Molecular biology of channel catfish brain cytochrome P450 aromatase (CYP19A2): cloning, preovulatory induction of gene expression, hormonal gene regulation and analysis of promoter region

Y Kazeto and J M Trant
Center of Marine Biotechnology, University of Maryland Biotechnology Institute, 701 East Pratt Street, Baltimore, MD 21202, USA

(Requests for offprints should be addressed to J M Trant; Email: trant@umbi.umd.edu)

Abstract

Cytochrome P450 aromatase (CYP19) converts androgens to estrogens. Unlike mammals, teleosts have two CYP19 genes, expressed differentially in ovary (CYP19A1) and neuronal tissues (CYP19A2). The primary purpose of this study was to demonstrate the potential involvement of CYP19A2 in the reproductive endocrinology of teleosts. Channel catfish CYP19A2 (ccCYP19A2) cDNAs were isolated from the brain using a PCR-based strategy. The ccCYP19A2 cDNA putatively encodes 500 amino acids which conferred aromatase activity in transfected COS-7 cells. Additionally, an alternatively spliced transcript was isolated which lacks the first 122 amino acids and is catalytically inactive. The brain and the pituitary were predominant sources of ccCYP19A2 transcript and the abundance in both tissues acutely increased prior to spawning. This preovulatory induction of ccCYP19A2 gene in the pituitary is remarkably similar to the pattern of gene expression for luteinizing hormone-β (LHβ). Estradiol-17β (E2) and testosterone enhanced the transcript abundance of ccCYP19A2 and LHβ in catfish pituitary cells cultured in vitro but the stimulatory effects of testosterone were abolished by an aromatase inhibitor, indicating an important role of E2, the product of CYP19A2 activity, in the regulation of CYP19A2 and LHβ. Structural and functional analysis of the 5′-flanking region of the gene suggested that the sequence from –1076 to –435 bp is critical for the basal promoter activity in the pituitary. This report demonstrates that CYP19A2 functions as an important factor in the reproductive endocrinology of teleosts through the brain-pituitary-gonadal axis.

Journal of Molecular Endocrinology (2005) 35, 571–583

Introduction

Estrogens, especially estradiol-17β (E2), function in many physiological processes including early development, sexual differentiation, onset of puberty and reproduction in all vertebrates studied to date. In non-mammalian vertebrates, E2 is involved in numerous reproductive events, for example hepatic vitellogenin (the precursor of yolk protein) synthesis (Wallace 1985) in the liver, gametogenesis (Billard 1992) and gonadal sex determination (Wibbels et al. 1991). Therefore, the precise regulation of E2 biosynthesis, controlled primarily through the expression of genes encoding steroidogenic enzymes, is apparently required for reproductive success. Cytochrome P450 aromatase (CYP19) is the terminal steroidogenic enzyme in the biosynthetic pathway of E2 and is responsible for the conversion of androgens into estrogens (Simpson et al. 1994). Even though the major sources of circulating E2 in mammals are ovary and placenta, in which CYP19 gene is highly expressed, it has been well documented that some peripheral (classical non-steroidogenic) tissues, such as adipose tissue, bone and brain, express this enzyme and that the locally synthesized E2 functions in a paracrine or autocrine manner (Simpson et al. 1994).

Originally described in humans (Simpson et al. 1997), the genomes of all mammals contain a single CYP19 gene, except for the pig in which three distinct genes encode three forms of CYP19 (Graddy et al. 2000). Tissue-specific expression is controlled by the use of a variety of tissue-specific promoters associated with alternatively spliced 5′-untranslated exons. In contrast there is increasing evidence that most teleosts (bony fish) possess two CYP19 genes: CYP19A1, which is primarily expressed in the gonads, and CYP19A2 which is mainly expressed in the brain. (Note that through discussions with Daniel Nebert (University of Cincinnati Medical Center, Cincinnati, OH, USA) and David Nelson (University of Tennessee, Memphis, TN, USA), the accepted rules of nomenclature for the superfamily of cytochromes P450 dictates these designations, but they should apply to the duplicated genes of the teleosts only. This nomenclature should be substituted for the previously published designations of CYP19a and CYP19b.) These gene products have been...
identified in a widely diverse list of teleost species, including the goldfish (Tchoudakov & Callard 1998), zebrafish (Kishida & Callard 2001, Trant et al. 2001), rainbow trout (Valle et al. 2002), tilapia (Kwon et al. 2001) and European sea bass (Blazquez & Pfiferrer 2004). Phylogenetic analyses of CYP19s isolated to date have clearly segregated the fish CYP19A1s, CYP19A2s and CYP19s from other vertebrate species into three distinct clusters (Blazquez & Pfiferrer 2004). It is evident that the CYP19 genes arose through gene duplication in goldfish (Tchoudakov & Callard 1998) and zebrafish (Kishida & Callard 2001, Trant et al. 2001) and have since diverged in function and primary structure. These CYP19 isoforms share approximately 60% amino acid sequence identity and map to different chromosomes (Trant et al. 2001).

