

Myosin Regulatory Light Chain Expression in Trout Muscle

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ABSTRACT Muscle's contractile properties can vary along different trajectories, including between muscle fiber types, along the body (within a muscle fiber type), and between developmental stages. This study explores the role of the regulatory myosin light chain (MLC2) in modulating contractile properties in rainbow trout myotomal muscle. Rainbow trout show longitudinal variations in muscle activation and relaxation, with faster contractile properties in the anterior myotome. The expression of two muscle proteins, troponin T and parvalbumin, vary along the length of trout in concert with shifts in muscle activation and relaxation. However, there is no longitudinal variation in myosin heavy chain in trout. This study explores the role of MLC2 (or regulatory light chain), part of the myosin hexamer, in contributing to longitudinal variations in contractile properties of trout swimming muscle. We cloned and sequenced two isoforms of MLC2 from trout muscle and used real-time quantitative polymerase chain reaction to assess the relative expression of these two isoforms in red and white muscle from different body positions of two ages of rainbow trout: parr and smolt. Longitudinal variations in slow (sMLC2) but not fast (fMLC2) regulatory light chain isoforms were observed in young trout parr but not older trout smolts. The differences in sMLC2 expression correlated with shifts in muscle contractile properties in the parr. *J. Exp. Zool.* 309A:64–72, 2008. © 2007 Wiley-Liss, Inc.

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The fish myotome is composed of two main muscle fiber types. White, glycolytic, muscle comprises the bulk of the myotome (upwards of 90 +%) and is recruited to power relatively fast swimming bouts (Rome et al., '84). Superficial, longitudinal bands of red or oxidative muscle are recruited for slow, steady swimming (Coughlin et al., 2004). These two muscle fiber types differ in terms of protein composition. Slow isoforms of most myofibrillar proteins are found in the red (slow-twitch) muscle, whereas fast isoforms are found in white (fast-twitch) muscle. The thick filament of skeletal muscle is composed primarily of the hexameric protein myosin. Myosin consists of four myosin light chain (MLC) and two myosin heavy chain (MHC) subunits that are the primary determinants of contractile performance (Moss et al., '95). The thin filament is composed of several different proteins, such as the troponin complex, tropomyosin, and actin. MHC, MLCs 1 and 3 (essential light chains), and MLC2 (regulatory light chain) are all expressed in slow and fast forms in vertebrate skeletal muscle (Moss et al., '95).

Thin filament proteins, such as troponin T, also exist in multiple isoforms (Berchtold et al., 2000).

Variations in the relative expression of different isoforms of myofibrillar proteins are known to affect muscle's contractile performance. There is a developmental change in muscle contractile properties in rainbow trout, *Oncorhynchus mykiss*: younger parr have faster contractile properties than older smolts (Coughlin et al., 2001). Further, this shift in physiological properties is correlated with a change in MHC expression. We previously have cloned and sequenced three MHC cDNAs from trout white, red, and ventricular muscle

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(Weaver et al., 2001). Using reverse-transcriptase/polymerase chain reaction (RT-PCR) with clone-specific primers, we assessed the presence/absence of each MHC isoform in red muscle of trout. The red muscle of parr were more likely than smolts to express both slow (red and ventricular) and fast (white) isoforms of MHC. The red muscle of older fish tended to express only the slow isoforms (Weaver et al., 2001).

Besides developmental variations in muscle contractile properties, trout show longitudinal variations in rates of muscle activation and relaxation. The anterior red and white muscle have faster rates of both muscle activation and relaxation (Coughlin et al., 2007), but there is no longitudinal variation in muscle shortening velocity (Coughlin et al., 2001). Although there were no observed longitudinal variations in MHC protein composition or mRNA expression (Coughlin et al., 2001; Weaver et al., 2001), longitudinal shifts in the expression of both the thin filament protein troponin T and the myoplasmic protein parvalbumin have been observed in trout muscle (Coughlin et al., 2005, 2007).

The focus of this study is the regulatory MLC2. Our objective was to use a very sensitive technique, real-time quantitative PCR (qPCR), to examine longitudinal variations in MLC2 mRNA expression in the red and white muscle of rainbow trout parr and smolts. Although the kinetic properties of muscle are predominantly a function of MHC, variations in MHC protein expression are known to be associated with variations in other muscle proteins, such as MLC2 (Schiaffino and Reggiani, '96). MLC2 or regulatory light chain is part of the myosin hexamer, the principal protein of muscle, and influences muscle contractile properties. Although the role of MHC in muscle activity is relatively clear, the function of MLCs remains a matter of debate (Lutz et al., 2001). MLC2 is thought to modulate cross-bridge kinetics (Gordon et al., 2000). The relative contribution of different MLC2 isoforms (i.e. fast vs. slow) to muscle composition may alter maximum shortening velocity of the muscle (V_o , determined via slack test) (Bottinelli and Reggiani, 2000), and reversible phosphorylation of MLC2 influences force production at submaximal Ca^{2+} levels (Schiaffino and Reggiani, '96; Olsson et al., 2004; Wang et al., 2006). Recent reports indicate that MLC2 "fine-tunes" cross-bridge kinetics but confirm that the MHC is the major determinant of muscle contractile properties (Andruchov et al., 2006).

