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A Novel Type of P450c17 Lacking the Lyase Activity Is Responsible for C21-Steroid Biosynthesis in the Fish Ovary and Head Kidney

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Cytochrome P450c17 is the single enzyme that mediates the 17α-hydroxylase and 17, 20 lyase activities during the biosynthesis of steroid hormones in the gonads and adrenal gland. However, the mechanism underlying its dual action continues to be a controversy in the field of steroidogenesis in fish. In an attempt to resolve this issue, we identified a novel type of *P450c17* (*P450c17-II*) by an *in silico* analysis from the genomes of six fish species. We cloned *P450c17-II* from tilapia and medaka, and comparison with the conventional *P450c17-I* revealed that they differ in gene structure and enzymatic activity. Enzymatic assays by thin-layer chromatography revealed that P450c17-II possesses only the 17α-hydroxylase activity without any 17, 20 lyase activity, unlike P450c17-I, which has both these activities. In testis, both *P450c17-I and -II* express in the interstitial cells. Remarkable differences, revealed by *in situ* hybridization, in the expression patterns of the *P450c17-I* and *-II* in the ovary and head kidney of tilapia during various stages of development strongly suggest that P450c17-I is responsible for the synthesis of estradiol-17β in the ovary, whereas P450c17-II is required for the production of C21 steroids such as cortisol in the head kidney. More interestingly, a temporally controlled switching is observable in the expression of these two genes during the steroidogenic shift from estradiol-17β to the C21 steroid, 17α, 20β-dihydroxy-4-pregnen-3-one (maturation-inducing hormone of fish oocytes) in the fish ovary, revealing a role for P450c17-II in the production of hormones that induce oocyte maturation in fish. (*Endocrinology* 148: 4282–4291, 2007)

CYTOCHROME P450C17 CATALYZES the 17α-hydroxylase activity required for glucocorticoids (cortisol) synthesis in the adrenal gland and the 17, 20 lyase activity required for sex steroid (androgens and estrogens) synthesis in the gonads. The mechanism behind this organ-specific action of P450c17 has been attributed to posttranslational modifications regulated by factors like, the abundance of the electron-donating protein P450 oxidoreductases, the presence of cytochrome b5, and the serine/threonine phosphorylation of P450c17 (1–5). Alternatively, the existence of P450c17 isozymes has also been proposed in mammals to explain the differential actions of P450c17 in the adrenal and gonad (6).

The vertebrate gonads in general experience a steroidogenic shift from estrogens to progestogens during the transition from the follicular phase to luteal/maturation phase (7). In mammals, the shift is from estradiol-17β to progesterone. Because the production of progesterone does not need the activity of P450c17, a cessation of the hydroxylase and lyase activities of P450c17 is sufficient to achieve the steroidogenic shift in mammals (8, 9). In fish, however, the situation is different. During the maturation of oocytes, postvitellogenic follicles have to synthesize 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-DP) (10) or 17, 20β, 21-trihydroxy-4-pregnen-3-one (11) [maturation-inducing hormones (MIHs) in fish] from 17α-hydroxyprogesterone. Because 17α-hydroxyprogesterone is produced from progesterone by the hydroxylase activity of P450c17, its lyase activity needs to be down-regulated in the fish ovary. Thus, the differential regulation of the activity of P450c17 is critical for the postvitellogenic oocytes to undergo the final maturation step, which is absolutely necessary for their fertilization and subsequent development. Because only a single P450c17 enzyme was found to exist in fish (12–18), the same mechanisms found in mammalian adrenals were thought to be responsible for the switching in the action of P450c17 during the steroidogenic shift in the gonads of fish (13, 14, 17, 19). However, no studies have proved this claim convincingly.

The cortisol produced by the hydroxylase activity of P450c17 in the interrenal cells of the head kidney (the piscine counterpart of the mammalian adrenal) is essential for the osmoregulation and energy metabolism in fish (20). No study has confirmed whether the same P450c17 is present in both the gonad and head kidney, except for ambiguous studies in

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Abbreviations: dah, Day after hatching; DHEA, dehydroepiandrosterone; 17α, 20β-DP, 17α, 20β-dihydroxy-4-pregnen-3-one; E2, estradiol-17β; EST, expressed sequence tag; FSGD, fish-specific genome duplication; HEK, human embryonic kidney; ISH, *in situ* hybridization; MIH, maturation-inducing hormone; NCBI, National Center for Biotechnology Information; ORF, open reading frame; RACE, rapid amplification of cDNA ends; *Scl*, sex characterless; TLC, thin-layer chromatography.

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zebrafish and rainbow trout (17, 21). The survival of a natural mutant of medaka [sex characterless (*Scl*)], in which the P450c17 function was abrogated due to a mutation in the gene, further complicates our understanding of the involvement of P450c17 in the production of cortisol because cortisol is vital to the existence of vertebrates including fish. Moreover, a mutation in the female *Scl* was shown to have impaired only the advanced stages of oogenesis and secondary sexual characteristics, which are dependent on estrogens (22). This indicates that the mutation in *P450c17* had affected the production of the sex steroids in the gonad but not the production of cortisol in the head kidney.

This led us to hypothesize that there could be a second *P450c17* gene, responsible for the production of cortisol in the head kidney of fish. Such a gene would also be responsible for the differential action of P450c17 during the steroidogenic shift from estrogen to the MIHs. Thus, we performed an *in silico* analysis and identified a novel *P450c17* gene in the genomes of fugu, medaka, stickleback, tetraodon, and zebrafish. Furthermore, we cloned *P450c17-II* from tilapia and medaka and studied the enzymatic activity and expression pattern of tilapia P450c17-II, in comparison with the conventional P450c17-I. Our data suggest that P450c17-I is required for the synthesis of estradiol-17β (E2) in the ovary, whereas P450c17-II is responsible for the production of cortisol in the head kidney and 17α, 20β-DP (the MIH of tilapia) in the ovary.

Materials and Methods

Animals

Tilapias were reared in 1-ton tanks with recirculating aerated fresh water. The fish were maintained at ambient temperature (26 C) under natural light conditions. Mature tilapias (XX) that spawn at an average of 14 d were used in the present study. All animal experiments conformed to the Guide for Care and Use of Laboratory Animals and were approved by the Institutional Committee of Laboratory Animal Experimentation (Institute for Basic Biology).