The brain of adult teleosts has relatively much higher aromatase activity (100–1000 times higher) than in adult mammals (Pasmank & Callard 1985). As suggested by the reports that neurally derived E₂ regulates neuronal differentiation, survival and function in mammals (Toran-Allerand 1996), this high level of CYP19A2 gene expression is thought to be related to the exceptional capability of the teleost brain to grow indefinitely, its neuroplasticity and its neuronal regeneration capability during adulthood (Fordano et al. 2001, Menuet et al. 2003). This hypothesis is contradicted by the fact that the rapidly growing brain of embryo/juvenile zebrafish expresses CYP19A2 poorly relative to the adult (Tong et al. 2001). Furthermore, this developmental pattern is quite different from mammals where brain aromatase activity is highest during prenatal organization and development and declines in adulthood (Lephart 1996). It has also been suggested that the expression of brain aromatase influences the brain-pituitary-gonadal axis of reproductive physiology, especially with respect to steroid feedback onto gonadotropin and/or gonadotropin-releasing hormone (GnRH) secretion (MacLusky & Naftolin 1981, Khan et al. 1999); however, little attention has been received to address this hypothesis. In addition to the brain, the teleost pituitary highly expresses the CYP19A2 gene (Gelinas et al. 1998, Menuet et al. 2003, Goto-Kazeto et al. 2004), whereas the mammalian pituitary is generally considered aromatase-negative although the rat pituitary is known to express this enzyme (Callard et al. 1983, Carretero et al. 1999). This suggests that CYP19A2 gene may be important for the function of the pituitary gland in teleosts.

The channel catfish, *Ictalurus punctatus*, was selected as a model teleost species to examine the association of the CYP19A2 gene with reproduction because many components of its reproductive endocrinology are well coordinated with gonadal development, which is typical for seasonal breeders with synchronous oocyte development. The channel catfish is a teleost model species in which the reproductive physiology (ovarian steroidogenesis, the expression of four steroidogenic enzymes, both gonadotropins and their receptors, etc.) has been well studied at the molecular level (Kumar et al. 2000, 2001a, 2001b, Kumar & Trant 2004). In this study, we characterized the channel catfish cDNAs encoding CYP19A2, the tissue distribution of expression and gene expression in the brain and the pituitary during a reproductive cycle, and examined its mode of gene regulation.

**Materials and methods**

**Animal and tissue collection**

Mature but inexperienced adult female channel catfish were directly captured from ponds of a local fish farm (Bowling Catfish Farms, Charles City, MD, USA). Up to six animals were collected at 4-week intervals for 15 months (Kumar et al. 2000). Few or no fish were available for the months from December through February because most catfish at this latitude are dormant in winter. Tissues were dissected in the field and immediately flash-frozen in liquid nitrogen. Pituitaries used for *in vitro* culture experiments were kept in ice-cold L-15 medium until processed. All frozen tissues were stored at −80°C prior to analysis.

**Isolation and sequence of channel catfish CYP19A2 cDNA**

Total RNA was extracted from the whole brain of a catfish with post-vitellogenic ovaries using Trizol reagent (Life Technologies, Gaithersburg, MD, USA) and subsequently enriched for poly(A⁺) RNA with Straight A’s mRNA Isolation System (Novagen, Madison, WI, USA). Brain cDNA was synthesized using PowerScript (BD Bioscience, Palo Alto, CA, USA) after being primed with a clamped oligo(dT) primer. PCR was carried out using a set of degenerate primers: 5'-CAGTG(T)GA(T)AG(GT)(GA)TAGA(G)(CA)ATGG(GT)(GA)AG(T)AGCAG(T)AGGTGT-3' (5' primer; P1) and 5'-TTC(GA)TCATC(AG)CC(AG)T(AG)A(CA)(ATG)A-3' (3' primer; P2). The PCR was performed at 94°C for 30 s, 50°C for 30 s and 72°C for 30 s for 40 cycles. The resultant PCR products were subjected to 1·2% agarose gel electrophoresis. A single amplicon was detected and inserted into a T/A cloning vector (pDrive; Qiagen, Valencia, CA, USA) and sequenced using an ABI 373 DNA sequencer (Perkin Elmer, Foster City, CA, USA).

Based on the sequence information of the presumed channel catfish CYP19A2 (ccCYP19A2) cDNA fragment, rapid amplification of cDNA ends (RACE) reactions were performed to generate the 5'- and 3'-termini using a SMART RACE cDNA Amplification kit (BD Biosience) according to the manufacturer’s instructions. Gene-specific primers used in the 5'- and 3'-RACE reactions were 5'-CCCCACACAGGCACGT

---

Journal of Molecular Endocrinology (2005) 35, 571–583

www.endocrinology-journals.org

Downloaded from Bioscientifica.com at 04/19/2022 05:57:54AM via free access
GGAGCCGGCTGAACT-3' (P3) and 5'-TGGACGCGAGGT GGAGCCGGCTGAACT-3' (P4), respectively. The RACE amplicons were subcloned and sequenced as described above. Finally, the complete open reading frames (ORFs) of ccCYP19A2 were generated by PCR with 5'-GTCAATCTTACGGAGTTTACGG-3' (P5) and 5'-GGCTGTATTATTGCTTGTCTGGGTG-3' (P6) primers using a proofreading Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA). Two resultant specific amplicons, designated the long and short forms of ccCYP19A2 (ccCYP19A2 and ccCYP19A2s; see the Results section), were generated and cloned into pBluescript (Stratagene), and three clones for each form were bi-directionally sequenced. As the multiple lengths of amplicons were seen when 5'-RACE PCR was conducted, the 5'-terminus regions of 15 additional clones were sequenced.

Analysis of 5'-terminus of the ccCYP19A2 gene

Catfish genomic DNA was analyzed by PCR to determine the structural relationship between multiple 5'-untranslated regions (5'-UTRs; see the Results section) and the ccCYP19A2 gene. Genomic DNA was extracted from catfish brain using a Blood & Cell Culture DNA Starter Kit (Qiagen) and was amplified by PCR with 5'-CCAGATTCACCAGAGGCTGTA GAGG-3' and 5'-GTACCCTTACCTGTCCAGACC AACAA-3' primers. The intron/exon boundary sites were determined by comparing the genomic sequence with the cDNA sequence obtained by 5'-RACE.

