Suppression of P450 aromatase gene expression in sex-reversed males produced by rearing genetically female larvae at a high water temperature during a period of sex differentiation in the Japanese flounder (*Paralichthys olivaceus*)

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ABSTRACT

The phenotypic sex of many teleost fishes including flounders can be experimentally altered by treating embryos or larvae with varied temperatures or sex-steroid hormones. To analyse the sex determination mechanism, especially the role of cytochrome P450 aromatase (P450arom), an enzyme that catalyses the conversion of androgens to estrogens, in temperature-dependent gonadal sex differentiation in the Japanese flounder, we generated two populations of larvae, both having XX (genetic females) but each growing up to display all phenotypic females or males, by rearing the larvae at normal (18 °C) or high (27 °C) water temperatures from days 30 to 100 after hatching respectively. The larvae (XX) were produced artificially by mating normal females (XX) with gynogenetic diploid males (XX) which had been sex-reversed to phenotypic males by 17α-methyltestosterone.

To study the role of P450arom in sex determination in the flounder, we first isolated a P450arom cDNA containing the complete open reading frame from the ovary. RT-PCR showed that P450arom mRNA was highly expressed in the ovary and spleen but weakly in the testis and brain. Semi-quantitative analyses of P450arom mRNA in gonads during sex differentiation showed that there was no difference in the levels of P450arom mRNA between the female and male groups when the gonad was sexually indifferent (day 50 after hatching). However, after the initiation of sex differentiation (day 60), the mRNA levels increased rapidly in the female group, whereas they decreased slightly in the male group. Similarly, estradiol-17β levels rose remarkably in the female group, yet remained constant in the male group.

These results suggest that induction of sex reversal of genetically female larvae to phenotypic males by rearing them at a high water temperature caused a suppression of P450arom gene expression. Furthermore, we suggest that the maintenance of P450arom mRNA at very low levels is a prerequisite for testicular differentiation, while the increased levels are indispensable for ovarian differentiation.
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INTRODUCTION

In vertebrates, including fishes, sex is often determined by genotype under normal circumstances. In some fishes, however, sex determination is greatly influenced by environmental factors (Conover & Kynard 1981, Rubin 1985, Sullivan & Schultz 1986). For example, the genetic sex determination mechanism in the Japanese flounder (*Paralichthys olivaceus*) is basically XX(female)–XY(male) type (Tabata 1991, Yamamoto *et al*. 1992). The female to male ratio in the Japanese

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flounder decreases drastically with exposure of the larvae to higher or lower water temperature than normal (17·5–22·5 °C) during sex differentiation (Yamamoto 1995, 1999). In addition, the phenotypic sex of many teleost fishes (Hunter & Donaldson 1983, Yamazaki 1983) including the flounder (Tanaka 1988, Tabata 1991) can be experimentally altered by treatment of the embryos or larvae with sex-steroid hormones, suggesting an important role of sex-steroid hormones in phenotypic sex differentiation in fishes.

Cytochrome P450 aromatase (P450arom) is a steroidogenic enzyme responsible for conversion of androgens to estrogens. In vertebrates, this enzyme is expressed in various tissues and plays an important physiological function by regulating estrogen biosynthesis (Pasmanik & Callard 1985, Matsumine *et al*. 1986, Sasano *et al*. 1997). Moreover, the estradiol-17β produced by the action of P450arom is essential for ovarian development (Wallace 1985). Recently, several reports have described the expression of P450arom during sexual differentiation in several species. P450arom mRNA is detected in the gonads of genetic females but not of males during sexual differentiation in the chicken (Yoshida *et al*. 1996, Smith *et al*. 1997). P450arom activity is significantly higher during ovarian differentiation than during testicular differentiation in reptiles and amphibians which exhibit temperature-dependent sex determination (Desvages & Pieau 1992, Desvages *et al*. 1993, Chardard *et al*. 1995). Positive immunoreaction against P450arom appears in differentiating ovaries, but not in differentiating testes in tilapia (*Oreochromis niloticus*) (Chang *et al*. 1997*a*). However, the role of P450arom in gonadal sex differeniation of fishes remains unclear, particularly in species with temperature-sensitive, phenotypic sexual differentiation.

To address the role of P450arom in gonadal sex differentiation in the Japanese flounder, we cloned a P450arom cDNA and examined mRNA expression in various adult organs. Furthermore, we analysed the expression level of P450arom mRNA in the flounder gonad and concentrations of sex-steroid hormones during sex differentiation in females (XX) reared under normal water temperature and in sex-reversed males (XX) reared under higher water temperature.

