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Cytochrome P450 aromatase in grey mullet: cDNA and promoter isolation; brain, pituitary and ovarian expression during puberty

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Abstract

In a study towards elucidating the role of aromatases during puberty in female grey mullet, the cDNAs of the brain (muCyp19b) and ovarian (muCyp19a) aromatase were isolated by RT-PCR and their relative expression levels were determined by quantitative real-time RT-PCR. The muCyp19a ORF of 1515 bp encoded 505 predicted amino acid residues, while that of muCyp19b was 1485 bp and encoded 495 predicted amino acid residues. The expression level of muCyp19b significantly increased in the brain as puberty advanced; however, its expression level in the pituitary increased only slightly with pubertal development. In the ovary, the muCyp19a expression level markedly increased as puberty progressed. The promoter regions of the two genes were also isolated and their functionality evaluated *in vitro* using luciferase as the reporter gene. The muCyp19a promoter sequence (650 bp) contained a consensus TATA box and putative transcription factor binding sites, including two half EREs, an SF-1, an AhR/Arnt, a PR and two GATA-3s. The muCyp19b promoter sequence (2500 bp) showed consensus TATA and CCAAT boxes and putative transcription binding sites, namely: a PR, an ERE, a half ERE, a SP-1, two GATA-binding factor, one half GATA-1, two C/EBPs, a GRE, a NFkappaB, three STATs, a PPAR/RXR, an Ahr/Arnt and a CRE. Basal activity of serially deleted promoter constructs transiently transfected into COS-7, αT3 and TE671 cells demonstrated the enhancing and silencing roles of the putative transcription factor binding sites. Quinpirole, a dopamine agonist, significantly reduced the promoter activity of muCyp19b in TE671. The results suggest tissue-specific regulation of the muCyp19 genes and a putative alternative promoter for muCyp19b.
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1. Introduction

The Cyp19 gene encodes cytochrome P450 aromatase, the enzyme that catalyses the conversion of androgens to estrogens (Simpson et al., 1994). In mammals, a single Cyp19 gene has been identified, except in pigs (Tong and Chung, 2003). In more recently evolved teleosts, Cyp19 occurs as two genes that are structurally and functionally distinct (Callard et al., 2001) and appears on different chromosomes (Harvey et al., 2003). Cyp19a (CYP19A1/P450aromA) is the isoform predominantly expressed in the ovary while Cyp19b (CYP19A2/P450aromB) is the main isoform expressed in the brain, as reported in the goldfish, *Carassius auratus* (Tchoudakova and Callard, 1998); zebrafish, *Danio rerio* (Kishida and Callard, 2001; Trant et al., 2001; Sawyer et al., 2006); European sea bass, *Dicentrarchus labrax* (Blázquez and Piferrer, 2004); Nile tilapia, *Oreochromis niloticus* (Chang et al., 2005); Atlantic halibut, *Hippoglossus hippoglossus* (van Nes et al., 2005; Matsuoka et al., 2006); killifish, *Fundulus heteroclitus* (Greytak et al., 2005) and zebrafish (Sawyer et al., 2006). Each isoform has its distinct regulatory gene promoter region, as shown in goldfish (Tchoudakova et al., 2001); zebrafish (Kazeto et al., 2001; Tong and Chung, 2003); barramundi, *Lates calcarifer*, barramundi cod, *Cromileptes altiveles* and goby, *Gobiodon histrio* (Gardner et al., 2005).

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We have chosen to closely examine Cyp19 gene expression as a means to follow the role of aromatases in the pubertal development of the grey mullet, *Mugil cephalus*. Estrogen, which is converted from testosterone by the aromatases, appears to have a dual, yet conflicting role in fish species where the reproductive function is inhibited by dopamine. In immature fish, estrogen is stimulatory both to the brain–pituitary–gonadal axis (BPG) (Huggard et al., 1996; Schulz and Goos, 1999; Zanuy et al., 1999; Okuzawa, 2002) and the brain–pituitary dopaminergic system (Linard et al., 1995), which inhibits the gonadotropin-releasing hormone (GnRH) and therefore the BPG axis (Yaron et al., 2003).

The inhibitory effect of dopamine on the reproductive function in fish is exerted through a dopamine D2 receptor (D_2R) subtype as was shown in goldfish (Chang et al., 1990), African catfish, *Clarias gariepinus* (Van Asselt et al., 1990), European eel, *Anguilla anguilla* (Vidal et al., 2004), tilapia (Levavi-Sivan et al., 2005) and grey mullet (Aizen et al., 2005). Estrogen up-regulates D_2R expression in tilapia (Levavi-Sivan et al., 2003, 2005), thus promoting the inhibitory effect of dopamine.

In order to further understand the role of the aromatases in pubertal development, we investigated the Cyp19a and Cyp19b gene expression in grey mullet undergoing puberty. The cDNAs encoding for muCyp19a and muCyp19b were isolated by RT-PCR and their relative expression levels in sexually maturing female fish characterized by real-time quantitative RT-PCR (QPCR). The corresponding promoter regions were also isolated by genome walking PCR and their basal activity evaluated *in vitro*, using luciferase as the reporter gene. We have further tested the effect of quinpirole, a dopamine agonist, on the promoter activity of muCyp19b.

