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Characterization of Two Types of Cytochrome P450 Aromatase in the Serial-sex Changing Gobiid Fish, *Trimma okinawae*

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ABSTRACT—To investigate the role of estrogen in the serial-sex changing fish *Trimma okinawae*, we isolated complementary DNAs encoding two distinct cytochrome P450 aromatase isoforms from adult ovary and brain (termed P450aromA and P450aromB, respectively). Sequence and phylogenic analyses showed that the goby *P450arom* forms belong to two separate CYP19 subfamilies. Transient expression of these cDNAs in HEK293 cells caused conversion of exogenous testosterone to estradiol-17β. RT-PCR showed that *P450aromA* was expressed in the brain, spleen, testis and ovary. *P450aromB* was expressed in the brain, liver, testis and ovary. *In situ* hybridization studies showed that *P450aromA* mRNA, but not P*450aromB* mRNA, was present in both ovary and testis. Positive signals were restricted to granulosa cells of vitellogenic follicles and interstitial cells of mature testis. Ovarian expression of both *P450arom* genes during the spawning cycle was examined by quantitative real-time RT-PCR. *P450aromA* transcripts increased during vitellogenesis and decreased prior to spawning. In contrast, *P450aromB* transcripts were barely detectable and did not correlate with ovarian development. These findings suggest that P450aromA, but not P450aromB, is involved in regulating ovarian vitellogenesis in goby.

Key words: aromatase genes, gonadal development and maturation, sex-changing fish, gobiid fish

INTRODUCTION

Despite numerous endocrine studies on sex change in teleosts, no general mechanisms mediating sex change have emerged. The gobiid fish, *Trimma okinawae*, possesses ovarian and testicular tissues simultaneously in its gonad. Furthermore, *T. okinawae* is able to change sex repeatedly in both directions depending on its social surroundings (Sunobe and Nakazono, 1993; Kuwamura *et al*., 1993). A dominant male controls a harem of several females. Removal of this dominant male results in sex change of the largest female in the harem. If placed with a larger male, the once dominant male reverts to a female. Almost all sequential sex-changing fish either protogynous or protandrous reversibly change sex in one direction. As sex change in both directions can be socially manipulated, *T. okinawae* provides an excellent animal model to elucidate the mechanisms of male to female and female to male sex change.

Steroid hormones produced by gonads play crucial roles in ovarian function, sex differentiation and sexual maturation and behavior in vertebrates in general (Fostier *et al*., 1983; Maclusky and Naftolin, 1981; Wilson *et al*., 1981). In hermaphrodite fishes, estrogen plays a particularly important role in natural and experimentally-induced sex change (Chang *et al*., 1998; Fostier *et al*., 1993; Hunter *et al*., 1983).

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Our concern is the physiological role of estrogen in serial-sex changing gobiid fish, *T. okinawae*. Estrogens are synthesized in the gonads and brain by an enzymatic step using testosterone as a substrate. The terminal enzyme in the steroidogenic pathway is cytochrome P450 aromatase (P450arom).

To investigate the role of estrogen in a serial-sex changing gonad, we focused on P450arom. As a first step, we isolated and characterized *P450arom* cDNAs from goby. Subsequently, localization of *P450arom* in the gonad was observed by *in situ* hybridization. Additionally, changes in *P450arom* expression were examined during the natural ovarian cycle using real-time quantitative RT-PCR.

MATERIALS AND METHODS

Experimental animals

Animals were collected from their natural habitat at Makurazaki, Kagoshima Prefecture, Japan and then kept in the laboratory. Fish were maintained under constant conditions (seawater at 27°C, 14L: 10D) in indoor tanks. Fish were fed commercial pellet and brine shrimp. Sex was determined by genital papillae (Sunobe and Nakazono, 1993; Grober and Sunobe 1996). Mature female and male fish were transferred to glass aquaria and reared under the above condition. The normal spawning cycle (~4 days) was seen with no evidence of sex change. In this study, samples were obtained after spawning to prevent contamination with sex-changing samples.

Sample collection and RNA extraction

Adult fish were sedated in an ice bath and sacrificed by decapitation. Following removal, majority of the gonad was frozen in liquid nitrogen and stored at –80°C. The remaining gonadal fragment was fixed in 4% paraformaldehyde in 0.1M phosphate buffer for approximately 14 hours. After dehydration and paraffin embedding, gonads were sectioned at 5 μm. Total RNA was extracted from frozen tissues using the RNeasy mini kit with the RNase-free DNase set (Qiagen) according to manufacturer's protocols.

Oligonucleotides

Oligonucleotides used as PCR primers are shown in Table 1. The degenerate primers of A-dn-Fw and -Rev were against highly conserved regions of the aromatase I-helix and heme-binding domains. Another degenerate primer pair (B-dn-Fw and -Rev) was designed on against highly conserved regions of *P450aromB* cDNA from channel catfish (AF417239), goldfish (U18974), zebrafish (NM_131642), Nile tilapia (AF295761) and rainbow trout (AJ311937). Gene-specific primers were synthesized based on our sequences. Primers actin-Fw and actin-Rev were selected on the goby *β-actin* cDNA sequence.

