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Analysis of zebrafish *cyp19* promoters☆

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Abstract

Cyp19 encodes P450 aromatase, the key enzyme catalyzing the conversion of androgens into estrogens. Estrogens play a crucial role in the anatomical, functional and behavioral characteristics of sexually dimorphic development. In zebrafish, two *cyp19* genes, *cyp19a* and *cyp19b*, expressed in ovary and brain, respectively, were found. We have isolated the promoter regions of the zebrafish *cyp19* genes from a bacterial artificial chromosome library to search for regulatory sequences that bind to transcription factors. Sequences like arylhydrocarbon receptor (AhR) recognition site, estrogen receptor recognition half sites (1/2ERE) and c-AMP responsive elements were found in the 5′-flanking regions of both *cyp19* genes. For ovarian-specific expression, we found binding sites for steroidogenic factor-1 (SF-1), GATA transcription factor 4 (GATA-4) and Wilm tumor 1 (WT1–KTS) on the promoter region of *cyp19a* but not *cyp19b*. For brain-specific expression of the *cyp19b* gene, sequences for recognition of chicken ovalbumin upstream promoter-transcription factor (COUP) and Ptx-1 were detected in the promoter. The importance of these putative control elements in ovary and brain-specific promoter has been assessed by sequence comparison among various species.

Keywords: Ovary; Brain; Promoter; Aromatase; *cyp19*; P450aro; Zebrafish

1. Introduction

Estrogens play crucial roles in the development of sexually dimorphic, anatomical, functional and behavioral characteristics that are vital for reproduction in vertebrates. Estrogen biosynthesis is catalyzed by an enzyme complex, one of which components is P450 aromatase encoded by the *cyp19* gene. The *cyp19* gene is highly conserved throughout the vertebrate phylum, such as human [1], mouse [2], rat [3], cow [4], birds like chicken [5] and zebra finch [6], reptile [7], and fishes like zebrafish [8] and medaka [9]. In mammals, *cyp19* is expressed in several types of tissues including gonad, skin, adipose tissue, placenta, and brain [10], while in fishes it is expressed mainly in the brain and the gonad. Except for pig [11] and fish [12,13], where multiple *cyp19* genes have been identified, there is only a single gene in most species. The tissue-specific expressions of *cyp19* in human [10], sheep [14], rabbit [15] and zebra finch [16] are achieved by the use of alternative transcription start sites that arise as a consequences of the use of tissue-specific promoters and alternative splicing.

So far, six promoter regions have been identified in human *CYP19* gene, namely, Ia, Ib, Ic, Id (II), Ie, and If; they are expressed in placenta, fetal liver/skin fibroblast, ovary, ovary/testis, placenta and brain, respectively [10,17–21]. The gonad-specific promoter is highly conserved in human, bovine, and even in avian [6,22,23], so is the brain-specific promoter sequence [23]. It indicates the conservation of regulatory elements that control expression of *cyp19* in gonad and brain across species. Extensive *cyp19* promoter analysis has been carried out for better understanding of the complex regulation of its expression in various tissues.

In both ovary and testis, the gonadotropins FSH and LH act through increasing concentrations of intracellular cyclic-AMP to induce expression [24]. A steroidogenic factor-1 (SF-1) binding site and a c-AMP responsive element located within 278 bp upstream of exon II were essential for the basal ovarian-specific transcriptional activity in human [25]. SF-1, also termed NR5A1 [26], is important for the expression of many steroidogenic genes. The gonad-specific promoter has been extensively studied because over-expression of *CYP19* mRNA in breast cancer and endometriosis is a consequence of the unusual utilization of promoter II [27,28]. Transcription factors WT1 and DAX1 inhibit *CYP19* expression in human endometriotic stroma cells [29]. Aside from CRE, which is required for basal cAMP induction, CCAAT/enhancer binding proteins, C/EBPβ is essential for ovarian follicle development in

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C/EBPβ-deficient mice [30]. C/EBPα and C/EBPβ also bind to the corresponding element on the promoter in endometriotic stromal cells to exert inductive and inhibitory actions, respectively [31]. Additions of RXR and PPARγ ligands inhibit *CYP19* mRNA transcription in human ovarian granulosa and breast adipose fibroblast cell line [32]. A silencing element S1 down regulates the *CYP19* activity in breast cells [33]. Recent study using the yeast one-hybrid approach showed that orphan nuclear receptors, EAR-2, COUP-TF1 and RARγ bind to S1 and down regulate transcription while ERRα-1 may have reverse action [34]. Apart from the *cyp19* expression in the granulosa cells of the ovary, it is also detected in Sertoli, Leydig and germ cells of testis [35,36]. An artificial promoter II with GATA repeats was found to bind GATA-1, -4, and -6 in association with (Friend of GATA) FOG in a number of testis-derived cell lines resulting in a decrease in *cyp19* mRNA [37,38].