Transient expression of ccCYP19A2 in COS-7 cells

The cDNAs containing the entire ORFs of ccCYP19A2 and ccCYP19A2s were ligated into a eukaryotic expression vector, pSI (Promega, Madison, WI, USA) and designated ccCYP19A2/pSI and ccCYP19A2s/pSI. COS-7 cells (ATCC, Manassas, VA, USA) were maintained following the ATCC's recommendations and dispensed into six-well tissue culture plates. Cells were cotransfected with a vector construct possessing the firefly luciferase gene downstream of a simian virus 40 (SV40) promoter and either ccCYP19A2/pSI, ccCYP19A2s/pSI or empty pSI using FuGENE 6 Transfection Reagent (Roche Diagnostic Corp, Indianapolis, IN, USA) according to the manufacturer's protocol. COS-7 cells transfected with the DNA constructs were incubated in complete growth medium (Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum (FBS)) for 36 h and then incubated with androstenedione (30 ng/ml; Sigma, St Louis, MO, USA) in serum-free medium (DMEM, 200 µg/ml BSA) for 3 or 10 h. After the incubation, media were collected and the estrone titer was measured by radioimmunoassay using a commercially available estrone RIA kit (Diagnostic System Laboratory, Webster, TX, USA). Additionally, luciferase activity was determined in the cells by a Luciferase Assay kit (Promega) and MicroLumat LB98P luminometer (EG&G Berthold, Stammwerk Wilbad, Germany) to verify constant transfection efficiencies in each experimental group. Three replicates of each experiment were carried out.

Primary culture of catfish pituitary cells

Channel catfish pituitary cells were maintained in primary cultured as described elsewhere (Rebers et al. 2000), with some modifications. In brief, pituitary glands were minced with a scalpel and treated with 0-1% type I collagenase (Sigma) in Ca2+- and Mg2+-free PBS for 2 h at room temperature with gentle shaking. Tissue fragments were allowed to settle for a few minutes and the supernatant was transferred into a new tube and centrifuged for 10 min at 200 g. The resultant cells were washed with PBS twice and resuspended in HEPES (10 mM)-buffered L-15 medium containing 10% charcoal-stripped FBS (Atlanta Biologicals, Norcross, GA, USA) and a 1% penicillin/streptomycin mixture (Life Technologies). Cells were plated at a density of 100 000 cells/well in 48-well tissue-culture plates and incubated for 48 h at 25˚C in medium supplemented with E2, testosterone (0.03–30 nM) or a combination of testosterone (0, 3 and 30 nM) and the aromatase inhibitor 1,4,6-androstatriene-3,17-dione (ATD; 10 µM). After incubation, cells were harvested, flash-frozen and stored at −80°C.

Isolation and characterization of the 5'-flanking region of ccCYP19A2

A catfish GenomeWalker library was constructed with a Universal GenomeWalker Kit (Kazeto et al. 2001; BD Bioscience) and used in a PCR-based strategy to isolate the promoter region of the ccCYP19A2 gene. Genome-walking PCRs using Advantage-GC genomic polymerase (BD Bioscience) were primed with 5'-CCCCC TACGCTCCATTACGAGCATGACATC-3' and then followed with a nested second amplification using a 5'-TACGCTCCATTACGAGCATGACATC-3' primer. The resulting single amplicon was T/A cloned and sequenced. Promoter analysis of the 5'-flanking region of the ccCYP19A2 gene was conducted at http://motif.genome.ad.jp/ with an 80% cut-off score. Additionally, consensus core sequences for transcription factor-binding sites of interest, such as cAMP-responsive element (CRE; TKACGTMA), estrogen-responsive element (ERE; AGGTCAANNTGACCT), glucocorticoid-/progestin-/androgen-responsive element (ARE; AGAAC ANNTGTCTTCT) and the responsive element for aryl hydrocarbon receptor and its nuclear transfer protein (AhR/Arnt; TYGCGTG) were manually searched using DNASIS-Mac v3.5 software.
Y Kazeto and J M Trant · Molecular biology of CYP19A2 in catfish

The membrane spanning region

The heme-binding region

The aromatic region

The nuclear localization signal

The basic region

The phosphatidylinositol binding region

The C-terminal domain

The proline-rich region

The bipartite nuclear localization signal

The pentapeptide repeats

The basic domain

The acidic domain
An amplicon spanning the promoter region and a portion of the ORF of ccCYP19A2 was generated by PCR using 5'-CATTTGAGGCTTGCTTGAA-3' and 5'-ATCCCCCTACCCTCCATTACAGCCATG-3' primers and Pfu Turbo DNA polymerase. The amplicon was T/A-subcloned and its sequence was confirmed. The DNA product was inserted upstream of a luciferase reporter gene in pGL3-Basic vector (Promega), a promoter-less vector. Several luciferase reporter constructs were generated in a similar manner using DNA products with progressively longer deletions of the upstream portion of the 5'-flanking region. In all constructs, orientation of inserts was verified by direct sequence analysis.

Aromatase-expressing rat pituitary cells, GH4C1 cells (ATCC), were maintained and grown in DMEM containing 17.5% FBS and antibiotics (penicillin and streptomycin) in 24-well tissue-culture plates. Cells were cotransfected with pRL-Null plasmid (a Renilla luciferase reporter plasmid; Promega) and either one of the ccCYP19A2-promoter–luciferase-reporter constructs or an empty pGL3-Basic vector using FuGENE 6 Transfection Reagent (Roche Diagnostic Corp). After transfection, the medium was replaced with new growth medium supplemented with charcoal-stripped FBS and the cells were incubated for 48 h. After incubation, medium was removed and the cellular enzymatic activity in each well. Three replicates of each transfection were carried out.