MATERIALS AND METHODS

Animals

Japanese flounder (*Paralichthys olivaceus*) were reared at natural sea water temperature (12–28 °C) in our laboratory.

We produced two populations of larvae, both having XX (genetic females), but each growing up to display all phenotypic females or males, making use of meiotic gynogenetic diploid. First, the meiotic gynogenetic diploid was induced by cold shock treatment (1 °C for 30 min, 4 min after insemination) of the eggs which had been inseminated by UV-irradiated (1000 erg/mm^2, 1 min) sperm. All the resultant larvae were genetically XX. The phenotypic sex was then reversed to male by feeding the larvae from days 30 to 100 after hatching with an artificial diet (Love Larva, Maruha Co., Yamaguchi, Japan) containing 17α-methyltestosterone (10 μg/g diet; Nakarai Tesque, Inc., Kyoto, Japan). These sex-reversed phenotypic males of meiotic gynogenetic diploid were then mated with normal females to produce all genetic female broods. These larvae (all XX) were reared at normal (18 °C) or high (27 °C) water temperatures from days 30 to 100 after hatching, the thermosensitive period for sex differentiation (Yamamoto 1995, 1999), to produce phenotypic females and males respectively. The percentage of the phenotypic females (males) among the resultant adult fishes reared at 18 °C (27 °C) was 100%, based on the morphological and histological observations of the adult gonads at 10 months of age (Yamamoto 1995).

Ten to twenty larvae or juveniles were sampled every 10 days after hatching. After measurements of the total body lengths, the samples were frozen at −80 °C for quantification of P450arom mRNA and sex-steroid hormones.

Construction of cDNA library

Total RNA was extracted by the guanidinium isothiocyanate–CsCl method (Chirgwin *et al*. 1979) from an ovary containing vitellogenic oocytes in a 2-year-old flounder. Poly(A)$^+$ RNA was purified by affinity chromatography on an oligo(dT)-cellulose column using an mRNA purification kit (Pharmacia Biotech, Tokyo, Japan). Double-stranded cDNA was synthesized according to the method of Gubler & Hoffman (1983) with the following modifications. Oligo(dT) primer with XhoI site at the 5′-end was used, and dCTP in the buffer for first-strand synthesis was substituted by 5-methyl dCTP for the protection of cDNA against the following XhoI treatment. After ligation with EcoRI adaptor (Stratagene, La Jolla, CA, USA), the cDNA was treated with XhoI and inserted into the EcoRI–XhoI site of λZAP phage vector (Uni-ZAP XR vector; Stratagene). The resulting constructs were packaged in Gigapack III packaging extract (Stratagene).

Isolation of P450arom cDNA

A flounder ovarian cDNA library was screened with an EcoRI–XhoI fragment (1·8 kbp) of tilapia P450arom cDNA (Chang *et al*. 1997*b*) as a probe which had been labeled with digoxigenin (DIG) by the method of Feinberg & Vogelstein (1983) using a DIG DNA labeling kit (Boehringer Mannheim, Tokyo, Japan). Plaque replicas on nylon membranes (Hybond-N^+; Amersham, Tokyo, Japan) were hybridized with the probe in DIG Easy Hyb solution (Boehringer Mannheim) at 42 °C for 16 h. The membranes were washed twice with 2 × SSC (0·3 M NaCl, 30 mM sodium citrate)/0·1% SDS at room temperature for 5 min and twice with 0·1 × SSC/0·1% SDS at 68 °C for 15 min. Detection of DIG was performed by immunoreaction with 1:10 000 diluted alkaline phosphatase-conjugated anti-DIG antibody, followed by treatment with 337·5 μg/ml nitroblue tetrazolium salt and 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate, using a DIG nucleic acid detection kit (Boehringer Mannheim).

DNA sequencing

The pBluescript phagemid containing the desired cDNA was excised from P450arom-positive phages by co-infection of *Escherichia coli* (XL1-Blue MRF′) with the positive phage and the helper phage R408 (Stratagene). Sequencing of both strands of the insert cDNA was performed by the dideoxy chain-termination method of Sanger *et al*. (1977) using the Cy5 AutoRead sequencing kit (Pharmacia Biotech) and an ALFexpress DNA sequencer (Pharmacia Biotech).