The brain-specific *cyp19* expression is most significant in teleosts [13]; it is believed to be related to adaptation of the animal to the environment [39]. Expression of *cyp19* in the hypothalamus and limbic region of the brain is believed to have a local influence. Estrogen produced in the brain can be further metabolized into a catechol component, which can also bind to estrogen receptors in the local or nearby region of the brain [40]. Sexual dimorphism has been described in its expression in rodent and avian brains. In human, the brain-specific expression of *CYP19* gene involves the use of promoter *If* [41]. The brain-specific promoter has also been identified in mouse, birds and fish [16,23]. A highly conserved 55 bp sequence in the brain-specific promoter was observed among mammals and bird [16]. However, such sequence was not found in fish promoters. Factors involved in the regulation of brain-specific expression of *cyp19* gene are not well understood. SF-1 binding site was not found in the brain-specific promoters identified to date. The expression of *cyp19* and *SF-1* overlaps in restricted region of the hypothalamus, but not in telencephalon and hippocampus of zebra finch, suggesting an SF-1 independent regulation of *cyp19* gene in avian brain [42]. Androgen was reported to increase *cyp19* expression in fish and birds brain, while both stimulatory and inhibitory effects were found in mammals [43–45]. Estrogen and xenoestrogen both upregulate the transcription of *cyp19b* in zerbfish brain [46]. Neurotransmitters involved in the PKC and PKG pathways increase the mouse *Cyp19* mRNA in embryonic diencephalons [47]. Recently, proteins that bind to brain-specific promoter *cis*-elements have been identified in mouse *Cyp19* gene [48]. The mechanism they may involve in the regulation of brain-specific expression is yet to be investigated.

2. *Cyp19* genes in fish

Cyp19 gene has been cloned and characterized in fish, such as tilapia [49], trout [50], catfish [51], goldfish [13], medaka [9] and zebrafish [12], etc. It is expressed mainly in gonad and brain. They play critical roles in the sex differentiation in gonochoristic fish [52]. *Cyp19* activity is elevated in natural sex differentiation of genetically female trout and female tilapia [53], while a repression is detected in XX female musculinized by elevation of temperature during the sex differentiation period [54]. A surge of elevated aromatase activity was observed during sex change of protandrous black porgy [52]. Japanese flounder treated with 17α-methyl testosterone or aromatase inhibitor, fadrozole, caused masculinization of genetic female flounders wherein suppression of *cyp19* expression in both cases were observed [55]. The mechanism involved in regulation of *cyp19* gene expression is not clear.

3. Promoters of zebrafish *cyp19* genes

Distinct *cyp19* genes were found expressed differentially in brain and ovary of zebrafish and goldfish. To investigate the molecular basis of tissue-specific expression of the genes, we isolated the promoter region of zebrafish *cyp19* genes from the bacterial artificial chromosome library. Fragments of the 2.5 and 3.3 kb size flanking the translation start sites of *cyp19a* and *cyp19b* have been sequenced. For *cyp19b*, an intron, which spans about 1.8 kb, is found 20nt upstream of coding region, and downstream of an untranslated exon I. Sequence analysis was carried out using Transfac data matrix at TESS website [56]. Within the 2.5 kb promoter region of zebrafish *cyp19a*, A TATA box as well as several potential regulatory elements has been identified (Fig. 1a). Among these putative regulatory elements, a putative steroidogenic factor-1 recognition site was identified in *cyp19a* but not in the 1.5 kb region of *cy19b* promoter that we have sequenced so far (Fig. 1b). SF-1 is known to regulate *cyp19* genes in human ovary [57] and rat granulosa cells [58]. This consensus sequence is also identified in the 5′-flanking region of goldfish *cyp19a* and medaka ovarian expressing *cyp19* gene [9,59] (Fig. 2). Recognition sites of arylhydrocarbon receptor (AhR) were also found both *cyp19* genes. AhR is expressed in brain and many other tissues. It is reported to be responsive to xenobiotics and may induce apoptosis in cells [60]. Estrogen receptor (ERE) half sites, inverted androgen receptor (ARE) half sites, cyclic-AMP responsive element (CRE) like sites, and CCAAT enhancer binding protein (C/EBP) recognition sites are also found in both promoters. CRE binding protein can induce estrogen and can induce *cyp19* expression in fish, whereas androgen has stimulatory effect in *cyp19* expression of fish brain but inhibitory effect in that of gonad. Putative GATA transcription factor 4 (GATA-4) and Wilm tumor 1 (WT1–KTS) binding sites were identified in the *cyp19a* promoter. These proteins are expressed in mammalian gonad and are important for sex differentiation [61–63]. The asterisks in Fig. 1b mark the transcription start sites in the untranslated exon I of *cyp19b*. Multiple transcription start sites have been reported in the brain-specific transcript in goldfish, medaka

