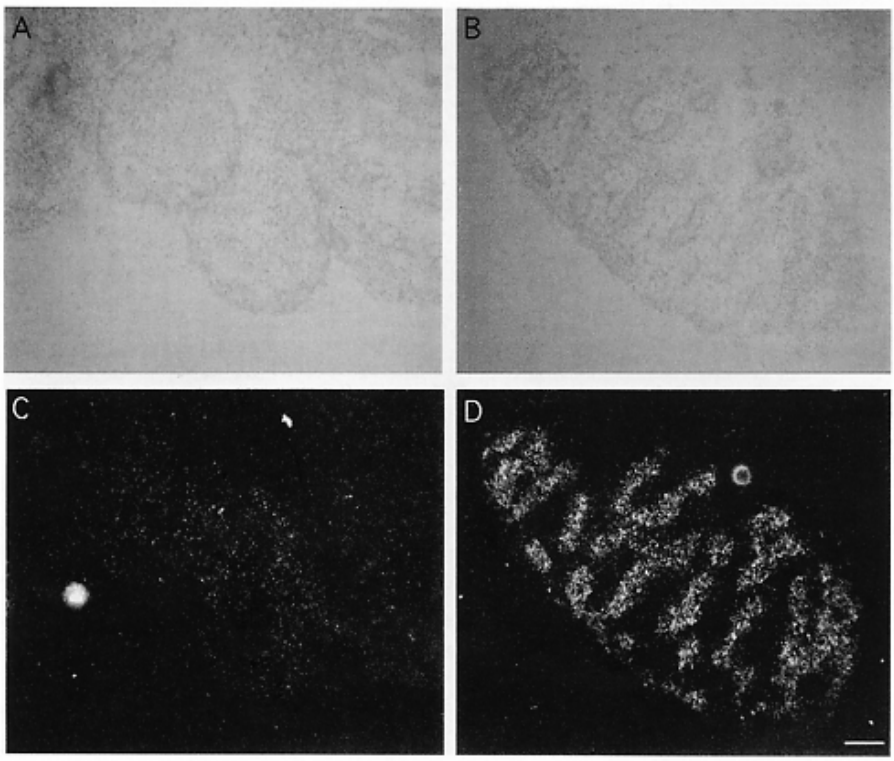


Fig. 8. Light- and dark-field pictures of an ovary (A,C) and a testis (B,D) from two embryos incubated at the threshold temperature that produces both sexes. Note the higher signal of ER mRNA per cell in the medullary cords of the testis. Bar, 50 μm.

also evident. In the embryos from 26°C, the testes as a group express more ER mRNA per cell compared to the ovaries in embryos from 31°C. The ER-expressing cells in the testes were concentrated in the medullary region in the sex cords. In the embryos from 29.2°C ($n = 16$), two-thirds of the gonads were ovaries, and these had a lower ER mRNA signal than did the testes from the same group (Fig. 8). In mammals, one very distinct difference in the functional aspects of the gonads is in the timing sequence of gametogenesis. Ovaries begin oocyte maturation before birth, and hold all the primary oocytes in meiotic prophase until puberty, after which they mature and are released in a specific, endocrine-regulated manner. Maturation of the sperm cells, however, is not initiated until well after birth, and sperm are then produced continuously. In addition to Leydig cells, spermatids and fully differentiated spermatozoa are known to produce estrogen in adult mice (Nitta *et al.* 1993). The germ-cell estrogen may serve as a regulatory signal sent back to the testicular cords for sperm maturation. Hence in the turtle, the high levels of ER in the embryonic testis, concentrated in the medullary cords, may be involved in the growth and regulation of spermatogenic cells.

Although the present study demonstrates that ER mRNA is expressed differentially relative to temperature, embryonic stage, and sex in the developing gonad, other studies indicate no such differences in the concentration of radiolabeled E2 during embryogenesis (Gahr *et al.* 1992). One explanation might be the presence in the AKG of other steroid-binding proteins that may bind to E2. Extracellular proteins comparable to mammalian serum albumin, sex-steroid binding protein (SSBP), or α-fetoprotein may have a role in E2 concentration (Bern 1990). An SSBP that binds E2 has been identified in the painted turtle (*Chrysemys picta*) by Salhanick and Callard (1980). Although other species show a high positive correlation between ER mRNA and ER protein levels, it is possible that in the red-eared slider, the ER mRNA levels may not reflect the ER protein levels. In the developing turtle there may be post-transcriptional regulatory processes involved in ER expression. Future assays with purified turtle ER will enable us to examine characteristics of estrogen binding to answer these questions.

Acknowledgements

The authors wish to thank C. T. Osborn III, for his tolerance and long hours during the multi-rack assays; K. Wennstrom, J. Godwin, L. Young, and Pat Randall for assistance with data analysis and interpretation; J. Branch for graphics; R. Baldwin with sequences and related figures; M. Ballinger with photography; C. Fitzpatrick, Y. Tasnif, A. Lindsey, T. Cole, H. Nguyen and H. Smith for assistance with animals, cryosectioning and assay preparation. This work was supported in part by an NSF predoctoral fellowship to JMB and NSF IBN 9723617 to DC.

References

Bern, H. A. 1990. The 'new' endocrinology: Its scope and its impact. *Am. Zool.* **30**, 877–885.

Crews, D. 1994. Temperature, steroids and sex determination. *J Endocrinol.* **142**, 1–8.

Crews, D. & Bergeron, J. M. 1994. Role of reductase and aromatase in sex determination in the red-eared slider turtle (*Trachemys scripta*), a turtle with temperature-dependent sex determination. *J. Endocrinol.* **143**, 279–289.