Cloning of goby aromatase cDNAs

Goby aromatase was cloned first by generating a partial cDNA by reverse transcriptional-polymerase chain reaction (RT-PCR) using degenerate primers. Following the amplifications of the 5'- and 3'-cDNA ends by rapid amplification of cDNA ends (RACE), a cDNA encoding the complete coding region was obtained by a single RT-PCR reaction.

Described briefly as above, one microgram of total RNA from ovary was reverse transcribed using an oligo(dT) primer and Superscript II reverse transcriptase (Gibco-BRL) according to manufacturer's instructions. PCR was performed using degenerate primers (A-dn-Fw and -Rev, B-dn-Fw and -Rev) designed from highly conserved regions of known *P450arom* sequences. RT-PCR cycle conditions were 25 cycles, with 94°C 1 min, 55°C 1 min, 72°C 1.5 min. The amplicons were T-A ligated to pGEM-T-easy vector (Promega, Madison, WI, USA) and sequenced.

Based on the sequence information of the presumptive aromatase cDNA amplicon, rapid amplification of cDNA ends (RACE) procedures were performed to isolate the 5' and 3' ends of the cDNA (SMART cDNA library construction Kit; Clontech). In each RACE procedure, the initial PCR amplification was followed by a nested amplification. Gene-specific primer sets (GSP series) in combination with adaptor primers were used, respectively, for 5'- and 3'-RACE. RACE PCR cycling conditions were 35 cycles with 94°C 30 sec, 64°C or 56°C 40 sec, 70°C 120 sec.

The complete open reading frames (ORF) of the goby *P450aromA* and *B* were generated from ovarian RNA by RT-PCR with primers targeting the untranslated portions immediately upstream (AORF-Fw and BORF-Fw) and downstream of the ORF (AORF-Rev and BORF-Rev). The resulting PCR products were subcloned into pBluescript II KS- (Stratagene, LaJolla, CA, USA) designated gfAA/pBlue and gfAB/pBlue and sequenced in both directions.

DNA sequencing analysis

The resulting plasmid DNA was purified by the alkaline lysis method (Sambrook and Russell, 2001). Sequencing was performed for both strands with an Applied Biosystem model 377 sequencer after labeling with dye terminator cycle sequencing kit (Applied Biosystems, Foster city, CA, USA) using vector and gene-specific primers. Sequence analysis was performed using DNASIS software (Hitachi Co, Ltd., Tokyo. Japan). Alignment of multiple protein sequence was performed using ClustalW multiple sequence alignment program and homology value (percent of amino acid sequence identity) was calculated by pair wise alignment. The phylogenetic tree was constructed using the Neighbor-joining method (Saitou and Nei, 1987) and viewed with TREE-view (Ver. 1.8). Program settings are detailed in the figure legend.

Transient expression in HEK293 cells

The cDNA inserts from goby gfAA/pBlue and gfAB/pBlue were subcloned into a cytomegalovirus expression vector (pcDNA3.1+, Invitrogen) at the EcoRI-XhoI restriction site and designated gfAA/pc and gfAB/pc. These clones were sequenced to confirm that the cDNA was inserted in the sense orientation. HEK293 cells were plated onto 6 cm tissue culture dish (Corning, Japan) in 4 ml Dulbecco's modified Eagle's medium (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10%(W/V) fetal calf serum (JRK, Japan) under 5% CO_2 until 80% confluent. One microgram of purified recombinant expression vector was transfected into HEK293 cells using TfxTM-50 reagent (Promega) according to the suppliers protocol, followed by incubation at 37°C for 2 hours in 4 ml culture medium. The cells were then cultured for an additional 12 hours. After adding testosterone at a final concentration of 10 ng/ml (Sigma Chemical Co., St Louis, MO, USA), the plates were incubated for 48 hours. Culture medium and cells were separated by centrifugation at 1000 r.p.m. for 10 min and supernatant was stored at –80°C until assay. To measure levels of estrogen produced by HEK293 cells, the culture medium was extracted twice with diethyl ether and evaporated by vacuum centrifugation. The amount of estradiol-17β was measured using an estradiol enzyme immuno assay kit (Cayman, USA). According to the supplier's information, the cross-reaction for testosterone is 0.1%.