We hypothesized that longitudinal body position will affect MLC2 expression in trout swimming muscle. Given that both trout red muscle and white muscle express both fast and slow isoforms of MLC2, we predicted that the kinetically faster anterior muscle would express relatively more of the fast MLC2 (fMLC2) isoform and less of the slow MLC2 (sMLC2) isoform for both fiber types.

MATERIALS AND METHODS

The experimental design of this project involved: (1) the cloning and sequencing of full-length sMLC2 and fMLC2 isoforms from trout muscle; and (2) the use of real-time qPCR using isoform-specific primers to assess the relative expression of each isoform in red and white muscle samples from two ages of trout (younger juvenile parr and older juvenile smolts).

Rainbow trout from the Huntsdale Fish Culture Station, Carlisle, PA, of the Fish and Boat Commission of the Commonwealth of Pennsylvania were maintained on Ziegler Trout Grower feed in a re-circulating aquarium system at 10°C. The parr used in this research had obvious parr markings and were 6–8 months old; the smolts had lost their parr markings, displayed a more silvery appearance, and were 18–20 months old. The sizes of fish used in each experiment are given below. All handling of experimental animals was reviewed by the Widener University Institutional Animal Care and Use Committee in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council.

Cloning of trout MLCs

MLC2 was cloned from muscle RNA in two fragments using RT-PCR and 5' RACE. A trout smolt (TL = 28.5 cm, Mass = ~300 g) was sacrificed by spinal transection, and tissue was dissected from red and white myotomal muscle. Samples were quick frozen in liquid nitrogen and weighed before RNA isolation. Total RNA was isolated from trout muscle using the Trizol Reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using the SUPERScript (Reverse Transcription) Preamplification System (Invitrogen). An MLC2-specific degenerate upstream or sense primer (Lutz, personal communication) was employed in conjunction with the downstream or antisense primer TW2 (targets the 3' poly-A tail of mRNA; Lutz et al., '98) to amplify MLC2-specific cDNA by PCR. Primers are listed in

TABLE 1. Primers used in the cloning and sequencing of MLC2 isoforms from trout muscle

Primer pairs	Primer type	Primer sequence (5' to 3')	
Consensus	Sense	TTTGAGACCTTCCTGCCCAT	“LCCON2” ¹
MLC2 primers	Antisense	GCGGATCCTTTTTTTTTTTTTTTTTT	“TW2” ²
RACE Primers			
fMLC2	Antisense—outer	ACTAACGATGACATCCTC	
	Antisense—inner	CTCCGAACATGGTGAGGA	
sMLC2	Antisense—outer	TCGGGGTCAGTACCTTTC	
	Antisense—inner	CTCGCCAAACATGGAGAG	
Full-length primers			
fMLC2	Sense	TCTTGTTGACCATCCAAACC	
	Antisense	ATCCCATATCCCCACGAAC	
sMLC2	Sense	TGAGATGTGGAAAAGGAAACGG	
	Antisense	ACATAATGGCACCCAAAAAGGC	

¹Gordon J. Lutz, personal communication

²Lutz et al. ('98).

Table 1. PCR was performed using the Perkin Elmer reagent kit and Taq polymerase as supplied by the manufacturer in $1 \times$ buffer, 0.2 mM dNTP, 4.5 mM MgCl₂, and a 52°C annealing temperature. cDNA products were subcloned into the TOPO TA Cloning Kit (pCR 2.1 TOPO Vector and TOP 10 (*Escherichia coli*, Invitrogen).

Plasmids containing MLC2 inserts were purified from potential clones using the Promega Wizard Plus Mini-prep Purification System (Promega, Madison, WI), and the presence of inserts was verified by EcoRI digestion. DNA sequences of MLC cDNAs were determined at the University of Delaware DNA Sequencing Core Facility using an ABI Prism 377 DNA Sequencer (Applied Biosystems, Foster City, CA) and the dye terminator chemistry, and processed using the program Chromas. The initial sequences were composed of the 3' end of the coding sequence and the 3' untranslated region. DNA sequence information was entered into BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>) for comparison with known MLC2 sequences. Two novel MLC2 cDNAs were sequenced. One of the MLC2 isoform sequences was termed fast MLC2 (fMLC2) because it was cloned from white muscle, and the one termed sMLC2 was cloned from red muscle.

