CLONING AND TISSUE-SPECIFIC EXPRESSION OF CYTOCHROME P-450 AROMATASE cDNA IN JAPANESE EEL *(Anguilla japonica)*

Kloning dan Ekspresi Spesifik Jaringan dari cDNA Sitokrom P-450 Aromatase pada Ikan Sidat Jepang *(Anguilla japonica)*

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ABSTRACT

The conversion of androgen to estrogen is catalyzed by an enzyme, cytochrome P-450 aromatase (P-450arom). The P-450arom cDNA fragment was cloned from brain and ovary of the primitive teleost, Japanese eel. The p-450arom cDNA fragment (1.1 kb) from brain and ovary of the Japanese eel are identical. The deduced amino acid from P-450arom cDNA in Japanese eel have conserved domains for common putative functional properties of P-450 superfamily. P-450arom mRNA was predominantly present in the brain and pituitary, weak in the ovarv and none in the other tissues.

Key Word : Aromatase. P-450arom, cDNA, Japanese eel, expression

ABSTRAK

Konversi androgen ke estrogen dikatalisis oleh enzim sitokrom P-450aromatase (P-450arom). Fragmen cDNA P-450arom telah diklon dari otak dan ovari ikan teleos primitif, sidat Jepang. Fragmen cDNA P-45arom (1,1 kb) dari otak dan ovari sangat identik. Asam amino deduksi dari cDNA P-450arom memiliki daerah konservatif untuk sifat-sifat fungsional dari super famili P-450arom. P-450arom mRNA dominan terdapat di otak dan pituitary, sedikit pada ovari dan tidak terekspresi pada jaringan lain.

Kata kunci : Aromatase, P-450arom, cDNA, ikan sidat Jepang, ekspresi

INTRODUCTION

The conversion of androgen to estrogen is catalyzed by an enzyme complex that consists of cytochrome P-450 aromatase (P-450arom), flavoprotein and NADPH (nicotinamide-adenine dinucleotide phosphate)-cytochrome P-450 reductase (Thompson & Siiteri 1974). In the ovary, the principal site of estrogen biosynthesis in mammals and salmonid fishes are the ovarian granulosa cells. In these animals, a two-cell-type model for the production of ovarian estrogen has been proposed. The thecal cells, under the influence of gonadotropin, secrete an androgen substrate (in the most case testosterone) which diffuses into the granulosa cells where are aromatase is exclusively localized (Leung & Amstrong 1980; Nagahama 1987). Thus androgen is converted to estrogen in the granulosa cells.

The brain of teleosts, including the goldfish, demonstrates exceptionally high P-450arom activity in the central nervous system (CNS), with levels from 100 to 1000 times greater than those measured in mammalian CNS (Callard *et al.* 1981). Furthermore, brain P-450arom activity in goldfish is distinctly higher than that in the ovary, regardless of time of year (Pasmanik & Callard 1988).

The duality of P-450arom gene have been shown in the modern fishes, and the two distinct P-450aroms showed different tissue-specific patterns in their expression. However, information is limited in modern fishes (Sudrajat 2000).

In order to understand the fundamental mechanism involved in the fish reproduction and to provide a broader basis for comparative study on fish P-450aroms, it would be important to clarify whether primitive teleost species show the duality of P-450arom gene as has been shown in relatively modern species, or not.

The Japanese eel belong to primitive teleost and thus good animal model to study the structure and expression of the P-450arom gene in an evolutionary point of view. Therefore, to clarify the duality of P-450arom gene in the primitive teleosts, I attemted to isolate the two distinct P-450arom cDNAs from this species. The expression of P-450arom genes was examined in various tissues. This article describes the cloning and expression of P-450arom cDNA in the Japanese eel.

MATERIAL AND METHODS

Animal and Tissues

The eels were purchased from a commercial dealer. The ovary was obtained from maturating fish (GSI 19,56). The brains were obtained from immature

female fish (GSI 0,23). This tissues were used for isolation of eel P-450arom cDNA. For tissue-specific expression analysis by RT-PCR, the brain, pituitary, testis, eye, gill, heart, intestine, spleen, skin, muscle, kidney and liver were sampled from two immature male eels (GSI 1,61 and 0,24), and the ovary from the maturing fish (GSI 19,56). This tissues were flash-frozen in liquid nitrogen and stored at -80°C until use.