Measurement of transcript abundance by real-time quantitative RT-PCR (rtqPCR)

Total RNA (1 µg) was extracted from whole brains that were collected from pond-raised fish throughout the year and from a variety of other tissues (listed in the Results section), and reverse-transcribed using Moloney murine leukemia virus reverse transcriptase (Life Technologies) primed with a clamped oligo(dT) primer. Due to the expected small amount of total RNA isolated from cultured catfish pituitary cells, total RNA was co-puriﬁcation with 4 µl polyacryl carrier (Molecular Research Center, Cincinnati, OH, USA). The preparation of cDNA from pituitaries collected throughout the reproductive cycle has been described by Kumar & Trant (2004) (Kazeto et al. 2003; 2005). The transcript abundance of the functional form of ccCYP19A2 was measured using a ﬂuorescence-based rtqRT-PCR method and normalized to the abundance of β-actin (GenBank accession no. AY555575). The rtqRT-PCR assays for ccCYP19A2 and catﬁsh β-actin have been described elsewhere (Kazeto et al. 2003). The primer/probe set for ccCYP19A2 was 5'-TGCACTGAC TCGGTATTGA-3' (forward), 5'-GGGAACATAGT TACTGCTCTTCAAGAC-3' (reverse) and FAM-5'-CACCCATCTACTGAGTGCAGC-3'-TAMRA (probe). The forward primer is located on the deleted portion of ccCYP19A2s, thus only the transcripts that give rise to the longer (functional) ccCYP19A2 are detected by this rtqRT-PCR assay. The primer/probe set for catfish β-actin was 5'-CAGCAATGAGAGGTT-3' (forward), 5'-TGGAGTTGA AAGGTGGTCTCATG-3' (reverse) and FAM-5'-CCA GCTATCCCTCTGGGATGCTGCTGATC-3'-TAMRA (probe). The transcript abundance for the β-subunit of lutinizing hormone (LHβ) was also measured by rtqRT-PCR in pituitary cells (Kumar & Trant 2004). The detailed procedures for designing and synthesizing the primer/probe sets, and to validate the rtqRT-PCR assay, have been fully described elsewhere (Trant et al. 2001).

Statistics

Fold changes in the transcript abundances were subjected to one-way analysis of variance (ANOVA) followed by Fisher’s protected least-significant difference (PLSD) post hoc test. Differences were considered significant at P<0.05.

Results

Isolation of the cDNAs encoding ccCYP19A2

PCR-based isolation of cDNAs encoding ccCYP19A2 (GenBank accession no. AF417239) containing the entire ORF were completed with the aid of 5'- and 3'-RACE PCRs. The nucleotide sequences of ccCYP19A2 cDNA with the 5'-UTR type 2 (see below) and the deduced amino acid sequence are illustrated in

Figure 1 The nucleotide and deduced amino acid sequences of the ccCYP19A2 cDNA. The target sequences of the primers used in the cloning strategy are shown in boxes in the nucleotide sequence with exception of those of the degenerate primers (P1 and P2) that are shown within the amino acid sequence. The amino acid sequences corresponding to putative structural regions (membrane-spanning region, I-helix, aromatic region and heme-binding region) are designated by an overline. Polyadenylation consensus sequences in the 3'-UTR are underlined. The nucleotide and the corresponding amino acid sequences of the region deleted by the alternative splicing is boxed. The arrowheads mark the identified (first and second sites) or putative intron/exon boundary sites. The asterisks denote the potential translation start sites for the long and short forms of ccCYP19A2. The open reading frames of the forms are composed of 1500 and 1131 bp, respectively.
Fig. 1. The length of the nucleotide sequence of ccCYP19A2 was 2533 bp and the lengths of the 5'-UTR, ORF (designated ccCYP19A2) and 3'-UTR were 294 bp, 1500 bp encoding 500 amino acid residues and 739 bp, respectively. A shorter form of ccCYP19A2 (designated ccCYP19A2s) was also isolated in which the region (bp 45–289) downstream of the translation start codon (ATG) was alternatively spliced out. The deleted nucleotide sequence encodes a portion of exon 4 and the entire exon 5. Furthermore, this alternative splicing resulted in a frame shift; thus the nucleotide sequences upstream of exon 5 would not be translated appropriately. However, there is a translation start codon 81 bp downstream of the deleted portion and therefore an ORF of 1131 bp (potentially encoding a 378-amino acid protein) was identified. The 3'-UTR contained multiple consensus polyadenylation signals, one AATAAA and three ATTAAA, located 12, 175, 487 and 535 nucleotides upstream from the poly(A) tail.

The amino acid sequence of ccCYP19A2 shared 62% identity with the ovarian form of the catfish P450 aromatase (CYP19A1; Trant 1994). When compared with other fish CYP19A2 forms (Tchoudakova & Callard 1998, Kishida & Callard 2001, Kwon et al. 2001, Valle et al. 2002), ccCYP19A2 was 67–72% identical. Moreover, three putative functional domains identified in mammalian CYP19 forms, the I-helix, aromatase-specific conserved region and the heme-binding region, were significantly more identical (97, 83 and 100% respectively) than the rest of the predicted CYP19A2 protein from zebrafish.

