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Analysis of myofibrillar proteins and transcripts in adult skeletal muscles of the American lobster *Homarus americanus*: variable expression of myosins, actin and troponins in fast, slow-twitch and slow-tonic fibres

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Summary

Skeletal muscles are diverse in their contractile properties, with many of these differences being directly related to the assemblages of myofibrillar isoforms characteristic of different fibers. Crustacean muscles are similar to other muscles in this respect, although the majority of information about differences in muscle organization comes from vertebrate species. In the present study, we examined the correlation between myofibrillar protein isoforms and the patterns of myofibrillar gene expression in fast, slow-phasic (S_1) and slow-tonic (S_2) fibers of the American lobster *Homarus americanus*. SDS-PAGE and western blotting were used to identify isoform assemblages of myosin heavy chain (MHC), P75, troponin T (TnT) and troponin I (TnI). RT-PCR was used to monitor expression of fast and slow (S_1) MHC, P75 and actin in different fiber types, and the MHC and actin levels were quantified by real-time PCR. Fast and slow fibers from the claw closers predominantly expressed fast and S_1 MHC, respectively, but also lower levels of the alternate MHC. By contrast, fast fibers from the deep abdominal muscle expressed fast MHC exclusively. In addition, slow muscles expressed significantly higher levels of actin than fast fibers. A distal bundle of fibers in the cutter claw closer muscle was found to be composed of a mixture of S_1 and S_2 fibers, many of which possessed a mixture of S_1 and S_2 MHC isoforms. This pattern supports the idea that S_1 and S_2 fibers represent extremes in a continuum of slow muscle phenotype. Overall, these patterns demonstrate that crustacean skeletal muscles cannot be strictly categorized into discrete fiber types, but a muscle's properties probably represent a point on a continuum of fiber types. This trend may result from differences in innervation pattern, as each muscle is controlled by a unique combination of phasic, tonic or both phasic and tonic motor nerves. In this respect, future studies examining how muscle phenotype correlates with innervation pattern may help account for variation in crustacean fiber types.

Key words: skeletal muscle, myosin heavy chain, actin, isoform, lobster, Crustacea, Arthropoda, *Homarus americanus*.

Introduction

Skeletal muscles differ significantly in their contractile properties, largely because of differences in myofibrillar protein isoforms among various fibers (Schiaffino and Reggiani, 1996). Crustacean muscle fibers are no different from other muscles in this respect. Crustacean muscles have been classified based on structural criteria (Silverman et al., 1987; Jahromi and Atwood, 1969, 1971), ATPase histochemistry (Oganowski and Lang, 1979; Silverman et al., 1987) and the specific assemblage of myofibrillar isoforms present in a particular fiber (Mykles, 1985a,b, 1988, 1997). Through these methods, crustacean muscles are classified as fast, slow twitch or phasic (S_1), or slow tonic (S_2). Fast fibers have sarcomere lengths of approximately 4 μm (Jahromi and Atwood, 1969, 1971; West, 1997), high myofibrillar ATPase activity (Oganowski and Lang, 1979; Silverman et al., 1987) and a number of distinct myofibrillar isoforms including P75 and a fast myosin heavy chain (MHC; Mykles, 1985a,b, 1997; Li and Mykles, 1990; Cotton and Mykles, 1993; Neil et al., 1993). Slow muscles have sarcomeres that range from 6 μm to 12 μm in length (Jahromi and Atwood, 1969, 1971; West, 1997), lower myofibrillar ATPase activity (Oganowski and Lang, 1979; Silverman et al., 1987) and a different set of protein isoforms including a slow MHC (Mykles, 1985a,b, 1997; Li and Mykles, 1990; Neil et al., 1993). MHC isoforms have received more attention than other myofibrillar proteins, as differences in shortening velocity are directly correlated with the rate of ATP hydrolysis by the myosin head (Schiaffino and Reggiani, 1996). As such, the type of MHC is considered to be the primary determinant of contractile properties among different muscle types (Schiaffino and Reggiani, 1996). In addition to distinct fast and slow MHC isoforms, crustaceans also appear to possess unique S_1 and S_2 MHCs, as indicated

by myosin ATPase activity (Mykles, 1988; Neil et al., 1993), partial protein hydrolysis fragments (Neil et al., 1993) and differences in contractile properties (Holmes et al., 1999). S_1 fibers have higher myosin ATPase activities than S_2 fibers (Mykles, 1988; Neil et al., 1993), which correlates with a twofold higher maximum shortening velocity (Holmes et al., 1999). A recent study of crayfish muscle fiber types identified up to four different MHC proteins (LaFramboise et al., 2000).