Complete sequences for the sMLC2 and fMLC2 isoform were generated using 5' RACE (Invitrogen) as previously described (e.g. Lutz et al., 2000). For RACE, the upstream primer UNI along with the nested upstream primers UNI-OUT and UNI-IN were employed (Lutz et al., 2000). Two nested downstream primers were generated for each MLC2 isoform from the 5' end of the initial

sequence fragments (listed as outer and inner antisense primers in Table 1). RACE PCR was followed by the same cloning and sequencing approach to find the cDNA sequence for the 5' untranslated region and the initial 100–200 bp of the MLC2 coding sequence. Complete sequences could then be determined initially by aligning the two fragments. New primers (Table 1) were then designed from this initial complete sequence so that the entire MLC2 cDNA for each isoform could be cloned and sequenced as one clone. Predicted protein sequences of the resulting complete sMLC2 and fMLC2 cDNA sequences were determined using the Baylor Search Launcher (<http://searchlauncher.bcm.tmc.edu/>). Molecular weights of the protein sequences were estimated using the Peptide Property Calculator (<http://www.basic.nwu.edu/biotools/ProteinCalc.html>).

MLC2 mRNA expression

Expression levels of sMLC2 and fMLC2 mRNAs along the length of trout were quantified using real time, quantitative PCR (qPCR). For both red and white muscle, sMLC2 and fMLC2 mRNA expression was compared for anterior, middle, and posterior muscle from smolts and parr ($n = 3$ for each; Parr, TL = 13.4 ± 1.5 cm, Mass = 48.3 ± 17.1 g; Smolt, TL = 23.1 ± 1.9 cm, Mass = 123.3 ± 30.6 g). Total RNA was extracted from muscle samples using the Trizol Reagent (Invitrogen, Carlsbad, CA) and treated with RNase free DNase I (Promega) in the presence of RNase inhibitor (Sigma Aldrich, St. Louis, MO) followed by ethanol precipitation (Sambrook et al., '89) to reduce

TABLE 2. Primers used in real-time quantitative PCR experiments with trout swimming muscle

Primer target	Primer type	Primer sequence (5' to 3')	Product size (bp)
α -Actin	Sense	AACGTGCCCATCTATGAGGGT	76
	Antisense	CAGTCAGATCGCGACCAGC	
fMLC2	Sense	CCCCATCAACTTCACGGTCT	71
	Antisense	TGACATCCTCGGGATCAGC	
sMLC2	Sense	TGACCCCCAGGAGACCATTAT	62
	Antisense	GGACTCCCTATCCCTCGGG	

genomic contamination. Total RNA (400 ng) from each sample was reverse transcribed using SuperScript III RNase H⁻ Reverse Transcriptase (Invitrogen) as described by the manufacturer. Negative controls for genomic contamination (“No RTs”) were prepared for each sample by processing the RNA as described above, without the addition of the SuperScript Reverse III Transcriptase.

MLC2 isoform-specific primers (Table 2) were designed by using the MLC2 sequence data and the Primer Express 2.0 software (Applied Biosystems, Foster City, CA). The isoform-specific PCR primers were then used in qPCR measurements of MLC2 mRNA expression. α -Actin primers were designed to test α -actin mRNA expression levels as a control; these were based on a published sequence of α -actin (NCBI Accession AF330142; Thiebaud et al., 2001). We expected α -actin levels to show little longitudinal variation.

qPCR reactions were performed according to the Brilliant SYBR Green QPCR Mix kit instructions (Stratagene, La Jolla, CA) and were carried out in consultation with Dr. Carl Schmidt at the University of Delaware using the ABI Prism 7900HT Sequence Detection System and default cycling conditions (95°C for 10 min; 40 cycles of 95°C for 30 sec; and 60°C for 1 min) for quantification. The ABI Prism SDS 2.0 software was used to analyze the raw fluorescence data. The threshold cycle (C_T) for each of the replicates of each sample was exported to Excel and further analyzed. Values used for statistical analysis (see below) for all samples are the mean value for five replicates of that sample. The C_T at which PCR product can be detected reflects the level of mRNA expression in the muscle samples. Lower C_T values indicate higher levels of expression. A higher initial level of MLC2 mRNA produces a higher quantity of cDNA (single-stranded), therefore it takes fewer PCR cycles to amplify the (double-stranded) MLC2 product to a level that is detectable by SYBR green fluorescence. α -Actin,

fMLC2, and sMLC2 primers were used to quantify mRNA levels using qPCR.