Identification of novel P450c17 from fully sequenced genomes and expressed sequence tags of vertebrates

Medaka (BAA13252), rainbow trout (CAA46675), Japanese eel (AAR88432), and zebrafish (NP_997971) P450c17-I sequences were used as the query sequences in TBLASTX or BLASTP searches carried out at National Center for Biotechnology Information (NCBI; http://www.ncbi.nlm.nih.gov) and University of California, Santa Cruz, Genome Bioinformatics (http://genome.ucsc.edu/) portals against the sequenced genomes and, as available, the genome-predicted proteins of the fish *Takifugu rubripes* (assembly 4 with 5.7× coverage), *Tetraodon nigroviridis* (assembly 1.1 with 8.3× coverage), *Oryzias latipes* (assembly 1.0 with 6.7× coverage), *Danio rerio* (Zv6 with 5–7× coverage), *Gasterosteus aculeatus* (assembly 1.0 with 11× coverage), the amphibian *Xenopus tropicalis* (assembly 4.1, 7.65× coverage, searched via DOE Joint Genome Institute), and the bird *Gallus gallus* (assembly 2.1 with 6.6× coverage). Each matching sequence returned with an expectation value less than e = 0.0001 was used to query the GenBank nonredundant protein database to establish the assignment as a P450c17 and identify which is closer to the mammalian P450c17 sequence. Sequences were also searched by TBLASTX against the database of expressed sequence tags (ESTs) at NCBI for ESTs from the corresponding organism to establish the existence of transcribed sequences corresponding to the open reading frame (ORF) predicted from genomic DNA. In some cases, EST sequences and comparisons with known *P450c17* were used to extend or correct the genome-predicted sequences.

The identified genomic contigs/scaffolds corresponding to *P450c17-I and -II* in fugu, tetraodon, stickleback, medaka, and zebrafish were as follows: fugu, *P450c17-I* (CAAB01002402) and *-II* (CAAB01005318.1); tetraodon, *P450c17-I* (CAAE01021244) and *-II* (GSTENT00036372001); stickleback, *P450c17-I* (chrVI:856070–859737, EST sequence DN731810) and *-II* (chrUn:29090145–29093435, EST sequences DW599621, DN655686, DN721050); medaka, *P450c17-I* (scaffold2317, golw_scaffold Hd-rR (200506)] and *-II* (scaffold730); and zebrafish, *P450c17-I* (NP_997971) and *-II* (NW_001512620). The deduced ORF of fugu, tetraodon (partial sequence), stickleback, medaka, and zebrafish were used for the following phylogenetic analysis.

Cloning of P450c17-I and -II from tilapia and P450c17-II from medaka and zebrafish

A fragment of *P450c17-II* was obtained from cDNA of tilapia testes using primers designed based on the conserved region of the identified fugu, tetraodon, and medaka *P450c17-II*. Subsequently four primers were designed to amplify a full-length cDNA sequence by 5′-rapid amplication of cDNA ends (RACE) and 3′-RACE.

The ORF of medaka *P450c17-I* was amplified from cDNA of the ovary by PCR based on a sequence downloaded from GenBank (BAA13252). The medaka *P450c17-II* ORF was isolated from testis cDNA by PCR according to the medaka genome sequence [scaffold730, golw_scaffold Hd-rR (200506)]. Zebrafish *P450c17-II* ORF was isolated from testis cDNA by PCR according to mRNA (XR_029319.1) derived from zebrafish genome sequence.

Analysis of domain architecture of novel P450c17

The domain architecture of the predicted and cloned novel P450c17 proteins was evaluated by searches against the Conserved Domain Database at NCBI to confirm they are members of the P450c17 family.

Phylogenetic analysis

Alignments of P450c17-I and -II amino acid sequences from six species of teleosts were prepared by the progressive, neighborhood-joining alignment method, Clustal X (23). The multiple sequence alignments are presented in Boxshade 3.2. The multiple alignment software Clustal X was also used to analyze homology and calculate phylogenetic trees by the neighborhood-joining method using Japanese eel P450 CYP1C1 (AAR15082) as an outgroup (23). Values on the trees represent bootstrap scores of 1000 trials, indicating the credibility of each branch. The GenBank accession nos. of P450c17 sequences used in this study are as follows: human P450c17-I (AAV38803), mouse P450c17-I (NP_031835), rat P450c17-I (NP_036885), chicken P450c17-I (NP_001001901), *Xenopus* P450c17-I (AAG42003), Japanese eel P450c17-I (AAR88432), zebrafish P450c17-I (NP_997971), tilapia P450c17-I (AB292401), medaka P450c17-I (BAA13252), rainbow trout P450c17-I (CAA46675), fugu P450c17-I (EF624004), tetraodon P450c17-I (CAAE010212); stickleback P450c17-I (EF624006), fugu P450c17-II (EF624005), medaka P450c17-II (EF423918), tetraodon P450c17-II (GSTENT00036372001), stickleback P450c17-II (EF624007), tilapia P450c17-II (EF423917), and zebrafish P450c17-II (EF624003).

Enzymatic assay by thin-layer chromatography (TLC)

The ORFs of tilapia *P450c17-I* and *-II* were subcloned into TOPO pcDNA 3.1 (Invitrogen, Carlsbad, CA) to obtain recombinant constructs. Human embryonic kidney (HEK) 293 cells were transfected with 4 μg pcDNA3.1 vector (Invitrogen) with or without the insert and were cultured in DMEM with 10% fetal bovine serum (JRH Biosciences, Lenexa, KS). These cells were incubated at 37 C and 5.0% CO_2 for 24 h. Then, serum-free medium with ^{14}C-progesterone (60,000 cpm/well) or ^{3}H-pregnenolone (2000,000 cpm/well) (PerkinElmer, Boston, MA) was used to replace the original medium. After radiolabeled substrates were added, the medium in each well was collected individually at 2, 4, and 8 h. The extraction of steroids, TLC assay, and quantification were carried out according to procedures described elsewhere (24). Lack of lyase activity in P450c17-II was ascertained by performing TLC with the substrate ^{3}H-17α-hydoxyprogesterone and in this case, the medium was collected at 24 h.

Three parallel samples were collected at each time point and quantified. The conversion rate was calculated as a percentage of the total

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radioactivity after extraction. Results were represented as mean ± SE of three independent measurements.

Tissue distribution analysis by RT-PCR

Total RNA was extracted, cDNA was synthesized, and RT-PCR was carried out to check the expression levels of tilapia *P450c17-I* and *-II* in various tissues according to methods described previously (25). Gene-specific primers were used for the RT-PCR analysis. Positive and negative controls were set up with plasmid DNA and water, respectively, as templates to validate the distribution pattern. A fragment of β-actin was amplified (as internal control) from tilapia to test the quality of the cDNAs used in the PCR. The PCR products were subjected to agarose gel (1.5%) electrophoresis.

In situ hybridization (ISH)

The whole bodies of XX and XY fry at 2, 5, 11, 20, 60 day after hatching (dah; after removal of the yolk and gut) and gonads and head kidneys of 8-month-old tilapia were fixed in 4% paraformaldehyde (Nacalai tesque, Kyoto, Japan) in 0.85× PBS at 4 C to check the expression of *P450c17-I* and *-II* in the gonad and head kidney by ISH as described previously (26). Similarly, gonads from regularly spawning fish were sampled and fixed at d 1, 3, 5, 8, 10, 12, and 14 of the spawning cycle. Probes of sense and antisense digoxigenin-labeled RNA strands were transcribed *in vitro* with an RNA labeling kit (Roche Diagnostics GmbH, Mannheim, Germany) from plasmid DNA containing ORFs of tilapia *P450c17-I*, *P450c17-II*, and *Cyp19a1* (ovarian type aromatase).

