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Hormonal Reversal and the Genetic Control of Sex Differentiation in *Xenopus*

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ABSTRACT—Administration of exogenous estradiol between stages 50 and 52 completely feminized the developing gonads of *Xenopus laevis*. However, when tadpoles were injected or cultured during the critical period with an inhibitor (CGS 16949A) of aromatase that prevents synthesis of estradiol from androgen, there were no detectable effects on the sexual differentiation of the gonads. Aromatase transcription in *Xenopus* gonads was then studied by the reverse-transcription polymerase chain reaction (PCR) method. In embryos at the beginning of the estradiol-sensitive period (stages 49 and 50), expression of the aromatase gene was not detected in the gonad. These results show that the period between stages 50 and 52 is the time when *Xenopus* is sensitive to sex reversal by estradiol and critical for sex determination, although estradiol synthesis may not be naturally involved in the gonad at this step.

INTRODUCTION

Many previous studies have attempted to elucidate the mechanisms of sex differentiation. In general, there are two such mechanisms in vertebrates: genotypic sex determination and temperature-dependent sex determination. Sexual differentiation to a male or a female is thought to depend on a switch mechanism that is triggered by a sex chromosome or a sex-determining gene. The sex of reptile embryos is controlled by temperature, but exogenous estradiol reverses the sex of male reptile embryos to yield apparently normal females or partially feminized males (Gutzke and Bull, 1986; Crews *et al.*, 1991). In bird embryos, exogenous estradiol also feminizes genetic males, but the effect is not permanent (Wolf, 1936; Scheib, 1983). Treatment of *Xenopus* tadpoles with estradiol can transform genotypic males into functional females (Witschi, 1967). Moreover, the sex of fish can also be reversed by sex hormone (Yamamoto, 1958). These findings are consistent with the hypothesis that estradiol may play a pivotal step in the sex determination cascade in these animals. Aromatase induces formation of estradiol from androgen. This reaction is irreversible and represents one-way regulation. In an ongoing series of investigations, aromatase inhibitors have been used in an attempt to block the endogenous production of aromatase during sex determination in a reptile, a bird, and a fish. The present study was conducted to determine the estradiol-sensitive period, to clarify the effects of a nonsteroidal aromatase inhibitor and to detect the transcription of the

aromatase gene in order to elucidate the role of exogenous estradiol during the stage at which *Xenopus* larvae are sensitive to the hormone.

MATERIALS AND METHODS

Embryos

Fertilized eggs of *Xenopus laevis* were obtained by injecting 200 IU of human chorionic gonadotropin into both the female and the male. They were dejellied with 2.5% sodium thioglycolate and allowed to develop at room temperature.

Treatment with estradiol and aromatase inhibitor

Tadpoles were placed in a solution of estradiol at 100 µg/L. Treatment with estradiol was started at stage 48, 49 or 50 and ended between stages 51 and 61. Thirty tadpoles were reared in plastic boxes with 5 L of control or sample solution. The solution was renewed every 2 days. After treatment, the tadpoles were reared without hormone for 1–2 months after metamorphosis. The aromatase inhibitor, CGS 16949A, was kindly provided by Novartis Co. Aliquots of the inhibitor at 12.5 µg/50 nl, 25 µg/50 nl or 50 µg/100 nl were injected into the abdominal cavity of the tadpoles at stage 49–50 via a micropipette. The treated tadpoles were then cultured in a solution of methyltestosterone at 100 µg/L. After stage 54, the tadpoles were fixed in Bouin's solution. In another experiment, the inhibitor was dissolved in water (100 µg/L, 200 µg/L, 500 µg/L, 800 µg/L or 1,200 µg/L), or dissolved simultaneously with aromatase inhibitor and methyltestosterone (aromatase inhibitor at 200 µg/L, 500 µg/L or 1,000 µg/L with methyltestosterone at 100 µg/L) and the tadpoles were reared in this solution between stages 50 and 52. The solution was renewed every day, and the tadpoles were then reared in water until stage 55–57. Animals at stages when their sex was distinguishable were immersed in Bouin's solution. The gonads were dissected from the tadpoles with the mesonephros attached, and these were then sectioned and stained. Sex was determined by examination of the fixed gonadal sections or from the external features of the gonads of male

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and female frogs (Chang and Witschi, 1956).