Identification and characterization of multiple 5'-UTRs of ccCYP19A2
Sequence analysis of 5'-terminal regions of 15 clones generated by 5'-RACE PCRs segregated the 5'-UTR into three groups. In order to examine the structural relationship between the 5'-UTRs and the gene, an upstream primer was designed using the sequence information of these clones and was used for PCRs along with a downstream primer located in the ORF. A schematic illustration of the structural relationship between the 5′-UTR and the corresponding region of the gene is shown in Fig. 2 (see Fig. 6, below, for the specific sequence information). The 5'-UTR type 1 is the least complex form: the UTR is 390 bp in length and is composed of a portion of intron 2 (69 bp), exon 3 (171 bp), intron 3 (96 bp) and a portion of exon 4 (54 bp) with the remaining 136 bp of exon 4 serving as the beginning of the translatable region. The 5′-UTR type 2 is the spliced form of the type 1: intron 3 is completely spliced out and the size of the UTR is 294 bp in length. Interestingly, the third form of the 5′-UTR designated as type 3 spans 4 exons. The first, second and third exons of the ccCYP19A2 gene comprise 96, 33 and 171 bp, respectively, of 5′-UTR type 3. The

Enzymatic activity of ccCYP19A2 CDNASs expressed in COS-7 cells
Recombinant ccCYP19A2 expressed in COS-7 cells catalyzed the conversion of androstenedione into estrone. Approximately 15% of the androstenedione (30 ng/ml) was converted into estrone (4·42 ± 0·29 ng/ml) in 3 h of incubation and over 30% (10·58 ± 0·67 ng/ml) in 10 h. In contrast, no activity was detected in COS-7 cells expressing ccCYP19A2s. Mock control groups did not exhibit any aromatase activity. Transfection efficiency in all experimental groups was confirmed to be consistent by determining firefly luciferase activity in the transfected cells.

Tissue-specific expression of the ccCYP19A2 gene
Tissue-specific distribution of ccCYP19A2 transcript (the one that encodes the functional enzyme) was determined by rtqRT-PCR (Fig. 3). The transcript for ccCYP19A2 was expressed predominantly in parts of the brain (the telencephalon and hypothalamus), pituitary and area surrounding the ventral aorta. In fish, this is the tissue that includes the thyroid follicles in fish. The transcript was also detected at lower levels in other parts of the brain: cerebellum, diencephalons (without the hypothalamus), pituitary and hindbrain. Expression in the intestine, liver and gonads was barely detectable.

Seasonal expression of the ccCYP19A2 gene in brain and pituitary
Detailed descriptions of the gonadosomatic index of the fish and water temperature have been reported
The abundance of the truncated form of cc (early to mid-July). Using traditional RT-PCR analysis around the end of June, prior to the time of spawning. The values rapidly increased from April and peaked and gradually increased from October to mid-spring. The somatic index showed the lowest value in the summer (cc of the active form, cc) of the reproductive cycle whereas the transcript abundance of cc protein, only the long transcript of cc expression and the lack of activity of the encoded shown). Owing to the lack of a dynamic pattern of gene regulation and involvement of ccCYP19A2 in LHβ gene expression in primary cultured pituitary cells

Both E₂ and testosterone (down to 0.3 nM) significantly increased the transcript abundance of both ccCYP19A2 and LHβ genes (Fig. 5). To determine whether testosterone itself modulated the gene expression of ccCYP19A2 and LHβ, cells were incubated with both testosterone and an aromatase inhibitor, ATD. ATD blocked the stimulatory effects of testosterone on ccCYP19A2 and LHβ transcription (Fig. 5).

Structure of the 5′-flanking region of the ccCYP19A2 gene

The 5′-terminus and the 5′-flanking region of the ccCYP19A2 gene (1648 bp; GenBank accession no. AY780360) were amplified by a GenomeWalker PCR using specific primers located in exon 4 of the gene (Fig. 6). The full length of the 5′-UTRs were putatively determined by sequence analysis of 5′-RACE amplicons. A TATA box was identified within 30 bp of the end of the 5′-UTR type 1 and 2 whereas there is no TATA-like consensus sequence in the 5′-flanking area of the 5′-UTR type 3. The 5′-flanking region of the ccCYP19A2 gene contained the consensus sequences of a palindromic ERE, a former half ERE and three half AREs.

Functional analysis of the 5′-flanking region of the ccCYP19A2 gene

The transcriptional activity of the promoter of the ccCYP19A2 gene was determined in a rat P450 aromatase-expressing cell line (GH4C1 cells) transfected with several deletion mutants of the 5′-flanking region of the gene fused upstream to the luciferase reporter gene in the pGL3-Basic vector. Transient transfection of the deletion constructs of the 5′-flanking region into GH4C1 cells resulted in substantial increases, up to 7-fold, in luciferase activity compared with a promoter-less cells.

The 5′-terminus and the 5′-flanking region of the ccCYP19A2 gene (1648 bp; GenBank accession no. AY780360) were amplified by a GenomeWalker PCR using specific primers located in exon 4 of the gene (Fig. 6). The full length of the 5′-UTRs were putatively determined by sequence analysis of 5′-RACE amplicons. A TATA box was identified within 30 bp of the end of the 5′-UTR type 1 and 2 whereas there is no TATA-like consensus sequence in the 5′-flanking area of the 5′-UTR type 3. The 5′-flanking region of the ccCYP19A2 gene contained the consensus sequences of a palindromic ERE, a former half ERE and three half AREs.

The transcriptional activity of the promoter of the ccCYP19A2 gene was determined in a rat P450 aromatase-expressing cell line (GH4C1 cells) transfected with several deletion mutants of the 5′-flanking region of the gene fused upstream to the luciferase reporter gene in the pGL3-Basic vector. Transient transfection of the deletion constructs of the 5′-flanking region into GH4C1 cells resulted in substantial increases, up to 7-fold, in luciferase activity compared with a promoter-less pGL3-Basic vector. The highest transcriptional activity was observed in the reporter construct containing the −1214/+442, −716/+442 and −687/+442 bp regions (Fig. 7). The transcriptional activity partially decreased when the 5′-flanking region was deleted up to the −116 bp position. Further deletion up to the −46 bp position resulted in a complete loss of promoter activity.