Oligonucleotide Primers

The oligonucleotides were used as PCR primers. Their structures are as follows: Primer 1, 5'-GG(A/T)AT(C/T)GG(C/G)AC(A/T)GC(C/G)AGTAA-3'; Primer 2, 5'-CTGTGGATGGGGATCGGC(A/T)-3'; and Primer 3, 5'-TCATCATCACCATGGCG ATG-3'. Primer 1 was constructed based on the known fish ovary-type P-450arom cDNAs. Primer 2 was constructed based on the goldfish brain-type P-450arom and mammalian P-450arom cDNAs. The Primer 3 was constructed as a common P-450arom based on the known P-450arom cDNAs.

Cloning of Eel P-450arom cDNA Fragment from Brain and Ovary

Total RNA was extracted and the first-strand cDNA synthesis methods as follows. The obtained cDNA fragment (1,1 kb) containing conserved heme-binding site and aromatic region of the P-450arom was used as a probe. Screening and plaque purification were performed according to standard method (Sambrook *et al.* 1989). From $3x10^5$ recombinant phage plaques of the Japanese eel ovarian cDNA library, ten positive clones were isolated. Positive phage clone was subcloned into pBluescript SK (-) according to *in vivo* excision system (Stratagene). After the insert size of this clones were checked, the longest clone (1,9 kb) was further sequenced. Subsequently, from 3x10 recombinant phage plaques of the Japanese eel brain cDNA library, six positive clones were isolated probing with the ovary-derived P-450arom cDNA (1,9 kb) from the Japanese eel ovarian cDNA library. After the insert size of this clones were checked, the longest clone (3,0 kb) was further sequenced.

The synthesized cDNA was amplified by PCR in 20 u,l reaction mixture containing 1 x PCR buffer (10 mM Tris-HCl, pH 8,3, 50 mM KC1, 1,5 mM $MgCl_2$, Takara), 200 uM of each dNTPs, 1 u,M of each primer, and 0,5 U *Taq* DNA polymerase (Takara). PCR amplification procedure was conducted in 40 cycles of 94°C for 1 min, 55 °C for 1 min and 72 °C for 2 min.Two pairs of primers, Primers 1 and 3, or Primers 2 and 3 were used for amplification. These primer pairs were predicated to amplify a 1,1 kb cDNA fragment. The amplified cDNA fragments were ligated in to pBluescript II SK (-) vector (Stratagene), then sequenced.

Sequencing and Sequence Analysis

The plasmid DNA was purified, and both strands of it were sequenced with T3 or T7 primer, using a SQ-5500 DNA sequencer (Hitachi). The known P-450arom amino acid sequences for teleosts, avians and mammals were entered and aligned using CLUSTAL W multiple sequence alignment program (Thomson *et al.* 1994) providing in Mc.Vector software (Oxford Molecular Ltd.). Alignment of amino acid sequences was carried out by introducing gaps to maximize the homology of the deduced amino acid sequences.

RT-PCR for Tissue Distribution Analysis

Total RNA was extracted from the brain, pituitary, ovary, testis, eye, gill, heart, intestine, spleen, skin, muscle, kidney and liver, according to manufacturer's protocol with RNA extraction solution (ISOGEN, Nippongene). The first-strand cDNA was generated according to the Ready-To-Go T-Primed First-Strand kit (Pharmacia Biotech). Five micrograms of total RNA was used as a template. A pair of primers, 5'-ATGAGCTGCACGAAGCAATG-3' (according to nucleotides 542-561) and 5'-ATGGCGATGTG TTTTCCCA-3' (reverse complement of nucleotides 1107-1124) of Japanese eel P-450arom cDNA fragment sequence. PCR was performed in 20 uJ reaction mixture containing an aliquot (3%) of the first-strand cDNA reaction, 1 uM of each primer, 200 uM of each dNTP, 1 x PCR buffer (100 mM Tris-HCl, pH 8,3, 50 mM KC1 1,5 mM $MgCl_2$, Takara), and 1U *TaqDNA* polymerase (Takara). PCR for 40 cycles was conducted under a cycle profile of 94°C for 30 sec, 55 °C for 30 sec and 72 °C for 30 sec. Each PCR reaction mixture was analyzed on a 3% agarose gel and stained with ethidium bromide.

