Cloning, expression and characterization of three types of 17 β -hydroxysteroid dehydrogenases from the Nile tilapia, *Oreochromis niloticus*

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Abstract

In order to elucidate the roles of 17 β -HSDs in fish gonadal steroidogenesis, three types of 17 β -HSDs (17 β -HSD1, 17 β -HSD8 and putative 17 β -HSD12) were cloned and characterized from the Nile tilapia, *Oreochromis niloticus*. The cloned cDNAs of 17 β -HSD type 1, 8 and 12 were 1504, 1006 and 1930 bp long, with open reading frames encoding proteins of 289, 256 and 314 aminoacids, respectively. Tissue distribution pattern analyzed by RT-PCR and Northern blot showed that 17 β -HSD1 was dominantly expressed in the ovary, while the putative 17 β -HSD12, one of the two duplicates found in fish, is a male specific enzyme and expressed exclusively in testis (detected by RT-PCR only). On the other hand, 17 β -HSD8 was expressed in the brain, gill, heart, liver, intestine, gonad, kidney and muscle of both male and female. Enzymatic assays of the three types of 17 β -HSDs were performed using recombinant proteins expressed in *E. coli* or HEK 293 cells. Tilapia 17 β -HSD1 expressed in *E. coli* had the preference for NADP(H) as cofactor and could catalyze the inter-conversion between estrone and estradiol efficiently as well as the inter-conversion between androstenedione and testosterone, but less efficiently. Tilapia 17 β -HSD8 recombinant protein expressed in HEK 293 cells could catalyze the conversion of testosterone to androstenedione, as well as the inter-conversion between estrone and estradiol. However, the putative 17 β -HSD12 expressed in *E. coli* or in HEK 293 cells showed no conversion to any of the four substrates tested in this study. Based on enzyme characterization and tissue distribution, it is plausible to attribute crucial roles to 17 β -HSDs in the gonadal steroidogenesis of teleosts.

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Introduction

17 β -hydroxysteroid dehydrogenases (17 β -HSDs) are remarkably multifunctional enzymes that modulate the synthesis and metabolism of sex steroids in gonadal tissues by catalyzing the conversion of 17-ketosteroids (dehydroepiandrosterone, androstenedione (A), and estrone (E₁)) into 17 β -hydroxysteroids (androstenediol, testosterone (T), and estradiol-17 β (E₂)), and vice versa. To date, multiple types of 17 β -HSDs, which belong to two protein superfamilies: short chain dehydrogenase/reductase (SDR) and aldo-ketoreductase (AKR), have been isolated and characterized in mammals (Puranen *et al.* 1997, Oppermann *et al.* 1999, Penning *et al.* 2003, Mindnich *et al.* 2004b). More importantly, each type of 17 β -HSDs differs from each other in substrate specificities, catalytic coenzyme preference, biochemical

properties and in particular, tissue distribution (Poirier 2003). Therefore, different isozymes might possess different physiological function in the steroidogenic pathway.

17 β -HSD1, which had been characterized in mouse, human, rat, chicken, Japanese eel and zebrafish (Poutanen *et al.* 1993, Akinola *et al.* 1996, 1998, Nokelainen *et al.* 1996, Wajima *et al.* 1999, Kazeto *et al.* 2000, Mindnich *et al.* 2004a), is a key enzyme dominantly catalyzing the final step of the synthesis of E₂. The enzymatic activity to convert A to T by 17 β -HSD1 was found only in mice and rats (Nokelainen *et al.* 1996, Akinola *et al.* 1996). In accordance to these, 17 β -HSD1 is abundantly expressed in the ovary of those species, as well as in the adrenal and mammary glands, uterus, placenta, and endometrium of mice (Poutanen *et al.* 1995).

The functional protein encoded by the Ke 6 gene has been determined to be 17 β -HSD8. Previously, the abnormal expression of Ke 6 gene has been closely associated with development of recessive polycystic kidney disease (Fomitcheva *et al.* 1998, Ramirez *et al.* 1998). In humans and mice, Ke 6 is linked to the major histocompatibility complex (MHC) class II region, whereas in zebrafish and medaka it is linked to MHC class I region (Sultmann *et al.* 2000, Matsuo *et al.* 2002). Mouse 17 β -HSD8 efficiently catalyzes the oxidation of E₂, T, and dihydrotestosterone and the reduction of E₁ to E₂. Therefore, 17 β -HSD8 could be essential for the development of both kidney and gonad by regulating the reduction or oxidation of E₂, T, and dihydrotestosterone, and vice versa maintaining the optimal levels of sex steroids within these organs (Fomitcheva *et al.* 1998).