In vitro gene regulation and involvement of ccCYP19A2 in LHβ gene expression in primary cultured pituitary cells

Both E₂ and testosterone (down to 0.3 nM) significantly increased the transcript abundance of both ccCYP19A2 and LHβ genes (Fig. 5). To determine whether testosterone itself modulated the gene expression of ccCYP19A2 and LHβ, cells were incubated with both testosterone and an aromatase inhibitor, ATD. ATD blocked the stimulatory effects of testosterone on ccCYP19A2 and LHβ transcription (Fig. 5).

Structure of the 5′-flanking region of the ccCYP19A2 gene

The 5′-terminus and the 5′-flanking region of the ccCYP19A2 gene (1648 bp; GenBank accession no. AY780360) were amplified by a GenomeWalker PCR using specific primers located in exon 4 of the gene (Fig. 6). The full length of the 5′-UTRs were putatively determined by sequence analysis of 5′-RACE amplicons. A TATA box was identified within 30 bp of the end of the 5′-UTR type 1 and 2 whereas there is no TATA-like consensus sequence in the 5′-flanking area of the 5′-UTR type 3. The 5′-flanking region of the ccCYP19A2 gene contained the consensus sequences of a palindromic ERE, a former half ERE and three half AREs.

Functional analysis of the 5′-flanking region of the ccCYP19A2 gene

The transcriptional activity of the promoter of the ccCYP19A2 gene was determined in a rat P450 aromatase-expressing cell line (GH4C1 cells) transfected with several deletion mutants of the 5′-flanking region of the gene fused upstream to the luciferase reporter gene in the pGL3-Basic vector. Transient transfection of the deletion constructs of the 5′-flanking region into GH4C1 cells resulted in substantial increases, up to 7-fold, in luciferase activity compared with a promoter-less pGL3-Basic vector. The highest transcriptional activity was observed in the reporter construct containing the −1214/+442, −716/+442 and −687/+442 bp regions (Fig. 7). The transcriptional activity partially decreased when the 5′-flanking region was deleted up to the −116 bp position. Further deletion up to the −46 bp position resulted in a complete loss of promoter activity.
Discussion

Due to recent intensive cloning studies of CYP19A2 cDNAs from a variety of teleost species (Tchoudakova & Callard 1998, Kishida & Callard 2001, Kwon et al. 2001, Valle et al. 2002, Blazquez et al. 2004) there is increasing evidence that the teleost genome contains at least two CYP19 loci, CYP19A1 and CYP19A2, encoding two distinct P450 aromatases that are expressed differentially in ovary and neural tissues. In contrast, it has been well demonstrated in almost all non-teleost vertebrates, with the notable exception of the pig (Graddy et al. 2000), that the CYP19 gene is present as a single-copy gene in the genome (Simpson et al. 1997). However, until this study there were no reports describing CYP19A2 in Siluroidei fish (the catfishes, which are a richly studied and intensively cultured taxon of teleost species), although the isolation and characterization of CYP19A1 has been fully described in channel catfish (Trant 1994).

In the present study, the cDNAs encoding the second form of channel catfish P450 aromatase, ccCYP19A2, were cloned from the brain and the pituitary glands during a complete reproductive cycle. Abundance of ccCYP19A2 transcript was determined by rtqRT-PCR analysis. The data were normalized to β-actin transcript abundance and expressed as a fold change relative to the mean of November 1996 for the brain and to the mean of October 1997 for the pituitary, which represent the lowest values calculated from a significant number of samples. The results represent means±S.E.M. The numbers in parentheses below the month labels are sample sizes. Different letters indicate statistical significant differences between means (P<0.05).

Figure 4 Seasonal fluctuations in transcript abundance of ccCYP19A2 in the catfish brain and the pituitary glands during a complete reproductive cycle. Abundance of ccCYP19A2 transcript was determined by rtqRT-PCR analysis. The data were normalized to β-actin transcript abundance and expressed as a fold change relative to the mean of November 1996 for the brain and to the mean of October 1997 for the pituitary, which represent the lowest values calculated from a significant number of samples. The results represent means±S.E.M. The numbers in parentheses below the month labels are sample sizes. Different letters indicate statistical significant differences between means (P<0.05).

Figure 5 Hormonal regulation of the gene expression of ccCYP19A2 and LHβ in catfish pituitary cells. Catfish pituitary cells were primary cultured in the presence of either E₂, testosterone (T) or a combination of testosterone and the aromatase inhibitor ATD (10 µM). The quantification of the transcript abundance of ccCYP19A2 and LHβ genes were determined by rtqRT-PCR analysis and normalized to the abundance of β-actin transcript. The values are expressed as a fold change in abundance relative to the means in the control group.