Real-time PCR

Gonads from regularly spawning fish (14 d/spawn) were sampled at d 1, 3, 5, 8, 10, 12, and 14 (2–4 h after spawning) of the spawning cycle. Five fish were used for each time point and three samples (replicates) were collected from each fish. Total RNA was extracted from all the samples, and cDNA was prepared for the real-time PCR. Real-time PCR was carried out according to the protocol of Platinum SYBR Green quantitative PCR SuperMix UDG (Invitrogen). β-Actin was used as an internal control. The relative expression levels (RNA abundance) were calculated by dividing the number of copies of the target gene by that of β-actin. Data were expressed as the mean ± SE of the 15 replicates. Furthermore, the results within each type (expressed as a function of days or ovarian stages) were analyzed with a Kruskall-Wallis ANOVA. When the ANOVAs were significantly different, data within each type and between the two types were compared using the Mann-Whitney test.

Primer sequences used for RT-PCR, RACE, and real-time PCR are listed in Table 1.

Results

Sequences of the two types of P450c17

An *in silico* search revealed the existence of two different *P450c17* genes in the genomes of fugu, medaka, stickleback, tetraodon, and zebrafish. The complete coding sequences were available for fugu, medaka, stickleback, and zebrafish, whereas only partial coding sequences were available for tetraodon due to sequence errors or low similarity at the 3′- and/or 5′-ends. Furthermore, using RT-PCR and RACE, both the *P450c17* cDNAs were cloned from tilapia, whereas only *P450c17-II* was cloned from medaka and zebrafish as *P450c17-I* from these two species has been cloned previously (13, 17). A blast analysis showed that tilapia P450c17-I was highly homologous to the P450c17-I of medaka (77.7%), Japanese eel (71.8%), zebrafish (69%), and fugu (82.4%) at the amino acid level. Whereas the tilapia P450c17-II showed a high degree of similarity to the P450c17-II of medaka (74.9%), fugu (70.6%), stickleback (68.7%), and zebrafish (63.0%), it was less homologous to the P450c17-I of Japanese eel (46.1%), zebrafish (47.4%), medaka (45.5%), fugu (45.9%), and tilapia (45.3%) at the amino acid level. These data demonstrated that P450c17-I and -II were having over 60% similarity with their corresponding homologues from other species of fish, whereas the similarity between the two types from different fishes tended to be around 45%. Nevertheless, alignment of the sequences revealed that the Ono-sequence, Ozols' tridecapeptide, and the Heme-binding regions, which were marked as box I, II, and III, respectively (Fig. 1), were highly

TABLE 1. Primer sequences used in cloning, real-time PCR, and tissue distribution analyses

Primer	Sequence (5′–3′)	Purpose
P450c17-I-F1	GACATHTTYGGRGCHGGVGTGGARAC	To amplify a fragment
P450c17-I-R1	CCHGC BCCRAAHGGCARGTARC	
P450c17-I-R2	CGGCCAGGGTCAAACCGCTCTGGGTTC	5′-RACE
P450c17-I-R3	CCACTCCTTCTCATCATGGTGCAGAGCC	
P450c17-I-F2	GGACAGGACGGTGGGTGACAGCCGCTC	3′-RACE
P450c17-I-F3	CGCCCCGTGGCACCACTACTCATCC	
P450c17-I-F	GATATGGCTTGGTTTTTGTGTCTGTGC	Tissue distribution and ORF amplification
P450c17-I-R	TTCTGCCTGGCACTTATGTCTTGCC	
P450c17-I-rF	CAGGGCATTGTGGACACTGT	Real-time PCR
P450c17-I-rR	TAGGTCCGCATTAGGAAAG	
P450c17-II-F1	AAGGACTTTGCTGGACGACCGAG	To amplify a fragment
P450c17-II-R1	TGTCCCAGTGATGGGGGTCGTGGTG	
P450c17-II-R2	GCCGTGTCGATAGGTCGCACTGAA	5′-RACE
P450c17-II-R3	CCACCTCGGACAGAACGATGTCCT	
P450c17-II-F2	CACCACGACCCCCATCACTGGGACA	3′-RACE
P450c17-II-F3	CTCTTCCTTTTCTTGTCCTCTCTGCTC	
P450c17-II-F	ATGCTCACCTGTCTCCTCTCG	Tissue distribution and ORF amplification
P450c17-II-R	TTTTCCTCCATCCTCCCATCC	
P450c17-II-rF	TGAGGATCCGGCCAGTGA	Real-time PCR
P450c17-II-rR	GCGTGGCCTCCAATG C	
mP450c17-I-F	ATGGCTTGGTTTCTGTGCCTGT	Medaka P450c17-I
mP450c17-I-R	TCAAGGATTTGCGGGGAATAACCC	ORF amplification
mP450c17-II-F	ATGCTCTCCTTTCTCCTCT	Medaka P450c17-II
mP450c17-II-R	TCATCTTGCCCCGCCCTCCCAC	ORF amplification
zP450c17-II-F	ATGTGTTCAGTGAGTGTGT	Zebrafish P450c17-II
zP450c17-II-R	TCAGTGTCGAGGTGTGACTGTG	ORF amplification

