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EXPRESSED SEQUENCE TAG ANALYSIS OF A 4 YEAR-OLD CHINESE STURGEON PITUITARY

CAO Hong[1,2], ZHOU Li[1] and GUI Jian-Fang[1]

(1. *State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan* 430072; 2. *Graduate School of the Chinese Academy of Sciences, Beijing* 100039)

Abstract: Expressed sequence tag (EST) analysis is an efficient tool for gene discovery and for profiling gene expression. In order to isolate specific functional genes involved in reproduction and endocrine regulation and to reveal their evolutionary mechanisms in Chinese sturgeon (*Acipenser sinensis* Gray), a chondrostean fish with a history of 140 million years, we constructed its pituitary cDNA library from a 4 year-old male. A total of 944 random clones were sequenced and compared with sequences in GenBank database. Among all the 944 EST clones, 802 (84.96%) clones were identified as 461 known genes, and additional 142 (15.04%) as unknown genes. Functional categorization indicated that the most abundantly expressed functional gene was the proopiomelanocortin (POMC), which accounted for almost 10.17% of the overall expression, indicating its important function in the pituitary. Interestingly, the expression patterns of 7 unknown genes were analyzed in various tissues, such as heart, liver, spleen, kidney, muscle, testes, ovary and pituitary. Three different categories of expression patterns were observed from them. Several unknown ESTs, such as EG009334, EG009337, EG009338 and EG009340, were detected to be pituitary-specific, or pituitary and ovary-specific genes. Further studies on their functions will be very useful for better understanding the mechanisms of sturgeon reproduction biology and endocrinology.

Key words: cDNA; Expressed sequence tag (EST); *Acipenser sinensis*; Pituitary

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Expressed sequence tag (EST) analysis is an efficient approach to identify new genes and profile gene expression in cells of a tissue[1—4]. Careful analysis of the sequence data can further provide functional, structural and evolutionary information[5]. Furthermore, sequence information from ESTs could also be used in many other applications such as the discovery of molecular markers[6—8] and the detection of gene loci that influence a quantitative trait locus (QTL), such as growth and reproduction[9, 10]. Recently, EST sequence resources are rapidly growing in molecular database. But most of the fish ESTs were generated from model fish such as zebrafish[11] and medaka[12] or commercial fish such as winter flounder[3], Japanese flounder[13], salmon[14, 15], channel catfish[16—19], red sea bream[20], tilapia[21], common carp[22], gilthead sea bream[23], and orange-spotted grouper[24].

Hypothalamus-pituitary-gonad is an important endocrine axis to regulate reproduction and sex differentiation. And pituitary is just the central role of this axis. Therefore, the screening and identification of genes expressed in pituitary will be able to provide useful in-

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Brief introduction of the author: Cao Hong (1978—), Male, born in Wuhan, Hubei Province; PhD of Institute of Hydrobiogy (CAS), Major in Genetics. E-mail: regancao@ihb.ac.cn

Corresponding author: Gui Jian-Fang, E-mail: jfgui@ihb.ac.cn. Tel.: +86-27-68780707, fax: +86-27-68780123

sight into the molecular mechanism of growth, reproduction, and sex differentiation behind the Chinese sturgeon. Also, the information will be helpful for us to make sound conservation program to protect this species.