Tissue expression of the goby aromatase genes

Tissue distribution of goby aromatases was examined by RT-PCR as follows: single-stranded cDNA was synthesized using Omniscript reverse transcriptase (Qiagen) from 0.25 μg of oligo(dT)-primed total RNA isolated from various tissues. PCR analysis of

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Table 1. Primer sets used for RT-PCR, 5' and 3'-RACE and real-time quantified RT-PCR for sequence and expression analysis of goby P450 aromtase A and B

Primer Name	Sequence	Nucleotide Position
A-dn-Fw	5'-CARTGYGTGCTGGAGATGGTGATYGCG-3'	972 → 998
A-dn-Rev	5'-CGYTACTTCCAGCCMTTYGGT-3'	774 → 754
B-dn-Fw	5'-AAAGCTCTTACTGGACCMGGYCTSCAG-3'	346 → 372
B-dn-Rev	5'-CTGGCCATGCGCTTCATYCCRCGA-3'	1536 → 1513
A-3GSP1	5'-ATACAGAACTCAGACCTGCCTCA-3'	1113 → 1135
A-3GSP2	5'-GTGATGGAGAGCTTCATAAACGA-3'	1143 → 1165
A-5GSP1	5'-GAGGGGTATTTTTCTCGAAGTTT-3'	1350 → 1328
A-5GSP2	5'-AAGTTTTCCAGGCTGAACTCATT-3'	1333 → 1311
A-5GSP3	5'-CGCGATCACCATCTCCAACAC-3'	998 → 978
A-5GSP4	5'-GTTGTCCAGTTTGTCGGCCTG-3'	905 → 885
B-3GSP1	5'-CGACCTTGACTTTGCAACAGAGCTGATTT-3'	873 → 901
B-3GSP2	5'-GCTGTTTTAGGGGAAGATGGAGCTGAAA-3'	1063 → 1090
B-5GSP1	5'-GTATTCAGTTTTATGCATCAGGCCGGTGT-3'	1269 → 1241
B-5GSP2	5'-TTATGCATCAGGCCGGTGTTGAGAATAAT-3'	1259 → 1231
A-ORF-Fw	5'-GAAAGCGCCATGACGAGCCAG-3'	39 → 59
A-ORF-Rev	5'-CGCGTTTGACGTCATGCACAAAC-3'	1703 → 1681
B-ORF-Fw	5'-CTCTACAACACAACATCTGGACAAC-3'	16 → 38
B-ORF-Rev	5'-GAATGCTTATGTACAGCGTATAC-3'	1602 → 1580
ART-Fw	5'-TAAGCGACACATACAGAACTCAGAC-3'	1103 → 1127
ART-Rev	5'-TCATGCACAAACTTTAAAAGATCAA-3'	1691 → 1667
BRT-Fw	5'-CTCTACAACACAACATCTGGACAAC-3'	549 → 573
BRT-Rev	5'-ATAATAAACGAAGCAGACAAGTTGGA-3'	869 → 844
RP-A-Fw	5'-GCGACACATACAGAACTCAG-3'	1106 → 1125
RP-A-Rev	5'-GTTGTAGCCGTCGATGACGTC-3'	1241 → 1221
RP-B-Fw	5'-CGTCAGAGAACGTCCGACAG-3'	926 → 945
RP-B-Rev	5'-TATGCATTCTTCCACTGCAG-3'	1047 → 1028
actin-Fw	5'-TTCTACAACGAGCTGCGTGTG-3'	
actin-Rev	5'-TGTCAGGATCTTCATGAGGTA-3'	

both *P450aroms* (primers ART-Fw and -Rev, BRT-Fw and -Rev, respectively) and *β-actin* (primers actin-Fw and -Rev) was carried out with the following cycle conditions: 30 cycles at 92°C for 30 sec, 55°C for 30 sec, and 72°C for 1 min. Ten-microliter aliquots of the PCR reactions were analyzed electrophoretically and the amplicons were visualized on UV transilluminator.

In situ hybridization analysis

Gonadal sections were hybridized with digoxygenin (DIG)-labelled goby *P450aroms* cRNA probe overnight at 58°C in hybridization buffer. Sense and antisense probes were labeled using standard transcription reactions and a DIG-labeling nucleotide mix (Boehringer Mannheim, Tokyo, Japan). Hybridization signals were detected with alkaline phosphatase-conjugate antibody according to manufacturer's protocol (Boehringer). Sections were incubated for 2 hours at room temperature with DIG antibody. After incubation sections were incubated with NBT/BCIP substrate (Boehringer) for detection of alkaline phosphatase activity.