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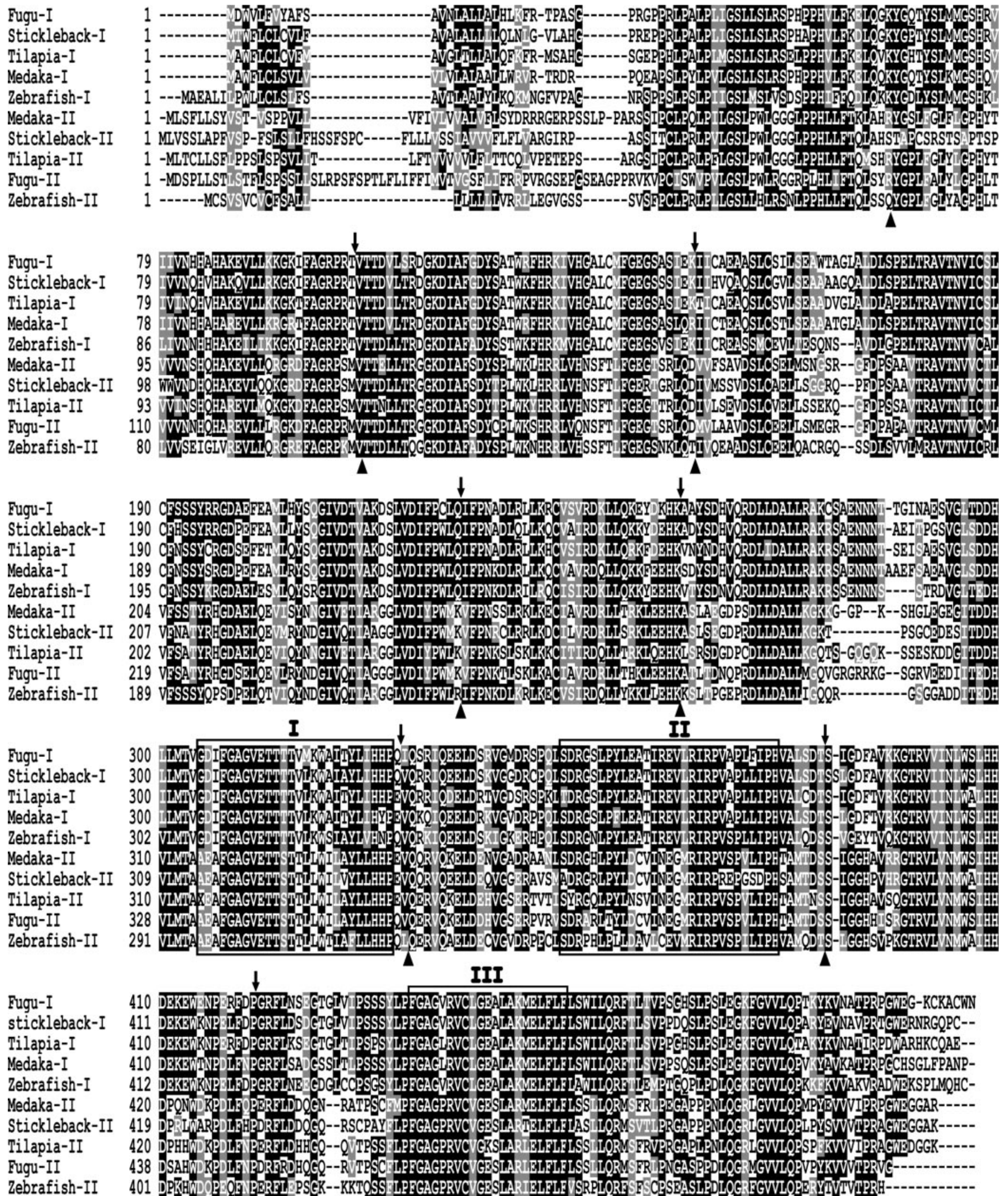

FIG. 1. Comparison of deduced amino acid sequences of P450c17-I and -II from tilapia, medaka, stickleback, fugu, and zebrafish. The intron positions of P450c17-I and -II are marked with *arrows* (above the aligned sequences) and *arrowheads* (below the aligned sequences), respectively. The putative conserved regions are *boxed*: I, Ono-sequence; II, Ozols' tridecapeptide region; and III, Heme-binding region. Refer to *Materials and Methods* for GenBank accession nos.

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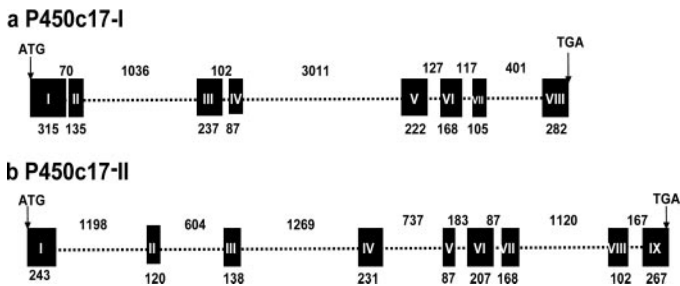

FIG. 2. Schematic representation of the gene structures of medaka *P450c17-I* (a) and *-II* (b). Exons are represented as *boxes* and marked with *Roman numerals*, whereas introns are indicated by *dotted lines*. The *numbers given under the boxes* and *above the dotted lines* represent the relative base pair sizes of the exons and introns, respectively.

conserved between P450c17-I and -II. The Ono-sequence is regarded as the signature sequence of the P450c17s (17, 27). The Ozols' tridecapeptide region includes the putative steroid-binding domain of cytochrome P450 family (28, 29). The heme-binding region is the binding site for heme iron, which mediates the catalysis of the substrate binding to P450c17 (3, 30).

A structural analysis of the genes of *P450c17-I* and *-II* of medaka revealed 8 and 9 exons, respectively (Fig. 2). This was further confirmed by analyzing the fugu and zebrafish *P450c17-I* and *-II* genes. This difference between *P450c17-I* and *-II* was caused by the insertion of an extra intron (intron 1) in the latter. The other introns of *P450c17-II* (introns 2–9) are located at more or less the same positions corresponding to introns 1–8 of *P450c17-I* (Fig. 1).

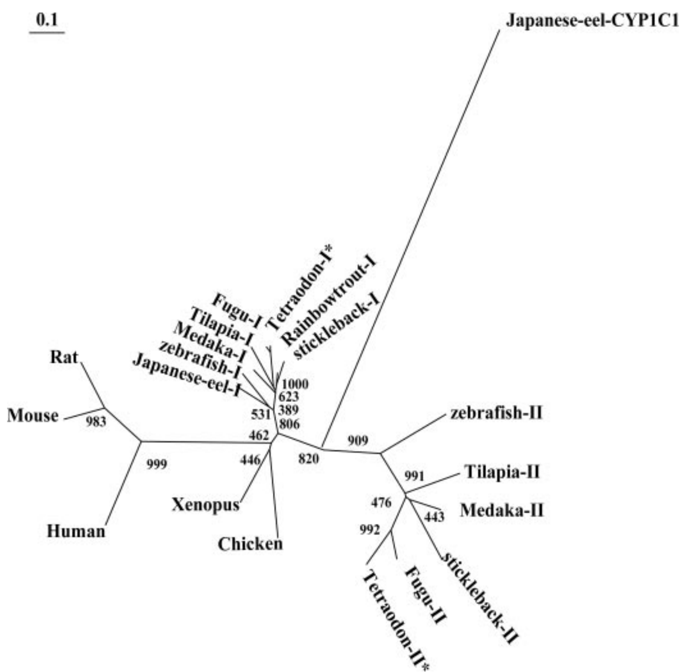

FIG. 3. Phylogenetic analysis of P450c17s of vertebrates using Japanese eel cytochrome P4501C1 as an outgroup. Values on the tree represent bootstrap scores of 1000 trials, indicating the credibility of each branch. Branch lengths are proportional to the number of amino acid changes on the branch. Partial sequences (*) may have artificially short branches. Refer to *Materials and Methods* for GenBank accession nos.