17 β -HSD12 is the most recent addition to this enzyme family and subsequent phylogenetic analysis revealed its close relationship to 17 β -HSD3 (Mindnich *et al.* 2004a). Interestingly, 17 β -HSD3, which is predominantly expressed in testis, is responsible for the conversion of A to T. Therefore, it is one of the crucial steroidogenic enzymes required for normal male sexual development (Baker *et al.* 1997, Bilbao *et al.* 1998, Moghrabi *et al.* 1998). Moreover, the orthologs of 17 β -HSD12 have been characterized in humans (AAP36605), mice (NP_062631), rats (NP_114455), *Xenopus* (BC041194) and zebrafish (A (AAH63943) and B (NP_955907)). It is ubiquitously expressed with highest levels in liver, muscle and kidney, both in humans and mice. Human and mice 17 β -HSD12s were demonstrated to have substrate specificity and function in fatty acid elongation (reduction of 3-ketostearoyl-CoA to 3-hydroxystearoyl-CoA). Putative homologs of 17 β -HSD12 have also been reported in yeast (YBR159w) and *Caenorhabditis elegans* (LET-767) (Beaudoin *et al.* 2002, Kuervers *et al.* 2003). Interestingly in the *C. elegans*, 17 β -HSD12 seems to be involved in the modification of sterol derivatives and its deletion affects embryogenesis, molting and female reproduction. However, in vertebrates, presence of catalytic activity towards steroids by 17 β -HSD12 has not been demonstrated so far.

17 β -HSDs are required for the production of E₂ and 11-Ketotestosterone with seemingly important roles in the process of sex differentiation and gametogenesis in fish (Guiguen 2000, Mindnich *et al.* 2004a). However, most of the data on 17 β -HSDs are from mammals, except for 17 β -HSD1 cloned and characterized from chickens, zebrafish and the Japanese eel. It is well known that sex steroids play important roles in the process of sex differentiation and gametogenesis of non-mammalian vertebrates (Nagahama 2000). In order to elucidate the roles of 17 β -HSDs and get more insights into the steroidogenic pathway in fish, we undertook the cloning and characterization of the three types of 17 β -HSDs in

the Nile tilapia, *Oreochromis niloticus*. To our knowledge, cDNA cloning and enzymatic characterization of 17 β -HSD type 8 has not been reported in any non-mammalian vertebrate.

Materials and methods

Animals

Tilapia was reared in 1 ton tanks with re-circulating aerated fresh water systems. Fish were maintained at ambient temperature (24 $\pm$ 1 $^{\circ}$ C) under natural light conditions. Mature tilapia (XX) that spawn once in every 2 weeks, at an average of 14–18 days, were used in the present study.

cDNA cloning of three types of tilapia 17 β -HSDs

Cloning of full length cDNA of 17 β -HSD1

A 548 bp cDNA fragment of 17 β -HSD1 was amplified from the ovary by RT-PCR with degenerate primers (1–1Fw, 1–1Rv) designed from the conserved region of human, mouse, chicken and Japanese eel 17 β -HSD1s. Tilapia ovarian follicular cDNA UNI-ZAP XR library was constructed according to the manufacturer's instruction (Stratagene, La Jolla, CA, USA). By using the ³²P-labeled cDNA fragment of 17 β -HSD1 obtained from RT-PCR as a probe, about 10⁶ independent plaques of tilapia ovary cDNA library were screened under high-stringency hybridization conditions and the positive clones were obtained after three rounds of screening. *In vitro* excision and rescue of pBluescript phagemids were performed according to the manufacturer's protocol to obtain the full-length cDNA sequence of 17 β -HSD1.

Cloning of 17 β -HSD8 and 17 β -HSD12 cDNAs

A 260 bp cDNA fragment of tilapia 17 β -HSD8, including 3'-untranslated region (UTR) and partial open reading frame (ORF), was obtained from tilapia EST (Expression Sequence Tag) clones. Subsequently, 5'-rapid amplification of cDNA end (RACE) was performed according to the manufacturer's instructions (SMART RACE cDNA Amplification Kit, Clontech, CA, USA) with gene specific primers (8–1Rv, 8–2Rv). Gene specific primers (8–3Fw, 8–4Rv) were designed basing on the 3'- and 5'-UTRs sequence to amplify the full length cDNA of 17 β -HSD8.

In order to obtain a fragment of 17 β -HSD12 cDNA, a blast search of fugu (*Fugu rubripes*) genome (<http://www.ncbi.nlm.nih.gov/BLAST/Genome/fugu.html>) was performed with human 17 β -HSD12 cDNA sequences. Through blast search we identified two 17 β -HSD12-like genes in fugu. We obtained their