RESULT

Isolation and Characterization of the Japanese Eel P-450arom cDNA

The P-450arom cDNA fragment (1,1 kb) was amplified with Primers 1 and 3 using the brain or ovarian cDNA as a template. However, no P-450arom cDNA fragment was amplified with Primers 2 and 3 using brain or ovarian cDNA as a template (data not shown). Furthermore, the isolated P-450arom cDNA sequence from the brain and ovary are identical. The isolated P-450arom cDNA fragment (1,1 kbp) from the Japanese eel is shown in Fig. 1. The deduced amino acid from the P-450arom cDNA in the Japanese eel showed 65%, 71%, 72% and 71% identity to the known ovary-type P-450aroms from goldfish, carp, bitterling and medaka, respectively, and showed 65%, 65%, 65% and 68% identity to the known brain-type P-450arom from goldfish, carp, bitterling and medaka, respectively homology analysis and the structural analysis of the

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GGT ATC GGC ACT GCG AGT AAT TAC TAC AAC GAA ACA TAT TGG GAC ATA GTC AGG GTG TGG ATA AAT GGA GAG GAG   75
G   I   G   T   A   S   N   Y   Y   N   E   T   Y   W   D   I   V   R   V   W   I   N   G   E   E    25

ACG ATC ATC CTT AGC AGG TCA TCT GCA GTA TAC CAG GTG CTG AGG AAG CCC CAG TAC ACC AGC AGG TTC GGC AGT  150
T   I   I   L   S   R   S   S   A   V   Y   Q   V   L   R   K   P   Q   Y   T   S   R   F   G   S    50

AAA CAA GGC CTG CGC TGT ATT GGC ATG CAT GAG AGA GGA ATC ATT TTC AAC AAC AAC ATA GAG CTC TGG AAG AAA  225
K   Q   G   L   R   C   I   G   M   H   E   R   G   I   I   F   N   N   N   I   E   L   W   K   K    75

GTC CGC ACC TAC TTT GCT AAA GCT CTA ACA GGT CCT GGG CTG CAG CGC ACG GTG GCC ATT TGC GTC GCC TCC ACA  300
V   R   T   Y   F   A   K   A   L   T   G   P   G   L   Q   R   T   V   A   I   C   V   A   S   T   100

GAC TCG CAC CTG GAC CAG CTG GAG GAG CTG ACC GAC CTC TCG GGC CAG GTG GAC ATC CTC AAC CTG CTG CGC TGC  375
D   S   H   L   D   Q   L   E   E   L   T   D   L   S   G   Q   V   D   I   L   N   L   L   R   C   125

ACC ATA GTG GAC ATC TCC AAC CAG ATG TTC CTC AGG GTG CCC CTC AAC GAA AAG GAG CTT CTG GTT AAA ATT CAG  450
T   I   V   D   I   S   N   Q   M   F   L   R   V   P   L   N   E   K   E   L   L   V   K   I   Q   150

AAG TAT CTT GAA GCC TGG CAG ACA GTT CTG ATC AGG CCA GAC TTT TTG TTC AAA TTT GAG TGG ATG TAC AAA GAA  525
K   Y   L   E   A   W   Q   T   V   L   I   R   P   D   F   L   F   K   F   E   W   M   Y   K   E   175

CAC AAG GAA GCA GCC CAT GAG CTG CAC GAA GCA ATG GAA ATA CTT GTG GAG AAA AAA CGC AAG GCT CTC GAG GAG  600
H   K   E   A   A   H   E   L   H   E   A   M   E   I   L   V   E   K   K   K   K   A   L   E   E   200

GCA GAG AAG TTG GAT GAT GCT GAC TTT GCC ACT GAT TTA ATA TTC GCT CAG AAC CAC GGG GAG CTG TCG GCA GAA  675
A   E   K   L   D   D   A   D   F   A   T   D   L   I   F   A   Q   N   H   G   E   L   S   A   E   225

AAC GTG CAA CAG TGC ATA CTG GAG ATG ATC ATA GCC GCA CCA GAC ACC ATG TCC ATC AGC CTG TTC TTC ATG CTC  750
N   V   Q   Q   C   I   L   E   M   I   I   A   A   P   D   T   M   S   I   S   L   F   F   M   L   250

ATG CTG CTC AAA CAG AAC CCA GAG GTA GAG CAG GAA ATA CTG AAG GAG CTG GAC ACT GTG ATC GGT GAC AAG AAA  825
M   L   L   K   Q   N   P   E   V   E   Q   E   I   L   K   E   L   D   T   V   I   G   D   K   K   275

GCA GAA AAC AGC AAC TTA CAA CAT CTA ATC ATC ATG GAG AGT TTC ATT AAT GAG TCC CTA CGC TAT CAT CCA GTG  900
A   E   N   S   N   L   Q   H   L   I   I   M   E   S   F   I   N   E   S   L   R   Y   H   P   V   300

