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Tissue-specific transcriptional initiation of the *CYP19* genes in rainbow trout, with analysis of splicing patterns and promoter sequences

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Abstract

The rainbow trout (*Oncorhynchus mykiss* Walbaum) genome contains three separate *CYP19* genes for distinct isoforms of cytochrome P450arom: *CYP19A* encoding the prevalently ovarian isoform P450aromA, and *CYP19B-I* and *II*, encoding forms I and II of the mainly cerebral variant P450aromB. RNA Ligase-Mediated 5′-Rapid Amplification of cDNA Ends analysis was used to determine the 5′-untranslated terminal regions (5′-UTRs) of the corresponding mRNAs, which are actually all expressed in the ovary, brain and gills. *CYP19A* is transcribed at different transcription start sites (TSSs) in each tissue, the most distal TSS being found in the brain, the intermediate one in the gills, and the proximal one in the ovary. *CYP19B-I* also displays tissue-specific TSSs, but transcripts undergo three distinct splicing patterns: the same pattern as previously reported for the brain and occurring also in the gills, and two novel patterns, established in the ovary and brain, which include two cryptic 3′-splice sites in intron 1, leading to the inclusion of intronic sequences of 92/94 and 66 b in the 5′-UTRs. Lastly, the *CYP19B-II* transcript in the ovary shows the same splicing pattern previously described for the brain. A PCR-based gene walking strategy was used to explore the promoter regions of the rainbow trout *CYP19* genes, which were found to contain potential binding sites for a variety of transcription factors.

Keywords: *Oncorhynchus mykiss*; *CYP19* genes; Promoter; Transcription start site; Splicing

1. Introduction

Cytochrome P450arom is a member of the cytochrome P450 superfamily and is the key component of the enzymatic aromatase complex converting androgens to estrogens in vertebrates. It is well-known that estrogens play regulatory roles in sex determination, gametogenesis, central nervous system (CNS) development, sex behaviour, and, in non-mammalian vertebrates, hepatic vitellogenin production (Simpson et al., 1994).

Cytochrome P450arom is expressed in a variety of tissues, including brain and ovary in all examined vertebrates (Callard and Tchoudakova, 1997). In the human and a number of higher primates, P450arom is expressed in multiple gonadal and extra-gonadal tissues: ovarian granulosa cells (McNatty et al., 1976), testicular Leydig cells (Tsai-Morris et al., 1985), placental syncytiotrophoblast (Fournet-Dulguerov et al., 1987), various sites in the brain (Roselli et al., 1985), and adipose stromal cells (Ackerman et al., 1981).

In mammals except pig (Simpson et al., 1997; Graddy et al., 2000), and in birds (Ramachandran et al., 1999), amphibians (Kuntz et al., 2004), reptiles (Jeyasuria and Place, 1998) and cartilaginous fish (Ijiri et al., 2000), a single gene (*CYP19*) has been isolated with a single protein product. In the human, analysis of transcripts and genomic sequences indicated that tissue-specific expression is regulated by multiple promoters associated with alternatively spliced, untranslated, first exons (Simpson et al., 1997).

By contrast, teleost fish have at least two separate *CYP19* loci, termed *CYP19A* and *CYP19B* (or *CYP19A1* and *CYP19A2*) encoding two distinct isoforms,

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P450aromA and P450aromB, which are preferentially expressed in the ovary and brain, respectively. The *CYP19B* gene has been found in *Carassius auratus* (Tchoudakova and Callard, 1998), *Danio rerio* (Kishida and Callard, 2001; Trant et al., 2001), *Oreochromis niloticus* (Kwon et al., 2001), *Oreochromis mossambicus* (Cruz and Canario, 1999), *Porichthys notatus* (Forlano et al., 2001), *Epinephelus akaara* (GenBank AC AY547353), *Epinephelus coioides* (AC AY510712), *Halichoeres tenuispinis* (AC AY489060), *Odontesthes bonariensis* (AC AY380061), *Oryzias latipes* (AC AY319970), and *Silurus meridionalis* (AC AY325907).

In a previous work, two neuronal P450aromB mRNAs, named forms I and II, were identified in the rainbow trout (*Oncorhynchus mykiss* Walbaum) (Dalla Valle et al., 2002a) and subsequently shown to be transcribed from two distinct genes, *CYP19B-I* and *CYP19B-II* (Dalla Valle et al., 2005). *CYP19B-I*, like the *CYP19B* gene of other teleosts and differently from teleost *CYP19A* genes (Kazeto et al., 2001; Chang et al., 2005), is made up of 10 exons and 9 introns. The first exon is untranslated and forms the first part of the 5′-UTR, while the second part and the ATG initiation codon are located in exon 2, whose coding region covers the first 36 amino acid residues that compose the transmembrane domain. In catfish, Kazeto and Trant (2005) found three different 5′-UTRs for *CYP19A2*, containing up to three untranslated first exons instead of the one reported for other teleost fish.