In many cases, fiber distribution in crustaceans is correlated with the type of motor innervation a muscle receives, but there is considerable variability in this pattern. Crustaceans possess motor nerves with a continuum of properties from purely phasic to purely tonic, with many forms being intermediate between these two extremes (Atwood, 1976). Phasic motoneurons are large, fire in brief bursts, are less fatigue resistant and form thin filiform endplates (Atwood, 1976; Bradacs et al., 1997). Tonic motoneurons are smaller, are active for prolonged periods, are fatigue resistant and have large and varicose terminals (Atwood, 1976; Bradacs et al., 1997). In addition, inhibitory neurons that modulate the neuromuscular responses are generally present (Atwood, 1976). Some muscles receive innervation predominantly from either phasic or tonic motor neurons, while other fibers are controlled by synapses from both nerves (Atwood, 1976). For example, many of the deep abdominal muscles receive only phasic motor axons, while the more superficial abdominal muscles receive purely tonic efferents (Atwood, 1976). Muscles in lobster claws, however, contain fibers that receive input from both nerve types, as well as some fibers that only receive input from the phasic or the tonic motor nerve (Atwood, 1976; Lang et al., 1980; Costello and Govind, 1983).

Unlike mammalian muscles, which are often composed of a mosaic of fiber types, crustacean muscles are often anatomically segregated, and the different muscle types are correlated with the type of excitatory innervation that they receive (Mykles, 1985a,b; Neil et al., 1993; Mykles et al., 2002). We have recently shown that myofibrillar isoform composition is correlated with synaptic efficacy in the claw and leg opener muscles of lobsters and crayfish (Mykles et al., 2002). In these muscles, S_1 and S_2 fibers form a segregated continuum along the length of the muscle. Proximal fibers are generally S_2 and show greater post-synaptic facilitation than the S_1 fibers that are located more distally. Similarly, Neil et al. (1993) demonstrated that the superficial abdominal flexor muscle in the Norway lobster, *Nephrops norvegiucus*, is comprised of S_1 and S_2 fibers that form distinct anatomical bundles from the medial to lateral positions. The correlation between muscle phenotype and innervation, combined with the distinct anatomical segregation of muscle types, makes crustacean systems desirable models for studying basic muscle function (LaFramboise et al., 2000).

Here, we extend our understanding of the molecular diversity associated with muscle phenotype among skeletal muscles in the American lobster *Homarus americanus*. Our primary focus is the different fiber types that form the claw closer muscles. Adult claws are dimorphic, with a large crusher claw and a slender cutter claw. The crusher claw is invested with slow (S_1) muscle fibers, while the cutter claw contains predominantly fast muscle fibers (Mykles, 1985a,b, 1997). This asymmetry is established in early juvenile stages and persists throughout the life of the animal (reviewed by Govind et al., 1987; Govind, 1992). In the present study, our objective was to more precisely characterize fully differentiated adult fast and slow claw muscles at the molecular level of organization. We extend our understanding of these muscles in terms of the MHC protein isoforms and the mRNA isoforms present in the fibers.

Materials and methods

Animals and tissue preparation

Adult lobsters (*Homarus americanus* Milne-Edwards) were purchased locally, and muscles were harvested the same day. Muscles used for RNA isolation were quickly frozen in liquid N_2 and then stored at –80°C until the time of RNA isolation (see below). Samples used for protein analyses were processed according to the methods of Mykles (1985b). Briefly, muscles were glycerinated in ice-cold buffer containing 20 mmol l^{-1} Tris-HCl (pH 7.5), 50% glycerol, 100 mmol l^{-1} KCl, 1 mmol l^{-1} EDTA and 0.1% Triton X-100 for 2–3 h with stirring. Single fibers or fiber bundles were removed from the muscle and solubilized in 250 μl of sodium dodecyl sulfate (SDS) sample buffer containing 62.5 mmol l^{-1} Tris-HCl (pH 6.8), 12.5% glycerol, 1.25% SDS and 1.25% β-mercaptoethanol. Muscle samples were left in this solution overnight at room temperature with occasional vortexing.

Muscle samples from the claws were taken from different regions of the closer muscles identified as central, ventral or distal (Fig.·1). In some cases, single fibers were harvested for analysis, while at other times a larger population of fibers was taken from a single region. Muscle samples from the tail were always a bundle of fibers.