Real-time quantitative RT-PCR

Abundance of goby aromatase transcript was determined as a measure of gene expression in ovary during the spawning cycle by real-time quantitative RT-PCR analysis (rtq-PCR) method using TaqMan technology published elsewhere (Perkin Elmer Applied Biosystems, 1997; Heid *et al.*, 1996). Samples were reverse transcribed from 500 ng of total RNA in a 20-μl reaction volume using random hexamer primers and Omniscript reverse transcriptase (Qiagen). Standard copy number of both *P450arom* genes was estimated based on molecular weight and absorbance of gfAA/pBlue and gfAB/pBlue plasmid. Two sets of primer RP-A-Fw and -Rev, RP-B-Fw and -Rev (*P450aromA*, *B* respectively) were designed according to Primer Express software (version 1.5, PE Applied Biosystems). The dilution templates for each of triplicate were tested in 25-μl of PCR mixture containing 2X SYBER Green master mix (PE Applied Biosystems) and 0.3 μM (in final concentration) of each primer. The PCR profile was as follows: one initial denaturation at 95°C for 15 min, 45 cycles of denaturation at 94°C for 15 sec, annealing at 52°C (both genes) for 15 sec, and extension at 72°C for 30 sec and final extension at 60°C for 5 min, followed by the dis-

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sociation curve analysis. The PCR amplifications and fluorescent detection were performed with ABI Prism Sequence Detector 7000. DNA contamination was checked by performing a control reaction in which there was no reverse transcribed RNA.

RESULTS

Isolation of two goby *P450arom* cDNAs

P450aromA

The degenerate RT-PCR generated an amplicon of the

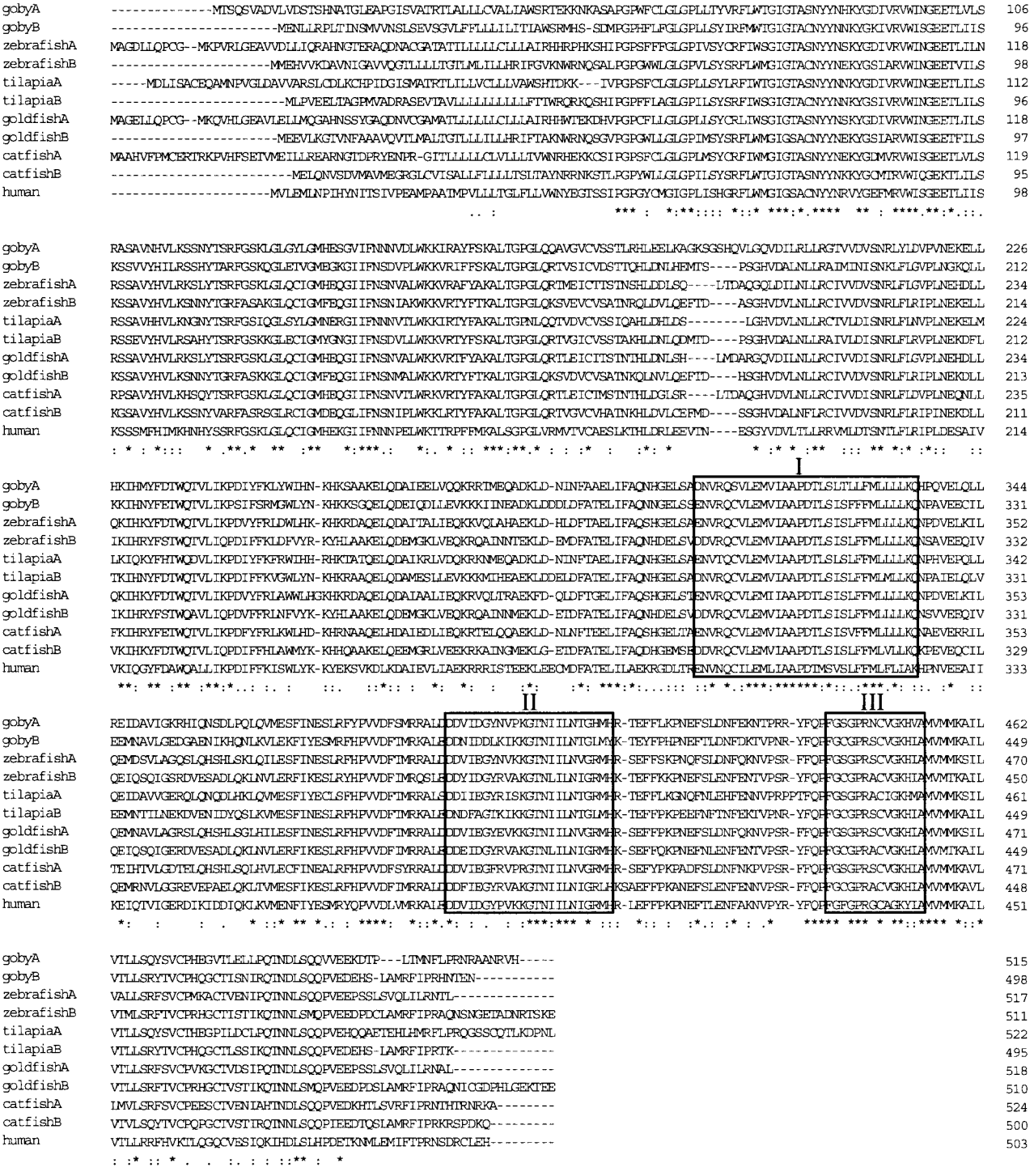

Fig. 1. Alignment of P450arom amino acid sequences. ClustalW multiple sequence alignment was used to compare the goby P450aromA and B, with P450arom forms derived from zebrafish A and B, Nile tilapia A and B, goldfish A and B, catfish A and B and human. Amino acids identical to those in other species are indicated by asterisks (*). Colon (:) indicates conserved substitution and dot (.) indicates semi-conserved substitution. Regions of high sequence homology are boxed and indicated by Roman numerals: I-helix (I); aromatase-specific conserved region (II); heme-binding region (III).

