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Gene Expression in the Muscles of Young and Mature Channel Catfish (*Ictalurus punctatus*) as Analyzed by Expressed Sequence Tags and Gene Filters

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To generate expressed sequence tags for genomics research involving genetic linkage analysis, to examine gene expression profiles in muscles of channel catfish in a non-normalized muscle cDNA library, to compare gene expression in young and mature channel catfish muscles using the EST reagents and gene filters to demonstrate the feasibility of functional genomics research in small laboratories. 102 randomly picked cDNA clones were analyzed from the catfish muscle cDNA library. Of the sequences generated, 90.2% of ESTs was identified as known genes by identity comparisons. These 92 clones of known gene products represent transcriptional products of 24 genes. The 10 clones of unknown gene products represent 8 genes. The major transcripts (70.1% of the analyzed ESTs) in the catfish muscle are from many major genes involved in muscle contraction, relaxation, energy metabolism and calcium binding such as alpha actin, creatine kinase, parvalbumin, myosin, troponins, and tropomyosins. Gene expression of the unique ESTs was comparatively studied in the young and adult catfish muscles. Significant differences were observed for aldolase, myostatin, myosin light chain, parvalbumin, and an unknown gene. While myosin light chain and an unknown gene (CM192) are down-regulated in the mature fish muscle, the aldolase, myostatin, and parvalbumin are significantly up-regulated in the mature fish muscle. Although the physiological significance of the changes in expression levels needs to be further addressed, this research demonstrates the feasibility and power of functional genomics in channel catfish. Channel catfish muscle gene expression profiles provide a valuable molecular muscle physiology blueprint for functional comparative genomics.

Keywords: Expressed sequence tag, Expression, Functional genomics, Mapping, Muscle

Introduction

The characteristics of an organism are determined by the genes expressed within it (Velculescu et al., 1995). Determination of the complete genomic sequences of a number of model organisms, including human, is now a real and attainable goal. However, temporal, spatial and relative abundance of gene expression needs to be determined for functional genomics. Several important advances such as serial analysis of gene expression (SAGE) (Velculescu et al., 1995) and expressed sequence tag (EST) analysis (Adams et al., 1991) have provided technological possibility for examination of gene expression at the whole genome level.

ESTs are partial cDNA sequences corresponding to mRNAs and generated from randomly selected library clones (Adams et al., 1991). ESTs are generated by a single pass sequencing at both/either the upstream and/or the downstream of cDNAs using arbitrarily selected cDNA libraries. Characterization of ESTs is a convenient and rapid way for identification of new genes in various organisms. Although it is slower than SAGE, its results are clean and easy to analyze and provide resources for development of gene chip technology (Johnston, 1998) specific to tissues, pathways, development stages, or the whole genome. EST approach is being used extensively to analyze expressed genes from organisms such as human (Adams et al., 1991; Wolfsberg and Landsman, 1997), *Caenorhabditis elegans* (Waterston et al., 1992), *Arabidopsis thaliana* (Hofte et al., 1993) *Plasmodium falciparum* (Chakrabarty et al., 1994), *Schitosoma mansoni* (Franco et al., 1995), *Entamoeba histolytica* (Azam et al., 1996), rice (Aliyeva et al.,1996), and fish (Gong et al.,1994; Inoue et al.,1997; Karsi et al.,1998). As of October 2002, database EST (dbEST) in NCBI contained 13,209,117 entries. Among the 273 species present, the most extensively studied species were human

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(42.4%) and mouse (27.7%). The number of fish ESTs in dbEST, excluding for zebrafish (1.1%), was 0.13% from channel catfish, japanese medaka, winter flounder, common carp, nile tilapia, and rainbow trout.

The EST approach is very important for generating type I markers (O'Brien, 1991; Liu et al., 1999b) for genomic research. Type I markers represent genes of known function and their mapping is essential as anchorage points for comparative gene mapping (Womack and Kata, 1995). The generation of human ESTs, for example, was a crucial step in the progress of the human genome project (Wolfsberg and Landsman, 1997), The availability of these human ESTs enabled the mapping of 16,000 genes in the human genome (Schuler et al., 1996).