Table 1 Sequence of primers used for PCR and RACE

Primer sequences	
Name	
(1-1Fw)	5'-T(GC)A T(CT)A CCG G(TC)T GCT CCT C(TG)G G(AC)A T-3'
(1-2Rv)	5'-GTG T(GT)(CG) AC(CT) G(GC)(GC) CC(GA) CAC T-3'
(1-3Fw)	5'-GGT CTC AGC TTG GCT GTC CGG CTT-3'
(1-4Rv)	5'-GAA TGT TGA AGT GTT GCA GGA GGA-3'
(1-5Fw)	5'-CGT CTC GCA TGG ATA AAA AGG TGG TGC-3'
(1-6Rv)	5'-CGG GAT CCC GTT GCT CCT CAG TTG AGA AA-3'
(8-1Rv)	5'-GCG CCA TCC TTC CCA GAG GCA CCA A-3'
(8-2Rv)	5'-AGA GGC AGC GTA GTT AGC CTG ACC GA-3'
(8-3Fw)	5'-ACA CAG AAC TCG GTT CTC CGT TCC-3'
(8-4Rv)	5'-CGC CTT TAT CTT CAT TCC CTC ACA-3'
(8-5Fw)	5'-GCC ACC ATG GCG GTT GCC ACA AGG-3'
(8-6Rv)	5'TTA ACC CAT GAA AAG TCC GCC TGT-3'
(12-1Fw)	5'-TGG CAT YGG RAA AGC YTA-3'
(12-2Rv)	5'-CAC ACT GGA YAR TRA TTC CTT T-3'
(12-3Fw)	5'-CTA CAG TAT CTA CCC TGC CAT AGC CA-3'
(12-4Fw)	5'-CCT TTG CTG ACG CTT TAC TCT GCC A-3'
(12-5Rv)	5'-TGG CTA TGG CAG GGT AGA TAC TGT AG-3'
(12-6Rv)	5'-GGT GGC AGA GTA AAG CGT CAG CAA AG-3'
(12-7Fw)	5'-GTT TGG CAG TTG AGC CAC TTC TTT GG-3'
(12-8Rv)	5'-CAC AAC ACA CTG AGT TTG TCG TCA C-3'
(12-9Fw)	5'-CAT GTC ATT TGC TGA CCT ACT GGC C-3'
(12-10Rv)	5'-CGC TCC CCC TTT TCA CCG GTC CAA T-3'
β -actin (Fw)	5'-GGC ATC ACA CCT TCT ACA ACG A-3'
β -actin (Rv)	5'-ACG CTC TGT CAG GAT CTT CA-3'

cDNA and amino acid sequences by carefully locating their intron and exon boundaries. Then, a pair of degenerate primers (12-1Fw, 12-2Rv) were designed in the conserved region of the nucleotide sequences of human, mouse, rat 17 β -HSD12 and putative fugu 17 β -HSD12s. A 516 bp fragment of putative 17 β -HSD12 was amplified from the testis of tilapia by RT-PCR with the degenerate primers. Subsequently, 5'- and 3'-RACE were performed by using four gene specific primers (12-3Fw, 12-4Fw, 12-5Rv, 12-6Rv). Finally, the full length putative 17 β -HSD12 cDNA was obtained from the testis of tilapia by using gene specific primers (12-7Fw, 12-8Rv) designed according to the sequence of 5'- and 3'-UTRs.

All the cDNA fragments obtained by PCR were purified by agarose gel electrophoresis, followed by subcloning into pGEM-T Easy Vector (Promega, Madison, WI, USA) for nucleotide sequencing. All clones were sequenced bidirectionally using vector-based and/or gene-specific primers with a fluorescence dye terminator in ABI PRISM 377 DNA Sequencer (Perkin-Elmer/Applied Biosystem, Chiba, Japan). The nucleotide sequences were analyzed using Lasergene software (DNASTAR, Madison, WI, USA) and BLAST network service of NCBI (National Center for Biotechnology Information www.ncbi.nlm.nih.gov/). The sequences of three types of tilapia 17 β -HSDs were deposited in GenBank under the accession numbers AY663853 (type 1), AY663855 (type 8) and AY663854 (putative type 12).

The sequences of all the primers used in this study are listed in Table 1.

Phylogenetic analysis

The multiple alignment software Clustal X (Thompson *et al.* 1997) was employed to analyze the homology and to calculate the phylogenetic trees by the neighbor-joining (NJ) method using androgen-regulated short-chain dehydrogenase/reductase (ARSDR) as outgroup. Values on the tree represent bootstrap scores out of 1000 trials, indicating the credibility of each branch. The sequences of fugu 17 β -HSDs used in the alignment were obtained from the fugu genome by the blast search with Japanese eel 17 β -HSD1, mouse 17 β -HSD8 and human 17 β -HSD12. The GenBank accession numbers of 17 β -HSD and ARSDR sequences used in this study are as follows: chicken-1 (BAA19567), human-1 (NP_000404), mouse-1 (NP_034605), rat-1 (NP_036983), fugu-1 (CAAB01001461.1), eel-1 (AAR88433), zebrafish-1 (AAP74564), tilapia-1 (AY663853), human-3 (NP_000188), rat-3 (NP_446459), mouse-3 (NP_032317), fugu-3 (CAAB01002155.1), zebrafish-3 (AAS58451), tetraodon-3 (CAF93282); human-8 (AAP36896), mouse-8 (P50171), pig-8 (AAD44802), medaka-8 (BAB83840), fugu-8 (CAAB01001220.1), zebrafish-8 (CAD54662), drosophila-8 (NP_570046), elegance-8 (NP_499346), tilapia-8 (AY663855);

human-12 (AAP36605), mouse-12 (NP_062631), frog-12 (AAH41194), tilapia-12 (AY663854), fugu-12A (exon 1 (CAAB01002105·1), other exons (CAAB 01006992·1) (CAAB01006435·1)), fugu-12B (CAAB01002155·1), zebrafish-12A (AAH63943), zebrafish-12B (NP_955907), tetraodon-12A (CAG01042), tetraodon-12B (CAF99943), Yeast YBR159W (AAS58452); drosophila-ARSDR1 (NP_610310), human-ARSDR1 (AP_057110), mouse-ARSDR1 (AP_067532).