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expected size (401 bp) from ovary. Ten clones of this amplicon were sequenced and all of the nucleotide sequences were *P450aromA*. A single set of nested 3' RACE reactions using sense primers 3GP1 and 3GP2 in combination with adapter primers amplified the entire 3' terminus of cDNA (including the poly-A tail). On the other hand, a nested 5' RACE reaction using the antisense primers 5GP1 and 5GP2 stopped short of the translation start codon. Therefore, a second 5'RACE using primers 5GP3 and 5GP4 was conducted to amplify the remainder of the 5' end and a portion of the untranslated region. Goby *P450aromA* cDNA contained 2126 nucleotides with a putative 1545 bp open reading frame (ORF) and polyadenylation signal, ATTAAA, located 30 bp upstream of the poly $(A)^+$ tail. This cDNA encoded a 515-amino acid protein with a calculated molecular mass of 58 KDa.

Fig. 2. Evolutionary relationships of the known CYP19 proteins. Phylogenic tree was constructed using the neighbor-joining method. Comparisons were made to amino acid sequence of zebrafish A (AF226620) and B (NM_131642), catfish A (S75715) and B(AF417239), rainbow trout A (1806325A) and B (CAC84574), goldfish A (AB009336) and B (U18974), medaka A (D82968), Nile tilapia A (U72071) and B (AF295761), European sea bass A (AJ311177), Japanese flounder A (AB017182), red seabream (AB051290), stingray (AAF04617), *Xenopus* (BAA90529), human (P11511), and chicken (J04047). The values are bootstrap probabilities estimated by 1000 replications. Horizontal line indicates genetic distance. A, P450aromA; B, P450aromB.

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P450aromB

The initial RT-PCR using the degenerate primers amplified a partial cDNA of the expected size (1038 bp) from ovary. Seventeen clones of this amplicon were sequenced and the nucleotide sequence of all clones was *P450aromB*. RT-PCR of brain mRNA also generated *P450aromB* amplicons. The RACE procedures led to the isolation of the 5' and 3' ends of the cDNA covering the ORF and untranslated region. Specific RT-PCR to generate a full-length clone using primer BRT-Fw and -Rev amplified a single amplicon. The nucleotide sequence of this clone was identical to that of RACE clones. The goby *P450aromB* cDNA contained 1790 nucleotides with a putative 1494 bp ORF. A putative polyadenylation signal is located 28 bp upstream of the poly-A tail. This cDNA encodes 498-amino acid protein with a calculated molecular mass of 57 KDa.

Sequence comparisons

Fig. 1 shows the deduced amino acid sequence of the two goby aromatase aligned with other reported P450arom forms of zebrafishA (AF226620); zebrafishB (NM_131642); Nile tilapiaA (U72071); Nile tilapiaB (AF295761); goldfishA (AB009336); goldfishB (U18974); catfishA (S75715); catfishB (AF417239) and human (P11511). Goby P450aromA was more closely related (60–73.5% identity) to isoform A of other teleosts than isoform B (58–68% identity). The two aromatases of goby shared only 55.8% overall identity. Identity to human aromatase is even lower (48, 51% respectively). Fig. 1 also depicts that putative functional domains, including the I-helix, the aromatase-specific region, and the heme-binding regions are highly conserved among vertebrates. Phylogenic analysis of the full-length goby P450aromA and B with other reported aromatases indicated that the teleost aromatases clearly segregated into P450aromA and B branches (Fig. 2). Goby P450aromA and B are grouped in teleost P450aromA and B branch, respectively.

Transient expression of *P450arom* cDNAs in HEK293 cells

Fig. 3 shows the production of estradiol-17β in HEK293 cells transfected with both gfAA/pc and gfAB/pc constructs using testosterone as substrate. Estradiol-17β was converted from testosterone in a linear fashion over 48 h of incubation. These results confirmed that goby cDNA

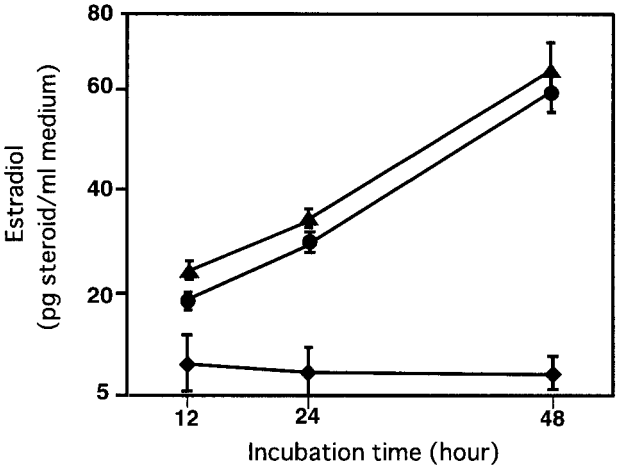

Fig. 3. Expression of functional P450aromA and B isozymes in nonsteroidogenic HEK293 cells, which were transiently transfected with pcDNA3.1+ *P450aromA* (▲), *P450aromB* (●) and mock construct (◆). Estradiol production was measured at 12, 24 and 48 hours after incubation.