ESTs are becoming important biological reagents for functional genomics such as gene chip or microarray technology. The most attractive aspect of functional genomics is its ability to determine gene expression changes of tens of thousands of genes in a complete view simultaneously.

We previously analyzed EST profiles in the channel catfish pituitary cDNA library (Karsi et al., 1998) and developed 11 polymorphic EST markers suitable for genetic linkage analysis using channel catfish (*Ictalurus punctatus*) and blue catfish (*Ictalurus furcatus*) hybrid system (Liu et al., 1999c). In this report, we present data from EST analysis of the channel catfish muscle cDNA library. Expression of the 32 genes in the young fish (9 months old) and in mature fish (four years old) are compared using a gene filter approach, demonstrating dramatic power for analysis of gene expression and functional genomics if the bio-reagents such as large numbers of ESTs are available.

Materials and Methods

RNA isolation

All experimental catfish (*Ictalurus punctatus*) were raised in troughs placed inside the hatchery of the Auburn University Fish Genetics Facility under the same conditions for 4 weeks before the tissues were harvested. Muscle tissues were collected from 5 young (9- months old) and 5 mature (4- years old) individuals. Approximately, equal weight of muscles was collected from each individual to have an averaged sample of the age groups and to avoid any unexpected variation in any single individual. Equal amount of muscles from all 5 fish was used for preparation of total RNA. Muscle tissues were frozen in liquid nitrogen for grinding with a mortar/pestle and then homogenized with a hand-hold tissue tearor (Model 985-370, Biospec Products, Inc., WI, USA) in RNA extraction buffer following the guanidium thiocyanate method (Chomczynski and Sacchi 1987). After preparation of the total RNA from the young and mature fish, the RNAs were labeled and stored separately. To make the cDNA library, equal amount of RNA from both age groups was mixed for preparation of poly $(A)^+$ RNA. Poly $(A)^+$ RNA was then purified by using the Oligotex Spin Column Kit from Qiagen according to manufacture's instructions (Qiagen Inc., Chatsworth, CA, USA).

Preparation of channel catfish muscle cDNA library

The muscle cDNA library was prepared from the poly $(A)^+$ RNA using λ Unizap cloning vector according to manufacturer's instructions (Stratagene, CA, USA). The primary cDNA libraries had a titer of 2.6×10^6 (pfu/ml) recombinant clones with a blue background of less than 0.3%. The primary cDNA libraries were then amplified once. The final library had a titer of about one billion plaques per milliliter.

The cDNA libraries prepared in λ Unizap vectors allow the conversion of the λ libraries into plasmid libraries by using the mass *in vivo* excision procedure (Stratagene, CA, USA). Briefly, 20 million plaque forming units (pfu) were used for each library representing clones equivalent to 10 initial cDNA libraries before amplification (primary recombinant clones). The XL1-blue plating bacteria were infected with 20 million phage (10 cells : 1 phage), and also infected with the ExAssist helper phage at 1:1 helper phage-to-cell ratio. Cells were incubated at 37°C for phage absorption. The infected cells were then grown in 20 ml LB broth for 3 hours with shaking at 37°C. During this time, phagemids were excised and secreted into medium. The cells were then heated to 70°C for 20 minutes and removed by centrifugation. The excised phagemids in the supernatant were titered. Any given number of clones can be grown on plates using the titer of the phagemids and SOLR strain of *E. coli* (Stratagene, CA, USA), which can only be infected by the phagemids, not by the λ phage. SOLR cells were infected with desired numbers of phagemids and incubated at 37°C for 15 min. The cells were then plated onto LB plates containing ampicillin at 50 µg/ml. Colonies were picked and cultures were grown in LB liquid culture for plasmid preparation.