Tissue distribution analysis of three types of 17 β -HSDs by RT-PCR

Total RNA (2·0–5·0 μ g) was extracted from different tissues of adult fish. Reverse transcription was performed by using Super Script II (Gibco BRL, Gaithersburg, MD, USA) and Oligo-dT18 at 42 °C for 1 h. DNase I (Invitrogen, Carlsbad, CA, USA) treatments were performed prior to reverse transcription to avoid contamination with genomic DNA. cDNA from various tissues was used as initial templates in RT-PCR for analysis of mRNA levels for different types of 17 β -HSDs. For this purpose, we designed gene specific primers (1–3Fw, 1–4Rv; 8–5Fw, 8–6Rv; 12–4Fw, 12–8Rv) from different exons of each type. The RT-PCR cycles were as follows: 94 °C for 2 min, followed by 30 to 36 cycles of 94 °C (30 s), 60 °C (30 s), and 72 °C (1 min), ending with 10 min of extension at 72 °C. Positive and negative controls were set up with respective plasmid DNA and water as templates to validate the distribution patterns.

A 342 bp tilapia β -actin fragment was amplified (as internal control) to test the quality of the cDNA used in the PCR reactions (Wang *et al.* 2002). All the PCR products were electrophoresed using 1·5% agarose gels and the gels were stained with ethidium bromide to visualize bands.

Northern blot analysis

Northern blot was performed as per the method reported previously (Wang *et al.* 2002, Jiang *et al.* 2003). Briefly, total RNA was extracted from various tissues, such as gonad, liver, intestine and brain of both male and female fish using ISOGEN solution as per the manufacturer's protocol (Nippon Gene, Toyama, Japan). Poly (A)⁺-RNAs were purified using Oligotex-dT30 (Takara, Otsu, Shiga, Japan). Then 5–8 μ g mRNAs were applied to each well and were electrophoresed on a 1·5% (w/v) formaldehyde denatured agarose gel, transferred onto Hybond-N+ nylon membrane (Amersham, Little Chalfont, Buckinghamshire, England) and baked at 80 °C for 2 h. The ORFs of three types of 17 β -HSDs were labeled individually using Random Extension Plus kit (Dupont, Wilmington,

DE, USA). Hybridization was carried out at 60 °C in a hybridization solution containing 6 $\times$ SSC, 5 $\times$ Denhardt's solution, 1% SDS and 200 μ g/ml denatured herring sperm. The membranes were washed at 60 °C with a series SSC-SDS solution. The membranes were exposed to image plates and signals were analyzed using a BAS 2000 Bio-Imager analyzer. Subsequently, the blot was stripped and rehybridized with a ³²P-labeled tilapia β -actin cDNA probe to serve as a positive control for loading variations.

Production of recombinant proteins of tilapia 17 β -HSDs

Two gene specific primers (1–5Fw, 1–6Rv) were designed to introduce two restriction enzyme digestion sites at two ends of the 17 β -HSD1 ORF. Both purified PCR product and pETBlue2 vector (Novagen, Madison, WI, USA) were double digested to generate compatible overhangs for subsequent ligation. The recombinant plasmid was sequenced to confirm the correct insert and was subsequently transformed into *E. coli* (DE3)pLacI strain for expression. The cells containing the recombinant construct were cultured at 37 °C under Isopropyl- β -D-thiogalactopy anoside (IPTG) induction (500 μ M) for 6–10 h to obtain maximum production of the recombinant protein. The cells were then pelleted and lysed by sonication in the presence of lysozyme (100 μ g/ml). After confirmation of the induction of recombinant protein by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE), total proteins from the cell lysate were saved for the subsequent analysis of enzyme activity. Radioactive steroid substrates and mock (cells transformed with pETBlue-2 vector minus insert) were loaded as controls in these experiments.

Functional expression of 17 β -HSD8 and putative 17 β -HSD12 was initially conducted using a bacterial expression system. However, we failed to get any enzymatic activity using the cell lysate. Assuming that some factors from the eukaryotic system are necessary for the normal function of these enzymes, we opted to use a mammalian expression system for the production of recombinant proteins of 17 β -HSD types 8 and 12. The ORFs of 17 β -HSD8 (8–5Fw, 8–6Rv) and 17 β -HSD12 (12–9Fw, 12–10Rv) were amplified and cloned into TOPO pcDNA 3·1 (pcDNA3·1/V5-His TOPO TA Expression Kit, Invitrogen, Carlsbad, CA, USA) to obtain respective recombinant constructs. After sequence confirmation, both constructs were transiently transfected into HEK 293 cells. Transient transfections were carried out using the Lipofectamine Reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Briefly, 3 $\times$ 10⁶ cells were seeded onto 10 cm tissue culture dishes before the day of transfection. For each culture dish, 24 μ g pcDNA 3·1 construct containing 17 β -HSD8 or 17 β -HSD12 was

Figure 1 Alignment of 17 β -HSD1 amino acid sequences of fish, chicken and human and 17 β -HSD8s of fish and human. BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html) was used to make this figure. Boxes I (coenzyme-binding motif), II (structurally conserved residues), III (catalytic center) and IV (motif for the determination of reaction direction) are the conserved motifs of the SDR superfamily. Refer to materials and methods for GenBank accession numbers.

transfected into the cells in 15 ml Dulbecco's modified eagle's medium with 10% fetal bovine serum, and cultured at 37 °C, 5·0% CO₂ for 24 h. Vector pc DNA3·1/LacZ construct was used as a control for transfection efficiency. Cells were collected and cell lysates were saved for subsequent enzyme assay.

