*Biology Bulletin, Vol. 32, No. 5, 2005, pp. 473–477. Translated from Izvestiya Akademii Nauk, Seriya Biologicheskaya, No. 5, 2005, pp. 573–577.*


**ANIMAL PHYSIOLOGY**

# Ontogenetic and Phylogenetic Analysis of Myosin Light Chain Proteins from Skeletal Muscles of Loach *Misgurnus fossilis*

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Received April 20, 2005

**Abstract**—mRNAs of all three types of myosin light chain proteins are expressed in skeletal muscles of both larval and adult stages of loach *Misgurnus fossilis* (Cobitidae) and these proteins are encoded by different genes (*mlc1*, *mlc2*, and *mlc3*). No difference was revealed between transcripts from larval stage and adult fish for all three MLC proteins. Our approach (RT-PCR with fish-specific *mlc1*, *mlc2,* and *mlc3* primers) failed to reveal the larval form of myosin light chain protein found previously by protein electrophoresis of loach fry muscle extract. Comparative analysis of the protein structure shows high homology of MLC1 and MLC3 proteins sharing a large EF-hand calcium-binding domain. Phylogenetic analysis of MLC1 from skeletal muscles of fish and other vertebrate species is concordant with the traditional phylogeny of the group. Within the Teleostei, loach MLC1 had the highest homology with other Cyprinidae, and least with Salmonidae fishes.

Analysis of gene and protein expression in the development of the muscular system includes two significant aspects. First, stage-specific embryonic, larval (or neonatal), and definitive contractile proteins are expressed from different genes in the course of myogenesis (Whalen *et al.*, 1978, 1981; Butler-Browne and Whalen, 1984; Holland *et al.*, 1995; Moutou *et al.*, 2001; Thiebaud *et al.*, 2001). Second, the genetic control of the synthesis of contractile proteins is achieved by different mechanisms in different animals: the proteins can be encoded by different genes or their isoforms are generated by alternative splicing (Rozek and Davidson, 1986; Nareiko, 1988; Nareiko and Ozernyuk; 1988; George *et al.*, 1989; Collier *et al.*, 1990; Focant *et al.*, 1992; Hirayama *et al.*, 1997; Ozernyuk, 1998, 2004; Moutou *et al.*, 2001; Thiebaud *et al.*, 2001; Ozernyuk *et al.*, 2004).

The control mechanisms of synthesis of contractile proteins are extensively studied using myosin proteins as a model. It was shown that myofibrillar isoforms of myosin heavy chain (MHC) are encoded by different genes in most animals but are generated by alternative splicing in *Drosophila* (Rozek and Davidson, 1986; George *et al.*, 1989; Collier *et al.*, 1990). Transcription regulation of myosin light chains (MLCs) also varies between animals. In lower vertebrates, MLCs are encoded by different genes, while they are synthesized both from different genes and by alternative splicing of the same transcript in higher vertebrates (Weeds and Lowley, 1971; Frank and Weeds, 1974; Matsuda *et al.*, 1981; Hirayama *et al.*, 1997; Xu *et al.*, 1999, 2000; Moutou *et al.*, 2001; Thiebaud *et al.*, 2001).

Specific embryonic, larval, and definitive MLC isoforms have been revealed in fish myofibrils previously (Nareiko, 1988; Focant *et al.*, 1992). In particular, three MLC types of loach skeletal muscle (alkaline MLC1 and MLC3 and regulatory DTNB–MLC2) are represented by proteins with molecular weights of 37 (MLC1), 26 (MLC2), and 17 kDa (MLC3) as well as by larva-specific MLC with molecular weight of 15.7 kDa (Nareiko, 1988; Nareiko and Ozernyuk, 1988). The problem of specific genetic control of MLC expression in fish seems substantial. In this context, this work studied specific expression of the genes encoding individual MLC isoforms in skeletal muscle of loach larvae and adults and comparatively analyzed cDNA sequences encoding these isoforms.