Data analysis

The threshold cycle (C_T) resulting from RT-PCR was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). For this analysis, α -actin was used as the internal control gene. The $2^{-\Delta C_T}$ method was employed to test if α -actin expression varied longitudinally (Livak and Schmittgen, 2001). Once it was determined that there was no statistically significant longitudinal variation in α -actin expression, the method described in Section 1.4 of Livak and Schmittgen (2001) could be employed. The basic equation employed was

$$\Delta\Delta C_T = (C_{T,MLC2\ isoform} - C_{T,actin})_{body\ position} - (C_{T,MLC2\ isoform} - C_{T,actin})_{anterior\ body\ position}. \quad (1)$$

For each MLC2 isoform, mRNA expression from each body position was analyzed relative to the anterior position. This approach is similar to that demonstrated in Figure 2 of Livak and Schmittgen (2001). This was done separately for each fiber type (red and white muscle) in each age of fish (parr and smolt). By definition, the anterior position will have a value of 0 and the middle and posterior positions will vary in either the positive or the negative directions. Statistical analysis was carried out by finding the value of $2^{-\Delta\Delta C_T}$, where the mean value of $2^{-\Delta\Delta C_T}$ for a given MLC2 isoform at a given body position is referred to as the mean fold change in gene expression (Livak and Schmittgen, 2001). The anterior position will have a mean change in gene expression of one, whereas middle and posterior muscle will be either greater than one or less than one, respectively, indicating higher or lower levels of expression of the MLC2 isoform in question. The $2^{-\Delta\Delta C_T}$ values were analyzed via analysis of variance (ANOVA) with body position as the independent value. There was a significant variation in α -actin

expression between fiber types and between fish ages, therefore we were unable to compare MLC2 isoform expression reliably along those trajectories.

RESULTS

Trout MLC cDNAs

Two isoforms of MLC2 were cloned, one from red or slow-twitch trout muscle (sMLC2) and one from white or fast-twitch trout muscle (fMLC2) (Fig. 1). These are available in the NCBI database as Accession numbers EU106632 for sMLC2 and EU106633 for fMLC2. The coding sequences were 78% similar between the sMLC2 and the fMLC2 cDNAs. The protein sequences reflect an evolutionarily conserved Ca^{2+} -binding region with high levels of similarity to other MLC2 protein sequences (Fig. 2).

The identity of the two isoforms (as fast or slow) is supported by the qPCR results. For instance, in white muscle, the mean C_T for fMLC2 was 23.1, whereas that for sMLC2 was 33.5. Analysis using the $2^{-C_T \text{fMLC2}} / 2^{-C_T \text{sMLC2}}$ ratio (Livak and Schmittgen, 2001) indicates that white muscle expresses >1000 times more of the fMLC2 mRNA isoform

than that of sMLC2. In red muscle, the mean C_T for all muscle samples for sMLC2 was 25.6 and that for fMLC2 was 27.8, indicating that red muscle expresses approximately 4.5 times more of the sMLC2 mRNA isoform than fMLC2.

Actin and MLC2 mRNA expression

The $2^{-\Delta C_T}$ analysis of α -actin expression indicated that α -actin expression was suitable for use as an internal control in experiments examining longitudinal patterns of MLC2 isoform expression (Fig. 3). There was no longitudinal variation in α -actin expression for either the red or white fiber types in either trout parr or smolts (ANOVA, parr red, $P = 0.432$; smolt red, $P = 0.886$; parr white, $P = 0.115$; smolt white, $P = 0.508$). The $2^{-\Delta \Delta C_T}$ method was then used to examine longitudinal variations in expression of each MLC2 isoform. Analysis of red muscle revealed a single longitudinal effect—sMLC2 expression was significantly greater in the posterior than in the anterior (Fig. 4; ANOVA, $P = 0.025$). No other effects were observed in red muscle (ANOVA, sMLC2 expression in smolts, $P = 0.732$; fMLC2