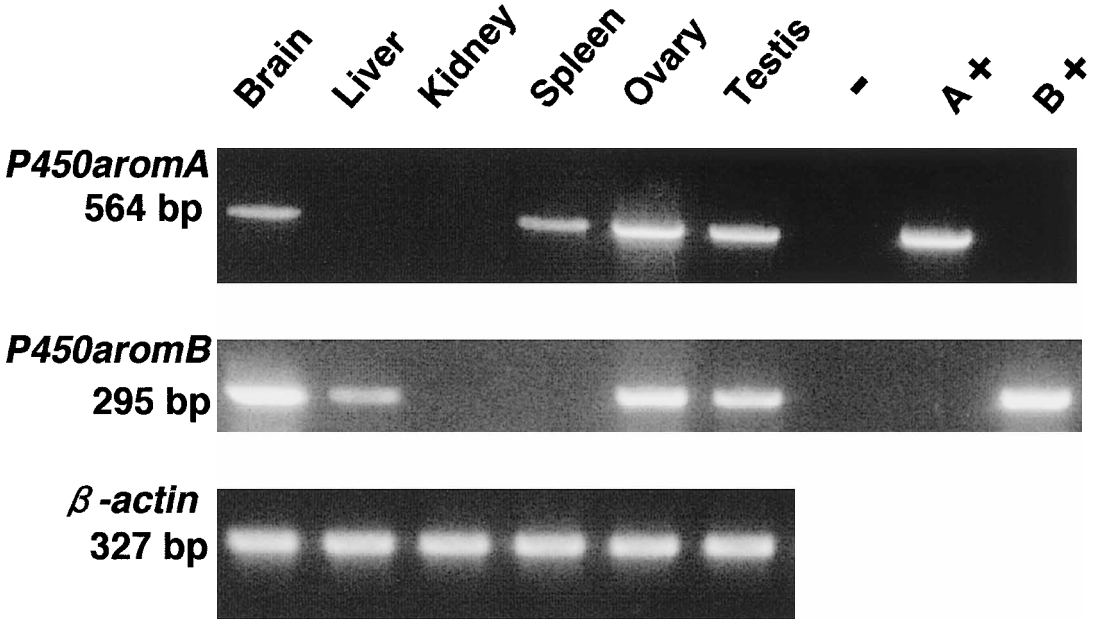

Fig. 4. RT-PCR analysis of tissue distribution patterns of goby *P450aromA* and *B* and *β-actin*. The positive control consists of a product amplified from the plasmid DNA.

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encoded P450aromA and B.

Tissue distribution pattern of *P450aromA* and *B*

Goby *P450aromA* was distributed in brain, spleen, ovary and testis. No signal could be detected in the liver and kidney (Fig. 4). *P450aromB* was expressed in brain, liver, ovary and testis. In contrast to *P450aromA*, *P450aromB* was undetectable in the spleen (Fig. 4). The expression of *β-actin* (Fig. 4) indicated the functional integrity of the cDNA template.

In situ hybridization of gonadal tissue

P450aromA transcripts were found in both cell layers of the ovarian follicle. In previtellogenic follicles, signals were seen only in the thecal cells (Fig. 5; AI and BI). In vitellogenic follicles, signals were more abundant in the granulosa cells, as compared to the thecal cells (Fig. 5; CI, DI and EI). *P450aromA* transcripts were also detected in interstitial cells of testis (Fig. 5; FI). Sense probes for *P450aromA* showed no signal (Fig. 5; AII–FII). In contrast to *P450aromA*, *P450aromB* mRNA was not detected in either testis or ovary (data not shown).