## MATERIALS AND METHODS

Experiments were carried out on larvae and adults of loach *Misgurnus fossilis.* White (fast) skeletal muscles of adults as well as muscles of 7-day-old larvae (the stage of transition to external feeding; eyes and integuments are pigmented; body length is 7–7.3 mm) were used.

Total RNA was isolated from muscle tissue by the guanidinium thiocyanate method using RNAzol (Cinna/Biotecx, United States) (Puissant and Houdebine, 1991). The mRNA fraction was isolated from the total RNA preparation using the Dynabeads mRNA Purification Kit (Dynal, Norway).

The isolated mRNA was used for cDNA synthesis. The first-strand cDNA was synthesized on the mRNA template from the muscle using the SuperScript reverse

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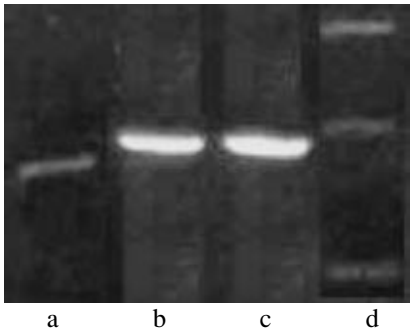


**Fig. 1.** Expression of genes *mlc1* (a), *mlc2* (b), and *mlc3* (c) from skeletal muscle of loach larvae; (d) molecular weight marker.

transcriptase and oligo(dT)$_{12–18}$ or random primers according to the manufacturer's instructions (Gibco BRL). The cDNA libraries were used in polymerase chain reaction (PCR) to produce the fragments informative about the structure of MLC genes.

PCR was carried out using a Taq-polymerase kit (Sileks M, Russia) on an OmniGene Thermal Cycler (Hybaid, United Kingdom). The durations, temperatures, and numbers of the reaction cycles depended on the size and nucleotide composition of the synthesized fragment. The primers were designed from the conserved DNA sequences of *mlc1, mlc2*, and *mlc3* available for carp and zebrafish. The cDNA sequences of carp were used (Hirayama *et al.*, 1997):

MLC1 direct, AAGCTCGATTTCACCCAGGAC;

reverse, CATAGTTGACACAGCCGTTTTC;

MLC2 direct, CCAAGAGGAGGGCAGGAG;

reverse, GTGTGATGACGTAGCAAATGTCTC;

MLC3 direct, CTCTGCTGACCAGATTGAGG;

reverse, CTCTCAGCGGACGGTTTCTTA.

PCR products were analyzed on a 1.5% agarose gel run using Tris-acetate buffer (Sambrook *et al.*, 1989). PCR fragments were eluted from agarose using a Geneclean Kit (Bio 101, United States) or a Kit for DNA Extraction from Agarose with Magnetic Particles (Sileks M, Russia). Sequencing was performed on an ABI 3100 sequencer using the PCR primers and a Big-Dye Kit v.1.1. Sequence fragments were merged and multiple alignments were generated using the DNAStar package (Lasergene, United States). The obtained sequences were deposited in GenBank under accession numbers DQ068406–DQ068408.

## RESULTS

MLC expression was studied by PCR analysis of cDNA from white (fast) skeletal muscle of adult loach in comparison with the expression pattern in larval muscle. The primers for PCR were constructed from cDNA structures of all three MLC types from white skeletal muscle of carp (Hirayama *et al.*, 1997). Our experiments demonstrated that all MLC types (MLC1, MLC2, and MLC3) encoded by different genes (*mlc1, mlc2*, and *mlc3*) were expressed in muscles of adult loach.