Phylogenetic analysis

A phylogenetic tree was constructed based on the amino acid sequences of vertebrate P450c17-I and -II. The sequences retrieved from the fugu, stickleback, and tetraodon genomes also were included in the phylogenetic analysis (Fig. 3). P450c17-II of fugu, medaka, stickleback, tetraodon, tilapia, and zebrafish clustered into one clade, which was distinct from the P450c17-I clade. The isolation of P450c17-II sequences from six species of teleosts, especially from zebrafish, which is phylogenetically far from fugu, medaka, and tilapia, indicates that the duplication of P450c17 is not restricted to a single clade of fish. The tree demonstrates clearly that this duplication is a phenomenon unique to the teleost fishes [ray-finned fish (*Actinopterygii*)].

Enzymatic activity determined by TLC

Enzymatic assays using TLC showed that tilapia P450c17-I possessed both 17α-hydroxylase and 17, 20-lyase activities as it efficiently catalyzed the reaction from progesterone to androstenedione through 17α-hydroxyprogesterone as well as the reaction from pregnenolone to dehydroepiandrosterone (DHEA) through 17α-hydroxypregnenolone. However, tilapia P450c17-II possessed only the hydroxylase activity. Although progesterone was converted to 17α-hydoxyprogesterone and pregnenolone to 17α-hydroxypregnenolone, the subsequent conversion of 17α-hydoxyprogesterone to androstenedione or 17α-hydroxypregnenolone to DHEA did not occur up to 8 h, indicating the absence of the lyase activity in P450c17-II (Fig. 4). Extension of incubation time to 16 and 24 h also did not have any effect (supplemental Fig. 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org). The absence of the lyase activity in P450c17-II was further confirmed by TLC using 17α-hydoxyprogesterone as substrate (Fig. 5).

Tissue distribution by RT-PCR

A tissue distribution analysis using RT-PCR revealed that tilapia *P450c17-I* and *-II* were expressed in the gonads. Very interestingly, the head kidney of tilapia exclusively expressed *P450c17-II* (Fig. 6).

Expression pattern of P450c17-I and -II in tilapia

Glucocorticoids (cortisol) are important for the physiological fitness of an organism and similarly, E2 is essential for the sexual differentiation of the fish gonads. Therefore, the ontogenic expression patterns of *P450c17-I* and *-II* in the head kidney and gonads were investigated by ISH using 2, 5, 11, 20, 60 dah and 8-month-old fish. At 5 dah, *P450c17-I* was expressed in both XX and XY gonads (Fig. 7, a and c), whereas no expression of *P450c17-II* was detected in the gonads (Fig. 7, b and d). In 8-month-old tilapia, *P450c17-I* and *P450c17-II* were expressed in the interstitial cells of the testis (Fig. 7, i and j). In the ovary, there was a strong expression of *P450c17-I* in the granulosa cells (Fig. 7g), whereas *P450c17-II* mRNA was detected only in the theca cells of the pre- and midvitellogenic oocyte (Fig. 7h). However, a weaker but persistent expression of *P450c17-I* also was seen in the theca

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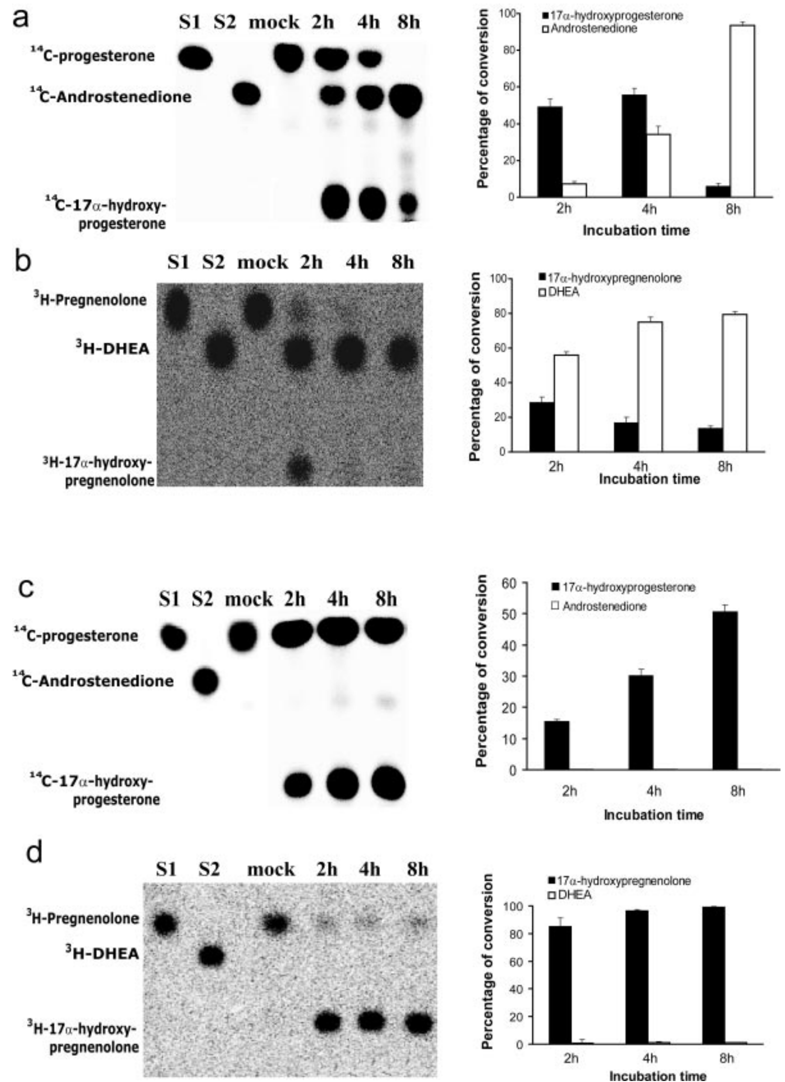

FIG. 4. Enzyme assay of P450c17-I and -II recombinant proteins expressed in HEK 293 cells by TLC followed by autoradiography. P450c17-I converts ^{14}C-progesterone and ^{3}H-pregnenolone to androstenedione and DHEA through 17α-hydroxyprogesterone and 17α-hydroxypregnenolone (a and b), whereas P450c17-II can convert only ^{14}C-progesterone and ^{3}H-pregnenolone to 17α-hydroxyprogesterone and 17α-hydroxypregnenolone (c and d). Standard sample (S1 and S2) and mock indicate radioisotope-labeled standard substrates and pcDNA3.1 vector without insert, respectively. The *right panel* indicates the conversion rate of substrates calculated as a percentage of the total radioactivity after extraction. The *bars* represent the mean ± SE of triplicates measurements.

cells of the ovary. In the head kidney, *P450c17-II* was strongly expressed in the interrenal cells (Fig. 7, f and l) throughout the stages examined, whereas no signal for *P450c17-I* expression was detected (Fig. 7, e and k) at any stage. Expression of P450c17-II is initiated in the XX and XY fish from 11 and 60 dah, respectively (data not shown), whereas P450c17-I is expressed in both the sexes throughout the developmental stages analyzed.