sMLC2 cDNA Complete Sequence

AAGTGAACAACTGAGAAGCTCCTGACATA**ATG**GCACCCAAAAAGGCCAAAGAAGAAGGCCA
GCAGAGGCCAGCTCCAATGTGTTCTCCATGTTTGAGCAAGCCAGATCCAGGAGTTCAA
GGAGGCTTTCACCATCATGGACCAGAACAGAGACGGTTTCATTGACAAGAATGACCTGA
GGACACATCTGTGCACTGGGCCGTCTTAACGTGGGTAATGATGAGCTGGACGAGATG
CTAAAGATGGCCCTGGACCCATCAACTTCACGGTCTTCTCTCCATGTTTGGCGAGAA
GTTGAAAGGTACT**AGACCCCAAGGAGACCATTA**TAAATGCCTTCAAGATCTTCGAC**CCCG**
AGGGACAGGGAGTCTCAAGGGAGAGGATATCAAATATTACATCATGTCTCAGGCGGAC
AAGTTCACCGAAGCTGAGGTTGAAGACATGTTCAAACTTCCCCTGGACGTCGCCGG
GAATCTGGACTACAAGAACCTGTGCTACGTTATCACCCACGGAGAGGACAAGGAGCGG
AG**TAA**ATGAGCCAATCACTAGCTCTCTAATCACCCAACTCACCTTGGACCGCCAC
TACTCATCGTCGCCCTGCACACCAGACCCGTTTCTTTCCACATCTCAATTTCCCA
CAAT

fMLC2 cDNA Complete Sequence

GGCGGCTTCACATCAGTCTCTTCTTGTGACCATCAAACCTCCATACATACCGTCT
CGAGATGGCACCCAAAGAAGGCCAAGAGGAGGGGAGCAGCAGAGGGCGGTTCTCCCA
ACGTGTTCTCCATGTTTGAGCAGAGCCAGATCCAGGAGTACAAGGAGGCTTTCACAATC
ATTGACCAGAACAGAGACGGTATCATCAGCAAGGATGACTTGAGGGACGTGCTGGCCTC
AATGGGCCAGTTGAATGTGAAGAATGAGGAGCTGGAAGCTATGGTCAAGGAGGCCAGCG
CCCCATCAACTTCACGGTCTTCTCACCATGTTTCGAGAGAAGCTCAAGGGG**GCTGAT**
CCCGAGGATGTCATCGTTAGTGCTCCTAAGGTCTTGGACCCCGATGCTACCGGTTTCAT
CAAGAAGGACTTCTTTCAGGAGCTCCTGACCACTCAGTGCAGACAGGTTCTGTCAGAGG
AGATGAAGAACCTGTGGGCTGCCTTCCCCCAGATGTGGCCGGCAACGTAGACTACAAG
CAATCTGTACGTCATCACACCGGAGAGGAGAAGGAGGAG**TAA**TGAAACAGACAGAA
GAAAAGAACAGCCTCCCTTGTCACTTCTACCTTCTGCTCCTCTCTTCTTCTTCTTATT
TTCTCATACCTTCATTCTCTTTATGTGTAATCATGTGCTCTGTCTCTCACTCACACAAA
TTCTCTAAAAGACTTGTCTCACTCAAGACATTTGTGTGAGAGCGGGTGTCTATGGGTT
GTCTATGTTTGTTCGTTGGGATATGGGATATTTTCAATAAAAAATGATCTTTAAAAAAA
AAAAAAAAA

Fig. 1. MLC2 cDNA sequences. Two complete sequences (slow and fast MLC2) are presented. The 5' and 3' untranslated regions are underlined, and the start and stop codons are indicated in red. The areas indicated in blue are upstream primers used in quantitative PCR (qPCR), whereas the areas indicated in green are the corresponding downstream primer sites (Table 2).

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sMLC2  1  MAPKKAKKKAEEA---SSNVFSMFEQAQIQEFKFAFTIMDQNRDGFIDKNDLRLDTFCALG
fMLC2  1  MAPKKAKRRGAAAEAGSSNVFSMFEQSQIQEYKFAFTIIDQNRDGIISKDDLRLDVLASMG
          *****  * *  *****  *****  *****  *****  * *  *****  *
          * *  * *  * *  * *  * *  * *  * *  * *  * *  * *  * *  * *  * *  * *

sMLC2  58  RLNVGNDELDEMLKMAPGPINFVFLSMFGEKLGKGTDPQETIINAFKIFDPEGQGVLKGE
fMLC2  61  QLNVKNEELEAMVKEASGPINFVFLTMFGEKLGADPEDVIVSAPKVLDPDATGFIKKD
          ***  * *  * *  * *  *****  *****  * *  * *  * *  * *  * *

sMLC2  118  DIKYYIMSQADKFTAEVEDMFTNFPPLDVAGNLDYKNLCYVITHGEDKE
fMLC2  121  FLQELLTTQCDRFSAEEMKNLWAAFPPDVAGNVYKQICVYITHGEEKE
          * * *  *  * *  *****  *****  * *  * *  * *  * *

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Fig. 2. Alignment of trout slow and fast MLC2 protein sequences, as predicted from cDNA sequences. *Identity in the two sequences. There is 60.4% identity between the full-length trout slow and fast isoforms of the regulatory light chain, and 73% identity within the Ca^{2+} -binding region of the molecule (indicated in red).