PCR amplification and RT-PCR analysis

Total RNA was isolated from 130 gonads with the attached mesonephros of tadpoles at stage 49–50, and from 0.1 g of the adult ovary of *Xenopus*, using RNazol B (Tel-Test, Inc.). The mRNA was purified using oligo (dT)-cellulose (Pharmacia Biotech), and reverse-transcribed into cDNA (Takara). To clone the aromatase gene expressed in the ovary, we designed three mixed primers based on the published protein sequences of aromatase (Tanaka *et al.*, 1992). For the first round of amplification, PCR (30 sec at 95°C, 30 sec at 37°C, 30 sec at 72°C for 30 cycles) was performed in 50 µl of buffer system with primers 1 and 2 (Table 1) and 1 µl of ovary cDNA. When primers 1 and 2 were used, the nucleotide fragment in the PCR product was 624 bp. A major band of about 620 bp was obtained together with minor bands. The PCR product was extracted from the main band on the polyacrylamide gel. For the second round of amplification, PCR was performed under the same conditions as those for the first round, except that primers 1 and 3 were employed, and the PCR product of the first round was used as a template. The product (540 bp) of the reaction was purified by polyacrylamide gel electrophoresis and ligated to the pGEM-T vector. One of the sequences apparently corresponded to the aromatase gene, and this was used as the oligonucleotide primer (primers 4 and 5) in the PCR (Table 1). PCR analysis of aromatase expression involved 25 cycles of denaturation at 94°C for 30 sec, annealing at 41°C for 30 sec and extension at 72°C for 30 sec. PCR analysis of EF-1 α expression was carried out under the same conditions as those for aromatase expression, except that the annealing temperature was 55°C.

Histology

All the embryos were fixed in Bouin's solution and the gonads with the attached mesonephros were cut into 10-µm-thick sections and stained with hematoxylin and eosin by routine procedures.

RESULTS

Treatment with estrogen

In order to identify the estradiol-sensitive period, we administered estradiol to tadpoles at various stages. Estradiol was suspended in water at 100 µg/L. The tadpoles were exposed to estradiol from stages 48–50 to stages 51–61 (Table 2). If exposure to estradiol was started at stage 48 and stopped at stage 51, the gonads developed into ovaries, testes and hermaphroditic organs. When tadpoles were cultured from stage 50 to stage 52 with estradiol, the gonads all developed into ovaries. Thus, in *Xenopus*, gonads treated with estradiol from stage 50 to 52 underwent conversion from testicular to ovarian development, and complete feminization required about 9 days.

Treatment with aromatase inhibitor

It is still not clear how estradiol treatment promotes feminization of gonads. To define the stage at which gonads were sensitive to naturally synthesized estradiol, we treated tadpoles with aromatase inhibitor. The inhibitor was injected into the abdominal cavity at various concentrations before the estradiol-sensitive period. The treated tadpoles were cultured in water containing methyltestosterone at 100 µg/L. Injection of tadpoles with the inhibitor resulted in very high mortality (84%–87%), whereas tadpoles injected with water did not die (Table 3). High embryonic mortality (40–70%) has also been reported in alligator embryos injected with the same reagent (Lance

Table 1. Primers used to cloning of aromatase gene and RT-PCR analysis.