Amplification of the 5′-end of P450arom cDNA

The 5′-end of P450arom cDNA was amplified by rapid amplification of cDNA ends (RACE) as described by Frohman *et al*. (1988), using a 5′-RACE system (Gibco BRL, Grand Island, NY, USA). First-strand cDNA was synthesized from 0·5 μg ovarian poly(A)$^+$ RNA using 200 units of an RnaseH deficient Moloney murine leukaemia virus reverse transcriptase (Superscript II RT; Gibco BRL) and a specific primer for flounder P450arom corresponding to nucleotides 498–517 (5′-ATGATGCCTC TCTCATACAT-3′: Fig. 1) in the reaction buffer (0·4 mM of each dNTP, 50 mM KCl, 2·5 mM $MgCl_2$, 20 mM Tris–HCl (pH 8·4), 10 mM dithiothreitol). After addition of oligo-dC to the 3′-end of the first-strand cDNA by treatment with terminal deoxynucleotide transferase, polymerase chain reaction (PCR) was performed with an abridged anchor primer from the kit (5′-GGCCACGCGTCGA CTAGTACGGGIIGGGIIGGGIIG-3′) and another specific primer for P450arom corresponding to nucleotides 469–488 (5′-GCTCAGTCCCTGCT TGCTCC-3′: Fig. 1) in the PCR mixture (0·2 mM each of dNTP, 50 mM KCl, 1·5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8·3), 1 unit Taq DNA polymerase (AmpliTaq Gold; Perkin Elmer, Foster City, CA, USA)). The product was then reamplified with the abridged universal amplification primer from the kit (5′-GGCCACGCGTCGACTAGTAC-3′) and a third specific primer for P450arom corresponding to nucleotides 407–426 (5′-TGGC TGATGCTCTGCTGAGG-3′: Fig. 1). PCR conditions were as follows: preheating at 95 °C for 10 min, 30 cycles of PCR at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min, and a final extension at 72 °C for 5 min. The resulting PCR product was electrophoresed on a 2% agarose gel, purified and inserted into the TA-vector pCR II (Invitrogen, San Diego, CA, USA). Determination and confirmation of base sequence of the PCR product were carried out by sequencing of four clones derived from the same PCR product.

Semi-quantification of P450arom mRNA

One microgram total RNA extracted from the juvenile gonads at 50, 60, 80 and 100 days after hatching, or various adult tissues was reverse-transcribed in 20 μl of the reaction mixture (1 mM of each dNTP, 50 mM KCl, 5 mM $MgCl_2$, 10 mM Tris–HCl (pH 8·3), 2·5 μM oligo $(dT)_{16}$, 1 unit RNase inhibitor, 2·5 units MuLV reverse transcriptase (Perkin Elmer)) at 42 °C for 30 min. Following dilution of the reaction mixture with 80 μl distilled water, 1 μl of the diluted mixture served as a template for a 20 μl PCR reaction containing 50 pmol of specific primers for P450arom and flounder elongation factor-1α (EF-1α). Primer sequences were based on the sequences of the flounder P450arom (Fig. 1) and the flounder EF-1α (DNA Data Bank of Japan, accession no. AB017183). Forward primers for P450arom and EF-1α were 5′-ATCGGATCCCTGCCTG TGAC-3′ and 5′-AGTTCGAGAAAGAAGCTG CC-3′ corresponding to nucleotides 73–92 and 4–23 respectively. Reverse primers for P450arom and EF-1α were 5′-TGGCTGATGCTCTGC TGAGG-3′ and 5′-ATCCAGAGCATCCAGC AGTG-3′ corresponding to nucleotides 407–426 and 562–581 respectively. After preheating of the PCR mixture at 95 °C for 10 min, 25 cycles