Plasmid preparation and sequencing analysis

Plasmid DNA was prepared by using the Qiagen Spin Column Mini-plasmid kit. Since the direction of cDNAs in the

Unizap vector is fixed, the direction of cDNAs in the plasmid vector is also fixed so that one knows which portion of the cDNA is sequenced with both the universal and reverse sequencing primers. Most cDNA clones were manually sequenced using the Sanger's dideoxy termination method (Sanger et al., 1977) with ABI prism BigDye^{TM} primer cycle sequencing kit (Applied Biosystems, Inc., CA, USA) until acquisition of the LI-COR automated sequencer, with which some clones were sequenced. Mini-preparation plasmid DNA (1 μl, about 200-500 ng) was used for all sequencing reactions. The profiles for cycling were: 94°C for 1 min., 55°C for 1 min, 72°C for 1 min. for 30 cycles. An initial two minutes of extra denaturation at 94°C were always used. DNA sequences were read and analyzed by using micro-computer software packages DNasis (Hitachi, version 2.0, Japan) and DNASTAR (DNA Star Inc., version 5.0 Madison, WI, USA). For most clones, readable sequences of about 350 bases were generated from a single pass sequencing using manual sequencing or at least 550 bases with the automated sequencer. Vector sequences were removed before searching for homologies using BLASTN and BLASTX servers, through Internet (NCBI, Bethesda, MD, USA). Matches were considered to be significant only when the probability (P) was less than 0.0001 and scores were greater than 160 for BLASTN and greater than 80 for BLASTX.

First-strand cDNA probe preparation

First-strand cDNA probes were made from Poly(A)$^+$ RNA or total RNA isolated from muscle tissues of the young (9 month old) and mature fish (4 year old). The probes were made in a typical reaction of reverse transcription using the MMLV reverse transcriptase (Life technologies, Inc., Bethesda, MD, USA). A typical reaction of 50 μl is composed of the following: 200 ng mRNA (or 10 μg total RNA), 1X first-strand buffer (Life Technologies, Inc., Bethesda, MD, USA), 20 units of RNase Block Ribonuclease Inhibitor (Stratagene, CA, USA), 500 ng of synthetic oligo $(dT)_{15}$ primer, 0.4 mM each of dATP, dGTP, dTTP; 50 μCi P^{32}-dCTP; and 40 units of reverse transcriptase. The reaction was incubated at 37°C for 1 hr. After completion of the reaction, the probes were cleaned by passing through a Sephadex G50 spin column (Sambrook et al., 1989). The probes were denatured by boiling for 5 min before being used for hybridization.

Dot blot analysis

After sequencing analysis, unique EST clones were used for dot blot analysis of gene expression. Equal amount of plasmid DNA (1 μg) was used from each clone for preparation of four sets of replicate "gene filters". The dot blot analysis was conducted as previously described (Liu et al., 1999a). Briefly, the plasmid DNA was denatured by mixing with an equal volume of 1 M sodium hydroxide and kept at room temperature for 5 min. An equal volume of 2 M ammonium acetate was added and vortexed to neutralize the samples. The samples were then loaded onto a nylon membrane by using a dot blot apparatus (BRL, Bethesda, MD, USA). One milliliter of 5X SSC was then added to each sample spot. The filter was removed and air dried before baking at 80°C for two hours prior to hybridization. Hybridization was conducted under the standard protocols (Sambrook et al., 1989). The hybridization signals were quantified by using the GS-525 Molecular Imaging System (BioRad, New York, USA) as described (Liu et al., 1997).

One set of filters was also stripped after hybridization for re-use in hybridization to the alternative probe. Filters were boiled in 0.5% SDS for 10 min. A Geiger counter was used to assist the process. The stripped filters were exposed to X-ray films overnight to assure complete removal of the probes before being used for the second hybridization. The stripped filter was pre-hybridized and then hybridized to the alternative probes.

Statistics analysis

All data were analyzed using SAS 6.12 (1996). For the genes that were expressed differentially in the young fish and the mature fish, the average values of hybridization signal (counts per minute) were compared and analyzed by paired T-test.