The Chinese sturgeon (*Acipenser sinensis* Gray) is a rare species which belonging to the group of chondrichthyans. It is a primitive teleost with a history of 140 million years. It mainly lives in the continental shelf of the Yellow Sea and the East China Sea and spawns in the upper Yangtze River and is one of the largest fish to enter fresh water. The construction of the Gezhouba Dam in 1981 in the Yichang section of the Yangtze blocked the spawning migration of Chinese sturgeon to the Yibin spawning reach. As a result of damming and over fishing, populations of the Chinese sturgeon have greatly declined in abundance[25]. To save this species from extinction, artificial propagation has been put into practice. Previous studies on Chinese sturgeon reproduction were mainly focused on the gonad ultrastructure and histology at different developmental stages[26—28], but little molecular information was known with regards to the reproduction regulation and sex differentiation. Our previous work of a 24 year-old female Chinese sturgeon's pituitary EST library found 8 reproduction or endocrine regulation related genes in Chinese sturgeon. Here, we described the study on gene identification by screening 944 clones from the pituitary cDNA library of a 4 year-old male Chinese sturgeon. In this study, several unknown ESTs were detected to be pituitary-specific, or pituitary and ovary-specific genes. This means they may include important information concerning about reproduction or sex differentiation. It would be interesting to study their function on molecule regulation mechanism of reproduction and endocrine regulation in Chinese sturgeon.

1 Materials and methods

1.1 RNA extraction and SMART cDNA synthesis

Pituitary was collected from a 4-year-old male Chinese sturgeon with 15.3 kilograms. This sturgeon was reared in the Yangtze River Fisheries Research Institute from eggs and was sampled in June 2005. Total RNAs were extracted using SV total RNA isolation system (Promega, USA). The RNA quantity was measured spectrophotometrically at A260 nm and the ratio of A260: A280 nm by biophotometer (Eppendorf). The RNA quality was assessed by gel-electrophoresis to ensure the integrality. The cDNAs were synthesized from 50ng of total RNAs according to previous reports[24] using the Switching Mechanism at 5′ end of RNA Transcript (SMART) cDNA Library Construction Kit (Clontech). Briefly, 50ng of total RNA was reverse-transcribed at 42℃ for 1h at the presence of both 3′BD SMART cDNA synthesis (CDS) primer II A (5′-AAGCAGTGGTATCAACGCAGAGTACTVN-3′) (N = A, C, G, or T; V = A, G, or C), and BD SMART II A oligonucleotide (5′-AAGCAGTGGTATCAACGCAGAGTACGCGGG-3′). Then 2 μL of first-strand reaction product was added into each 100 μL long-distance PCR system containing 0.2 μM PCR primer (5′-AAGCAGTGGTATCAACGCAGAGT-3′). The LD PCR parameters were 95°C for 15s and 65°C for 30s and 68°C for 6min respectively on Perkin-Elmer PCR System 2400 for 20 cycles. Five microliters of the amplified products were separated by electrophoresis on 1% agarose gels.

1.2 Plasmid preparation and sequencing analysis

The cDNAs were ligated to pMD-18T vector (Promega) and the plasmids were used to transform *E. coli* DH5α super competent cells. The plasmid cDNA library was plated to appropriate density to pick individual colonies. 944 colonies (with insert lengths of > 150 base pairs) were randomly picked for single-pass sequencing of the 5′-termini from the above constructed SMART cDNA plasmid library. DNA sequencing was performed using dRhodamine terminator cycle sequencing Kit and ABI PRISMTM 310 Genetic Analyzer (Perkin Elmer). In order to identify sequences, all ESTs were compared with nucleotide and protein sequence databases of NCBI using BLAST through Internet (http://www.ncbi.nlm.nih.gov). All sequences were compared by BLASTN and BLASTX with the sequences in GenBank, orthologs of known genes were identified on the basis of three criteria: percentage match (usually >50%), the length of match (usually >100 bp or >50 amino acids) and matches were con-

sidered to be significant only when the probability (P) was less than 10^{-4}.

1.3 Semi-quantitative RT-PCR

The isolated RNAs were respectively reverse-transcribed with M-MLV Reverse Transcriptase (Promega) and oligo (dT) 15 (Promega) as described by the manufacturer. Total volume for each reaction is 25μL containing 15μL of the isolated RNAs, 10 mM of each dNTP, 200 units of M-MLV RT, and 40 units of rRNasin® Ribonuclease Inhibitor with 5 X M-MLV buffer (Promega). The reaction mixture was incubated at 37℃ for 1.5h.