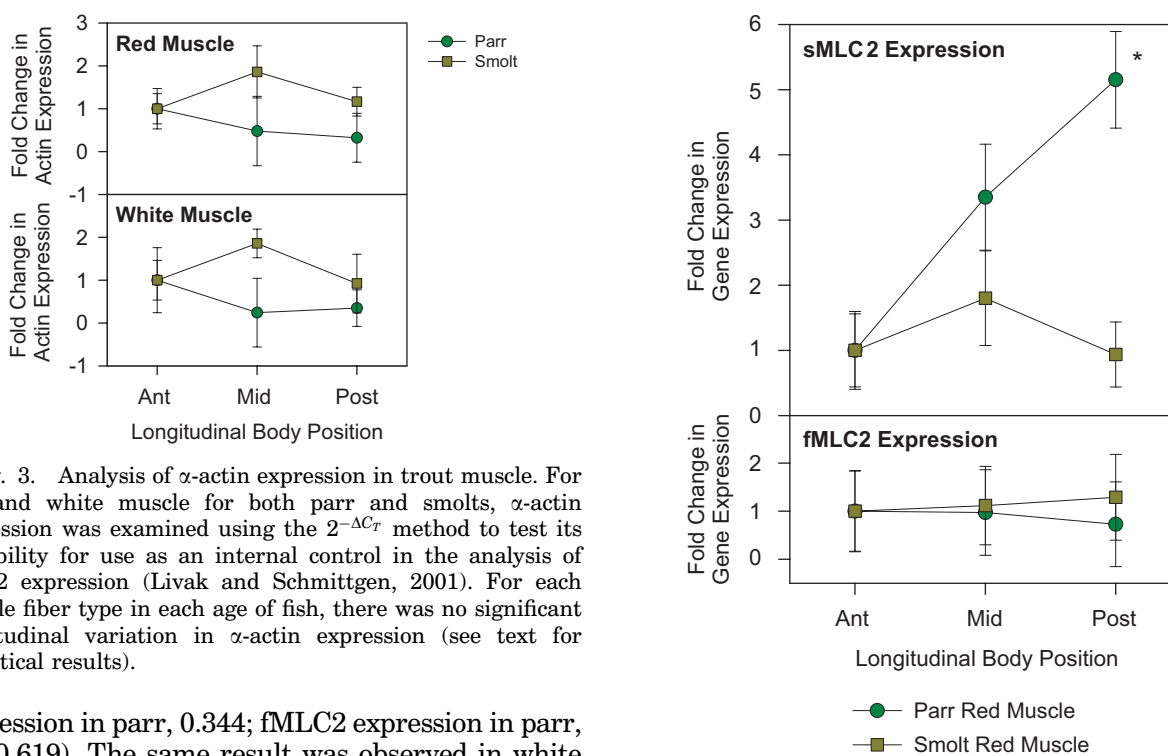


Fig. 3. Analysis of α -actin expression in trout muscle. For red and white muscle for both parr and smolts, α -actin expression was examined using the $2^{-\Delta C_T}$ method to test its suitability for use as an internal control in the analysis of MLC2 expression (Livak and Schmittgen, 2001). For each muscle fiber type in each age of fish, there was no significant longitudinal variation in α -actin expression (see text for statistical results).

expression in parr, 0.344; fMLC2 expression in parr, $P = 0.619$). The same result was observed in white muscle: sMLC2 expression was significantly greater in the posterior than in the anterior (Fig. 5; ANOVA, $P = 0.045$). No other effects were observed in white muscle (ANOVA, sMLC2 expression in smolts, $P = 0.470$; fMLC2 expression in parr, 0.402; fMLC2 expression in smolts, $P = 0.440$).

DISCUSSION

We report two novel cDNA sequences of MLC2 or the regulatory light chain from rainbow trout. The Ca^{2+} -binding region of trout sMLC2 is 100% identical to that of human cardiac muscle. Similarly, the same region in fMLC2 is 100% identical to MLC2 cDNA sequences from the fast muscle of a number of fish species (e.g. sea bream, carp, and zebrafish, Moutou et al., 2001) but interestingly differs by one amino acid from the MLC2 sequence from the

Fig. 4. Analysis of sMLC2 and fMLC2 isoform expression in red muscle. The expression levels of the two MLC2 isoforms were examined in the red muscle of both parr and smolts using the $2^{-\Delta\Delta C_T}$ method with α -actin as the internal control (Livak and Schmittgen, 2001). There was a significant longitudinal body position effect on sMLC2 expression in parr red muscle—the posterior muscle expressed significantly more sMLC2 than the anterior muscle. No other effects were observed in red muscle.

rainbow trout congeneric *Oncorhynchus kisutch* (Hill et al., 2000). The predicted size of fMLC2 is 18,967 Da, whereas that for sMLC2 is 18,978 Da.