CIP19 loci, CYP19A1 and CYP19A2, encoding two distinct P450 aromatases that are expressed differentially in ovary and neural tissues. In contrast, it has been well demonstrated in almost all non-teleost vertebrates, with the notable exception of the pig (Graddy et al. 2000), that the CYP19 gene is present as a single-copy gene in the genome (Simpson et al. 1997). However, until this study there were no reports describing CYP19A2 in Siluroidei fish (the catfishes, which are a richly studied and intensively cultured taxon of teleost species), although the isolation and characterization of CYP19A1 has been fully described in channel catfish (Trant 1994).
Figure 6 Nucleotide sequence and structural features of the 5'-terminus and 5'-flanking regions of the ccCYP19A2 gene. Sequences of putative cis-acting elements of interest are boxed and labeled. Transcribed sequences in the three types of the 5'-UTRs are underlined. Sequences of introns 1–3 are indicated in lower case. All numbering is relative to the putative transcription initiation site for the 5'-UTR types 1 and 2, designated −1.
The remaining nucleotide sequences are the same in the two forms, and therefore these forms appear to be derived from alternative splicing of the same gene. Two forms of CYP19A2, named form I and II, were also found in rainbow trout brain and form II lacks the first 32 amino acids of form I although it remains unknown whether both of these forms are derived from the same gene or if the truncated form is catalytically active (Valle et al. 2002). Like catfish, an alternative aromatase transcript lacking the initial coding exons 2 and 3 (corresponding to exons 4 and 5 in the ccCYP19A2 gene) has been identified in mammalian brain (Kato et al. 1997, Roselli & Resko 2001). The truncated form of the recombinant human aromatase was not functional using an ‘in cell’ assay system although the truncated enzyme was active when reconstituted with its redox partner, NADPH-cytochrome P450 reductase (Kao et al. 1999). It was not determined whether the activity of the truncated form of the catfish aromatase can be resurrected in a reconstituted system. More importantly, the significance (if any) of these alternative transcripts or the potential resultant proteins is unknown. It is interesting that the alternative splicing of CYP19 genes in the brain has been maintained in the vertebrates despite the evolutionary distance of the genes.

Three types of 5’-UTR, types 1, 2 and 3, were identified and characterized by comparing those sequences with the structure of the corresponding genomic region. Type 1 is an unprocessed 5’-UTR showing the same structure as the corresponding region of the gene whereas the 5’-UTR type 2 is identical to type 1 except intron 3 (96 bp) is spliced out. In zebrafish (Kazeto et al. 2001) and goldfish (Tchoudakova et al. 2001) the 5’-terminus of the CYP19A2 gene possesses an untranslated exon 1 and an intron (intron 1) and only the transcript without intron 1, namely the spliced form of the 5’-UTR, has been identified. Furthermore, similar structural features have been demonstrated in the brain transcript of CYP19 in human (Honda et al. 1994) and the Atlantic stingray (Ijiri et al. 2000), in which there is a single CYP19 gene in the genome. Therefore, the 5’-UTR type 2 of ccCYP19A2 appears to be a typical form of the 5’-UTR of the brain-specific CYP19 transcript. In contrast, three untranslated exons were identified in the 5’-UTR type 3 of ccCYP19A2, which is the first time this has been demonstrated in any vertebrate. These structural differences of the 5’-UTR may affect the translatability of the transcript and/or the transcriptional activity of each type of transcript driven by the different 5’-flanking region of the gene.

Tissue-specific gene expression of the functional transcript of ccCYP19A2 was examined by rtqRT-PCR analysis. The brain and the pituitary were the major sources of the transcript as demonstrated in other species of teleost (Gelinas et al. 1998, Menuet et al. 2003, Goto-Kazeto et al. 2004) with the telencephalon and hypothalamus expressing the CYP19A2 gene most dominantly. In situ hybridization analyses illustrated high expression of the CYP19A2 gene in the ventral region of the telencephalon and hypothalamus in zebrafish (Goto-Kazeto et al. 2004), rainbow trout (Menuet et al. 2003) and plainfin midshipman (Forlano et al. 2001). Therefore this regional expression pattern of CYP19A2 in the brain appears to be common in teleost fish.

The tissues surrounding the ventral aorta (the site for thyroid follicles in bony fish) was also identified as a novel site for CYP19A2 expression in catfish. The high level of gene expression in the ventral aorta region was confirmed in two additional female catfish by rtqRT-PCR analysis and further by a conventional RT-PCR reaction using a different set of primers (data not shown). CYP19 expression was demonstrated in the thyroid gland of humans (Dalla Valle et al. 1998). It has been established in mammals that E2 enhances the expression of the tgb gene that controls the synthesis of thyroglobulin (del Senno et al. 1989) and modulates the proliferation and function of thyrocytes (Banu et al. 2002, Filipovic et al. 2003). Although not studied, E2 may play a role in thyroid function in teleost and ccCYP19A2 may contribute significantly to the regional E2 concentration within the area of the thyroid follicles, especially during periods when systemic E2 titers are low. Thyroid follicles would be a possible source for the ccCYP19A2 transcript; however, this region is comprised of multiple tissue types, including blood vessels, nerves,
connective tissues and potentially the thymus. The specific site for ccCYP19A2 transcript in the tissues surrounding the ventral aorta remains to be established.

The seasonal changes in the transcript abundance of ccCYP19 in the brain and the pituitary had a similar pattern of expression in that transcript abundance dramatically increased prior to spawning and quickly decreased thereafter. More importantly, the patterns of brain and pituitary expression of the ccCYP19A2 gene and the three subunits of pituitary gonadotropins (glycoprotein-α, follicle-stimulating hormone β and LHβ) were determined simultaneously in the same tissues (Kumar & Trant 2004). This is the first report to compare CYP19A2 transcript abundance with those of the gonadotropin subunits from any vertebrate.

It should be noted in this study of the seasonal changes in expression that there are some time points with very small sample sizes (n≤3); however, the transcript abundance was relatively constant, with a small standard error. Even though it would be best to have the confidence afforded by a larger number of samples, the lack of variation in these data permits some degree of conjecture as to the biological significance of the seasonal changes in the expression of ccCYP19A2.