Name (References)	Sequences	Sizes of products
Aromatase (Mixed primers: Tanaka <i>et al.</i> , 1992)	1 TA(CT)TT(CT)(AG)A(CT)(AG)C(AGCT)TGGCA(AG)	624
	2 TTCATCAT(AGCT)ACCAT(AGCT)GC(AGT)AT	
	3 CC(ATGC)AT(AG)TT(ATGC)A(AG)(AGT)AT(AGT)AT(AG)TT	
	4 TATTTCGATACATGGCAG	533
	5 TTGAAGATTATGTTAGTG	
EF-1 α (Triege <i>et al.</i> , 1989)	6 GGAGCATCAATGATAGTGAC	226
	7 GGAAAGTCCACAACAACCTGG	

Table 2. Effects of estradiol on gonadal differentiation in *Xenopus*. Tadpoles were placed in a suspension of estradiol (100 µg/L). Tadpoles treated of various stages were reared in water until they developed into frogs and were then fixed. Sex was determined by observations of gonadal sections and external features of gonads. (): Sex was decided on the basis of external features.

Timing of treatment (stages)	Total no. of tadpoles	Ovaries	Testes	Hermaphroditic organs
48–56	40	13 (27)	0	0
48–51	28	2 (10)	1 (5)	4 (6)
49–52	24	14 (10)	0	0
49–53	28	16 (12)	0	0
50–61	23	6 (17)	0	0

Table 3. Effects of injection of the aromatase inhibitor on gonadal differentiation in *Xenopus*. A solution of the aromatase inhibitor at 12.5 µg/50 nl, 25 µg/50 nl and 50 µg/100 nl was injected. After 24 h, the surviving tadpoles were counted. After stage 55–57, tadpoles were fixed, cross sections of gonads were examined and the sex was decided. Mortality after injection of water or aromatase inhibitor is shown in parenthesis.

Dose (µg)	Original no. of tadpoles	No. of surviving tadpoles (% mortality)
Water	50	50 (0%)
12.5	80	13 (84%)
25	119	15 (87%)
50	23	3 (87%)

and Bogart, 1991). Disturbance of embryos at an early sensitive stage obviously resulted in death through an unknown mechanism. The gonadal structure of surviving tadpoles was not obviously modified (Fig. 1). After sexual differentiation, in the case of the ovary, an ovarian cavity was formed and, in some parts of the cortex, multiplying oogonia formed a mass (Fig. 1A). In the case of the testis, germ cells appeared in the medullary tissue and formation of the seminiferous tubules was apparent (Fig. 1B). For application of the inhibitor, tadpoles were also placed in a solution of the inhibitor at various concentrations (Table 4). Embryos treated with any concentration of the inhibitor showed no sex reversal. Tadpoles treated with aromatase inhibitor at 1.2 mg/L showed altered somatic growth. When the control tadpoles had reached stage 55–56, about 70% of the treated tadpoles showed growth retardation (stage 51–52), although application of the aromatase inhibitor had no effect on sex determination. Furthermore, no morphological change related to sex occurred after simultaneous application of aromatase inhibitor and methyltestosterone (Table 5). Thus, neither method of inhibitor administration affected the sex ratio.

In previous studies, methods of treatment and doses of aromatase inhibitor have differed among the animal species employed. Injection of developing turtle embryos with CGS