Results and Discussion

Known genes and novel genes from the catfish muscle expressed sequence tags

Of the 102 clones sequenced, 92 clones were from known genes and 10 clones from unknown genes. The high proportion of known ESTs is related to the highly specialized functions of the muscle and the fact that a few major transcripts account for the large percentage of the sequenced muscle ESTs. Multiple clones were sequenced for 14 genes ranging from 2-17 clones per gene (Table 1). Thus, the 92 known ESTs represent gene products from 24 genes and the 10 clones of unknown genes represented 8 different genes. The

Table 1. ESTs of the channel catfish muscle

Clone #	Accession number	Most homo logous to	Putative identification	Overlap	Probability	Number of gene
CM177	AF227793	AF055289	Creatine kinase	1226	0	17
CM160	AF227795	X97824	Parvalbumin beta	386	3e-28	15
CM194	AF227796	D50028	Alpha actin	503	0e-164	13
CM224	AF227797	AF081462	Myosin light chain 2	533	0	13
CM169	AF227798	M24635	Alpha tropomyosin	563	1e-162	4
CM188	AF227799	D85139	Myosin light chain 1a	255	2e-28	3
CM204	AF227800	AF072687	Troponin T	585	1e-74	4
CM185	AF227801	AB003078	Troponin C	308	4e-53	2
CM168	AF227802	AJ238272	Ribosomal protein S24	371	1e-57	2
CM083	AF227803	M29033	Ribosomal protein L5b	333	4e-21	2
CM173	AF227804	X16504	Muscle specific enolase	183	2e-31	2
CM304	AF227805	U75686	Polyadenylate binding protein	226	6e-55	1
CM317	AF227806	AF019626	Myostatin	752	0	2
CM320	AF227807	Z93780	Carbamoyl phosphate synthase	64	6e-16	1
CM190	AF227808	AB004737	Alpha-globin	59	8e-06	1
CM321	AF227809	M11941	Triosephosphate isomerase	85	9e-12	1
CM174	AF227810	AJ006883	Glyceraldehyde-3-phosphate-dehydrogenase	322	8e-62	1
CM176	AF227811	NC000860	Mitochondrion	369	3e-42	1
CM163	AF227812	AJ133768	ZASP protein	114	6e-16	1
CM164	AF227813	NM005165	Aldolase C	267	2e-34	1
CM005	AF227814	AF165216	Tropomodulin	549	3e-56	1
CM084	AF227815	AB003335	Y-box binding protein 1	501	1e-79	2
CM329	AF227816	AF074720	Ribosomal protein L17	341	3e-95	1
CM170	AF227817	D16478	Mitochondrial long-chain enoyl-CoA hydratase	97	2e-06	1
CM191	AF227818		Unknown			1
CM162	AF227819		Unknown			1
CM192	AF227820		Unknown			3
CM233	AF227821		Unknown			1
CM310	AF227822		Unknown			1
CM236	AF227823		Unknown			1
CM318	AF227824		Unknown			1
CM319	AF227825		Unknown			1
Total						102

abundance of the major transcripts in the muscle tissue necessitates the construction of normalized cDNA library for discovery of the catfish genes and for cataloguing the ESTs for functional genomics study.

Most abundantly expressed muscle genes

The expression profiles as revealed by the EST analysis are summarized in Fig. 1. Clearly, the genes involved in muscle contraction and relaxation accounts for the major gene expression activities in the muscle. Over 70.1% of ESTs were from genes involved in muscle movement, energy metabolism, and calcium binding. These genes include parvalbumin, creatine kinase (which is an enzyme, but are here categorized as a protein of muscle contraction because its main function is in energy metabolism for the muscle), alpha actin, myosin, tropomysin, troponin, and tropomodulin. The enzymes account for 6.9%, ribosomal proteins for 4.9%, and regulatory proteins for 4.9%. With exception of 9.8% of ESTs, whose identity and functions are not known, proteins categorized as "other proteins" (not included in the categories of muscle movement, enzymes, regulatory, and ribosomal proteins) accounts for only 2.9% of the major transcriptional activity. The total number of genes in the human is believed to be approximately 26,000-38,000 and that of a fruit fly is about 13,600 (Paabo, 2001). In any one cell type, perhaps 10,000 genes are expressed at levels of from 200,000 copies to 1

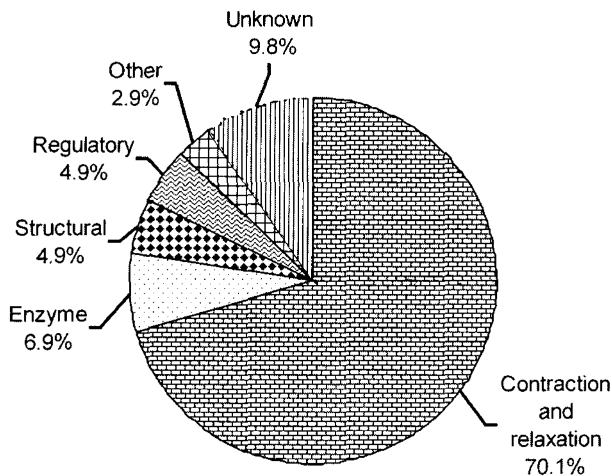

Fig. 1. Gene expression profiles in the channel catfish muscle as revealed by expressed sequence tag (EST) analysis.

