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Two types of aromatase with different encoding genes, tissue distribution and developmental expression in Nile tilapia (*Oreochromis niloticus*)

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Abstract

We isolated a novel type of aromatase cDNA from a Nile tilapia (*Oreochromis niloticus*) ovary cDNA library. Because this aromatase is phylogenetically related to brain aromatase (CYP19b) of goldfish, zebrafish and sea bass, we named it tilapia CYP19b (tCYP19b). *tCYP19b* encodes a protein that is predicted to consist of 495 residues and have 63.8% homology with the aromatase (tCYP19a) we previously isolated from the same source. In vitro transient transfection of cultured COS7 cells demonstrated that tCYP19b codes a functional protein to catalyze estrogen production from an androgen substrate. RT-PCR and Northern hybridization analysis showed that tCYP19b was expressed at a high level in the brain and at a low level in a wide variety of other tissues, whereas tCYP19a was mainly present in the ovary and its level significantly increased during the vitellogenic stage. RT-PCR also detected tCYP19b expression in brain and gonad tissues of both female and male tilapia during sex differentiation, but tCYP19a was only found in the ovary of the fry at that period. These results suggest that tCYP19a plays a key role in sex differentiation and ovarian development. We also isolated genes of two tilapia aromatases. Based on the location of the transcription initiation site, we predicted that there is one promoter for tCYP19a and three promoters for tCYP19b. Although the two aromatase isoforms have similar gene structures in the coding region, we found that the binding regions of SF-1/Ad4 BP region, WT1-KTS and SRY, which are sex-determining factors in mammals, are present in the 5′ flank region of tCYP19a but not tCYP19b. A similar situation is present in promoters of zebrafish and goldfish aromatase isoforms. This data indicates that CYP19a plays a decisive role in sex differentiation of those species. The unique presence of the ERE motif in the tCYP19b promoter and the high expression of tCYP19b in the brain support that CYP19b is mainly involved in estrogen-mediated neural estrogen synthesis.

Keywords: Aromatase; Estrogen; Tilapia; Sex differentiation; Ovary

1. Introduction

Aromatase cytochrome P450 (P450arom; CYP19) is a product of the CYP19 gene and a terminal enzyme in the estrogen biosynthetic pathway that catalyzes the formation of estrogen from androgen. Estrogen is essential for gonad development and other diverse physiological processes, ranging from normal growth to reproductive behavior.

Aromatase was originally cloned from the human placenta in 1988 (Corbin et al., 1988; Harada, 1988; Simpson et al., 1987). Subsequently, aromatases of various animals have been isolated based on the sequence of human placental P450arom. Thus, these aromatases have comparable cDNA sequences. For several years, it was thought that P450arom was encoded by a single

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gene and its transcript underwent alternative splicing in different tissues. However, in 1995, Corbin isolated a functional isoform of aromatase from the porcine ovary that exhibits 87% amino acid homology with the known form cloned from the placenta (Corbin et al., 1995). Shortly thereafter, a third aromatase isoform was isolated by Choi et al. from porcine peri-implantation blastocysts (Choi et al., 1996). These aromatase isoforms showed the different substrate specificity, expression level, enzymatic activity, and mode of regulation, implying that they play diverse role(s) in reproductive events (Choi et al., 1996, 1997a,b; Cloley et al., 1997; Corbin et al., 1995, 1999, 2000; Graddy et al., 2000).

Multiple forms of aromatase were also found in teleost including goldfish, zebrafish and sea bass (Blazquez and Piferrer, 2004; Callard and Tchoudakova, 1997; Chiang et al., 2001a,b; Gelinas et al., 1998; Kishida and Callard, 2001a; Tchoudakova and Callard, 1998; Tong et al., 2001). The intraspecific aromatase genes are located on different chromosomes, and encode enzymatic proteins that have about 60% sequence homology with each other. Studies demonstrated that aromatase isoforms have different tissue distribution, response to exogenous estrogen and expression pattern during gonad ontogeny. The aromatase that is mainly expressed in the ovary has been designated CYP19a/P450aromA/CYP19A1. The type that is expressed at a high level in the brain and is strongly induced by estrogen has been designated CYP19b/P450aromB/CYP19A2.

In 1997, we characterized an aromatase cDNA obtained from ovarian tissue of Nile tilapia (*Oreochromis niloticus*) (Chang et al., 1997). During that study, we also obtained an novel aromatase PCR fragment (Fig. 1) that did not identically match the aromatase cDNA sequence we reported to GenBank (Accession No. U72071), although we performed the RT-PCR with the same primer set and ovarian mRNA template as we did in the published paper. Those results indicate the presence of intraspecific aromatase isoforms in tilapia ovary. Thus, the primary purpose of the present study was to identify this novel aromatase isoform and its gene structure. Because this novel tilapia aromatase is expressed at a high level in brain tissue and is highly homologous to CYP19b of goldfish and zebra fish, we designated it tilapia CYP19b (tCYP19b) or brain-type aromatase. The aromatase we previously cloned from ovary tissue was designated tilapia CYP19a (tCYP19a) or ovary-type aromatase, due to its high level of expression in vitellogenic ovaries.

2. Materials and methods

2.1. Animals and tissues

Adult Nile tilapia (*Oreochromis niloticus*) and their new hatchlings were reared under natural light in laboratory glass aquaria with aerated water at 24 °C. Ovarian tissues (at stage of pre-vitellogenisis, early vitellogenesis, late vitellogenesis, and final maturation) and tissues from the brain, heart, gills, muscle, kidneys, liver, intestine, spleen, blood, and testes were collected and briefly rinsed in ice-cold goldfish Ringer's solution (Kagawa et al., 1984). To study expression of aromatase during sex differentiation, brain tissues were harvested from genetic female (XX) and male (XY) tilapia at 1, 10, 15, 20, 25, and 35 days after hatching. Gonad tissues were collected from fry around the period of the sex differentiation (15, 20, 25, and 35 days after hatching). Genetic females (XX) and males (XY) were obtained by artificial fertilization of eggs from a normal female (XX) with sperms from a sex-reversed psuedomale (XX) and a super male (YY), respectively (Kobayashi et al., 2003). Many studies have indicated that Nile tilapia undergoes the histological sex differentiation at 19–21 days after hatching (Kobayashi et al., 2003; Nakamura and Nagahama, 1985, 1989, 1993, 1996).

2.2. Preparation of total RNA and mRNA

The total RNA of various tilapia tissues was prepared with ISOGENE (Nippongene, Japan) according to the manufacturer's protocol. Poly(A)$^+$ RNA was isolated from the total RNA using an oligotex-dT30 super kit (JSR, Japan).

2.3. Construction of cDNA library

cDNA libraries were constructed with mRNA extracted from mature tilapia ovaries, using a ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold cloning kit (Stratagene) according to the manufacturer's protocol (Chang et al., 1997).

2.4. Construction of a genomic DNA library

Genomic DNA was extracted from tilapia liver using the Wizard Genomic DNA Purification Kit (Promega). After partial digestion with Sau3AI (Takara) and size-fractionation on 1.5% TAE agarose gels, excised DNA fragments greater than 10 kb in length were purified using a Geneclean II DNA Purification kit (BIO101, USA). The DNA fragments were ligated into Xho fill-in Lambda FIX II vectors (Stratagene) and packaged into Gigapack III gold packaging extracts (Stratagene).

2.5. Isolation of tCYP19b isoform from cDNA library

To specifically isolate tilapia tCYP19b cDNA instead of the previously obtained tCYP19a, we designed two oligo-nucleotides based on DNA sequence comparison between tCYP19a (Chang et al., 1997) and the