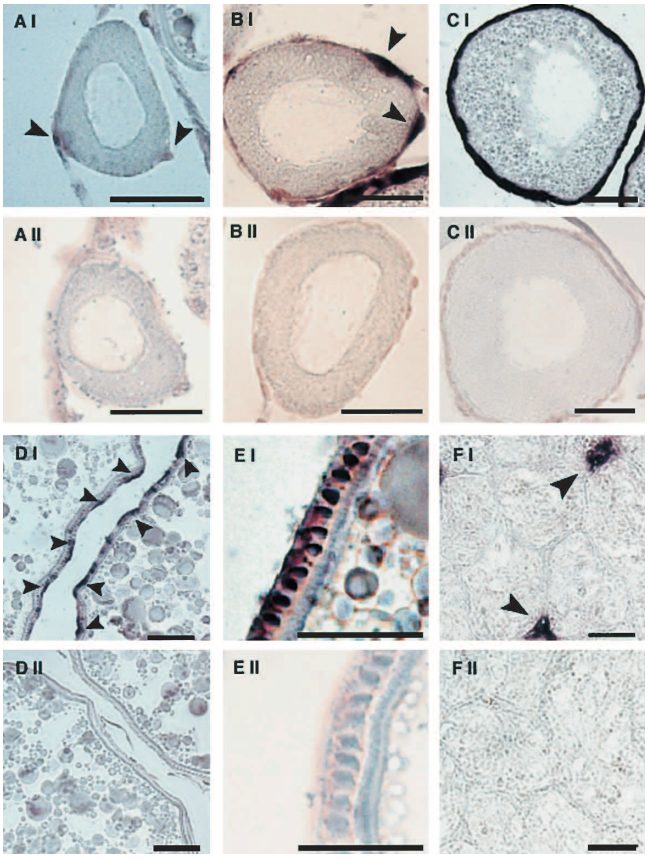

Fig. 5. *In situ* hybridization signals showing the presence of *P450aromA* transcripts in ovarian follicles (arrowheads, AI–EI) and testis (FI). Sense probes as control (AII–FII) are shown. Bars indicate 20 μm.

Quantitative analysis of *P450aromA* and *B* transcripts in ovarian follicles

Expression levels of *P450aromA* and *B* mRNA during the spawning cycle were examined (Fig. 6). Both forms (*P450aromA* and *B*) were detected in the ovary; however, *P450aromA* transcripts were at least more than 100-fold greater than *P450aromB* transcripts. *P450aromA* transcript levels changed markedly with progression of oocyte growth. Maximum levels of *P450aromA* transcripts were detected during late vitellogenesis on Day 3. Thereafter, levels sharply declined with maturation on Day 4. In contrast, the levels of *P450aromB* did not change during oocyte development.

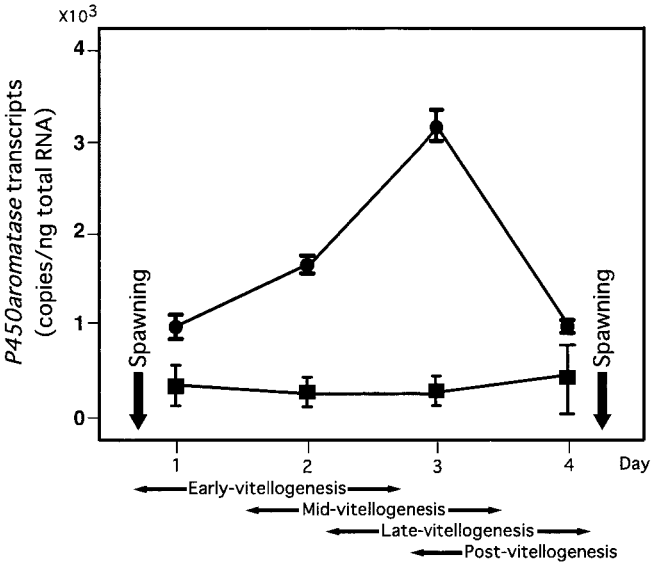

Fig. 6. Expression of *P450aromA* (●) and *B* (■) transcripts in ovary during the spawning cycle. Abundance of the transcripts was measured by real-time quantitative RT-PCR.

DISCUSSION

Most teleosts have at least two *CYP19* genes (P450arom), which encode structurally and functionally distinct P450arom isozymes in ovary (*CYP19a*/P450aromA) and brain (*CYP19b*/P450aromB) (Callard and Tchoudakova, 1997; Chiang *et al*., 2001; Gelinas *et al*., 1998; Kwon *et al*., 2001). In this study, we isolated two forms of the aromatase cDNA from the goby, *T. okinawae*. Sequence comparison and phylogenic analysis indicate that two isoforms of fish P450arom are orthologs of the previously identified mammalian and avian aromatase and members of paralogous clades within teleost A- and B-lineages. The A- and B-isoforms of goby are only about 58% identical, indicating a long evolutionary history as separate genes.

In addition to sequence analysis, the ability to catalyze the transformation of androgen to estrogen was checked in HEK293 cells transfected with two *CYP19* genes. We confirmed that both isoforms have aromatization ability as is the case with goldfish (Tchoudakova *et al*., 1998) and zebrafish

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(Chiang *et al*., 2001).