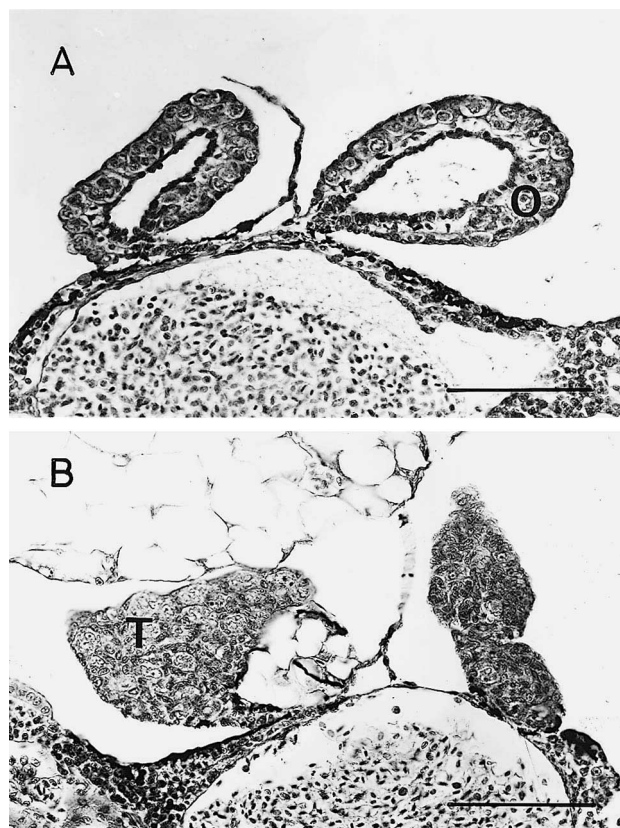


Fig. 1. The ovary and testis of a female (A) and a male (B) tadpole treated with aromatase inhibitor. Aliquots of the aromatase inhibitor were injected into the abdominal cavity of the tadpoles. After stage 54–55, the tadpoles were fixed, and cross-sections of gonads were examined. Abbreviations: O, ovary; T, testis. Scale bar, 100 μ m.

Table 4. Effects of treatment with the aromatase inhibitor on gonadal differentiation in *Xenopus*. Tadpoles were placed in a solution of the inhibitor at 100 μ g/L, 200 μ g/L, 500 μ g/L, 800 μ g/L or 1,200 μ g/L. Sex was determined from observations of cross sections of gonads.

Dose	Female	Male
100 (μ g/L)	16	8
200	10	12
500	12	11
800	10	8
1200	6	4

Table 5. Effects of treatment with the aromatase inhibitor and testosterone on gonadal differentiation in *Xenopus*. Tadpoles were placed in a solution of the inhibitor at 200 μ g/L, 500 μ g/L or 1,000 μ g/L containing methyltestosterone at 100 μ g/L. Sex was determined from observations of cross sections of the gonads.

Dose	Female	Male
200 (μ g/L)	11	13
500	15	15
1000	12	16

16949A resulted in male sex determination (Wibbels and Crews, 1994). The sex ratios (males:females) produced by the various treatments were control 9:15, 1 μ g 24:5, 10 μ g 20:5, and 100 μ g 27:0. Sex ratios in all groups treated with aromatase inhibitor were significantly different from that in the control group. Injection of lizard embryos with CGS16949A at 1 μ g/egg caused 100% masculinization (Wennstrom and Crews 1995). For chicken, injection of CGS 16949A at doses exceeding 50 μ g/egg resulted in a male phenotype in almost all hatchlings (Elbrecht and Smith 1992). When genetically female fish embryos received only a single 2-h immersion treatment with simultaneous application of 1 mg of aromatase inhibitor and 100 μ g of testosterone, they developed into perfect males indistinguishable from normal ones (Piferrer *et al.*, 1994). Rat ovary aromatase is inhibited *in vivo* by CGS 16949A at a dose as low as 3 μ g/kg (Steele *et al.*, 1987). These reports show that the doses of aromatase inhibitor used in our experiment are sufficient to prevent aromatase activity, but insufficient for sex reversal.

PCR amplification and RT-PCR analysis

To clone the aromatase gene, we designed PCR primers based on the published protein sequences for aromatases (Tanaka *et al.*, 1992). PCR amplification of ovary cDNA and subcloning of the products into pGEM-T led to the isolation of part of the aromatase gene. Fig. 2 shows the predicted amino acid sequence of *Xenopus* aromatase compared with those of chicken, rat and trout; the amino acid sequence identity is 75%, 67% and 59%, respectively.

