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Molecular and expression analysis of apolipoprotein E gene in the Chinese sturgeon, *Acipenser sinensis*

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ABSTRACT

Apolipoproteins are carrier proteins that bind to lipids to form lipoprotein particles and have been shown to play an important role in lipid metabolism. In this study, a full-length cDNA for apolipoprotein E, named *AsapoE*, was cloned from the Chinese sturgeon (*Acipenser sinensis*). This cDNA sequence is 1289 bp in length, and codes for a polypeptide of 274 amino acid residues, which is 45% and 42% identical to that of the rainbow trout and zebrafish, respectively, and 39%, 30%, and 29% identical to frog, mouse, and human respectively. The predicted *As*ApoE protein has a conserved amphipathic α-helix region with the potential to bind to lipids. RT-PCR analysis reveals that *AsapoE* is expressed in all tissues examined with a preferential expression in the kidney and liver. During the embryo development stage, *AsapoE* mRNA is low but still detectable at gastrula stage embryos; then *AsapoE* mRNAs reach a higher level in muscle contraction stage embryos, this relatively stable expression persists during the following embryogenic stages and declines 1 day after hatching. These results will serve as a basis for comparative studies on vertebrate *apoE* genes.

1. Introduction

The protein components of plasma lipoproteins are known as apolipoproteins, which are mainly synthesized in the liver and the intestine (Havel, 1975). Apolipoproteins bind to lipids and play important roles in lipid transport and lipid admission in the circulation system (Havel, 1975). In mammals, over 10 exchangeable and soluble apolipoproteins (including ApoC-I, ApoC-II, ApoC-III, ApoC-IV, ApoA-I, ApoA-II, ApoA-IV, ApoB, ApoD, ApoE, and ApoM) were characterized by the occurrence of repeated amphipathic helical regions that were considered to be structural units essential for lipid-binding properties (Segrest et al., 1994; Allan et al., 1995; Xu and Dahlbäck, 1999). These apolipoproteins, except for ApoB, are encoded by a multigene family in the genomes of vertebrates, and have evolved through whole-gene duplication, intraexonic amplification of repeating units, and intragenic deletions (Li et al., 1988).

In humans, the major physiological role of APOE consists of mediating the cellular recognition and internalization of lipoproteins with members of the low-density lipoprotein receptor super-family (Schneider et al., 1997). ApoE was also found in the peripheral and central nervous systems, and may play a role in the normal brain function and cognition, as well as in disorders such as Alzheimer's disease, brain injury, and stroke (Higgins et al., 1997; Horsburgh et al., 2000). However, little information has been dedicated in ApoE in lower vertebrates. In contrast to mammals that mainly use carbohydrates (Watanabe, 1982), most fish utilize lipids as the major source of energy. Lipid metabolism seems to be more important for homeostasis maintenance in fish than in homeotherms (Kondo et al., 2005). Only several reports have been published on the structures of ApoE from fish, such as zebrafish (Babin et al., 1997), rainbow trout (Durliat et al., 2000), turbot (Poupard et al., 2000), pufferfish (Kondo et al., 2005), and spotted barbel (Kim et al., 2009).

Chinese sturgeon (*Acipenser sinensis*) is one of Acipenseriformes, a group of cartilage ganoid with an evolutionary history of over 200 million years (Wei et al., 1997; Birstein et al., 1997). Comparable to other sturgeon species, its stock has declined dramatically due to overfishing, loss of natural habitat for reproduction and other anthropogenic interferences (Wei et al., 1997; Billard and Lecointre, 2001; Wei, 2003; Chen, 2007). To save this species and to be able to develop an aquaculture industry in the future, artificial propagation has been attempted since 1983. This task is rather difficult, because so far little is known about the regulation mechanism of growth and reproduction of the Chinese sturgeon. Recently, we have established a systematic molecular study in the Chinese sturgeon, and identified some important genes relative to growth, development, and reproduction, such as somatostatin 1 and 2 (Li et al., 2009), three gonadotropin subunits *common* α, *FSH*β and *LH*β (Cao et al., 2009), growth hormone/prolactin family, thyroid-stimulating hormone subunit β (*TSH*β) (in preparation), and insulin-like growth factor I (*IGF-I*). In the present study, the *apoE* cDNA was cloned from the liver

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samples of a 2-year-old Chinese sturgeon female. Moreover, the expression pattern analyses were performed by using tissues from the central nervous system and peripheral tissues as well as in the embryonic stages and larval development of the Chinese sturgeon.