2. Materials and methods

2.1. Isolation of aromatase cDNA sequences

Brain and gonadal tissues were collected from maturing grey mullet held in captivity at the Bribie Island Aquaculture Research Centre, following the procedures approved by the Department of Primary Industries and Fisheries Animal Ethics Committee (approval number Bribie/032/07/02). Dissected tissues were fixed in RNA*Later* (Ambion, Austin, TX) and stored at −80 °C until used. Total RNA was extracted with TriZOL Reagent (Invitrogen, Carlsbad, CA). RNA quality and quantity were evaluated by spectrophotometry and by a denaturing formaldehyde agarose gel. Reverse transcription was carried out on 200 ng of total RNA with Powerscript Reverse Transcriptase (Clontech, Palo Alto, CA) according to the manufacturer's protocol for 5′ and 3′ first strand cDNA synthesis.

The oligonucleotides used in PCR amplifications for the isolation of muCyp19 cDNA sequences are shown in Table 1. Partial muCyp19a cDNA was first amplified by RT-PCR using degenerate primers 1 and 2 from 1 μl of the synthesized first strand 5′ ovarian cDNA. The 25 μl PCR reaction contained 2.5 μl 10× PCR buffer, 5 mM $MgCl_2$, 200 μM dNTPs, 1 μM forward and reverse primers, and 0.3 units of Taq DNA polymerase (Invitrogen). Thermal cycling comprised of initial denaturation at 94 °C for 1 min; followed by 30–35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 4 min; with final extension at 72 °C for 10 min. A semi-nested PCR using degenerate primers 1 and 3 was performed from the PCR reaction diluted 50×, obtaining the expected 650 bp amplicon. This PCR product was purified from an agarose gel with the Perfectprep Gel Purification System (Eppendorf, Hamburg, Germany) and subcloned into pGEM-T Easy plasmid vector (Promega, Madison, WI). Colonies obtained from transformed XL-1 blue *E. coli* cells (Stratagene, La Jolla, CA) were screened by PCR using primers 3 and 4 (190 bp amplicon). Plasmid DNA from positive colonies was purified with SV Minipreps Wizard Plus Purification System (Promega), then labeled with Big Dye Terminators 3.1 (PE Applied Biosystems, Foster City, CA), and sequenced on an ABI 377 automatic sequencer (PE Applied Biosystems). The identity of the partial sequence was ascertained by BLAST tool (http://www.ncbi.nlm.nih.gov).

From the partial cDNA sequence obtained, gene-specific primers were designed for 3′ and 5′ RACE-PCR following the SMART™-RACE PCR pro-

Table 1
Primer sequences used for the isolation of muCyp19 cDNAs by RT-PCR and isolation of promoter regions by genome walking PCR and for the construction of reporter plasmids by PCR

Primer number	Position	Direction	5′–3′ sequence
1	89–95[a]	Forward	GTNTGGATHAAYGGNGARGARAC
2	444–449[a]	Reverse	TTCATCATNACCATNGC
3	307–316[a]	Reverse	GTRTCNGGNGCNGCDATNACCATYTC
4	250–256[a]	Forward	CAGGAGYTVCAVGATGCHAT
5	204–212	Forward	CTGCGCTGCACCGTGGTCGACATCTCC
6	435–441	Forward	CCTCGTCGTTACTTCCAGCCGTTC
7	497–505	Forward	GACCAACAACCTCTCCCAGCAGCCCG
8	1–9	Forward	GGATCTGATCTCCGCTTGTGAACAGGC
9	212–204	Reverse	GGAGATGTCGACCACGGTGCAGCGCAG
10	104–110[a]	Forward	GTSTAYCAYGTYYTGARGAG
11	385–393	Forward	ACGGCACGGCAATCAGGAAAGGAACC
12	242–254	Reverse	CTTTGCCTGCTCTCTTATGCTTGTTGTA
13	19–11	Reverse	CATGGCGTCCAAAGCTACAGGAGTCAC
14	2–10	Reverse	GCCTGTTCACAAGCGGAGATCAGATCC
15	30–23	Reverse	GCAGCAGGAGTAGTAGAAGAGAGGCG
16	1–8	Reverse	CTTCTCCAGCAGCAGCAGCATCATCC
17	−165 to −140	Forward	CCAGATCCTATAAACCCGAGGTCACAC
18	+698 to +724	Reverse	TGTAGTTTCCCTGCCTTTGCCAC
19	−878 to −851	Forward	GAGCCTTCAGAGAGCTTTCAGTAAATGA
20	97–71[b]	Reverse	GTCTTCCATGGTGGCTTTACCAACAG

[a] Position number of degenerate primers was based on conserved regions of brain aromatase cDNAs available at the GenBank database. Primers 1–3 were designed by Gardner et al. (2005).
[b] This primer was derived from the sequence immediately downstream of the multiple cloning region of the pGL3-basic vector.

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