All of the resultant cDNAs were respectively diluted 1∶2, and then used as templates for PCR with Taq DNA polymerase (MBI, Fermentas). Seven pairs of primers were synthesized (Sangon, Shanghai, China), and used to identify tissue distribution. Amplification reactions were performed in volume of 25μL containing 1μL cDNA as template, 0.2μM each primer, 0.5 units Taq polymerase (MBI, Fermentas), 5 mM of each dNTP, 1 × Buffer for Taq polymerase (MBI, Fermentas). The details about each PCR cycle were included in Tab. 1.

As a positive control for the RT-PCR analysis, β-actin (β-actin-F: 5′-CACTGTGCCCATCTACGAG-3′ and β-actin-R: 5′-CCATCTCCTGCTCGAAGTC-3′) was amplified to determine the template concentration for PCR reaction efficiency under the same reaction conditions as the 7 unknown ESTs. Briefly, alternate cycle numbers from 30 to 40 ensured that the semi-quantitative RT-PCR products were in a linear range of accumulation performed ten duplicate reactions. After the cycle numbers were optimized to 40 cycles for the 7 unknown ESTs and β-actin, the semi-quantitative RT-PCR assays were used to evaluate expression level of the 7 unknown ESTs.

2 Results

2.1 Gene identification from the 944 expressed sequence tags

After elimination of the vector sequence, 944 DNA sequences were chosen and analyzed by BLAST searches through Internet using NCBI database. The average EST length was 553 bp. Of all the expressed sequence tags, 802 (84.96%) clones of known genes represent transcripts of 461 genes, and additional 142 (15.04%) clones were identified as unknown ESTs (Tab. 2).

Tab.1 The length of 7 unknown ESTs, oligonucleotide primer sequences and conditions used in RT-PCR anaysis

Categories	Accession No.	The length of unknown ESTs	The length of prediction ORF	Sequence (5′-3′)	Length of amplified DNA fragment (bp)	Annealing temperature (°C)	Cycle number of RT-PCR
1	EG009335	991	159	GCTTTGGAGAGAGCAGTCGGTTCA	851	51	40
				TCGCGTAATCAGCTCCCTTCTA			
	EG009336	597	243	ACTCCCACAATGCTCTACTAGCC	479	50	40
				CTGGTTGTGTTGGTATGGAATGA			
	EG009339	580	165	GGGAAGAAGACACAGGCTAAAGGC	329	58	40
				AGAGACCCAACGACGGGCGATT			
2	EG009337	1358	381	GCCAGCAGTTTGTGAAGCAGCCATTC	1184	51	40
				GCAAGGGTGAGGCATTCAGC			
	EG009340	589	174	ATCAGCAGACACAGCCCTCCGCA	455	58	40
				CCTCACTTCCGAAAGGGCACTAA			
3	EG009338	527	222	GGTCTGTGAAGGTGCTTAGC	419	53	40
				CACAATGCCATCTAACCACTGAAG			
	EG009334	512	273	TCAGGGAGGAAGAGAAGCGTGGT	416	53	40
				CTGAACCAAGCAACATGACCCAC			

Tab. 2 Gene categorization of 944 Chinese sturgeon pituitary ESTs

Category		*Number of clones sequenced*	*Number of genes*	*Redundancy factor*	*Expression (%)*
1	Gene involved in the protein translation machinery	38	22	1.73	4.03
2	Cellular structural genes	16	12	1.34	1.69
3	Enzymes	26	22	1.18	2.75
4	Transcriptional factors, DNA repair and DNA-binding	13	9	1.44	1.38
5	Genes involved in immune system	7	7	1.00	0.74
6	Ionic channels, metal metabolism, sorting proteins and transporters	26	17	1.53	2.75
7	Proto-oncogenes, tumor-related proteins, tumor suppressors	5	3	1.67	0.53
8	Hormones, receptors, and regulatory proteins	133	14	9.50	14.09
9	Development and differentiation-related proteins	15	9	1.67	1.59
10	Stress induced proteins	3	2	1.50	0.32
11	Genes involved in lipid metabolism	1	1	1.00	0.11
12	Genes homologous to human mental disease-related genes	1	1	1.00	0.11
13	Genes homologous to sequences of unknown functions	341	329	1.04	36.12
14	Mitochondrial genes	161	10	16.10	17.06
15	Other genes	16	3	5.33	1.69
Subtotal		802	461	1.74	84.96
Unknown clones		142	—	—	15.04
Total		944	—	—	—