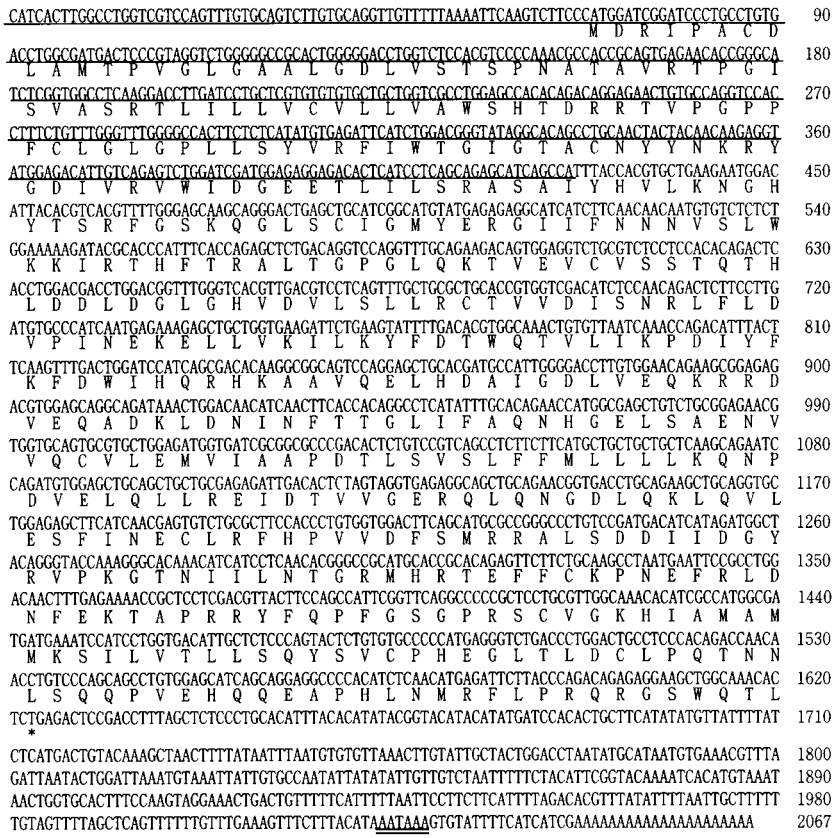

FIGURE 1. Nucleotide and deduced amino acid sequences of Japanese flounder ovarian P450arom cDNA. The portion of the cDNA fragment (426 bp) determined by 5′-RACE is underlined. The polyadenylation signal is double underlined.

of PCR were performed at 94 °C for 30 s, 59 °C for 30 s, 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. The PCR products were electrophoresed on a 2% agarose gel, transferred to nylon membrane (Hybond-N^+; Amersham), and fixed to the membrane by baking the membrane at 80 °C for 2 h. Following hybridization with either the DIG-labeled 5′-RACE product or EF-1α cDNA in DIG Easy Hyb solution at 42 °C for 16 h, membranes were washed and visualized according to the methods described above.

For quantification of P450arom mRNA during the period of sex differentiation, the intensity of the hybridization signal was determined by image analyzer software (PDI Inc., Huntington Station, NY, USA). The relative amounts of P450arom mRNA in the gonads at various stages were normalized to EF-1α. The mean difference between female and male groups was analysed by Student's *t*-test.

Determination of the amounts of sex-steroid hormones in a whole body

Larvae or juveniles were homogenized in 10 ml phosphate-buffered saline (137 mM NaCl, 2·68 mM KCl, 8·1 mM Na_2HPO_4, 1·47 mM KH_2PO_4; pH7·4)/g body weight. Estradiol-17β and testosterone were extracted three times from the homogenates into diethyl ether, evaporated by vacuum centrifugation and concentrations were determined according to the enzyme immunoassay described by Van Weemen *et al*. (1979), using enzyme immunoassay kits (Cayman Chemical Co., Ann Arbor, MI, USA). The mean difference between female and male groups was analysed by Student's *t*-test.

RESULTS

Isolation of P450arom cDNA from the Japanese flounder ovary

To isolate P450arom cDNA from Japanese flounder, one million independent cDNA clones in the ovarian

cDNA library were screened with tilapia ovarian P450arom cDNA as a probe, and two positive clones were obtained. The longer clone was 1672 bp which contained the 3′-untranslated region of 444 bp. Compared with the open reading frame (ORF) length (more than 1500 bp) of other P450arom cDNAs, the ORF (1228 bp) of this clone did not seem sufficient for the complete ORF. Therefore, the 5′-end of the cDNA fragment (426 bp) was isolated by 5′-RACE. As there was no difference in the overlapping region (31 bp) of the 5′-RACE clone and library clone, the 5′-RACE clone was considered to be the 5′-end of flounder P450arom. The combined size of the two clones was 2067 bp (Fig. 1) which contained an ORF (69–1623 bp) of 1554 bp encoding a potential protein of 518 amino acid residues. However, there was another potential initiation codon 30 bp downstream of the first ATG. Both start sites resulted in a sequence similar to the consensus sequence proposed by Kozak (1986). The true initiation codon remains uncertain for flounder P450arom. The 3′-untranslated region was 444 bp long and terminated with a poly(A)$^+$ tail. The polyadenylation signal, AATAAA, was 18 bp from the first A of the poly(A) track.