Enzyme assay by thin layer chromatography (TLC)

For each reaction, 10 μ l total cell lysate was diluted with 180 μ l buffer A (50 mM potassium phosphate buffer (pH 7·4) containing 1 mM EDTA and 20% (W/V) glycerol) and incubated with 3 μ l of ¹⁴C-labeled substrates ([4-¹⁴C]-T, [4-¹⁴C]-A, [4-¹⁴C]-E₁, [4-¹⁴C]-E₂ in ethanol) (0·02 mCi/ml) (PerkinElmer, Boston, MA, USA) and 2 μ l

coenzyme (100 mM) (NAD(P)H for reducing reaction and NAD(P)⁺ for oxidizing reaction)). The reaction mixtures were incubated at 37 °C for 4 h, and the reactions were terminated by adding 200 μ l dichloromethane. The mixtures were shaken vigorously and centrifuged at 3000 r.p.m. for 10 min. The lower organic layer was saved and the upper aqueous layer was re-extracted with 200 μ l dichloromethane. The combined organic layers were subjected to evaporation under nitrogen. The residual steroids were dissolved in 50 μ l ethanol and developed on silica gel TLC plate (5729, E. Merck, Darmstadt, Germany) by using dichloromethane/ethylacetate/methanol (85:15:3 by volume) as the developing solvent system. The TLC plate was then air-dried and the radioactive spots were located by autoradiography, and

Figure 2 Alignment of the amino acid sequences of 17 β -HSD12s from mammals, frog and fish. Boxes I, II and III are the conserved motifs of the SDR superfamily as mentioned in Fig. 1. Refer to Materials and methods for GenBank accession numbers.

visualized by BAS 5000 bio-image analyzer (Fuji, Tokyo, Japan). The signal densities were quantified using Fuji-MacBAS software (V1.0).

The measurements with different substrates were done with the same lysate for the same enzyme in order to reduce the variation. For each substrate, the same amount of total radioactivity was added to each reaction tube. Conversion of A to T, T to A, E₁ to E₂ and E₂ to E₁ in the presence of NAD(H) or NADP(H) was calculated as a percentage of total radioactivity after extraction. Results are represented as means $\pm$ S.E. of three independent measurements.

Results

Molecular cloning of three type 17 β -HSDs

After three rounds of library screening, nine positive clones of putative tilapia 17 β -HSD1 were recovered. Subsequent sequence and blast analysis proved that they all encoded true tilapia 17 β -HSD1. It had high homology to 17 β -HSD1 of human (46.6%), mouse (46.1%), chicken (51.4%), Japanese eel (67.6%) and fugu

(83.4%) at amino acid level. Based on the analysis of tilapia EST database (Wang, Ijiri and Nagahama unpublished data) and subsequent 5'-RACE, putative tilapia 17 β -HSD8 was obtained, and further analysis with blast revealed high similarity at amino acid level to its counterparts in human (65.6%), mouse (55.9%), zebrafish (79.3%) and fugu (82.1%). Tilapia putative 17 β -HSD12 cDNA was obtained by RT-PCR and RACE. Its deduced amino acid sequences showed high identity with 17 β -HSD12 of human (40.3%), mouse (41.7%), zebrafish (about 41% to both type A and B), fugu (39.5% to type A, 60.3% to type B) and to 17 β -HSD3 of human (38.9%), mouse (37.4%) and zebrafish (34.5%).

The cloned cDNAs of tilapia 17 β -HSD type 1, 8 and 12 were 1504, 1006 and 1930 bp long, encoding proteins of 289, 256 and 314 aminoacids, respectively.

Phylogenetic analysis

17 β -HSDs of the SDR family share several amino acid sequence motifs. These motifs include TGxxxGxG (a part of the Rossmann fold for cofactor binding), NAG

Figure 3 Phylogenetic analysis of three types of 17 β -HSDs of vertebrates, insects and yeast using androgen-regulated short-chain dehydrogenase/reductase (ARSDR) as the outgroup. Numbers are the bootstrap values from 1000 replicates indicating credibility. Branch lengths are proportional to the number of amino acid changes on the branch. Partial sequences (*) may have artificially short branches. Close distance between tilapia and fugu 17 beta-HSD12s might be an artefact due to partial sequence known in fugu. Refer to Materials and methods for GenBank accession numbers.

(a region between cofactor binding and active sites for structural stabilization), YxxSK (active center) and PGxxxT (C-terminal to active site, determination of reaction direction). Outside these conserved regions sequence similarity might be as low as 20% (reviewed in Mindnich *et al.* 2004b).