2.2 Functional categorization of the known genes

The 461 known genes were categorized into 15 groups according to categories described previously [4]. Among the known genes, the genes homologous to known sequences of unknown functions are the largest group, which accounts for 36.12%. The following groups are: 22 in genes involved in the protein translation machinery (16.7%); 12 in cellular structural genes (9.1%); 22 in enzymes (16.7%); 9 in transcriptional factors, DNA repair andDNA-binding proteins (6.8%); 7 in genes involved in immune systems (5.3%); 17 in ionic channels, metal metabolism, sorting proteins, and transporters (12.9%); 3 in proto-oncogenes, tumor-related proteins, tumor suppressors (2.3%); 14 in hormones, receptors, and regulatory proteins (10.6%); 9 in development and differentiation-related proteins (6.3%); 2 in stress induced proteins (1.5%); 1 in genes involved in lipid metabolism (0.8%); 1 in genes homologous to human mental disease related genes (0.8%); 10 in mitochondrial genes (7.6%) and 3 genes (2.3%) lacking enough information to be classified. A summary of the identified known function genes was shown in Tab. 2. The 132 distinct known function genes are isolated and reported for the first time in Chinese sturgeon. The cDNA sequences of known genes reported in the present study have been deposited into GenBank database at NCBI with accession numbers from EC268297 to EC268399 and EC324431 to EC324459.

2.3 Abundantly expressed genes in the pituitary

Of the 944 EST clones identified by BLASTN and BLASTX, the most abundant cDNA was POMC (Proopiomelanocortin), which was sequenced 96 times, accounting for almost 10.2% of the transcripts in the pituitary, indicating its important function in the pituitary. The top 10 abundant ESTs were: POMC (10.2%), 18s rRNA (8.4%), mitochondrion gene (5.1%), growth hormone (1.9%), AfuG microsatellite gene (1.4%), cytochrome c (1.3%), 28s rRNA (0.8%), secretograin III (0.6%), cytochrome b (0.5%), rRNA S39 (0.5%). These top 10 genes accounted for almost 30.7% of the 944 se-

quenced clones (Fig. 1). Among the top 10 most abundantly expressed genes, 3 were related to ribosomal proteins (18s rRNA, 28s rRNA, and rRNA S39 ribosomal protein).

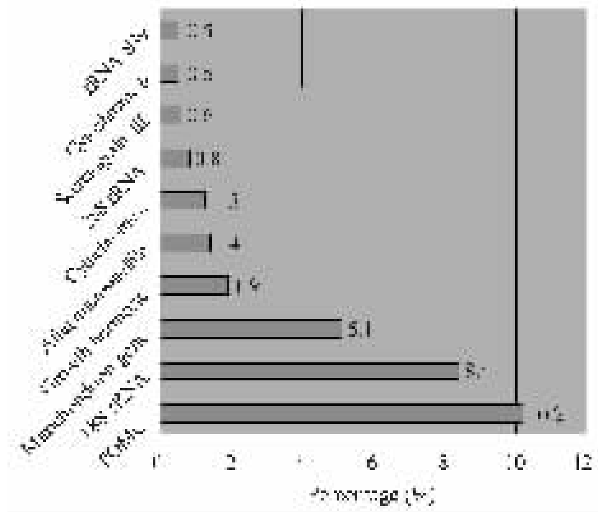

Fig. 1　Expression percentages of 10 most abundantly expressed genes in the Chinese sturgeon pituitary