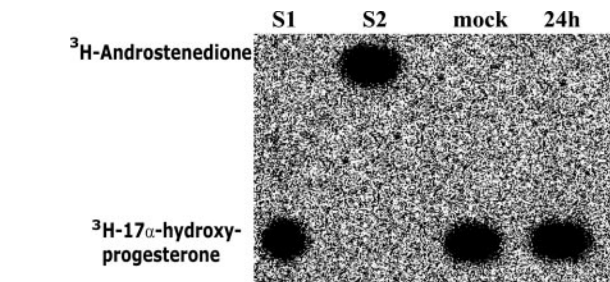

FIG. 5. Enzyme assay of P450c17-II recombinant protein expressed in HEK 293 cells by TLC followed by autoradiography. P450c17-II cannot convert ^{3}H-17α-hydroxyprogesterone to androstenedione, even after 24 h of incubation, demonstrating the absence of lyase activity of this enzyme. Standard sample (S1, S2) and mock indicate radioisotope-labeled standard substrates and pcDNA3.1 vector without insert, respectively.

Expression pattern of tilapia P450c17-I and -II during the spawning cycle

Furthermore, gonads of regularly spawning fish were subjected to ISH to examine the performance of these two genes during the growth and maturation of oocytes in tilapia. *P450c17-I* was mainly expressed in the granulosa cells during the early and midvitellogenic stages. Its expression increased sharply from d 3 to 5, tapered off by d 8, and had ceased

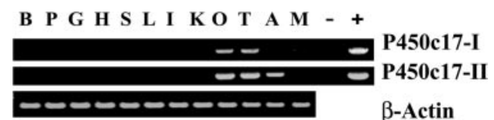

FIG. 6. RT-PCR analysis of tilapia *P450c17-I* and *-II* from various tissues of adult tilapia. B, Brain; P, pituitary; G, gill; H, heart; S, spleen; L, liver; I, intestine; K, kidney; O, ovary; T, testis; A, head kidney (equivalent to adrenal of mammals); M, muscle; −, negative control; +, positive control. *Lower panel* shows β-actin (internal control).

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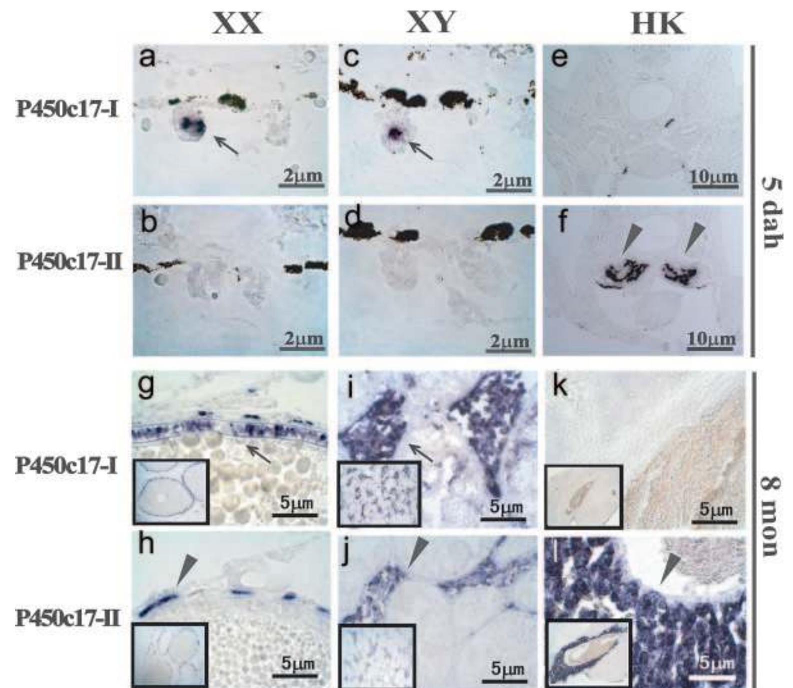

FIG. 7. Expression of *P450c17-I* and *-II* in the gonad and head kidney of tilapia detected by ISH. At 5 dah, *P450c17-I* was expressed in both XX and XY gonad (a and c), whereas no expression of *P450c17-II* was detected in the gonad of the same day (b and d). In 8-month-old tilapia, both *P450c17-I* and *-II* mRNA were detected in the ovary [granulosa (g) and theca cells (h), respectively] and testis (interstitial cells) (i and j). *P450c17-II* was strongly expressed in the interrenal cells of the head kidney (f and l), whereas no signal of *P450c17-I* expression was detected (e and k) in both 5 dah and 8-month-old fish. *Arrows* indicate P450c17-I and *arrowheads* indicate P450c17-II.

completely by d 14 (Fig. 8, b–g) of the spawning cycle. In contrast, *P450c17-II* exhibited a very interesting pattern of expression. Although present in the theca cells throughout the spawning cycle (Fig. 8, h–n), it also started to appear in the granulosa cells by d 8 (Fig. 6k), precisely when *P450c17-I* levels were declining in the granulosa cells (Fig. 8d). The expression of *P450c17-II* in the granulosa cells culminated by d 12 and then completely disappeared by d 14 (Fig. 8, m and n). The expression pattern of aromatase in the granulosa cells was not entirely the same as that of *P450c17-I* during the spawning cycle (Fig. 8, o–u). A decline in the expression of aromatase occurred only after d 10 (Fig. 8s). However, the expression of aromatase in theca cells was similar to the pattern of *P450c17-II* expression, being present throughout the spawning cycle.

The quantitative analysis of *P450c17-I* and *-II* by real-time PCR during the spawning cycle corroborated the results of ISH. There were two peaks on d 3–5 and 11–12 corresponding to *P450c17-I* and *-II*, respectively (Fig. 9).

Discussion

In fish, 17α, 20β-DP and cortisol are indispensable for the final maturation of oocytes and osmoregulation, respectively. The hydroxylase activity of the steroidogenic enzyme P450c17 is crucial for these two major physiological events (10, 20). In this study, we isolated and characterized a novel type of P450c17 (P450c17-II) that orchestrates the above two events in fish. Our data from comparative analyses of the gene structure, enzymatic activity, and expression of P450c17-I and -II demonstrate that P450c17-II lacks the lyase activity, in stark contrast to the conventional P450c17-I, and possesses a very distinct expression pattern, proving it to be a gene with greater biological significance among fish.