CYP19B-II is made of almost the same sequence as *CYP19B-I*, but lacks the first two exons and thus encodes a protein deprived of the transmembrane segment required for anchoring to the endoplasmic reticulum. Moreover, it is interrupted by only one intron, corresponding to the third intron of the *CYP19B-I* gene. A similar form, lacking the first 122 amino acid residues encoded by *CYP19A2*, and thus of the membrane-spanning region, was found by Kazeto and Trant (2005) in catfish but, according to the authors, this form derives from alternative splicing and not from a different gene.

To understand the mechanism of the differential expression of trout *CYP19* genes, we determined the transcriptional initiation sites, alternative splicings and 5′-UTRs of the transcripts in brain, ovary and gills, in which all aromatase isoforms are expressed. We also report on the promoter sequences of trout *CYP19* genes and their putative transcriptional regulatory elements.

2. Materials and methods

2.1. Preparation of genomic DNA and total RNA

Genomic DNA was isolated from liver of rainbow trout with DNeasy™ Tissue Kit (Qiagen, Milan, Italy), following the manufacturer's instructions. Total RNA from brain, ovary and gills of rainbow trout was extracted using the TRIZOL reagent (Invitrogen Life Technologies, Milan, Italy), according to the manufacturer's instructions.

2.2. RNA Ligase-Mediated 5′-Rapid Amplification of cDNA ends (RLM 5′-RACE)

RLM 5′-RACE was carried out using the First-Choice RLM-RACE Kit (Ambion, Milan, Italy) following the manufacturer's recommendations. Briefly, 10 μg of tissue total RNA was treated with calf intestinal phosphatase to remove the 5′-phosphate from truncated RNAs, leaving a 5′-OH end. Total RNA was then treated with tobacco acid pyrophosphatase to remove the 5′-cap from full-length mRNAs, leaving a 5′-phosphate to which a 5′-RACE RNA adapter oligonucleotide was ligated with T4 RNA ligase. Ligated mRNAs were then reverse-transcribed with random decamers.

5′-RACE cDNAs were PCR-amplified using the 5′-RACE outer primer and a specific 3′-reverse primer. The amplification procedure consisted of 2 min at 95 °C, followed by touch-down PCR with annealing temperatures decreasing from 68 to 56 °C over 12 cycles and the final 28 cycles at 56 °C. The extension phase of the last cycle was prolonged by 10 min. Diluted products were subjected to a second round of amplification, using the 5′-RACE inner primer and a second gene-specific 3′-reverse primer. All primers used in these analyses are listed in Table 1.

Amplified products were analysed in 1% agarose gel stained with ethidium bromide. Single bands were gel-purified, ligated into the TA cloning vector pGEM-T Easy (Promega, Milan, Italy) and sequenced.

2.3. PCR cloning of promoter sequences and 3′-UTR

Cloning of genomic sequences was performed with the Universal GenomeWalker Kit (Clontech, Celbio, Milan, Italy) according to the manufacturer's procedure. Briefly, aliquots of genomic DNA (2.5 μg) were digested for 16 h with the following blunt-end restriction endonucleases: *Dra*I, *Eco*RV, *Pvu*II, and *Stu*I. After inactivation, the four digested DNA preparations were separately ligated to the GenomicWalker adaptor. Three rounds of PCR were performed with the BD Advantage 2 PCR kit (Clontech). Adaptor-ligated DNA fragments were used as templates for primary PCR amplification, with the outer adaptor primer (AP1) and a gene-specific 3′-outer primer (GSP1). Each PCR reaction contained: DNA template, BD Advantage 2 PCR Buffer 1×, 0.2 mM dNTP mix, 0.2 μM of each primer, and BD Advantage 2 Polymerase mix 1×. The amplification procedure consisted of 2 min at 95 °C, followed by touch-down PCR with annealing temperatures decreasing from 68 to 58 °C over 10 cycles and the final 30 cycles at 58 °C. DNA synthesis was performed at 68 °C for 3 min in each cycles. The extension phase of the last cycle was prolonged by 10 min.

Table 1
Oligonucleotides used in the RLM 5′-RACE analysis

Gene target	Primer	Sequence	Nucleotide position (5′ → 3′)	Exon position
CYP19A	AromA-rev-1	5′-GAGGTGTAACGCCCCTGTC-3′	+398 → +380	3
	AromA-rev-2	5′-GTCGGCTATGACGGTGTC-3′	+69 → +52	1
CYP19B-I	AromB-rev-1	5′-TGGGCACTCTTCAGGAC-3′	+296 → +280	4
	AromB-I-rev-2	5′-GGTATGTGTGAGGCGTTAG-3′	+107 → +89	2
CYP19B-II	AromB-rev-1	5′-TGGGCACTCTTCAGGAC-3′	+194 → +178	2
	AromB-II-rev-2	5′-AGGGTTCATCTTCGACCA-3′	−246 → −263	1