Alignment of human, chicken and fish 17 β -HSD type 1 and 8 (Fig. 1), and human, frog and fish 17 β -HSD type 12 amino acid sequences (Fig. 2) revealed that the

three types of tilapia 17 β -HSDs possess the conserved motifs, such as TGxxxGIG (Box I), NAG (Box II) and YxxSK (Box III) of the SDR superfamily, except PGxxxT (Box IV). The latter is conserved only in types 1 and 8. Box I (TGCSSGIG) and Box III (YCASK) of tilapia 17 β -HSD1 are completely conserved as in all other vertebrates. This is also true for tilapia 17 β -HSD8 (Box I: TGGGSGIG, Box III: YAASK). However, the two boxes (Box I: TGATSGIG and Box III:

Figure 4 RT-PCR analysis of 17β-HSD1, 17β-HSD8 and 17β-HSD12 from various tissues of adult tilapia. B, brain; P, pituitary; G, gill; H, heart; S, spleen; L, liver; I, intestine; O, ovary; K, kidney; M, muscle; T, testis; 1, 2, and 3 markers; +, positive control; –,negative control. Lower panel is for β-actin as internal control.

Figure 5 Northern blot analysis of poly (A)⁺-RNA (5–8 μg) from various tissues of tilapia by using ³²P-17β-HSD1 (a) and ³²P-17β-HSD8 (b) cDNA fragments. T, testis; O, ovary; Im, male intestine; If, female intestine; Lm, male liver; Lf, female liver; Bm, male brain; Bf, female brain. The lower panel shows the same membrane after stripping and hybridization with the tilapia ³²P-β-actin probe.

Y SATK) in putative tilapia 17β-HSD12 are identical to those of fugu 12B yet showed some sequence differences from conventional 17β-HSD12s cloned from tetrapod and other fish 12As and 12Bs. The structurally conserved residues (Box II) showed some variation in three types of 17β-HSDs. These residues are NAG, xAG and NVG in type 1, type 8 and type 12, respectively. According to the alignment of the amino acid sequences of three types of 17β-HSDs cloned from vertebrates and insects, including sequences retrieved from the fugu genome, a phylogenetic tree was

constructed (Fig. 3). Tilapia 17β-HSD1 and 17β-HSD8 were grouped into the corresponding phylogenetic clades as expected. Interestingly, two 17β-HSD12 genes were found in fugu, tetraodon and zebrafish genomes. Phylogenetic analysis of putative tilapia 17β-HSD12 with those in fugu, tetraodon and zebrafish indicated that these 17β-HSD12s cluster into two distinct clades. The two 17β-HSD12s from tetraodon and zebrafish, as well as one of the two 17β-HSD12s identified from fugu genomes (we named it 12A) were grouped under one clade. This clade was also homologous to the 17β-HSD12s found in other vertebrates including

Figure 6 (a) Enzyme assay of 17β-HSD1 recombinant protein expressed in *E. coli* cells. Conversion of androstenedione (A) to testosterone (T), T to A, estrone (E₁) to estradiol (E₂) and E₂ to E₁ in presence of NAD(H) or NADP(H) was examined by thin layer chromatography (TLC) followed by autoradiography. As cell lysate instead of purified recombinant protein was used in the assay, the influence of native cofactors cannot be ruled out. Standard sample, radioisotope labeled standard substrates and mock, pETBlue 2 vector without insert. (b) Conversion calculated as a percentage of the total radioactivity after extraction. Results are represented as means±s.e. of three independent measurements.

mammals. The other clade included the tilapia and fugu (we named it 12B) 17 β -HSD12s.

Tissue distribution analysis by RT-PCR

Analysis of the tissue distribution pattern by RT-PCR revealed that tilapia 17 β -HSD1 was expressed dominantly in the ovary while putative tilapia 17 β -HSD12 was expressed exclusively in the testis. A weak expression of 17 β -HSD1 was detected in the testis. On the other hand, tilapia 17 β -HSD8 was ubiquitously expressed in tissues such as brain, gill, heart, liver, intestine, gonad, kidney and muscle of both sexes (Fig. 4).

Northern blot

Results of Northern blot analysis of 17 β -HSD1 and 17 β -HSD8 were corroborative to those obtained by RT-PCR. A single band of ~ 1.5 kb 17 β -HSD1 transcript was detected in the ovary of tilapia, whereas no signal was found in testis (Fig. 5a). On the other hand, a 2.0 kb transcript of 17 β -HSD8 was observed in brain, liver, intestine and gonad of both sexes. Additionally, a transcript of ~ 1.0 kb was also detected in the ovary, intestine of female and liver of both sexes (Fig. 5b). However, no signal of putative 17 β -HSD12 was observed in this study when hybridized with mRNAs obtained from the gonads of both sexes, most probably due to the low sensitivity of Northern blot when compared with RT-PCR.

Enzymatic assay by TLC

Enzymatic assay of 17 β -HSD1 was performed using the 17 β -HSD1 recombinant protein expressed in *E. coli*. Tilapia 17 β -HSD1 showed preference for NADP(H) and it could inter-convert E_1 and E_2 efficiently, and also A and T, though less efficiently (Fig. 6a). The percentages of conversion from A to T, T to A, E_1 to E_2 and E_2 to E_1 , calculated as mean of triplicates, were ~ 8.25 , 34.55 , 51.23 and 98.68% , respectively in the presence of NAD(H). However, the corresponding conversion rates were higher in the presence of NADP(H), ~ 39.06 , 39.87 , 70.82 and 98.85% , respectively (Fig. 6b).