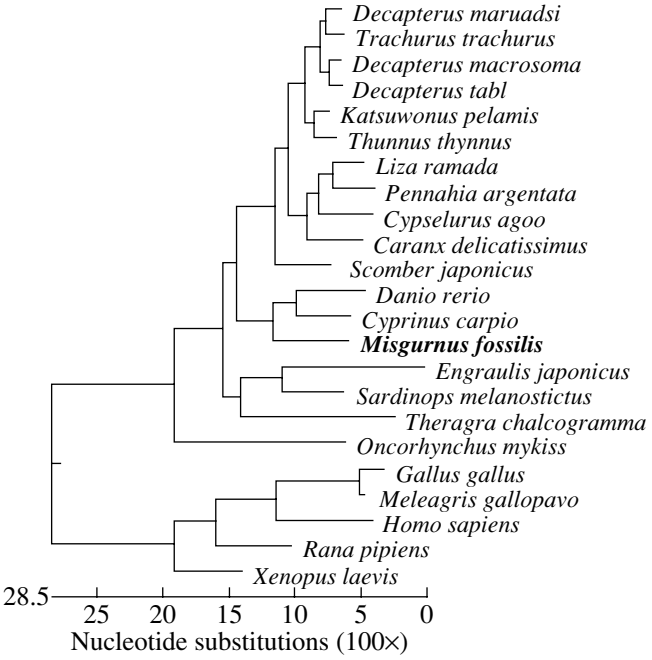


**Fig. 2.** Phylogenetic reconstruction based on amino acid sequence of MLC1 from muscle of vertebrates.

For comparison of MLC expression at different stages of myogenesis, PCR analysis of cDNA from larval muscle was carried out with the primers used in the PCR analysis of MLC expression in skeletal muscle of adult loach. Similar to adult muscles, all three MLC types were expressed in myofibrils of the larvae (Fig. 1). This approach failed to detect the gene encoding the larva-specific MLC that has been revealed in this fish species previously (Nareiko, 1988).

In order to determine structural features of *mlc* genes from larval and adult muscles of loach, the corresponding cDNAs were sequenced. Sequence analysis demonstrated that the obtained PCR fragments are *mlc1, mlc2*, and *mlc3* genes. No differences were revealed in cDNA sequences of these genes in the larval and adult muscles.

The deduced amino acid sequences of MLC1, MLC2, and MLC3 from muscles of adult loach were also compared. A similarity was revealed for MLC1 and MLC3 within the C-terminus and region of EF-hand calcium-binding domain. The degree of similarity between MLC1 and MLC3 was 77.3%, while it was much lower between MLC2 and MLC1 or MLC3.

MLC structure was also comparatively analyzed in various fish, amphibian, and bird species as well as humans (by the example of MLC1) as shown in Fig. 2 and table. The degree of similarity between the compared MLCs considerably varied. Loach (Cobitidae) MLC1 showed the highest homology to MLC1 in representatives of Cyprinidae, zebrafish and carp, both of which belong to the order Cypriniformes. A lower homology was observed for representatives of other fish orders, Clupeiformes (Carangidae and Scombridae), and the lowest homology was observed for Salmoniformes (Salmonidae).

Amino acid sequences of MLC1 used in phylogenetic analysis

| Species | GenBank accession number | Reference |
|---|---|---|
| **Fish** | | |
| *Decapterus tabl* | AB072808 | GenBank |
| *Decapterus macrosoma* | AB072806 | GenBank |
| *Decapterus maruadsi* | AB072803 | GenBank |
| *Scomber japonicus* | AB072811 | GenBank |
| *Euthynnus pelamis* | AB042037 | GenBank |
| *Thunnus thynnus* | AB042034 | GenBank |
| *Trachurus trachurus* | AB042046 | GenBank |
| *Theragra chalcogramma* | AB042054 | GenBank |
| *Engraulis japonicus* | AB042052 | GenBank |
| *Sardinops melanostictus* | AB04209 | GenBank |
| *Cypselurus agoo* | AB042043 | GenBank |
| *Pennahia argentata* | AB042040 | GenBank |
| *Caranx delicatissimus* | AB072800 | GenBank |
| *Oncorhynchus mykiss* | AF330140 | Thiebaud *et al.*, 2001 |
| *Tetraodon nigroviridis* | CR732903 | Genoscope database (http://www.genoscope.cns.fr) |
| *Misgurnus fossilis* | | This work |
| **Amphibians** | | |
| *Xenopus laevis* | AAH41529 | Klein *et al*., 2002 |
| *Rana pipiens* | AAU86906 | Robinson *et al*., 2005 |
| **Birds** | | |
| *Gallus gallus* | NM_205479 | Nabeshima *et al*., 1988 |
| *Meleagris gallopavo* | AAR85986 | Chaves *et al*., unpublished |
| **Mammals** | | |
| *Homo sapiens* | BC005318 | Strausberg *et al*., 2002 |