Longitudinal variation in fish muscle

Many fish species, including rainbow trout, display longitudinal variations in the contractile properties of their myotomal muscle, including

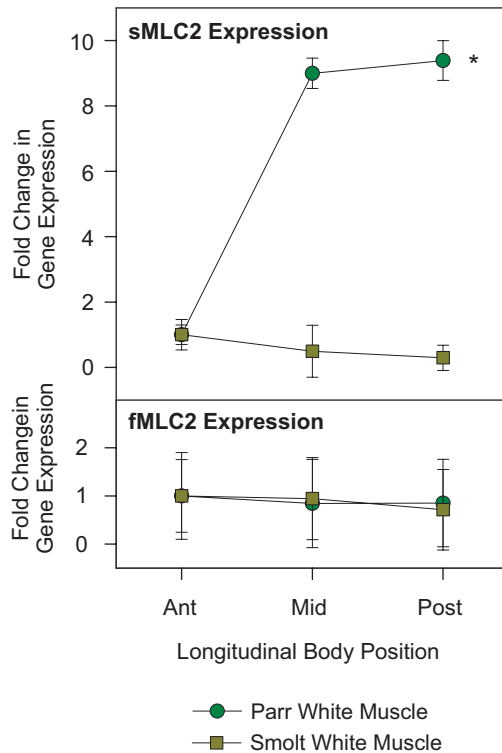


Fig. 5. Analysis of sMLC2 and fMLC2 isoform expression in white muscle. The expression levels of the two MLC2 isoforms were examined in the white muscle of both parr and smolts using the $2^{-\Delta\Delta C_T}$ method with α -actin as the internal control (Livak and Schmittgen, 2001). There was a significant longitudinal body position effect on sMLC2 expression in parr white muscle—the posterior muscle expressed significantly more sMLC2 than the anterior muscle. No other effects were observed in white muscle.

both red and white fiber types (Coughlin, 2002a; Coughlin et al., 2007). In rainbow trout red muscle, the anterior muscle displays faster rates of activation and relaxation from contraction, but there are no longitudinal differences in V_{max} or MHC expression (Coughlin et al., 2001). This study suggests a role of MLC2 isoform expression in modulating contractile properties along the length of trout—but specifically for younger juvenile parr. In addition, only the “slow” isoform of MLC2 (sMLC2) was determined to vary in terms of relative amount expressed along the length of parr red and white muscle. The kinetically slower muscle in the posterior myotome expressed greater amounts of sMLC2 as compared with the anterior myotome. This was true for both red and white muscle. The relative amount of sMLC2 expressed did not vary longitudinally in the older juvenile smolts, and fMLC2 expression did not vary along the length of the

myotome of either parr or smolts. Our predictions were partly realized—sMLC2 mRNA expression is regulated in a manner consistent with the modulation of contractile properties, but only in younger trout juveniles. A correlation between mRNA expression and protein composition of muscle has been established for MHC, at least in mammals (Pette et al., '99; Stevens et al., '99a). Although this correlation can be disrupted under specific conditions (e.g. limb suspension experiments in mice; Stevens et al., '99b), we believe that the qPCR results for shifts in sMLC2 mRNA expression along the length of young rainbow trout parr indicate variations in the relative amounts of sMLC2 vs. fMLC2 protein in the swimming muscle of these fish.

Two other proteins also play a role in the longitudinal variation in trout red muscle activation and relaxation: troponin T and parvalbumin. In a study of rainbow trout smolts, Coughlin et al. (2005) showed that trout red muscle shows shifts in the relative expression of two isoforms of troponin T from anterior to posterior in a manner that corresponds with longitudinal variation in muscle activation. Faster anterior muscle expresses relatively greater amounts of a smaller TnT isoform (TnT2) compared with the posterior muscle, which is dominated by the larger TnT1. Variations in TnT expression appear to regulate muscle activation in fish white muscle as well (Thys et al., '98, 2001). Differences in Ca^{2+} sensitivity by the different isoforms of TnT appear to affect muscle activation (Gordon et al., 2000).