Enzymatic assays of 17 β -HSD8 and putative 17 β -HSD12 were performed after expressing their recombinant proteins in HEK 293 cells. 17 β -HSD8 could catalyze the conversion from T to A, as well as the

inter-conversion between E_1 and E_2 in the presence of NAD(H) (Fig. 7a). The percentages of conversion from T to A, E_1 to E_2 and E_2 to E_1 , expressed as the means of three separate experiments, were 37.85 , 12.53 and 24.73% respectively (Fig. 7b).

However, the putative 17 β -HSD12 expressed in *E. coli* cells did not show any conversion to any of the substrates tested in this study. The results were unchanged even after using the HEK 293 cells expressing 17 β -HSD12 recombinant protein.

Discussion

Previous reports showed that 17 β -HSD1s of human, mouse, rat, chicken, zebrafish and the Japanese eel were able to inter-convert E_1 and E_2 with preferential reduction of E_1 than oxidation of E_2 (Poutanen *et al.* 1993, Luu-The *et al.* 1995, Nokelainen *et al.* 1996, Akinola *et al.* 1996, Wajima *et al.* 1999, Kazeto *et al.* 2000, Mindnich *et al.* 2004a). The amino acid residues Ser142, Tyr155, Lys159, His221 and Glu282, form part of the active center of 17 β -HSD1 in humans and interact with the 3-OH of steroids' A-ring (Puranen *et al.* 1997, Huang *et al.* 2001). These residues are highly conserved in tilapia 17 β -HSD1 and therefore, it is reasonable to contemplate that tilapia 17 β -HSD1 catalyzes the conversion between E_1 and E_2 . Our results clearly demonstrate the inter-conversion of E_1 to E_2 . Moreover, tilapia 17 β -HSD1 can also catalyze the conversion between A and T but less efficiently. Both rat and mouse 17 β -HSD1 could catalyze the conversion from A to T as efficiently as E_1 to E_2 (Akinola *et al.* 1996, Nokelainen *et al.* 1996). However, to our knowledge, our report is the first of its kind to demonstrate the catalytic activity of tilapia 17 β -HSD1 to convert T to A. Our data demonstrated that tilapia 17 β -HSD1 is indeed a multifunctional enzyme that might be involved in many different reactions *in vivo* in the steroidogenic pathway depending on its spatial and temporal expression pattern.

Our data also showed that tilapia 17 β -HSD1 had preference to NADPH as cofactor and higher percentages of conversion were detected in reactions in the presence of NADPH (NADP⁺) rather than of NADH (NAD⁺). This is in accordance with the results obtained with human 17 β -HSD1 (Mazza *et al.* 1998, Lin *et al.* 2000). Residues that are critical for cofactor specificity in human 17 β -HSD1 (Leu36 and Ser121) are highly conserved in tilapia.

Figure 7 (a) Enzyme assay of 17 β -HSD8 recombinant protein expressed in HEK293 cells. Conversion of androstenedione (A) to testosterone (T), T to A, estrone (E_1) to estradiol (E_2) and E_2 to E_1 in presence of NAD(H) was examined by thin layer chromatography (TLC) followed by autoradiography. As cell lysate instead of purified recombinant protein was used in the assay, the influence of native cofactors cannot be ruled out. Standard sample, radioisotope labeled standard substrates and mock, pcDNA3.1 vector without insert. (b) Conversion calculated as a percentage of the total radioactivity after extraction. Results are represented as means $\pm$ S.E. of three independent measurements.

Our former study showed that abundant expression of tilapia 17 β -HSD1 was detected from day 0 to day 11 of the tilapia ovarian spawning cycle with the expression being sharply down regulated to undetectable levels at the day of spawning, day 14 (Zhou *et al.* 2003). The expression pattern of 17 β -HSD1 indicates that this enzyme plays an essential role in the process of oocyte growth and vitellogenesis, which is consistent to the putative function attributed for cytochrome P450 aromatase (Yoshiura *et al.* 2003). In teleosts, the production of steroids in ovary follows the classical two cell type model system (Nagahama 2000). E₂ and 17 α , 20 β -dihydroxy-4-pregnen-3-one (17 α ,20 β -DP) are known to be important for the oocyte growth and maturation, respectively (Nagahama 2000). A dramatic shift in the steroidogenic pathway from E₂ to 17 α , 20 β -DP production occurs just prior to the oocyte maturation. Consistently, the expression of two enzymes involved in the synthesis of E₂, 17 β -HSD1 (Zhou *et al.* 2003) and cytochrome P450 aromatase (Yoshiura *et al.* 2003), were greatly decreased. On the other hand, the expression of 20 β -HSD, the enzyme required for the production of 17 α , 20 β -DP was up regulated just before meiotic maturation (Senthilkumaran *et al.* 2002). These results together warrant the pivotal role played by 17 β -HSD1 synergistically with cytochrome P450 aromatase during vitellogenesis. Ontogeny of 17 β -HSD1 revealed its initial expression around 35–50 days after hatching (dah) (data not shown), which is around the initiation of oogenesis, however, late in terms of sex differentiation. Therefore, this enzyme seems unlikely to be involved in the early estrogen or androgen biosynthesis during sex differentiation while its role in gametogenesis seems essential.