GTG GAC TTC ACC ATG AGG AAA TCC TTG GAG GAT GAT GTC ATC GAA GGC TAC AAG GTT TTC AAG GGA ACG AAC ATC  975
V   D   F   T   M   R   K   S   L   E   D   D   V   I   E   G   Y   K   V   F   K   G   T   N   I   325

ATC CTT AAT GTT GGG CGT ATG CAT AAA TGT GAA TTC TTC TCC AAA CCC AAT GAA TTC AGC CTA GAG AAC TTT GAA 1050
I   L   N   V   G   R   M   H   K   C   E   F   F   S   K   P   N   E   F   S   L   E   N   F   E   350

AAA ACT GTG CCC AAC CGT TTC TTC CAG CCC TTC GGT TCC GGG CCA CGT TCG TGC GCT GGG AAA CAC ATC GCC ATG 1125
K   T   V   P   N   R   F   F   Q   P   F   G   S   G   P   R   S   C   A   G   K   H   I   A   M   375

GTG ATG ATG                                                                                       1134
V   M   M                                                                                         378
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Figure 1. The nucleotide and deduced amino acid sequences for the Japanese eel (*Anguilla japonica*) P-450arom cDNA fragment. The numbers on the right refer to the nucleotide and amino acid sequences.

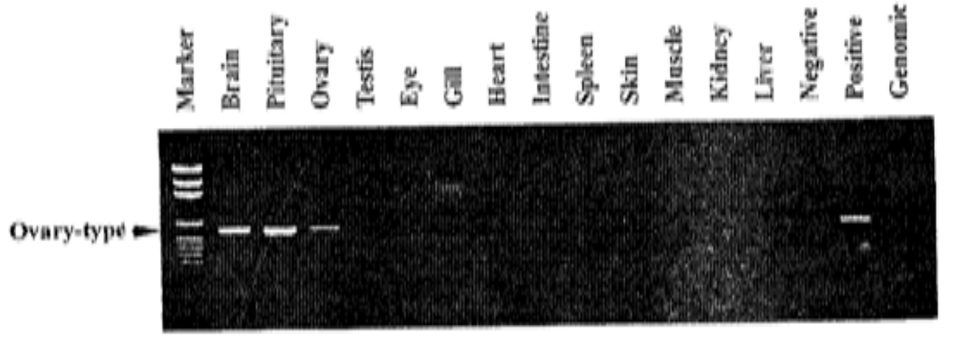

Figure 2. Tissue-specific distribution of the Japanese eel (*Anguilla japonica*) P-450arom mRNA. The RT-PCR products of expected size (582 bp) were obtained by the primer sets specific for eel P-450arom. pGEM was used as a size marker. Negative, PCR without template, Genomic, PCR with genomic as a template.

deduced amino acid sequences (Fig. 2) indicate that the P-450arom isolated from the Japanese eel brain and ovary was similar to the cyprinid and medaka ovary-type P-450aroms.

Expression of Japanese Eel P-450arom mRNA in Various Tissues

Tissue distribution of Japanese eel P-450arom mRNA was examined by RT-PCR. The results revealed that P-450arom mRNA was predominantly present in the brain and pituitary, weak in the ovary and none in the other tissues (Fig. 2). No expected product was detected from negative control.

DISCUSSION

In this article, I tried to isolate P-450arom cDNA from the Japanese eel and only one type of P-450arom cDNA fragment was isolated from the Japanese eel brain and ovary. The P-450arom of primitive teleost (Japanese eel) showed higher homology to the ovary-type P-450arom than the brain-type aromatase.

In the Japanese eel, P-450arom gene was abundantly expressed in the brain compared to the ovary. Although this result indicate that the P-450arom gene is strongly expressed in the brain, low expression in the ovary is probably due to late maturity of materials used, since GSI was 19,56%. Further investigation using mature females at various developmental stage are necessary to compare the expression levels of P-450arom gene in both tissues.

In this article, only one type of P-450arom cDNA could be isolated from the brain and ovary of Japanese eel (primitive teleost). On this aspect, there may be two posibilities; 1) primitive teleost possess two distinct types of P-450arom gene, but the effort for isolation of two P-450arom cDNAs was not sufficient, and 2) primitive teleost possess only one type of P-450arom as in mammals. Since the ovary-type and brain-type P-450arom cDNAs were respectively cloned from ovary and brain of Japanese eel, the second possibility is though to be true case. The tissue-specific expression patterns of P-450arom genes also support this hypothesis. Regulatory mechanisms of P-450arom gene expression in modern and primitive teleost will be an interesting theme in future.

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