Crews, D., Bergeron, J. M., Flores, D. *et al.* 1994. Temperature-dependent sex determination: Proximate mechanisms, functional outcomes, and practical applications. *Dev. Genet.* **15**, 297–312.

Dorizzi, M., Richard-Mercier, N., Desvages, G., Girondot, M. & Pieau, C. 1994. Masculinization of gonads by aromatase inhibitors in a turtle with temperature-dependent sex determination. *Differentiation* **47**, 9–17.

Evans, R. M. 1988. The steroid and thyroid hormone receptor superfamily. *Science* **240**, 889–895.

Gahr, M., Wibbels, T. & Crews, D. 1992. Site of estrogen uptake in embryonic *Trachemys scripta*, a turtle with temperature-dependent sex determination. *Biol. Reprod.* **46**, 458–463.

Georges, A., Limpus, C. & Stoutjesdijk, R. 1994. Hatchling sex in the marine turtle *Caretta caretta* is determined by proportion of development at a temperature, not daily duration of exposure. *J. Exp. Zool.* **270**, 432–444.

Greco, T. L., Furlow, J. D., Duello, T. M. & Gorski, J. 1992. Immunodetection of estrogen receptors in fetal and neonatal male mouse reproductive tracts. *Endocrinology* **130**, 421–429.

Green, S., Walter, P., Kumar, V. *et al.* 1986. Human estrogen receptor cDNA: Sequence, expression, and homology to v-erb-A. *Nature* **320**, 134–139.

Greene, G. L., Gilna, P., Waterfield, M., Baker, A., Hort, Y. & Shine, J. 1986. Sequence and expression of human estrogen receptor complementary DNA. *Science* **231**, 1150–1154.

Koike, S., Sakai, M. & Muramatsu, M. 1987. Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Res.* **15**, 2499–2513.

Krust, A., Green, S., Argos, P. *et al.* 1986. The chicken estrogen receptor sequence: Homology with v-erb-A and the human estrogen and glucocorticoid receptors. *EMBO J.* **5**, 891–897.

Kuiper, G. G. J. M., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J-Å. 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *PNAS* **93**, 5925–5930.

Lauber, A. H., Mobbs, C. V., Muramatsu, M. & Pfaff, D. W. 1991. Estrogen receptor messenger RNA expression in rat hypothalamus as a function of genetic sex and estrogen dose. *Endocrinology* **129**, 3180–3186.

Lubahn, D. B., Moyer, J. S., Golding, T. S., Couse, J. F., Korach, K. S. & Smithies, O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disrution of the mouse estrogen receptor gene. *PNAS* **90**, 11162–11166.

McLaren, A. 1991. Development of the mammalian gonad: The fate of the supporting cell lineage. *Bioessays* **66**, 149–179.

Maxwell, B. L., McDonnell, D. P., Conneely, O. M., Schulz, T. Z., Greene, G. L. & O'Malley, B. W. 1987. Structural organization and regulation of the chicken estrogen receptor. *Mol. Endocrinol.* **1**, 25–35.

Merchant-Larios, H., Ruiz-Ramirez, S., Moreno-Mendoza, N. & Marmolejo-Valencia, A. 1997. Correlation between thermosensitive period, estradiol response and gonad differentiation in the sea turtle *Lepidochelys olivacea*. *Gen. Comp. Endocrinol.* (in press).

Nitta, H., Bunick, D., Hess, R. A. *et al.* 1993. Germ cells of the mouse testis express P450 aromatase. *Endocrinology* **132**, 1396–1401.

Pakdel, F., LeFuellec, C., Vaillant, C., LeRoux, M. G. & Valotaire, Y. 1989. Identification and estrogen induction of two estrogen receptors (ER) messenger ribonucleic acids in the rainbow trout liver: Sequence homology with other ERs. *Mol. Endocrinol.* **3**, 44–51.

Pieau, C., Girondot, M., Desvages, G., Dorizzi, M., Richard-Mercier, N. & Zaborski, P. 1994. Environmental control of gonadal differentiation. In *The Differences Between the Sexes* (Eds R. V. Short & E. Balaban), pp. 433–448. Cambridge University Press, Cambridge.

Rhen, T. & Lang, J. W. 1994. Temperature-dependent sex determination in the snapping turtle: Manipulation of the embryonic sex steroid environment. *Gen. Comp. Endocrinol.* **96**, 243–254.

Salame-Mendez, A., Herrera, J., Moreno-Mendoza, N. & Merchant-Larios, H. 1997. The brain as a sensor of temperature during sex determination in the sea turtle *Lepidochelys olivacea*. *Gen. Comp. Endocrinol.* (in press).

Salhanick, A. C. R. & Callard, I. P. 1980. A sex-steroid binding protein in the plasma of the freshwater turtle, *Chrysemys picta*. *Gen. Comp. Endocrinol.* **42**, 163–166.

Weiler, I. J., Lew, D. & Shapiro, D. J. 1987. The *Xenopus laevis* estrogen receptor: Sequence homology with human and avian receptors and identification of multiple estrogen receptor messenger ribonucleic-acids. *Mol. Endocrinol.* **1**, 355–362.

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

Wibbels, T. & Crews, D. 1994. Putative aromatase inhibitor induces male sex determination in a female unisexual lizard and in a turtle with TSD. *J. Endocrinol.* **141**, 295–299.

Yntema, C. L. 1968. A series of stages in the embryonic development of *Chelydra serpentina*. *J. Morphol.* **125**, 219–252.