2.4　Tissue distribution of 7 unknown ESTs

The cDNA sequences of 7 unknown genes have been deposited into GenBank database at NCBI with accession numbers from Eg009334 to Eg009340. To analyze the 7 unknown ESTs, 7 pairs of primers (Tab. 1) were synthesized and used to detect the tissue distribution by RT-PCR. Their RT-PCR analysis revealed 3 different categories of expression patterns. The first category includes 3 ESTs that are detected in some tissues. For example, EG009339 mRNA was expressed abundantly in pituitary and ovary, slightly in heart, liver, spleen, and muscle, and no signals were detected from other analyzed tissues (Fig. 2e). The second categories (2 ESTs) were very important and interesting because they only expressed in pituitary. As shown in Fig. 3c, the EG009337 mRNA was expressed in pituitary, and no signals can be detected in any other tissues. The third category were also very attractive, they were described as pattern of EG009338 and EG009334, which were pituitary and ovary specific gene (Fig. 3d and 3g).

3　Discussion

Expressed sequence tag (EST) analysis, which survey sequences contained in cDNA libraries, is a

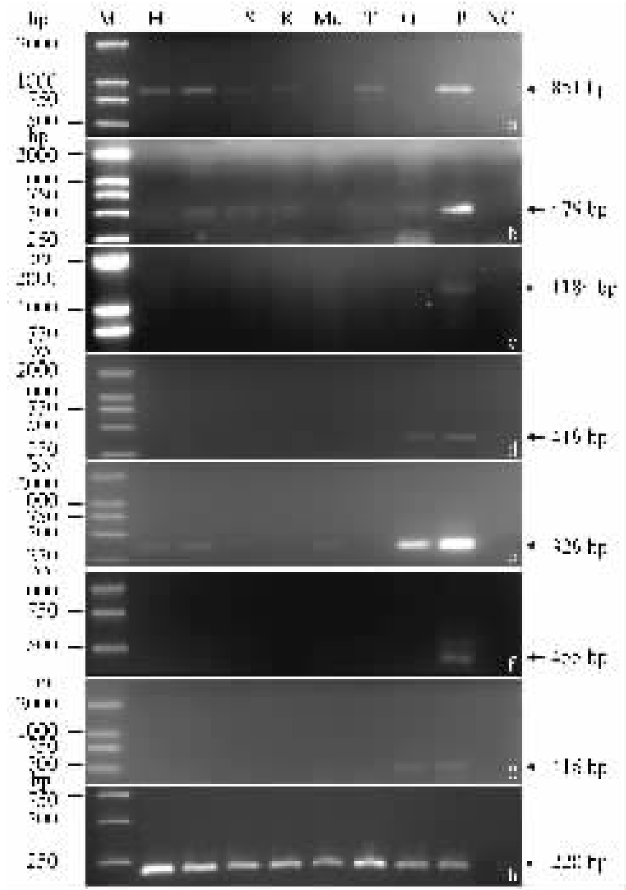

Fig. 2　RT-PCR detection of 7 typical unknown ESTs (a-g, as shown on the above) in different tissues, such as heart (H), liver (L), spleen (S), kidney (K), muscle (Mu), testis (T), ovary (O) and pituitary (P). β-actin (h) was used as control. NC: negative control of PCR. M is the 2kb DNA Ladder marker (TaKaRa). Arrows indicate the amplified fragments, and their sizes are shown on the right

powerful approach to identify new genes and profile gene expression in cells of a tissue[1-4]. In this study, we described our primary work using an EST approach for the identification of genes in the pituitary cDNA library of the 4 year-old male Chinese sturgeon. Among the 944 sequenced clones, we identified 461 known genes (802 clones) and only identified 132 known function genes from the 944 ESTs. Some reasons may be useful to explain the fact why only 132 known function genes were identified from the 944 ESTs. For example, many genes could be novel and therefore there are no orthologs existing in the GenBank, and there exists low sequence conservation among many sturgeon genes with those in the GenBank. Therefore, longer fragments should be sequenced to reveal the homologous sequences[20].