2. Materials and methods

2.1. Animals and samples

All Chinese sturgeons used in this study were cultured in Taihu Station, Yangtze River Fisheries Research Institute, Chinese Academy of Fisheries Science. Deep anaesthesia was induced by a 0.05% solution of MS-222 (Sigma-Aldrich, USA).The tissues (including liver, kidney, spleen, fat, heart, ovary, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, medulla oblongata and spinal cord) sampled from three 2-year-old female Chinese sturgeons (about 1.05 m in length and 4.5 kg in mass) were collected within 30 min of exsanguination by tailing and immediately dipped into liquid nitrogen and stored at −80 °C. To study the ontogenetic expression profiles, fertilized embryos and larvae from different developmental stages (including the first cleavage, multicellular stage, blastula stage, gastrula stage, blastopore stage, tail bud stage, rudiment of heart stage, muscle contract stage, heartbeat stage, head to tail stage, pre-hatching stage and 1-day larvae) were identified, collected in liquid nitrogen and stored at −80 °C. The experimental procedures are based on the standards of the Chinese Council on Animal Care.

2.2. RNA extraction and SMART cDNA synthesis

Total RNA was extracted using SV total RNA isolation system kit (Promega, USA). The purity of total RNA was checked by the A260: A280 nm ratio (Eppendorf Biometer, Germany). Double-stranded cDNAs were synthesized and amplified using the Switching Mechanism at the 5′-end of RNA Transcript (SMART) cDNA Library synthesis Kit (Clontech, USA) as already described in Li et al. (2005).

2.3. Cloning and sequencing

The *AsapoE* cDNA was amplified by 3′- and 5′-RACE (rapid amplification of cDNA ends) as described previously (Li et al., 2005). Degenerate sense and antisense primers (*apoE*-F and *apoE*-R, Table 1) were designed and synthesized (Sangon, China) according to two conserved regions revealed from amino acid sequence alignment (Fig. 2A) of the known ApoE proteins available on GenBank, a feature of the NCBI website. The 3′-end of *AsapoE* cDNA was amplified using sense primers (*AsapoE*-F1, Table 1) and a PCR anchor primer corresponding to the terminal anchor sequence of the SMART cDNA (3′-AP, Table 1). The 5′-end of the *AsapoE* cDNA was amplified with a 5′ PCR anchor primer (5′-AP, Table 1) and specific antisense primer (*AsapoE*-R1, Table 1). All PCRs were performed on a PTC-100 thermal cycler (Bio-Rad, USA) by denaturation at 94 °C for 4 min, followed by 35 cycles of amplification at 94 °C for 30 s, 56 °C for 40 s and 72 °C for 2 min and an additional elongation at 72 °C for 7 min after the last cycle. The PCR mixture contained 1U Taq DNA polymerase (MBI, Fermentas, Canada) together with 0.2 mM of each dNTPs (Pharmacia, USA), a suitable reaction buffer (MBI), 1.5 mM $MgCl_2$, 15 pmol of each primer and 2 μL diluted SMART cDNA. The amplified DNAs were visualized by electrophoresis of ethidium bromide stained agarose gel, cloned into pMD18-T vector (Takara, Japan), and sequenced (Sangon).

Table 1
Primers used for PCR amplification of *AsapoE* and *Asβ-actin*.