It is apparent from the sequences and structure of the medaka *P450c17-I* and *-II* genes that the two are completely different. The phylogenetic analysis revealed that the teleost P450c17-I is more closely related to the tetrapod P450c17, indicating it to be an archetype of the tetrapod P450c17s. The duplication of *P450c17* appears to be a unique incident that occurred only in the course of the evolution of fish. The existence of *P450c17-II* in the six species of fishes analyzed in this study makes the presence of this gene in other fishes highly probable. A structural analysis of the genes of *P450c17-I* and *-II* of fugu, medaka, and zebrafish revealed 8 and 9 exons, respectively. The extra exon appears to be generated by the insertion of an additional intron in *P450c17-II*, in a position that aligns to the middle of the first exon of *P450c17-I*. The other introns of *P450c17-II* are located more or less at the same positions as *P450c17-I.* These gene structures are quite conserved among all vertebrate *P450c17* genes (15, 31). The conservation of gene structures and P450c17 signature domains indicate that in fish, *P450c17-II* was derived from duplication of *P450c17-I.*

Genomic data indicate that the genomes of most vertebrates (including human) had undergone two whole genome duplications in their evolutionary history, the 2 round hypothesis (32), whereas the genomes of most bony fishes had undergone an additional duplication [the fish-specific genome duplication (FSGD) or the 3 round duplication] at the origin of modern fish (33–35). FSGD probably occurred after the bichirs (*Polypteriformes*), sturgeons (*Acipenseriformes*), gars and bowfins (*Semionotiformes*) branched off from the fish stem lineage (34). In the present study, two P450c17 types encoded by two different genes were found in six different teleosts species: fugu, tetraodon, stickleback, tilapia (*Percomorpha*), medaka (*Atherinomorpha*), and zebrafish (*Ostariophysi*), which belong to three different clades (36). Phylogenetic analysis of all known P450c17 sequences revealed that the occurrence of two P450c17 types exists only in teleost fish. We did an *in silico* search on the genomes of *Xenopus*,

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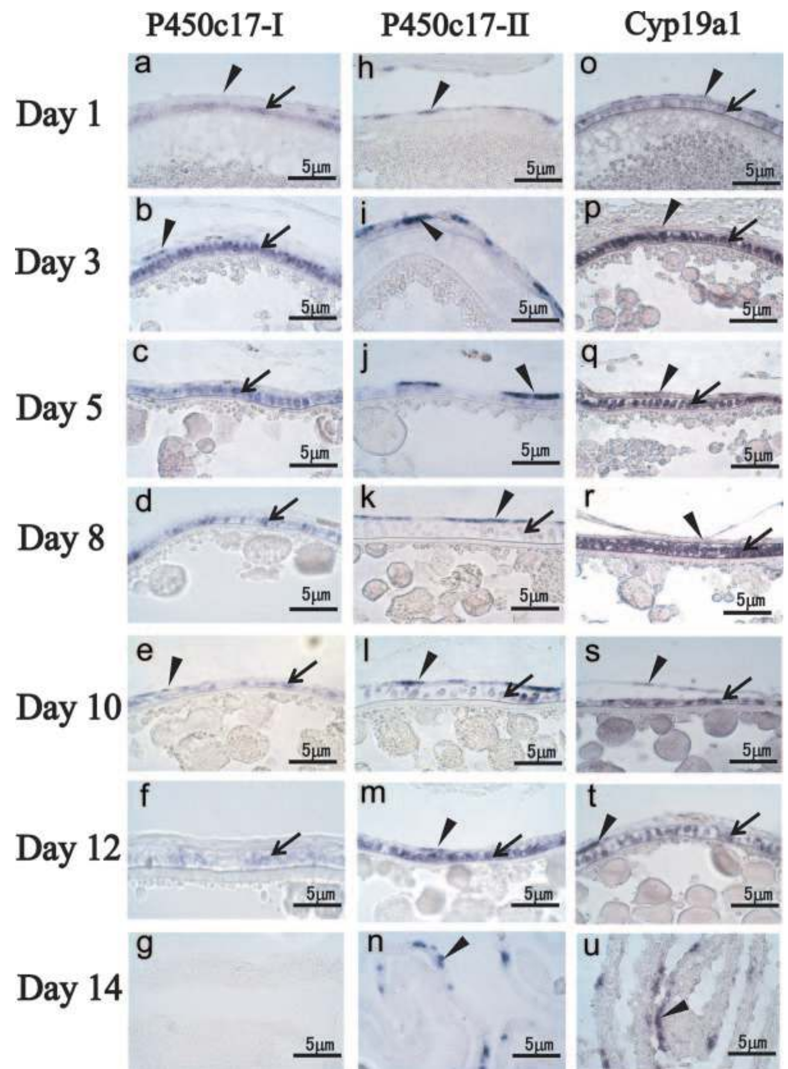

FIG. 8. Expression of *P450c17-I*, *-II*, and *Cyp19a1* in the granulosa cells and/or theca cells during the spawning cycle of female tilapia. Ovaries collected from regularly spawning female tilapia on d 1, 3, 5, 8, 10, 12, and 14 were subjected to ISH using the amplified RNA probes for *P450c17-I*, *-II*, and *Cyp19a1*. a–g, *P450c17-I* expression was found in the granulosa cells in the early and midvitellogenic follicles on d 1–8 (a–d), with the expression at its peak on d 3–5 (b and c). Thenceforth, the expression tapered off and had completely ceased by d 14 (e–g). h–n, Expression of *P450c17-II* persisted throughout the spawning cycle especially in the theca cells. However, a simultaneous increase in the expression of *P450c17-II* was observed in the granulosa cells from d 8 (k), precisely when *P450c17-I* started to decrease. The expression reached its peak by d 12 (m) and disappeared on d 14 (n). o–u, Expression of *Cyp19a1*. Expression pattern of *Cyp19a1* in granulosa cells followed a similar pattern of *P450c17-I* expression during the entire spawning cycle but declined from d 10, whereas the expression of *Cyp19a1* remained unchangeable in the theca cells, like the *P450c17-II*. *Arrows* and *arrowheads* indicate the signal in granulosa cells and theca cells, respectively.

chicken, mouse, rat, and human but failed to obtain any P450c17-II-like sequences. Hence, we propose that the P450c17-II sequences probably originated from the FSGD event associated with the emergence of modern teleosts, and therefore most teleosts with the possible exception of bichirs, sturgeons, gars, and bowfins should possess both P450c17 types unless they had experienced a secondary loss after the FSGD event. This claim remains rather speculative as further experimental evidence is yet to be obtained.

Because the nucleotide and amino acid sequences of the two types of P450c17 are clearly different, we further examined the enzymatic activity of P450c17-II by transfection into intact HEK 293 cells and compared it with that of P450c17-I under the same experimental conditions. The inability of P450c17-II to convert 17α-hydroxyprogesterone to androstenedione or 17α-hydroxypregnenolone to DHEA proves that it lacks the lyase activity, making it different from P450c17-I functionally also. HEK 293 cells were regarded as a cell line rich with cytochrome b5 (4). Even the human P450c17 displayed lyase activity when transfected into this cell line (37), without the addition of any exogenous cytochrome b5. Our data clearly showed that P450c17-II lacks any lyase activity under the above experimental conditions, whereas P450c17-I possesses both the activities. This experiment indicates that P450c17-II does not have any lyase activity even in the presence of cytochrome b5.