Previous reports showed that down regulation of the Ke 6 gene is associated with three different recessive murine models of polycystic kidney disease, ovarian under-development and testicular abnormalities (reviewed in Fomitcheva *et al.* 1998). The enzymatic (17 β -HSD) property of Ke 6 to promote either estradiol synthesis (reduction) or inactivation (oxidation) of estradiol and testosterone could be important in the development of both the kidney and gonads as it may maintain optimal levels of sex steroids within these organs (Fomitcheva *et al.* 1998). In this study, enzyme assay by TLC showed that the recombinant protein of 17 β -HSD8 could catalyze the conversion from T to A, as well as the inter-conversion between E₁ and E₂ in tilapia. Additionally, the expression of 17 β -HSD8 in gonads was found as early as 5 dah (data not shown), before the morphological sex differentiation, by EST sequencing and RT-PCR. Therefore, regulation of the intracellular levels of these steroids by Ke 6 and other 17 β -HSDs might play an important role in the development of tilapia gonads. The ubiquitous expression pattern of 17 β -HSD8, revealed by northern blot and RT-PCR might further indicate its role

in the metabolism and regulation of peripheral steroids. Two transcripts were observed in our northern blot. As we performed northern blot in very high stringency conditions, we believe both of the two transcripts should be specific to the 17 β -HSD8 ORF probe. The 3'-UTR of 17 β -HSD8 was obtained by sequencing of the EST clones, which contained relatively short inserts. After 5'-RACE, the 17 β -HSD8 cDNA we obtained is 1006 bp, which matches the 1 kb Northern blot band in size. The 2 kb band might be an alternatively spliced form of 17 β -HSD8 with a relatively longer 3'-UTR. Ubiquitous distribution of the long form 17 β -HSD8 in the Northern blot is in accordance with the tissue distribution data obtained by RT-PCR. On the other hand, the expression of the short form is restricted to some tissues, such as ovary, female intestine and liver of both sexes, but not expressed in the brain of both sexes and male intestine. The functional significance of differential expression of these two differently spliced forms remains elusive.

Recently, two 17 β -HSD12s were successfully cloned from zebrafish (Mindnich *et al.* 2004a). Through blast search we also retrieved two 17 β -HSD12-like genes in both fugu and tetraodon genomes. The presence of two distinct types of 17 β -HSD12s appears to be unique for fish and it might be due to gene duplication. However, phylogenetic analysis showed that the two 17 β -HSD12s of zebrafish and tetraodon, together with all 17 β -HSD12s from tetrapods were grouped in one clade whereas the putative tilapia 17 β -HSD12 and fugu 17 β -HSD12B were clustered into another clade. Therefore, our data cannot exclude the other possibility that putative tilapia 17 β -HSD12 and fugu 17 β -HSD12B in fact belong to an unidentified type of SDR, which shows high homology to both types 12 and 3 identified from tetraodon, zebrafish and some mammals. Tilapia 17 β -HSD type 12 expressed exclusively in the testis of 50 day-old fish, which is in consistent with the results obtained from mammalian 17 β -HSD type 3 (Baker *et al.* 1997, Mustonen *et al.* 1997). On the contrary, all 17 β -HSD type 12s, from zebrafish to human, were expressed ubiquitously in a wide variety of adult tissues such as brain, gonad, skin, liver and intestine in both sexes. Furthermore, both zebrafish 17 β -HSD12 paralogs are expressed throughout embryogenesis. These data seem to suggest that putative tilapia 17 β -HSD12 is similar to 17 β -HSD3 but different from the conventional 17 β -HSD12 in gene expression pattern. However, unlike mammalian 17 β -HSD3, enzyme assays of the putative tilapia 17 β -HSD12 expressed in both HEK293 cells and *E. coli* cells did not show any conversion from A to T or vice versa in this study. As the expression of putative tilapia 17 β -HSD12 recombinant protein was confirmed by SDS-PAGE and western blot (data not shown), the failure in enzyme assay can only be attributed to its unknown substrate specificity. Now that 17 β -HSD12 cloned from yeast to mammal exhibited a

conserved function in the fatty acid metabolism (Beaudoin *et al.* 2002, Kuervers *et al.* 2003, Moon & Horton 2003), further characterization of putative tilapia 17 β -HSD12 will be necessary to understand the molecular evolution and function of SDR family genes in fish.

In conclusion, three types of 17 β -HSDs were cloned from the Nile tilapia in this study. Phylogenetic analysis, gene expression and enzyme characterization confirmed that the putative 17 β -HSD type 1 and 8 cDNA encode genuine tilapia 17 β -HSD homologues. However, the putative 17 β -HSD12 we cloned might be a paralog of the 17 β -HSD12 duplicates found in fish or an unidentified SDR member in vertebrates. Further characterization of this clone will delineate the molecular evolution and function of 17 β -HSDs in vertebrates.

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