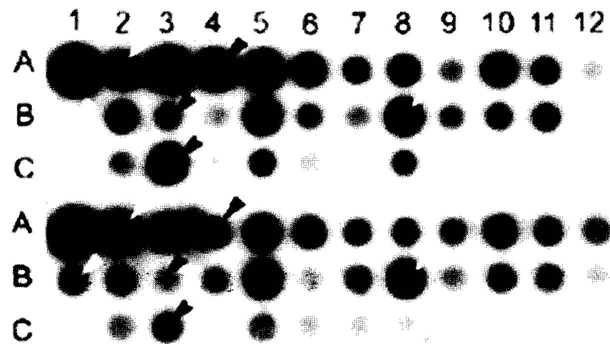

Fig. 2. Dot blot analysis of gene expression in the catfish muscle tissues of 9- month old and 4-year old individuals. Plasmid DNA containing various cDNAs was immobilized onto a nylon membrane and hybridized to the first-strand cDNA probes from young (upper) and mature (lower) individuals. Genes represented by the dots are: A1, creatine kinase; A2, parvalbumin beta; A3, α-actin; A4, myosin light chain; A5, α-tropomyosin; A6, myosin light chain 1; A7, troponin T; A8, troponin C; A9, ribosomal protein S19; A10, ribosomal protein L5b; A11, muscle specific enolase; A12, polyadenylate binding protein; B1, myostatin; B2, carbamoyl phosphate synthase III; B3, alpha globin; B4, triosephosphate isomerase; B5, glyceraldehyde-3-phosphate dehydrogenase; B6, mitochondion protein; B7, KIAA0613 protein; B8, aldolase; B9, tropomodulin; B10, Y-box-binding protein 1; B11, ribosomal protein L17; B12, mitochondrion long chain enoyl ; C1-C8: all are unknown genes: C1: CM162; C2, CM191; C3, CM192; C4, CM233; C5, CM236; C6, CM310; C6, CM318; C8, CM319. White arrows indicate genes that were expressed at significantly higher levels in the mature fish than in the young fish; black arrows indicate genes that were expressed at significantly lower levels in the mature fish than in the young fish.

copy or less per cell (Patanjali et al., 1991). In the catfish, a few dozen of genes accounts for the major transcriptional activities of the muscle. Clearly, the transcriptional activities are extremely polarized in the specialized muscle tissue.

Among these major transcripts, creatine kinase, parvalbumin beta, skeletal alpha actin, myosin, tropomyosins and troponins are among the most abundantly expressed genes (Table 1). The expression of creatine kinase was the highest (16.6%), followed by parvalbumin (14.7%), and skeletal alpha actin (12.7%). The large proportion of these 3 genes of the muscle transcriptional activity is consistent with their relative importance in muscle functions of contraction and relaxation. Creatine kinase plays a key role in the energy metabolism. Three types of creatine kinase have been identified in vertebrate (e.g., Sun et al., 1998; for review see: Willimann, 1994): M creatine kinase (M-CK), B creatine kinase (B-CK), and mitochondrial creatine kinase. M-CK is expressed in muscles and B-CK is expressed in brain. The CK cDNAs sequenced for the catfish muscles are thus M-CK cDNAs.

The gene expression profiles in the muscles provide valuable information for the strength of promoters involved in transcription of these genes although gene copy numbers are not known. Assuming a single copy gene is responsible for the transcription of creatine kinase, its strong promoter may be useful for purposes of driving strong, muscle-specific gene expression in transgenic studies. Similarly, promoters of alpha actin and parvalbumin may also be valuable resources for genetic engineering.