Because the novel type of P450c17 was found to have only the hydroxylase activity, we further checked whether this is the gene responsible for the production of cortisol by analyzing its expression pattern in the head kidney of tilapia during different developmental stages. Surprisingly, only *P450c17-II* was found to be expressed in the interrenal cells of the head kidney from the very early stages (5 dah) to adulthood (8 months old). One of the most important physiological functions of the interrenal cell is to produce cortisol (38). Our data suggest that only P450c17-II is responsible for cortisol production in the interrenal cells, making its presence crucial to the existence of the individual concerned. Although this study has not provided any functional data to support this further due to the unavailability of knockout strategies in fish, inferences on the function of P450c17-II can be drawn from the study of the *Scl* mutant (22). Despite ablation of the protein, and thus the function of P450c17-I, the *Scl* embryos survived like normal embryos. This shows that

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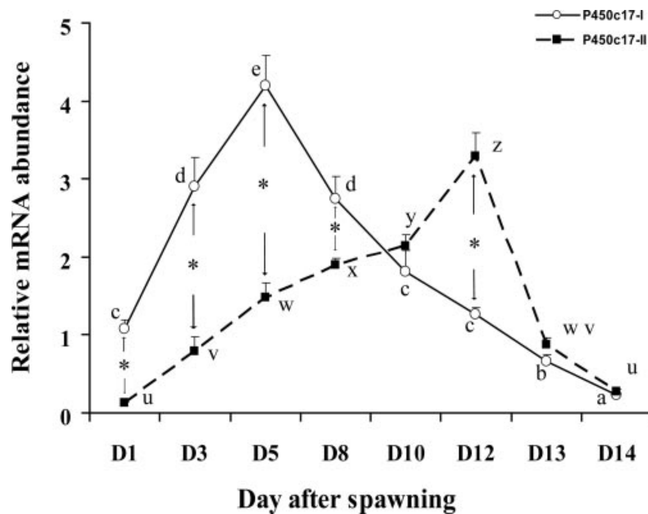

FIG. 9. Expression pattern of tilapia *P450c17-I* and *-II* during the 14-d spawning cycle analyzed by real-time PCR. The *solid line* represents *P450c17-I* expression and the *dotted line*, *P450c17-II* expression. Each *point* on the curve represents the relative mRNA expression at each time point in the spawning cycle. Each *value* represents the mean ± SE of at least 15 samples obtained from five different fish. Mean *values with different letters* in each type of *P450c17* are significantly different ($P < 0.05$). The *asterisks* indicate significant differences between the expression levels of *P450c17-I* and *-II* for the same day or the same ovarian stage.

they produced cortisol to regulate the osmoregulation. Cloning and sequencing of *P450c17-II* from the *Scl* mutant further confirmed that the fish had an intact *P450c17-II* gene. This is a staunch proof to the notion that these fish survived successfully due to the existence of another *P450c17* gene to orchestrate the synthesis of cortisol in their head kidneys.

Thereafter we checked the expression patterns of both *P450c17-I* and *-II* in the gonads during gonadal differentiation and the reproductive cycle. We used the tilapia for this purpose because it is a good model in this context due to the availability of all -XX and -XY genetic populations, making the analysis more robust with respect to the sexuality of the samples being tested. Moreover, the tilapia has a reproductive cycle, spanning 14 d on an average, well suited for the profiling of gene expression during the various stages of oogenesis and maturation.

P450c17-I was expressed in the gonads of both XX and XY tilapia from 5 dah, the time when gonadal sex differentiation becomes apparent in this species (39). It continued to be expressed strongly in the gonads of both the adult male and female, reemphasizing its requirement in the production of androgen and E2. In contrast, *P450c17-II* was not found in the gonads of either XX or XY fish during the early stages of gonadal differentiation (5 dah). Because it lacks the lyase activity, its expression may not be required in the gonads during the initiation of sexual differentiation. The difference in the temporal expression patterns of *P450c17-I* and *-II* suggests that they are required for apparently different functions in the gonads.

The data from the ISH and quantitative analysis during the spawning cycle of tilapia suggest that P450c17-I is needed mainly for oocyte growth, whereas P450c17-II is required for oocyte maturation. The expression of *P450c17-I* in the granulosa cells reached its peak around d 3–5, signifying its importance during early and midvitellogenesis for the production of estrogen. *P450c17-I* started to decline in the granulosa cells from around d 8, probably to reduce the production of the precursors of E2. A simultaneous initiation of *P450c17-II* expression was observed in the granulosa cells may be to orchestrate the synthesis of 17α, 20β-DP. Before d 8, *P450c17-II* expression was confined only to the theca cells. Furthermore, *P450c17-I* was reduced by d 12 significantly, and there was a synchronous culmination in the expression of *P450c17-II* in the granulosa cells. The significant decline in the expression of *P450c17-I* between d 8 and 12 and the sharp increase in the expression of *P450c17-II* at the same time indicate that the steroidogenic shift from estrogen to 17α, 20β-DP does occur somewhere between d 8 and 12. A previous study on the steroid hormone profiles of tilapia during a spawning cycle of 14–15 d has shown peaks in estrogen and 17α, 20β-DP, respectively, at 9 and 12 d after spawning (40), providing support to our expression data.

The present study using tilapia has resolved a longstanding issue in the field of steroidogenesis with respect to the differential regulation of the two enzymatic activities displayed by the fish P450c17 during oocyte maturation (13, 14, 17, 19). Recently 17α, 20β-DP has been suggested to be an essential factor for the initiation of the meiosis in spermatogenic cells of the Japanese eel (41). Very intriguingly, we could notice in tilapia gonads that the initiation of *P450c17-II* expression is sexually dimorphic. In XX gonad its expression starts at 11 dah, whereas in XY gonad the expression is initiated from 60 dah only. This sex-specific stage-dependency in the initiation of *P450c17-II* expression matches with the pattern of meiosis, which starts in the XX and XY tilapia gonad at around 35 and 75 dah, respectively. We assume that *P450c17-II* expression may have a role in the production of 17α, 20β-DP which in turn initiates the meiosis in tilapia, as suggested in Japanese eel. In this context, our finding might open another research area for P450c17-II in the field of fish reproductive physiology.

Acknowledgments

The sequences reported in this paper have been deposited in the GenBank database [accession no. of P450c17-I: AB292401 (tilapia-I), EF624004 (fugu-I), EF624006 (stickleback-I); P450c17-II: EF423917 (tilapia-II), EF423918 (medaka-II), EF624003 (zebrafish-II), EF624005 (fugu-II), EF624007 (stickleback-II)].

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