The deduced amino acid sequence of the Japanese flounder P450arom was compared with other species (Fig. 2). The overall homology between flounder P450arom and human (Corbin *et al*. 1988, Harada 1988), rat (Hickey *et al*. 1990, Lephart *et al*. 1990), mouse (Terashima *et al*. 1991), bovine (Hinshelwood *et al*. 1993) and chicken (McPhaul *et al*. 1988) P450arom is 50·3, 51·2, 51·9, 50·9 and 50·4% respectively. The homology between flounder and rainbow trout (Tanaka *et al*. 1992), catfish (Trant 1994), medaka (Tanaka *et al*. 1995) or tilapia (Chang *et al*. 1997*b*) is significantly higher (74·9, 61·5, 76·7, 76·2% respectively). The membrane-spanning (37 to 56 residues), α-helix (302 to 336 residues), Ozols peptide (361 to 383 residues), aromatic (419 to 430 residues) and heme-binding (444 to 467 residues) regions in the flounder P450arom are easily identified by comparison with the corresponding regions in other species.

Tissue-specific expression of P450arom mRNA in adult Japanese flounder

Expression of P450arom mRNA in various adult tissues was examined by RT-PCR. P450arom mRNA was expressed highly in the ovary and spleen, but weakly in the testis and brain (Fig. 3). No difference in the expression pattern in brain or spleen was observed between sexes (data not shown). Signal was barely detectable in the liver, kidney, heart and muscle of both sexes. When a random primer and a different primer set were used for the reverse transcription reaction and PCR, respectively, the expression pattern of P450arom mRNA was similar (data not shown).

Differential expression of P450arom mRNA in gonads during sex differentiation

The expression pattern of P450arom mRNA during gonadal sex differentiation in both sexes was analysed semi-quantitatively by RT-PCR (Fig. 4). On day 50 after hatching there was no difference in the levels of P450arom mRNA between female and male groups. After day 60, significant differences ($P<0·01$) in the expression level of P450arom mRNA between the female and male groups were seen. The level of P450arom mRNA in the female group increased rapidly, whereas it decreased slowly in the male group.

Sex-steroid hormone levels during development

To investigate steroid hormone levels during development, whole body concentrations of estradiol-17β and testosterone were measured (Fig. 5). There were no differences in testosterone levels between the female and male groups during development. Concomitant with the change of P450arom mRNA levels, estradiol-17β levels in the female group were higher than in the male group after day 60 following hatching. Subsequently, on day 100 there was a significant difference between the female and male groups ($P<0·05$).

DISCUSSION

In the present study, we isolated P450arom cDNA containing the complete ORF from the Japanese flounder ovary. Similar to rainbow trout, medaka and tilapia, the predicted amino acid sequence of the flounder P450arom has two potential initiating codons. Catfish P450arom has three potential initiating codons. However, the true initiation codon remains uncertain in teleost fish P450arom. The deduced amino acid sequence size of flounder P450arom is 518 amino acid residues if the first potential initiation codon is used or 508 residues if the second codon is used. The deduced amino acid sequence sizes of other teleost fish P450arom cDNA range from 517 to 524 residues if the first potential initiation codon is used or 507–517 residues if the second codon is used. In comparison with the deduced amino acid sequence size of mammalian and chicken P450arom (502–508 residues), teleost fish P450arom tends to be longer.