Analysis of myofibrillar proteins

For analysis of myofibrillar isoform assemblages and western blotting, SDS-PAGE was performed using a discontinuous gel system as described in Mykles (1985b). Briefly, 10% separating gels (37.5:1 acrylamide:*N*,*N*′-methylenebisacrylamide) were used to separate approximately 4–6 μg of myofibrillar proteins using a Mini-Protean II gel system (Bio-Rad, Hercules, CA, USA) and were stained with Coomassie blue or silver (Wray et al., 1981). Protein concentrations were determined empirically from silver-stained gels. For western blots, samples were separated as described but, instead of staining the proteins, they were electophoretically transferred to a PVDF (polyvinylidine fluoride) membrane. Following transfer, the membrane was blocked with 2% non-fat milk in Tris-buffered saline (TBS: 20 mmol l^{-1} Tris-HCl, pH 7.5, 500 mmol l^{-1} NaCl) overnight and then incubated for 1 h with polyclonal antibodies raised in rabbits against purified P75, troponin T (TnT) or troponin I (TnI) at 1:20 000 dilutions of antiserum in 0.05% Tween in

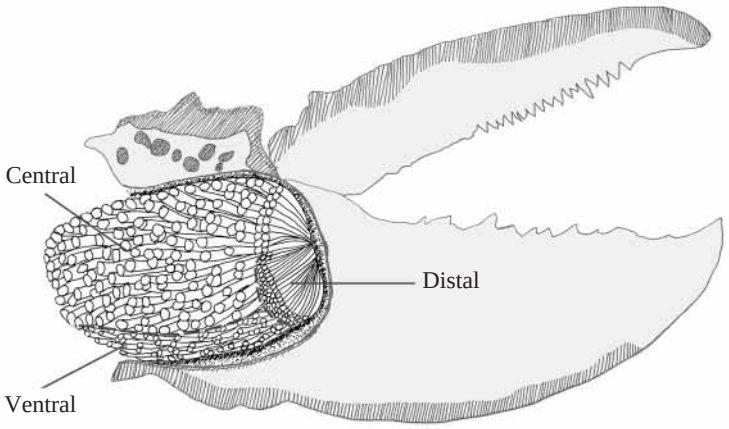

Fig. 1. Diagram of cutter claw with lateral portion of exoskeleton removed to reveal the arrangement of the closer muscle. The central region of the muscle is composed of large fibers that are the fast phenotype. The ventral and distal regions of the closer are slow, with the ventral region composed of S_1 fibers and the distal region composed of S_2 fibers. Previous work by Lang et al. (1980) demonstrated that these regions are controlled by phasic (central), tonic (distal) or both (ventral) motor neurons. The same study showed that the distal region of both claws possessed highly oxidative fibers. The basic anatomy of the crusher claw is similar to that of the cutter but is composed entirely of S_1 fibers.

TBS (TTBS; Sohn et al., 2000; Mykles et al., 2002). After several washes in TTBS, blots were incubated with biotinylated anti-rabbit IgG (1:5000) for 1 h followed by avidin/biotinylated horseradish peroxidase complex (ABC reagent; Vectastain, Vector Labs, Burlingame, CA, USA; 1:1000 dilution in TTBS) and chemiluminescent detection (Covi et al., 1999).

MHCs from different muscle samples were resolved according to the methods of Talmadge and Roy (1993), with the modification that 0.1% β-mercaptoethanol was added to the upper buffer (Blough et al., 1996; LaFramboise, 2000). Samples were separated on 8% resolving gels (50:1 acrylamide:*N*,*N*′-methylenebisacrylamide) on 15 cm gels (Bio-Rad Protean II) at 200 V for at least 48 h. Rabbit MHC (Sigma, St Louis, MO, USA) was loaded as a standard on each gel. The gel apparatus was packed in ice and run in a refrigerated cabinet at 4–5°C. Gels were stained with Coomassie blue or silver (Wray et al., 1981). The relative amounts of specific proteins in bands from stained gels and blots were determined using scanning densitometry (NIH Image 1.62).

Isolation and sequencing of cDNA encoding lobster slow MHC and P75

A cDNA encoding lobster S_1 MHC was obtained by the methods of Cotton and Mykles (1993). Briefly, a cDNA library was made by Invitrogen from mRNA purified from lobster crusher claw muscle and cloned into a pcDNAII plasmid vector. Cells were grown for 8 h on LB ampicillin plates, transferred to dried isopropyl-1-thio-β-D-galactoside (IPTG)-soaked nylon membranes and grown overnight on fresh LB ampicillin plates. The cells were then lysed with 5% SDS and the proteins were fixed to the membranes by microwaving. Membranes were then blocked with 10% non-fat milk and incubated with polyclonal anti-MHC, and the bound antibodies were detected with a Vectastain kit as described by Mattson and Mykles (1993). Clones that showed a positive reaction with the anti-MHC antibodies were selected for sequencing. Both strands of the slow MHC cDNA were sequenced by Davis Sequencing (Davis, CA, USA) using SP6 and T7 primers, followed by sequence-specific primers.