The non-quantitative RT-PCR analysis of aromatase expression in various adult tissues of *T. okinawae* revealed that *P450aromA* expressed in spleen and *P450aromB* in liver similar to other species; Japanese flounder (Kitano *et al*., 1999), Nile tilapia (Kwon *et al*., 2001) and human fetus (Price *et al*., 1992) in spleen whereas *Xenopus* (Miyashita *et al*., 2000), Nile tilapia (Kown *et al*., 2001) and human (Harada *et al*., 1993; Toda *et al*., 1994; also review by Simpson *et al*., 2002) in liver. These results suggest that the spleen and liver are capable of producing estrogen from precursors. However, the potential function of locally-produced steroids in these organs remain unknown. Our RT-PCR analysis also detected both *P450arom* transcripts in testis, ovary and brain. The overlapping expression of both forms in gonads and brain is consistent with the results obtained in zebrafish (Kishida and Callard, 2001; Trant *et al*., 2001), Nile tilapia (Kwon *et al*., 2001) and rainbow trout (Valle *et al*., 2002). The expression of aromatase gene in brain suggests an important role for sexual behavior (Matsumoto *et al*., 2003) and development of the central nervous system (Maclusky *et al*., 1981). Changes in the expression of both forms of *P450arom* genes in brain during sex change in *T. okinawae* is of particular interest.

Our *in situ* hybridization studies determined the cellular location of *P450arom* within the goby ovarian tissues. The most abundant transcripts for *P450aromA* could be seen in the granulosa cells of mid- and late-vitellogenesis and weak signals were seen in thecal cells throughout vitellogenesis. Aromatase activity was reported to be present in ovarian follicles of several teleosts including Nile tilapia (Chang *et al*., 1997), amago salmon (Young *et al*., 1983) and medaka (Fukada *et al*., 1996). However, these studies did not examine which type of aromatase is localized in ovarian follicles. Thus, the present study was the first to show that A-type of aromatase, but not B-type, is localized in granulosa cells in teleosts.

Real-time quantitative RT-PCR revealed that *P450aromB* transcripts were very low in ovary and showed no marked changes during ovarian development. The low levels of *P450aromB* transcripts may explain why our *in situ* hybridization did not detect the transcripts in ovary. In fact, using the same probes, we were able to detect *P450aromB* transcripts in brain (unpublished data). In any case, the expression profile of *P450aromB* during the spawning cycle suggests that this form of P450arom does not play a major role in regulating ovarian development.

The present study clearly showed the differential expression of two forms of *P450arom* transcripts in ovary. The amounts of *P450aromA* transcripts increased significantly during vitellogenesis and declined during late/post vitellogenesis. In contrast, *P450aromB* transcripts were very low (RT-PCR) or hardly detectable (*in situ* hybridization) throughout vitellogenesis. Study of the 5'-flanking region of both *P450arom* genes may help to determine the mechanism of this tissue-specific expression. We recently showed that in the Nile tilapia, *Oreochromis niloticus*, the expression of *P450aromA* and *Ad4BP/SF-1* increased in parallel with ovarian growth (Yoshiura *et al*., 2003). These findings suggest that the form of *P450arom* responsible for estrogen synthesis during vitellogenesis is *P450aromA*, and *Ad4BP/SF-1* plays an important role in regulating the expression pattern of *P450aromA* gene in ovarian follicles. It was also shown that in mammals, decrease in *P450arom* transcripts in granulosa cells was associated with a reduction in the mRNA of *SF-1* (Fitzpatrick *et al*., 1997). Further studies are required to analyze the role of Ad4BP/SF-1 in relation to ovarian development and also with reference to P450arom in *T. okinawae*.

Surprisingly, our *in situ* hybridization analysis revealed that *P450aromA* transcripts could be detected in the interstitial cells of mature testis. Expression of aromatase in the testis agrees with earlier findings in dogfish (Callard *et al*., 1985), channel catfish (Trant *et al*., 1997), rainbow trout (Belvedere *et al*., 1998), European sea bass (Valle *et al*., 2002) and Atlantic stingray (Ijiri *et al*., 2000). However, the situation between these species and *T. okinawae* in terms of the mode of gonadal formation is different. *T. okinawae* is unique in having both ovary and testis regardless of sexual phase (Sunobe and Nakazono, 1997). The functional significance of *P450aromB* in the testis of the sex changing goby remains to be studied. In mammals, estrogens have recently been shown to play a physiological role in the regulation of spermatogenesis (see a review by Carreau *et al*., 2003). The expression profiles of *P450aromA* in the testis during the spermatogenesis as well as sex change need to be determined. It is also important to determine whether expressions of ovarian and testicular P450aromA genes are regulated by a similar or different mechanism.

In summary, we cloned, sequenced and characterized two *P450arom* cDNAs from *T. okinawae*. *P450aromA* transcripts, but not *P450aromB*, exhibited distinct changes during ovarian development, suggesting an important role for *P450aromA* in the synthesis of estrogen during active vitellogenesis. These cDNA probes will be useful for future studies on the roles of aromatase in gonadal development and even sex behavior during the serial-sex change in *Trimma okinawae*.

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