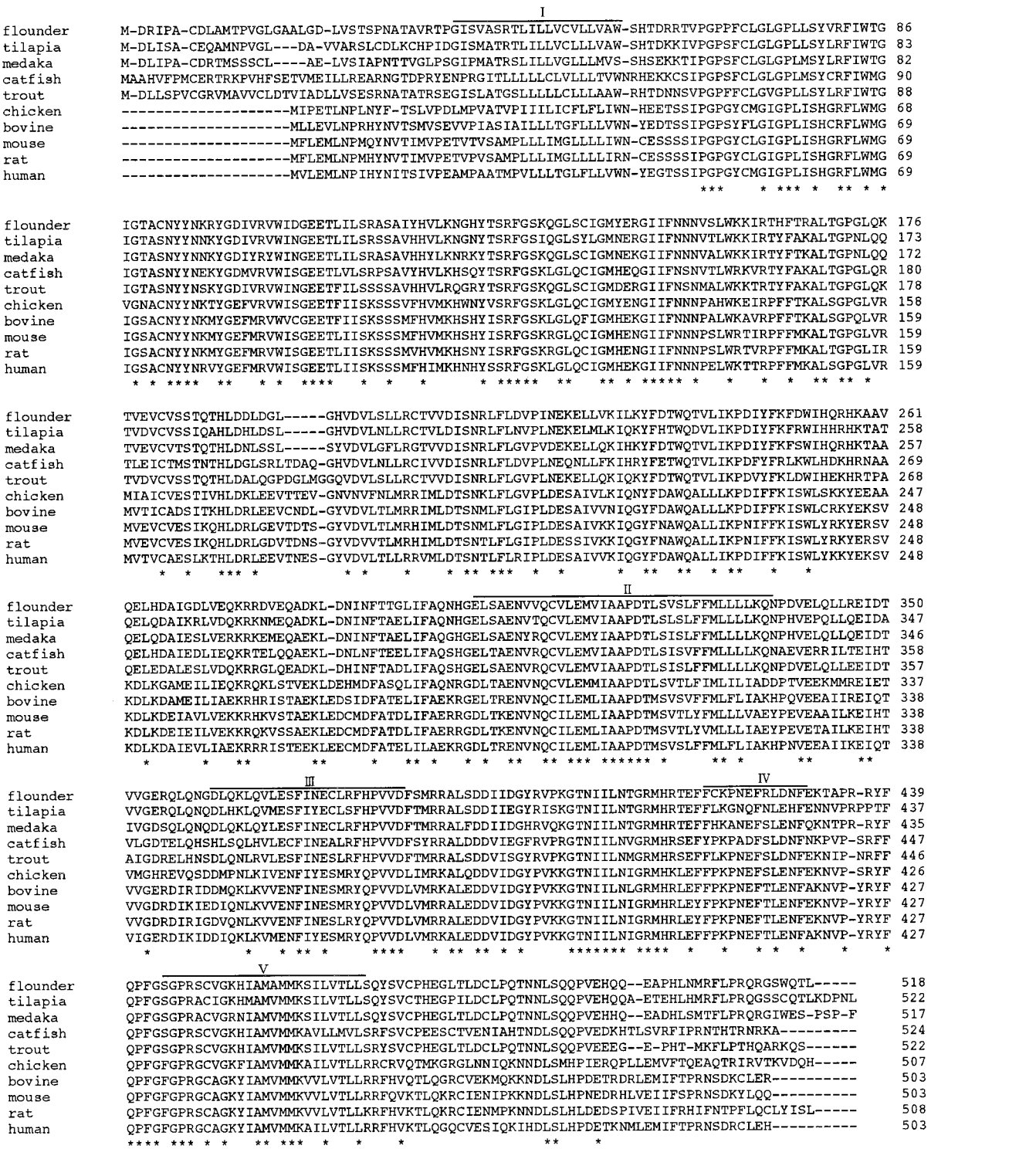

FIGURE 2. Alignment of the amino acid sequence of Japanese flounder P450arom with tilapia, medaka, catfish, rainbow trout, chicken, bovine, mouse, rat and human. Asterisks indicate identical amino acid residues. Roman numerals indicate the membrane-spanning region (I), the helix region (II), the Ozols peptide region (III), the aromatic region (IV) and the heme-binding region (V).

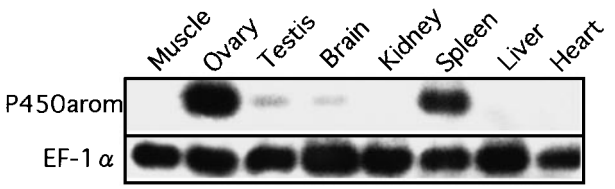

FIGURE 3. Tissue-specific expression of P450arom transcript in adult Japanese flounder. EF-1α serves as an internal control for loading differences. RT-PCR amplified fragments specific for P450arom and EF-1α are predicted to be 344 bp and 577 bp respectively. PCR products were fractionated on a 2% agarose gel and confirmed by Southern blotting with labeled P450arom or EF-1α cDNA.

RT-PCR shows that the flounder P450arom mRNA is expressed highly in the ovary and spleen but weakly in the testis and brain. All transcripts are detected as a single band. In catfish, the expression of P450arom mRNA is observed as a single band in the testis and brain, but as double bands in the ovary (Trant *et al*. 1997). Recently, two CYP19 genes encoding different P450arom isozymes in brain and ovary have been identified in goldfish. RT-PCR analysis has shown that brain- and ovary-type P450arom cDNA variants are found in the forebrain, whereas only the ovary-type variant is detected in the ovary (Callard & Tchoudakova 1997, Tchoudakova & Callard 1998). In the present study, there was no difference in the sequences (344 bp) of RT-PCR products among the ovary, spleen, testis and brain (data not shown), suggesting that none of the RT-PCR products is a brain-type variant. It therefore remains to be clarified whether the brain-type variant exists in the flounder.