A cDNA encoding approximately 1500 bp of lobster muscle P75 was obtained as described above but from a cDNA library made from fast muscle cDNA (Cotton and Mykles, 1993). Positive clones were selected as described above, but the library was screened with the polyclonal anti-P75 antibody. A single strand of DNA was sequenced from the SP6 primer, followed by sequence-specific primers. The complementary strand from the T7 primer did not yield any usable sequence. More information about this cDNA will be published once we obtain the full sequence. The sequence for the fast MHC was selected from the same library and has been published previously (Cotton and Mykles, 1993).

RT-PCR of myofibrillar protein mRNAs

Total RNA from lobster muscles was isolated using TRIZOL reagent (Invitrogen). Tissues (approximately 150 mg) were homogenized in 2 ml TRIZOL using a hand-held glass homogenizer until the tissue was completely homogenized. Alternatively, single muscle fibers were homogenized in 1 ml TRIZOL using the same protocol. Insoluble materials were removed by centrifugation at 12 000 ***g*** for 10 min at 4°C. Chloroform (0.2 ml per 1 ml TRIZOL reagent) was added to each supernatant following a 5 min incubation at room temperature. Samples were shaken by hand for 30 s, allowed to sit at room temperature for 5 min and then centrifuged at 12 000 ***g*** for 10 min at 4°C. RNA in the aqueous phase was precipitated by the addition of isopropanol (0.5 ml per 1 ml TRIZOL reagent) followed by incubation at room temperature for 10 min. RNA was collected by centrifugation at 12 000 ***g*** for 10 min at 4°C and then washed with 75% ethanol. After air-drying, samples were dissolved in water and stored at –80°C.

RNA samples were treated with DNase (Invitrogen) for 15 min at room temperature to remove any genomic DNA contaminating the samples. First-strand cDNA was synthesized from total RNA using SuperScript II RNase H- reverse transcriptase (Invitrogen) using oligo(dT) primers. The reaction contained 2.5 μg of oligo(dT) 12–18, 2.5 mmol l^{-1} dNTP, 1× First-Strand Buffer, 5 mmol l^{-1} dithiothreitol (DTT), 2.5 units of RNase inhibitor, 1–2 μg of RNA and 200 units of SuperScript II RNase H- reverse transcriptase. MHC primers (fast or slow) were synthesized from the fast and S_1 MHC

Table 1. *PCR primer sequences (5′→3′)*

Sequence	Forward	Reverse
Fast MHC	GCATTCGTGAACTTGAAGGTC	AACCTTATAGCCCATGAATCTAGG
S_1 MHC	CGAAACCAATGCTCTGAAGAACACC	GTAAGCATCAAGTACCGTCCAAGG
P75	AACGAGATCACCACTGACCC	CATGGTAGTCTTGCCGGTTT
Actin*	TACATCCAGACCAGTAGCAAGCAC	TGGGTCATCTTCTCACGGTTGG

*Primers for actin are the same as used by Koenders et al. (2002); GenBank accession #AF399872.
MHC, myosin heavy chain.

sequences so that the reverse primers complemented a sequence in the 3′-UTR, while the forward primers complemented a sequence in the open reading frame (Table 1; Fig. 2). The fast MHC primers amplified a 315 bp product, while the S_1 MHC primers amplified a 453 bp product. P75 primers were synthesized based on the partial sequence of the P75 cDNA and yielded a 176 bp product (Table 1). Plasmid DNA containing the specific cDNA sequences for the fast MHC, S_1 MHC or P75 were used as template for PCR to optimize reaction conditions. The actin primers were designed to amplify α-actin from lobster skeletal muscles as previously described, yielding a 401 bp product (Table 1; Koenders et al., 2002; GenBank accession no. AF399872). Under these conditions, the primer pairs were found to be highly specific, amplifying single bands of the expected product lengths. Hot start ExTaq (Takara, Otsu, Shiga, Japan) was added to tubes containing 1 μmol l^{-1} of each primer and 2 μl of the first-strand cDNA reaction in each PCR reaction (20 μl total volume). PCR amplification consisted of denaturation of the template and activation of ExTaq (95°C for 120 s) followed by amplification of the target cDNA (35 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s). Products were separated on a 1% agarose gel and visualized with ethidium bromide staining. For figures, products from the individual reactions (fast MHC, S_1 MHC and P75) were combined before separation on agarose gels.