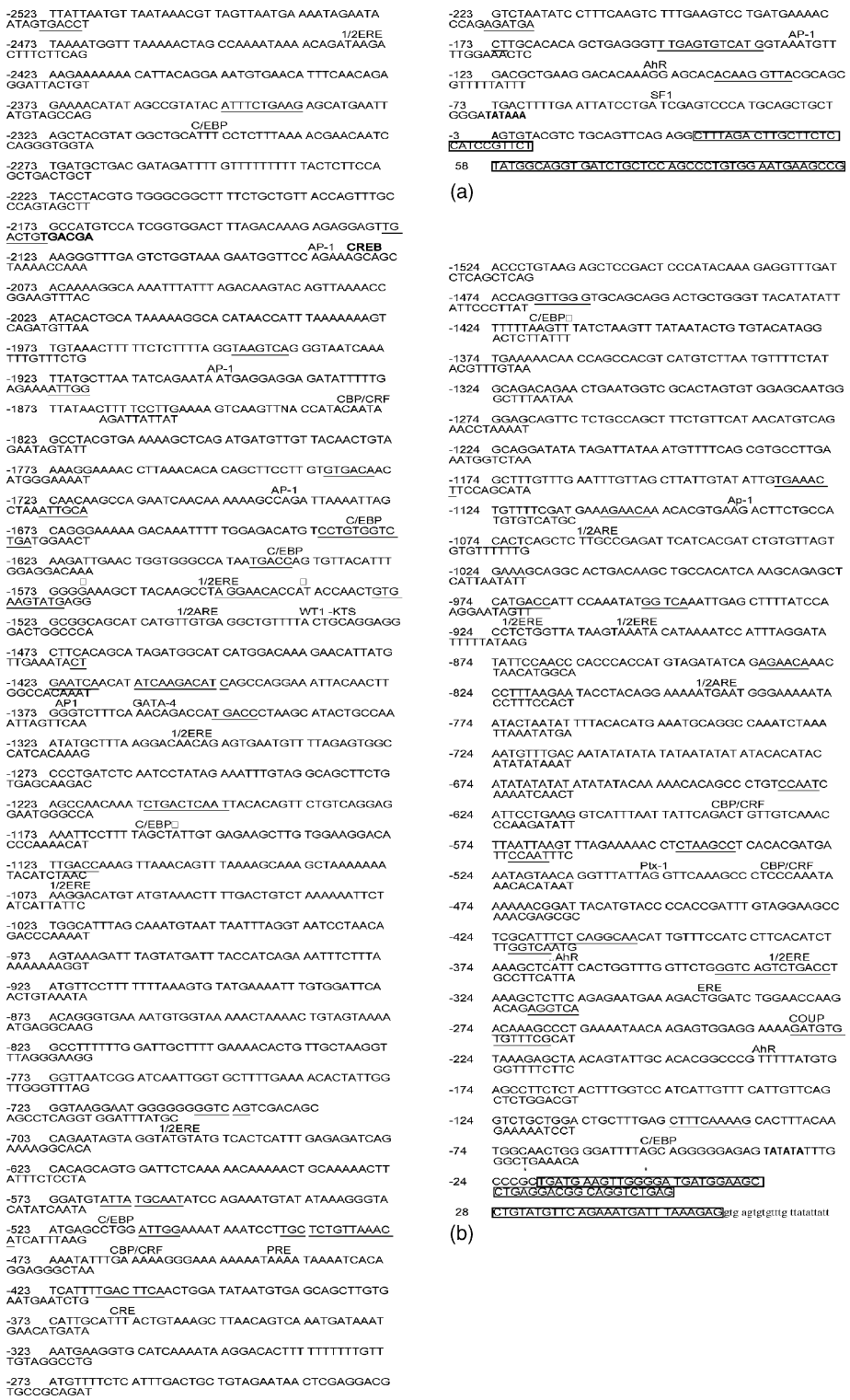

Fig. 1. Sequence of the promoter regions of zebrafish *cyp19* genes. Exons I are indicated in boxes and the translated region is distinguished by a shaded box. Sequences in lower case indicate intron. TATA box are shown in bold. Sequences of the putative binding sites of the transcription factors of interest are underlined and labeled: the 5′ flanking regions of zebrafish (a) *cyp19a* gene and (b) *cyp19b* gene.

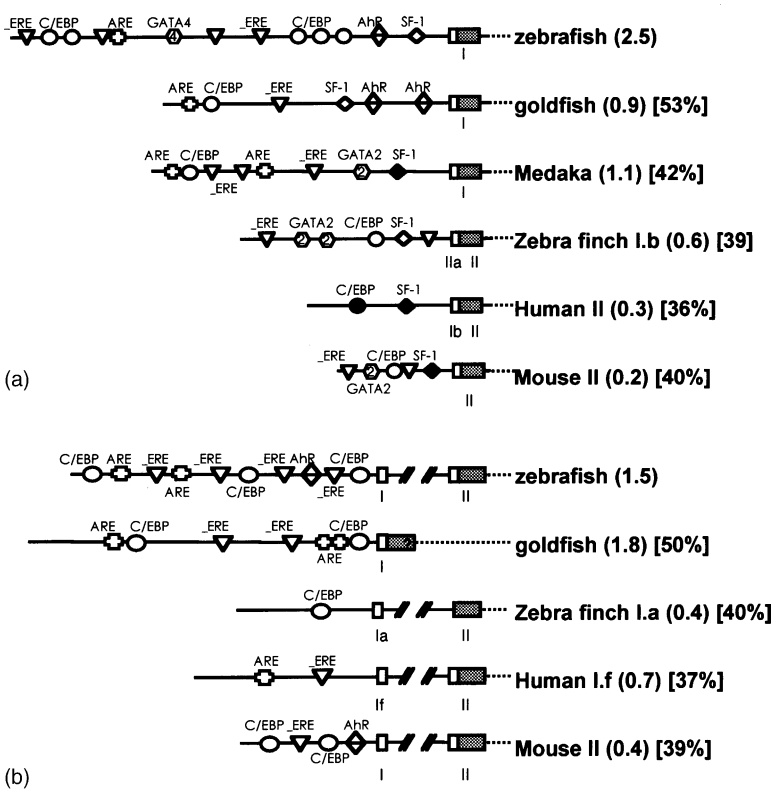

Fig. 2. Comparison of potential transcription factor binding sites of *cyp19* from various species: (a) ovary and (b) brain-specific *cyp19* promoters for zebrafish, goldfish, medaka, zebra finch, human and mouse. Numbers in parentheses indicate the approximate length of the promoters. Numbers in square brackets indicates the local percentage of sequence similarity within the tissue-specific group using default parameters in gap of the GCG program. Untranslated and translated exons are indicated in open and shaded boxes, respectively, with the exon numbers shown below. Filled symbols represent experimentally proven protein transcription factors binding sites. SF-1, steroidogenic factor-1; C/EBP, CAAT-enhancer binding protein; 1/2ERE, estrogen receptor recognition half sites (1/2ERE); ARE, androgen receptor binding element; AhR, arylhydrocarbon receptor binding site; GATA, GATA-2 or GATA-4 binding sites.

and zebrafish [9,59,64], but only one is found in the *cyp19a* transcript.

The promoter sequences of *cyp19* gene are highly conserved in mammal. There is a 90% similarity among, human, mouse and bovine ovarian-specific promoter and likewise for the brain-specific ones (Fig. 2). The zebrafish *cyp19* promoters are less similar to the mammalian promoter (less than 40% similarity), but show about 40–52% similarity to the goldfish and medaka promoters. The ovarian-specific gene of zebrafish does not involve an untranslated exon I. This shows evolutionary conservation with mammals and bird, where ovarian-specific transcript start from exon II using a promoter directly upstream of exon II. The binding sites of steroidogenic factor-1, are also conserved among mammals, birds and fish. Despite the evolution of two distinct *cyp19* genes in fishes, similarity in gene structure and splicing mechanism is observed within the vertebrate phylum.

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