Expression of the aromatase gene was examined during the estradiol-sensitive period in gonads of *Xenopus* embryos, using reverse transcriptase and PCR (Fig. 3). In gonads between stages 49 and 50, aromatase transcription was not

Xenopus,	YFDTWQALLKPDNFFKISCLYKKYERAANDLKEAIELLI EQKRQKLSSSEKLD EMD FSS ELIFAQNHGDLT AENV	219	295
Chicken,	YFD AWQALLKPDIFFKISWL SKKYEEAAKDLKGAMEILIEQKRQKLSTVEKLD EHMDFASQLIFAQNRGDLT AENV	220	296
Rat,	YFN AWQALLKPNIFFKISWLYRKYERSVKDLKDEIEILVEKKRQKVSSAEKLEDCMD FATDLIFAERRGDLT KENV	241	316
Trout,	YFDTWQT VLIKPDVYFKLDWIHEKHRRAAQELED AIESLV DQKRRLQEADKLDHIN FTADLIFAQSHGELS AENV		
	NQCILEMLIAAPDTMSVSLF FMLVLIAQH PKIEEGIMNEMDKVIGDRDVESNDIPNLKILESFIYESMRYLPVVDLVMRKALEDD		380
	NQCVLEMMIAAPDTLSVTLFIMLILIAADPTVEEKMMREIETVMGHRVQSDDMPNLKIENFIYESMRYQPVDLIMRKALQDD		381
	NQCILEMLIAAPDTMSVTLYM LLLIAEYPEVETAILKEIHTVVGDRDIRIGDVQNLKVVENFINESLRYQPVDLVMRRALEDD		401
	RQCVLEMVIAAPDTLSISLFFMLLLLKQNPDELQ LLEEIDTAIGDRELHNSDLQNLRVLESFINESLRFHPVVDFTMRRALEDD		
	% Homology		
	IIDGYVVKKG TNIIFN	100	
	396		
	VIDGYPVKKG TNIILN	75	
	397		
	VIDGYPVKKG TNIILN	67	
	417		
	VISGYRVPKG TNIILN	59	

Fig. 2. Comparison of *Xenopus*, chicken, rat and trout aromatase polypeptide sequences. Aromatase cDNA from *Xenopus* ovary was isolated by the RT-PCR method and predicted peptide sequences of aromatase cDNA from chicken, rat and trout were compared. Amino acids are numbered from the first methionine. Positions with variations in amino acids are indicated by “•”, while “–” represents a gap inserted to optimise alignment of sequences. The sources of the aromatase sequences are chicken (McPhaul *et al.*, 1988), rat (Lephart *et al.*, 1990) and trout (Tanaka *et al.*, 1992).

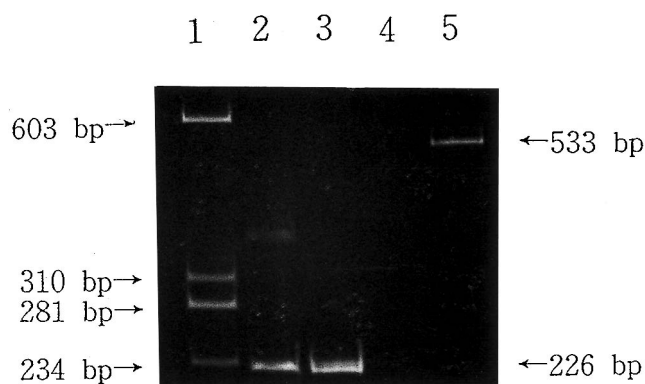


Fig. 3. RT-PCR analysis. The cDNA of ovary and gonad were used as templates for PCR using oligonucleotide primers based on sequences corresponding to the regions conserved in the genes for aromatase and EF-1 α protein. The products were separated on 5% polyacrylamide gel and visualized by ethidium bromide staining. Lane 1: ϕ X174-HaeIII-digested DNA as a molecular weight marker; lane 2: gonad cDNA and EF-1 α primers 6 and 7; lane 3: ovary cDNA and EF-1 α primers 6 and 7; lane 4: gonad cDNA and aromatase primers 4 and 5; lane 5: ovary cDNA and aromatase primers 4 and 5.

detected (Fig. 3, lane 4), whereas aromatase transcription was detected in the ovary of adult *Xenopus* (Fig. 3, lane 5). As the control primers, the sequence of elongation factor 1 alpha (EF-1 α) (primers 6, and 7) was used for RT-PCR analysis. The expression of EF-1 α , in terms of the amount of RNA, was similar in the gonad (Fig. 3, lane 2) and ovary (Fig. 3, lane 3).