POMC (proopiomelanocortin) is the first abundantly expressed gene, which accounts for 10.17% of the Chinese sturgeon pituitary ESTs. POMC is typically composed of segments for adrenocorticotropic hormone (ACTH), lipotropic hormone (LPH) and N-terminal peptide, and each segment contains one melanophore-stimulating hormone (MSH), although a single endorphin (END) also exists in the LPH segment[29]. These segments can mediate many physiological processes such as skin coloration, steroidogenesis, energy balance and food intake[30]. Moreover, in relation to reproduction, transcriptional activation and/or repression of POMC have been reported in several studies in late gestation and during active labor in mammals[31—35]. It is generally recognized that the opioid peptides inhibit GnRH-mediated LH release and interfere with the GtH stimulatory effect on gonadal sex steroid production[36]. In our previous work of a 24 year-old female Chinese sturgeon's pituitary EST library (data not shown), the POMC is the second abundant gene which accounting for 7.26% of the whole EST library. Our result suggested that POMC should be highly expressed in the pituitary of Chinese sturgeon. It would be interesting to do further works to reveal the POMC's molecular mechanisms and its characteristic in this ancient ray-finned fish from an evolutionary point of view.

Three different categories of expression patterns were observed from 7 unknown genes, and some interested genes were revealed from the expression pattern analysis. For example, the EG009338 gene and EG009334 gene only express in the ovary and pituitary (Fig. 3 d and g); the EG009337 gene and EG009340 gene were pituitary specific genes. These suggested that they might be very important during reproduction regulation and sex differentiation. Further investigation on these genes will provide useful sight for physiological functions of pituitary in the Chinese sturgeon.

Chinese sturgeon is an endangered species which under the first rank protection in China now because some dames such as the Three Gorges Dam and the Gezhouba Dam blocked the migratory route of them. Although the success of artificial propagation based on the wild sturgeon captured just below the dam was achieved in 1983, little molecular information was known with regards to the reproduction regulation and sex differentiation. In this study, some reproduction relevant genes were identified and isolated. Further studies on their functions will be very useful for better understanding the mechanisms of its reproduction biology.

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4 龄中华鲟垂体的 EST 分析

曹 宏[1,2] 周 莉[1] 桂建芳[1]

(1. 中国科学院水生生物研究所,淡水生态与生物技术国家重点实验室,武汉 430072; 2. 中国科学院研究生院,北京 100039)

摘要:表达序列标签 (Expressed sequence tag, EST) 是鉴定基因表达规律和发现新基因的一种有效的分子生物学手段。为了能在中华鲟(*Acipenser sinensis* Gray) 中发现与生长和生殖内分泌调控相关的基因,我们构建了中华鲟垂体的 SMART cDNA 质粒文库。垂体是调节生长和生殖内分泌的重要器官。在本研究中,通过测序筛选得到了 944 个 EST 克隆,将所得 EST 与 GenBank 数据库中的序列进行比对, 结果表明,802 (84.96%) 个克隆可以找到同源序列,共代表 461 个基因, 其中含 132 个已知功能基因;而 142 (15.04%) 个克隆不能找到同源序列。研究发现,在所有基因中,阿黑皮素原基因 (Proopiomelanocortin, POMC) 是出现次数最高的基因,占总 EST 数的 10.17% , 显示出其在垂体中的重要地位。我们还发现了 7 个未知功能的基因并重点研究了其在心脏、肝脏、脾脏、肾脏、肌肉、精巢、卵巢和垂体等组织中的表达特异性。结果发现,4 个基因:EG009334、EG009337、EG009338 和 EG009340 为垂体特异性表达或垂体和卵巢特异性表达。对这些基因进一步的功能研究将有利于我们更好地了解中华鲟生长和生殖内分泌调控的分子机制。

关键词:cDNA;表达序列标签;中华鲟;垂体