Primers	Sequence (5′-3′)
apoE-F	ACYCGANGGNCTMAHCVDAG
apoE-R	ASCHTCTHCTNBANCYTVCG
AsapoE-F1	GCTCCCGGATCAGCATGTACAC
AsapoE-R1	TGGAGGTCATCGGCGTAGATG
AsapoE-F2	CGAGAAGTTGGAACCCTACACC
AsapoE-R2	AAGAGGGCTATTGGCCATTCTG
3′-AP	CGAGGAGGACGACATGTTTTTTTTTTTT
5′-AP	AGTCAATGTAGGATGGACAGG
Asβ-actin-F	TCCGTGACATCAAGGAGAAGC
Asβ-actin-R	TACCGCAAGATTCCATACCC

2.4. Spatial and temporal expression analyses of the AsapoE gene

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA extracted from different tissues (including liver, kidney, spleen, fat, heart, ovary, pituitary, hypothalamus, telencephalon, midbrain, cerebellum, medulla oblongata and spinal cord) from three 2-year-old female Chinese sturgeons were isolated using SV Total RNA Isolation System kit according to the manufacturer's instructions (Promega, USA). Total RNAs of embryos at the first cleavage, multicellular stage, blastula stage, gastrula stage, blastopore stage, tail bud stage, rudiment of heart stage, muscle contract stage, heartbeat stage, head to tail stage, pre-hatching stage and 1-day larvae were isolated. The quality and purity of the RNAs were checked by electrophoresis of the samples in a 1% agarose gel with ethidium bromide staining, and the RNA purity was checked by the ratio A260: A280 nm (Eppendorf).

Total RNAs (1 μg) were reverse-transcribed with 200 units Moloney murine leukemia virus reverse transcriptase (Promega, USA) and anchoring primer oligo $d(T)_{15}$ (Promega, USA) as described by the manufacturer. All of the resultant cDNAs were respectively diluted 1:10, and then used as PCR templates. The primer pairs, *AsapoE*-F2/*AsApoE*-R2 (Table 1, arrows in Fig. 1), were designed to detect the different expression patterns of *AsApoE*. Amplification reactions were performed in a volume of 25 μL containing 1 μL cDNA as template DNA, 0.5 μM of each primer, 0.5 U Taq polymerase (MBI), 0.1 μM of each dNTP (Pharmacia, USA), 1× buffer for Taq polymerase (MBI). Each PCR cycle included denaturation at 94 °C for 30 s, annealing at 55 °C for 40 s, and extension at 72 °C for 50 s. The RT-PCR analysis was carried out as described previously (Li et al., 2010). Briefly, 10 duplicate reactions were performed by alternate cycle numbers from 15 to 33 to ensure that the semi-quantitative RT-PCR products were in a linear range of accumulation. After the cycle number was optimized (31 cycles), temporal and spatial expression analysis of the *AsapoE* were completed by RT-PCR from the different samples. As a positive control for the RT-PCR analysis, *Asβ-actin* was amplified by primers *Asβ*-actin-F/*Asβ*-actin-R (Table 1) to determine the template concentration and to provide a semi-quantitative external control for PCR reaction efficiency under the same reaction conditions as *AsapoE*. About 25% of each PCR product was separated by electrophoresis on 1.5% agarose gel with 0.5 μg/mL ethidium bromide in tris-borate ethylenediaminetetraacetic (TBE) acid buffer, and the separated PCR products were visualized under ultraviolet (UV) light.

2.5. Database and sequence analysis

Nucleotide sequence identity was performed using the BLAST algorithm (NCBI). The glycosylation sites, phosphorylation sites, and the cleavage site for the putative signal peptide were predicted using the ExPASy Molecular Biology Server (http://www.expasy.pku.edu.cn). Multiple alignments were performed with the MAP method at BCM Search Launcher web servers (http://www.searchlauncher.bcm.tmc.edu/) and the printing output was shaded by BOXSHADE 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The secondary structure was predicted by using PHD (http://www.Predictprotein.org). Phylogenetic analysis was performed using Mega4.1 molecular evolutionary genetic analysis software package by bootstrap analysis using neighbour-joining method (1,000 replicates).