DISCUSSION

Treatment with steroid hormones has been shown to affect sex determination in a variety of vertebrates. Administration of estradiol to *Xenopus* embryos causes them to become females. It has been reported that between tadpole stages 52 and 55, exogenous estradiol converts testicular development to ovarian development (Chang and Witschi, 1956). When estradiol treatment was initiated at stages 44–50 and was continued for 3 months (stages 56–67), 100% sex reversal of tadpoles was induced (Villalpando and Merchant-Larios, 1990). In our study, the stage at which tadpoles were sensitive to estradiol (stage 50) fell within the reported stage range, although the period for which we conducted estradiol treat-

ment (about 9 days) differed. We found that sensitivity to exogenous estradiol occurred at stage 50–52. Sexual differentiation is a sequential and orderly process, and the step that is sensitive to exogenous estradiol in the sex determination cascade is probably important during the natural development of tadpoles.

Treatment of developing embryos with a nonsteroidal aromatase inhibitor induces male sex determination in reptiles, birds and fish. (Elbrecht and Smith, 1992; Wartenberg *et al.*, 1992; Wibbels and Crews, 1994; Piferrer *et al.*, 1994; Smith *et al.*, 1995). If aromatase inhibitor acts during the estradiol-sensitive stage in *Xenopus* embryos, our findings provide strong support for the hypothesis that estradiol might also be involved in natural sex determination and gonadal differentiation in *Xenopus*. The importance of testosterone conversion to estrogen by aromatase might not be restricted to sex determination in reptiles, birds and fish. We examined whether the synthesis of estradiol is conserved as a common component in the sex determination of amphibians. However, administration of aromatase inhibitor to *Xenopus* tadpoles did not appear to affect gonadal differentiation: the gonads showed a sex ratio of approximately 1:1.

In chicken, although 100% of embryos treated with aromatase inhibitor developed a male phenotype upon hatching, only 50% developed into permanent males (Elbrecht and Smith, 1992). In the alligator, the same aromatase inhibitor prevented normal ovarian development, but did not masculinize females (Lance and Bogart, 1991). These results suggest that other regulatory components may be necessary for promotion of normal male sexual differentiation. Thus, the effects of aromatase inhibitor differ among species.

Furthermore, aromatase transcription was not detected in gonads between stages 49 and 50. Thus, the aromatase gene may not be expressed in the gonads at this step of sex determination. It has also been reported that *Xenopus* tadpoles are capable of steroidogenic activity in the interrenal region after stage 47, but are not capable of this activity in the gonads between stages 50 and 52 (Rao *et al.*, 1969; Kang *et al.*, 1995). Steroid metabolism and production in the gonads of *Rana catesbeiana* also occur after gonadal differentiation (Hsu *et al.*, 1985). The step of aromatase expression in the gonad must lie close to the beginning of the female sex-determination pathway in birds, reptiles, salamanders and fish (Desvages and Pieau 1992; Piferrer *et al.*, 1994; Chardard *et al.*, 1995; Smith *et al.*, 1997). The mechanism of natural feminization that occurs between stages 50 and 52 in *Xenopus* embryos might differ from this. It has been reported that the yolk of alligator embryos contain a high concentration of steroid, and that the yolk may provide the estradiol which initiate gonadal development (Conley *et al.*, 1997). Also, the estradiol that influences sex differentiation in *Xenopus* tadpoles may be of maternal origin.

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