Parvalbumin contributes to the regulation of longitudinal variations in muscle relaxation in trout in both parr and smolts (Coughlin et al., 2007). The faster anterior muscle expresses more parvalbumin than the posterior muscle for both ages of rainbow trout. Parvalbumin buffers Ca^{2+} in muscle, enhancing relaxation by lowering myoplasmic $[Ca^{2+}]$ (Berchtold et al., 2000). In other species, muscle relaxation along the length of the fish is affected by the relative expression of two parvalbumin isoforms. Two isoforms of parvalbumin (Parv1 and Parv2) have been identified in the myotomal muscle of a number of fish species. The faster anterior muscle has been shown to express relatively more of the smaller isoform (Parv2) compared with the posterior muscle in sheephead, *Archosargus probatocephalus* (Wilwert et al., 2006), and brook trout, *Salvelinus fontinalis*, but not rainbow trout (Coughlin et al., 2007). We are examining how the two isoforms of parvalbumin expressed in the swimming muscle

of a number of fish species differentially affect muscle relaxation.

Longitudinal variations in muscle contractile properties in fishes appear to result from complex shifts in expression of several muscle proteins. These proteins include, but surely are not limited to, MLC2, TnT, and parvalbumin. Although the roles of TnT and parvalbumin are understood to some degree (e.g. Berchtold et al., 2000), the specific function of MLC2 in muscle contraction remains an area of ongoing inquiry.

MLC2 in fish skeletal muscle

This study demonstrated longitudinal variation in sMLC2 mRNA expression in trout myotomal muscle. Changes in MLC2 mRNA expression were observed under circumstances where MHC variation was not observed in previous studies (Coughlin et al., 2001; Weaver et al., 2001). Work on the role of MLC2 in muscle contractions in mammalian muscle indicates that MLC2 affects various aspects of contraction, including shortening velocity (Bottinelli and Reggiani, 2000; Sherwood et al., 2004) and rates of tension development and total tension generation (Diffie et al., '95; Wang et al., 2006). In trout muscle, because MLC2 does appear to vary independently from MHC expression and does not appear to affect shortening velocity, MLC2 may affect rates of myosin head attachment, as its expression correlates with variations in muscle activation. The specific effects of varying the relative contribution of MLC2 isoforms should be examined in the future.

Fish modulate longitudinal variations of contractile properties of muscle fibers through a variety of means that are independent of MHC expression, including shifts in the expression of MLC2, troponin T, and parvalbumin isoforms. It remains a question as to why sMLC2 expression is regulated along the length of trout parr but not smolts. The answer may relate to developmental shifts in muscle contraction and protein expression in trout. Rainbow trout muscle contractile properties shift developmentally, with a general slowing of muscle from younger juvenile trout parr to older juvenile trout smolts. The younger fish have faster rates of muscle activation and relaxation and faster maximum muscle shortening velocity (Coughlin et al., 2001). It has previously been shown that shifts in MHC expression are associated with this parr-smolt transformation (Coughlin, 2002b). In addition, there are shifts in parvalbumin expression between parr and smolts,

with the red and white muscle in parr expressing higher levels of parvalbumin than in smolts (Coughlin et al., 2007). As the fish grow and muscle's contractile properties slow, the shifts in the expression of isoforms of MHC and parvalbumin (and perhaps TnT, although it has not been studied developmentally in trout) may be adequate for establishing longitudinal patterns of swimming muscle contractile properties. Ultimately, the proteins expressed and the contractile properties that result contribute to differences in the kinematics of swimming between parr and smolts (Coughlin et al., 2001). Because swimming kinematics (e.g. tailbeat frequency) and muscle contractile properties differ developmentally, longitudinal patterns of protein expression would likely differ developmentally as well.

Unfortunately, α -actin cannot be used as an internal control for $2^{-\Delta\Delta C_T}$ analysis of MLC2 variation developmentally, as its expression appears to be regulated by both fiber type and age (Thiebaud et al., 2001). Repetition of the current experiment with different control genes may reveal developmental shifts in the mRNA levels of the MLC2 isoforms. However, examination of sMLC2 expression along the length of trout parr red and white muscle (Figs. 4 and 5, top panels) suggests differences in sMLC2 between parr and smolts—there appears to be a greater expression of sMLC2 mRNA in parr compared with smolts.

This work presents two novel MLC2 cDNA sequences. Knowledge of the cDNA sequences of the sMLC2 and fMLC2 isoforms allowed the use of qPCR to assess longitudinal patterns of MLC2 mRNA expression in trout muscle. There are shifts in sMLC2 expression along the length of red and white muscle in trout parr or young juveniles, but not in the older trout smolts. We continue to examine the role of MLC2 and other myofibrillar proteins in regulating trout muscle contractile properties.

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