The hypothalamic area of the brain, one of the main sites for CYP19A2 expression, is also a major site for the expression of type I GnRH. GnRH plays an important role in gonadal maturation through the induction of gonadotropin expression and secretion in vertebrates, including teleosts (Senthilkumaran et al. 1999, Dubois et al. 2001a). It was also demonstrated in African catfish, Clarias gariepinus, that testosterone (an aromatizable androgen), but not 11-ketotestosterone (a non-aromatizable androgen), enhanced gene expression of type I GnRH in the brain (Dubois et al. 2001b). Considering these findings together with the preovulatory induction of the ccCYP19A2 gene expression in the catfish brain, CYP19A2 could be an important agent in the regulation of type I GnRH in catfish. Furthermore, the preovulatory induction of the ccCYP19A2 gene in the pituitary correlate well with the seasonal dynamics of LHβ subunit gene expression (Fig. 4; Kumar & Trant 2004), thus suggesting the involvement of CYP19A2 in LHβ gene regulation. It should be noted that the channel catfish is the first vertebrate species in which the 5′-flanking region of the ccCYP19A2 gene contains ERE-related consensus sequences, as would be predicted by the ability of E2 to upregulate CYP19A2 expression in the pituitary. Upregulation of transcription or activity of CYP19A2 by estrogen has been well established in the brain of adult (Gelinas et al. 1998) and juvenile fish (Mennet et al. 2005). Furthermore, ERE-related sequences, including a highly conserved palindrome ERE, has been identified in zebrafish (Kazeto et al. 2001) and goldfish (Tchoudakova et al. 2001). These findings strongly suggest that estrogen would be a comprehensive regulator for the expression of CYP19A2 gene in teleosts and the effects of estrogen on CYP19A2 expression would be, at least in part, dependent upon direct transcriptional modulation of the gene. The 5′-flanking region of the CYP19A2 gene in zebrafish and/or goldfish showed other consensus sequences for
transcriptional factor-binding sites potentially related to reproductive physiology or endocrine disruption, such as CRE and AhR/Arnt; however, there are no potential binding sites for these transcriptional factors in the 5′-flanking region of the catfish CYP19A2 gene. Therefore, these factors may not be common regulators of the CYP19A2 gene in teleosts even if they are functional regulatory elements in the zebrafish and/or goldfish.

Promoter activity of several selected segments of the 5′-flanking region of ccCYP19A2 gene was quantified by a luciferase reporter assay system. Even though the use of catfish pituitary cells in primary culture would be the most relevant model for promoter analysis of the ccCYP19A2 gene, primary cultures of catfish pituitary cells resisted transfection, thereby prompting us to use an aromatase-expressing rat pituitary cell line, GH4C1. The entire 5′-flanking region of the gene showed strong transcriptional activity in GH4C1 cells and this high transcriptional activity was maintained when the 5′-flanking region was truncated to the −687 bp position (Fig. 7). Further deletion of the 5′-flanking region caused decreases in the activity and the construct containing a minimal fragment, −46/+52 bp, of the region did not show any promoter activity. These findings suggest that the sequence from −687 to −46 bp is critical for the basal promoter activity in catfish pituitary. However, we still cannot rule out the possibility that the basal expression of ccCYP19 in the catfish pituitary is not well reflected by the promoter activity in GH4C1 cells. The promoter structure and the regulation of CYP19 gene in the rat pituitary have not been described.

In conclusion, an active form of CYP19A2, an inactive form (derived from alternative splicing) and multiple 5′-UTRs were identified in the channel catfish. Furthermore, it was demonstrated for the first time in the pituitary gland that estrogen upregulates transcription of the CYP19A2 gene. The gene expression of CYP19A2 in the catfish brain and pituitary correlates positively with the reproductive status of the ovary. Moreover, in vitro experiments with pituitary cells clearly demonstrated that CYP19A2 is specifically involved in the regulation of the LHβ subunit gene expression through the aromatization of testosterone. These findings clearly establish that the CYP19A2 gene functions as an important factor for the reproductive endocrinology in teleost through the brain-pituitary-gonadal axis.

Acknowledgement

We thank Dr Shigeho Ijiri (National Institute for Basic Biology, Okazaki, Japan) for designing and providing degenerate primers (P1 and P2), Dr Ramasamy Sampath Kumar and Dr Wie Goto-Kazeto (Center of Marine Biotechnology, Baltimore, MD, USA) for technical assistance and thoughtful discussion. This is contribution number 05–108 from the Center of Marine Biotechnology. This work was supported by a grant from the USDA (Enhancing Reproductive Efficiency; 00–35203–9105) to J M T. Y K was supported by a fellowship from the Japan Society for the Promotion of Science. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

References


Ijiri S, Berard C & Trant JM 2000 Characterization of gonadal and extra-gonadal forms of the cDNA encoding the Atlantic stingray (Dasyatis sabina) cytochrome P450 aromatase (CYP19). Molecular & Cellular Endocrinology 164 169–181.


Kazeto Y, Place AR & Trant JM 2004 Effects of endocrine disrupting chemicals on the expression of CYP19 genes in zebrafish (Danio rerio) juvenile. Aquatic Toxicology 69 25–34.


Kishida M & Callard GV 2001 Distinct cytochrome P450 aromatase isoforms in zebrafish (Danio rerio) brain and ovary are differentially programmed and estrogen regulated during early development. Endocrinology 142 740–750.

Kumar RS, Ijiri S & Trant JM 2000 Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish (Ictalurus punctatus) ovary throughout a reproductive cycle. Biology of Reproduction 63 1676–1682.


Kumar RS & Trant JM 2004 Hypophysical gene expression profiles of FSH-β, LH-β, and glycoprotein hormone-α subunits in Ictalurus punctatus throughout a reproductive cycle. General & Comparative Endocrinology 136 82–89.


Pasmanik M & Callard GV 1983 Aromatase and 5α-reductase in the teleost brain, spinal cord, and pituitary gland. General & Comparative Endocrinology 60 244–251.


Trant JM 1994 Isolation and characterization of the cDNA encoding the channel catfish (Ictalurus punctatus) form of cytochrome P450 arom. General & Comparative Endocrinology 95 155–168.


Received 12 July 2005
Accepted 6 September 2005