Expression of the P450arom gene in vertebrate spleen has only been reported in human fetal spleen (Price *et al*. 1992). Although it is known that P450arom plays important physiological functions in various tissues by regulating estrogen biosynthesis, the role of P450arom in spleen remains unclear. However, the presence of an mRNA encoding P450 cholesterol side-chain cleavage was detected in the spleen, suggesting a steroidogenic function similar to gonads and the adrenal cortex (K-I Morohashi, personal communication). The significance of P450arom mRNA expression in the flounder spleen needs to be studied further.

The expression of P450arom mRNA in the gonads during sexual differentiation was analysed by RT-PCR. Previous histological observations showed that ovarian and testicular differentiation in the flounder were initiated in juveniles about 30 mm

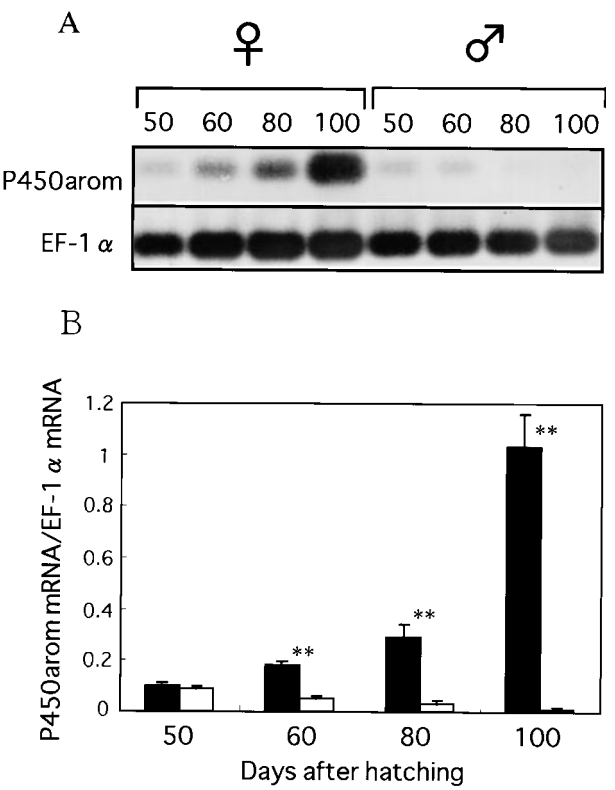

FIGURE 4. Differential expression of P450arom transcripts in female and male gonads during sex differentiation. (A) P450arom transcript levels in total RNA isolated from ten pooled gonads from either the female or male group were examined by RT-PCR at 50, 60, 80 or 100 days after hatching. RT-PCR and Southern blotting were done as described in Fig. 3. (B) Relative amounts of P450arom mRNA in female (solid bars) and male (open bars) gonads were normalized to EF-1α. Vertical bars indicate the mean ± standard error of the five replicates. $^{**}P<0{\cdot}01$ compared with the male group (Student's *t*-test).

in total length (TL) and 37 mm in TL respectively (Tanaka 1987, Yamamoto 1995, 1999). In the present study, the avarage TLs of the female and male groups at day 60 after hatching were 31·6 mm and 39·0 mm respectively. Thus, this period is thought to be the time of initial sex differentiation for both sexes. On day 50 after hatching when the gonad is sexually indifferent, there was no difference in the levels of P450arom mRNA between the female and male groups. After the initiation of sex differentiation (day 60), the level of P450arom mRNA increased rapidly in the female group, whereas it decreased slowly in the male group. In this study, females were produced artificially by mating normal females with gynogenetic diploid