One of the advantages of EST analysis using non-normalized libraries is its ability to produce expression profiles. The assumption is that the frequency of cDNAs in a cDNA library is a reflection of mRNA abundance in the mRNA pool. This has been demonstrated for catfish pituitary genes (Karsi et al., 1998). If the EST analysis is for the purpose of EST cataloguing for the development of bio-reagents, repeated sequencing of highly expressed genes is not desirable. Normalized cDNA libraries (Patanjali et al., 1991; Sasaki et al., 1994) are therefore very much required for characterization of large numbers of unique ESTs. Construction of normalized cDNA libraries of catfish is underway in our laboratory.

Gene expression in muscle tissues at different ages

To demonstrate the feasibility of functional genomics studies in small laboratories like ours using "gene filters, gene expression was analyzed through RNAs isolated from young fish (9-month) and mature fish (4-year old). Plasmid DNA containing unique ESTs was spotted to two sets of nylon membranes and hybridized to the first-strand cDNA probes. Here the target plasmid DNA (1 μg) was in excess for the probe. The hybridization signal should depend on the mRNA concentration of the gene. As shown in Fig. 2, many differences can be detected in terms of levels of gene expression in

Table 2. Genes expressed at significantly different levels in the young and mature catfish muscles

Gene identity	Expression in young (9- month)(%)	Expression in mature (4- year)(%)	Change	P
Globin	0.73	0.09	7.8x down	0.0077
Myosin light chain	2.57	0.45	5.6x down	0.0053
Parvalbumin	0.57	2.23	3.9x up	0.0075
Myostatin	0.34	1.05	3.1x up	0.0089
Aldolase	1.67	4.03	2.41x up	0.0025
CM192 (unknown gene)	6.74	1.78	3.8x down	3.6E-5

the 2 age groups. Initially, the experiments were conducted by stripping away the first-strand cDNA probe made from the muscle RNA of the young fish and re-probed with the first-strand cDNA probe made from the muscle RNA of the mature fish. To assure that the differences in radioactive signal were from the relative proportion of the probe due to its mRNA concentration in the RNA pool, but not from any errors made during the stripping process, four sets of replicate filters (8 filters) were prepared and hybridized to the two probes, respectively. The same results were obtained. Four replicates were conducted and the consistent differences of signals were confirmed (not shown).

Several genes showed significant levels of difference in their transcriptional levels at the 2 ages. Among many noticeable differences, parvalbumin, myosin light chain, myostatin, and aldolase showed significant differences at the two ages (P<0.01). Parvalbumin (3.9 X), myostatin (3.1 X), and alsolase (2.4 X) are up-regulated in mature fish, while myosin light chain (5.6 X) and an unknown gene (MC192, 3.8 X) are down-regulated in mature fish (Table 2). Additionally, globin gene representation in the mature fish muscle was 7.8 times less than in the muscle of the young fish. Globin gene expression is specific to the blood cells. Therefore, the globin ESTs must have been from blood contamination in the muscle tissues. The noticed difference in globin representation in the 2 age groups may be because the blood circulation is higher in the muscle tissues of the young fish than in the mature fish.

The observed up-regulation of aldolase is consistent with the developmental regulation of the muscle-specific aldolase gene. Skeletal muscles undergo important modifications leading to the appearance of different types of myofibers that exhibit distinct contractile and metabolic properties. This maturation process results from the activation of the expression of different sets of contractile proteins and metabolic enzymes, which are specific to the different types of myofibers. The muscle-specific aldolase is expressed mainly in fast-twitch glycolytic fibers in adult body muscles (Spitz et al., 1999). At the transcriptional level, this up-regulation is controlled by a nuclear receptor-binding element in the aldolase promoter (Spitz et al., 1999). The increased expression of myostatin in mature fish muscle is expected because its main function is to inhibit muscle growth (McPherron et al., 1997).

These "gene filter" studies demonstrate that gene expression can be determined using a "bulk" genomics approach. Four replicates and statistic analysis indicated that gene expression levels revealed by gene filter analysis were highly reproducible. Whole genomic signatures of gene expression, however, depend on development of great numbers of ESTs.

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