## DISCUSSION

The most substantial features of biochemical differentiation of the muscular system are associated with the presence of specific embryonic, larval, and definitive forms of contractile proteins as well as with the functioning of different mechanisms of genetic control of muscle protein expression in different animals. These properties have been studied most extensively for myosin molecules. Embryonic and larval (neonatal) forms of myosin light and heavy chains were first found in birds and mammals (Whalen *et al.*, 1978, 1981; Hoh and Yeoh, 1979; Gauthier *et al.*, 1982; Lowey *et al.*, 1982; Takano-Ohmuro *et al.*, 1982; Crow *et al.*, 1983; Butler-Browne and Whalen, 1984). Later these myosin forms were revealed in lower vertebrates including fishes (Nareiko, 1988; Focant *et al.*, 1992). These isoforms are present together with the definitive ones in the embryonic and larval myofibrils.

The problem of genes encoding the embryonic and larval isoforms of myosin, and MLC genes in particular, is substantial. In this work we used primers designed from cDNA sequences of all three MLC types from definitive white skeletal muscle of carp (Hirayama *et al.*, 1997). At the same time, larval muscles of loach proved to have transcripts specific for MLC1, MLC2, and MLC3 while the templates for the synthesis of the larval MLC have not been revealed using this approach.

An important feature of contractile proteins is different mechanisms controlling their expression in different animals. For instance, the control of MLC expression essentially differs between lower and higher vertebrates. The mechanism of MLC synthesis was initially studied in higher vertebrates (Weeds and Lowey, 1971; Frank and Weeds, 1974; Matsuda *et al.*, 1981). In these animals, MLC1 and MLC3 are formed by alternative splicing. Primary structure analysis of these isoforms demonstrated that MLC1 and MLC3 include 190 and 150 amino acids, respectively. The sequence of C-terminal 141-amino acids proved identical in these MLCs, while the N-terminal part differed, which was considered as the result of alternative splicing (Weeds and Lowey, 1971; Frank and Weeds, 1974).

Another mechanism of MLC synthesis was revealed in lower vertebrates and fish, in particular. In this work, all three MLC types proved to be encoded by different genes. The expression of MLC types from different genes was also demonstrated for white skeletal muscle of other fish species, carp *Cyprinus carpio* (Hirayama *et al.*, 1997), rainbow trout *Oncorhynchus mykiss* (Thiebaud *et al.*, 2001), and gilthead seabream *Sparus aurata* (Moutou *et al.*, 2001). Hence, all three MLC types are encoded by independent genes in fish.

Primary structure analysis of MLCs from skeletal muscle of adult and larval loaches made possible a number of important conclusions. First of all, the obtained PCR fragments represent the genes of MLC1, MLC2, and MLC3. The primary structure of each MLC type proved identical in the skeletal muscle of the larvae and adult fish. The nucleotide sequence encoding the specific larval MLC, previously revealed in larval muscle of loach (Nareiko, 1988), has not been revealed

by the primers used in this work. Thus, the proposal of high similarity between the larval MLC and MLC1, MLC2, or MLC3 (as well as of its being a product of alternative splicing of one of these genes) has not been confirmed.

Comparative analysis of the primary structure of MLCs from the skeletal muscle of loach demonstrated a similarity between MLC1 and MLC3 resulting from the presence of an EF-hand calcium-binding domain. High conservation of these structures in most calcium-binding proteins seems natural considering the function of this domain.

Comparative analysis of MLC1 gene structure from various fish, amphibian, and bird species as well as humans demonstrated the correspondence of the sequence similarity with the accepted taxonomy of these animal groups. Among fish species, loach MLC1 showed the highest and lowest homology to MLC1 in representatives of Cypriniformes and Salmoniformes, respectively.

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