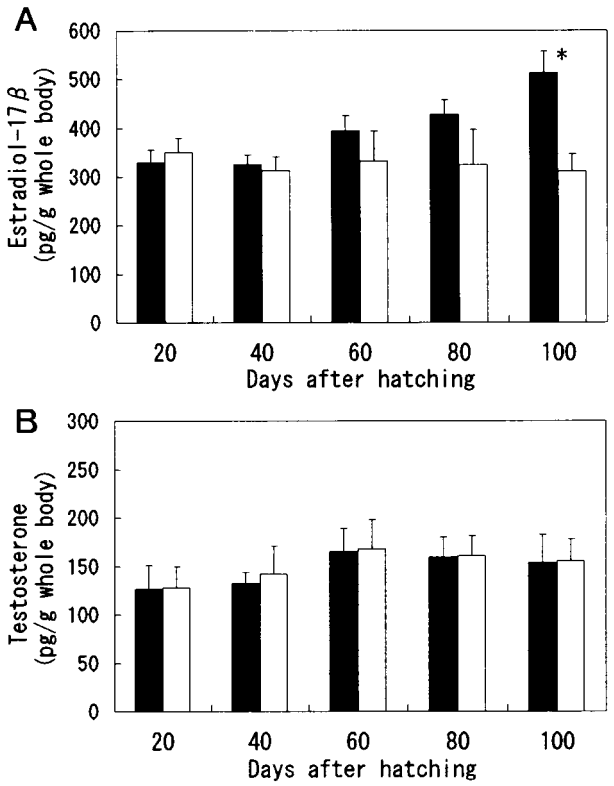

FIGURE 5. Whole body concentrations of estradiol-17β (A) and testosterone (B) in the female (solid bars) and male (open bars) groups during development. Sex-steroid hormones extracted from five to twenty pooled larvae or juveniles on day 20, 40, 60, 80 or 100 after hatching were measured by enzyme immunoassay. Vertical bars indicate the mean ± standard error of the five replicates. *$P<0{\cdot}05$ compared with the male group (Student's *t*-test).

males, reared at normal water temperature (18 °C), resulting in all genetic females (XX). Males were produced by rearing the same genetic females as described above at higher water temperature (27 °C) during sex differentiation, resulting in conversion to all phenotypic males. Thus, we present, to the best of our knowledge, the first demonstration that the expression of P450arom mRNA in genetically identical (XX) gonads is inhibited by rearing the larvae at a high water temperature, resulting in conversion of genetically female larvae into phenotypic males.

In reptiles with temperature-dependent sex determination (TSD), P450arom mRNA levels in both sexes have been examined during sexual differentiation (Jeyasuria & Place 1997). In embryos of the diamondback terrapin (*Malaclemys terrapin*), P450arom mRNA levels in female adrenal/kidney/gonadal complexes are significantly higher than in male complexes at the sexually differentiated stage. Thus, temperature appears to play an important role in the regulation of P450arom gene expression in fishes and reptiles with TSD. Studies on the regulation of P450arom gene expression by temperature would provide insight into the sex determination mechanisms in lower vertebrates.

Estradiol-17β levels in the whole body of the female group were elevated relative to the male group after sex differentiation was initiated. In contrast, no differences in the whole body testosterone levels in the female and male groups were observed throughout the course of sex differentiation. P450arom catalyses the conversion from testosterone to estradiol-17β. Consequently, the change of estradiol-17β levels in female and male groups during sex differentiation is consistent with the changes in P450arom mRNA levels. The rate of increase in estradiol-17β levels in the female group, however, was moderate relative to the marked change in P450arom mRNA levels. There was basal estradiol-17β before and after sex differentiation. In addition to the ovary, a significant amount of P450arom mRNA was expressed in the spleen. Since there were no differences in P450arom mRNA levels in brain and spleen between the female and male groups during the sex differentiation (data not shown), the increase of estradiol-17β levels above basal levels in the female group may be derived from the increased P450arom mRNA levels in ovaries. In tilapia, P450arom-like immunoreactivity appears in differentiating ovaries, but not in differentiating testes (Chang *et al*. 1997*a*). In the embryos of the European pond turtle (*Emys orbicularis*), aromatase activity remains low in differentiating testes, but increases in differentiating ovaries (Desvages & Pieau 1992). In the marine turtle (*Dermochelys coriacea*), P450arom activity and estrogen content increase in gonads of the embryos incubated at a feminizing temperature, whereas they remain low in gonads of the embryos incubated at a masculinizing temperature (Desvages *et al*. 1993). In the larvae of urodele amphibia (*Pleurodeles waltl*), aromatase activity is significantly higher in the gonads of genetic females (ZW) than of genetic males (ZZ) during sex differentiation. The gonads of sex-reversed males (ZW) produced by exposure of the larvae to a high temperature have low aromatase activity similar to those of genetic males (Chardard *et al*. 1995). These results suggest that endogenous estrogen plays a key role in gonadal differentiation of fishes, amphibians and reptiles with TSD.

In chinook salmon (*Oncorhynchus tshawytscha*), brief treatment with an aromatase inhibitor during sex differentiation causes genetic females to develop

into normal phenotypic males (Piferrer *et al*. 1994). This result indicates that P450arom activity and estradiol-17β biosynthesis are indispensable for sex differentiation into females in some teleost fishes. Based on increases seen during ovarian differentiation, we suggest that expression of P450arom mRNA and estradiol-17β biosynthesis is indispensable for ovarian differentiation. In contrast, the expression of P450arom mRNA and estradiol-17β biosynthesis is inhibited during testicular differentiation, suggesting suppression as a prerequisite for testicular differentiation. Future studies will focus on the effect of aromatase inhibitors in the sex differentiation of the flounder.

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