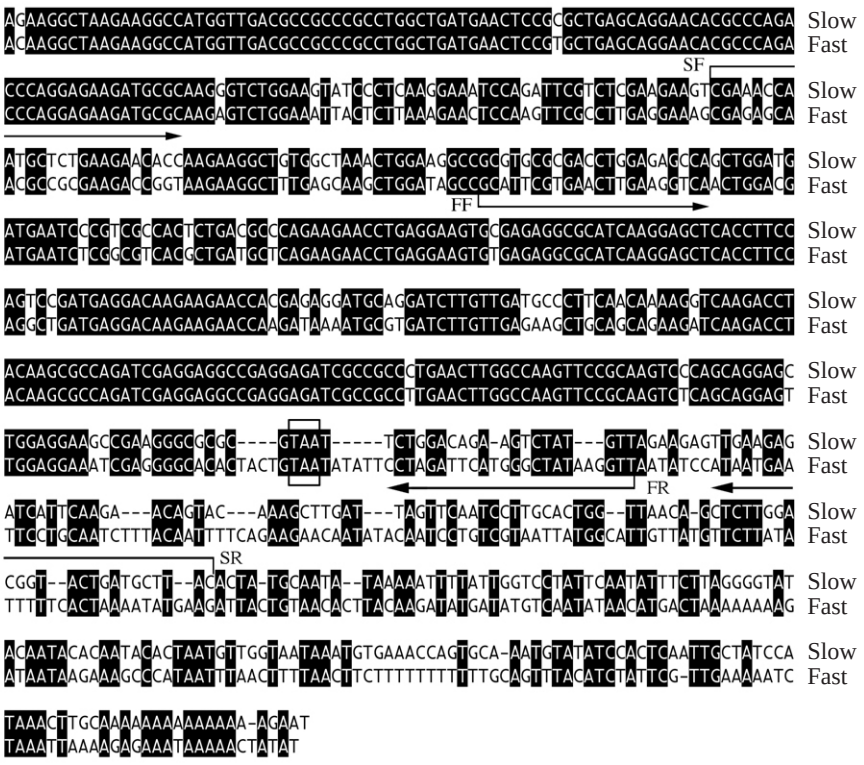

Fig. 2. Alignment of cDNA sequences encoding part of the open reading frame (ORF) and 3′ untranslated region (UTR) for fast and slow (S_1) MHCs to show the regions amplified by PCR. Bases that match one another are shaded, and the stop codon is enclosed in a box. The S_1 MHC sequence and the fast MHC sequence share 79% identity within the ORF. The 3′ UTR follows the stop codon and has lower sequence identity. Fast forward (FF), fast reverse (FR), slow forward (SF) and slow reverse (SR) MHC primer positions are indicated by arrows adjacent to the fast and slow sequences, respectively. Reverse PCR primers were designed to anneal to specific sequences in the 3′ UTR, while forward primers were designed to anneal to regions of relatively low sequence identity in the ORF. The fast MHC sequence has been published previously (Cotton and Mykles, 1993). The GenBank accession numbers for the fast and S_1 MHC sequences are U03091 and AY232598, respectively.

Real-time PCR was used to quantify the levels of fast MHC, S_1 MHC and actin mRNA from different muscle tissues. The above protocol was used for the generation of cDNA from isolated RNA samples. Single cDNA samples were divided for use in separate reactions to measure the copy numbers of each of the above target sequences. Thus, at least three distinct transcripts were monitored for each sample and served as internal controls. The Light Cycler DNA Master SYBR Green I reaction mix for PCR (Roche Molecular Biochemicals, Indianapolis, IN, USA) was used for the amplification of target cDNA with a Cepheid Smart Cycler Instrument (Yu and Mykles, 2003). PCR master mix containing 1× Light Cycler DNA Master SYBR Green I, 2.5 mmol l^{-1} $MgCl_2$, 0.5 μmol l^{-1} of each primer and 5 μl of the first-strand cDNA

reaction were added to 25 μl sample tubes. PCR amplification consisted of denaturation of template and activation of the HS Taq (95°C for 5 min) followed by amplification of the cDNA target (30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 6 s and extension at 72°C for 20 s). A standard curve was constructed for each sequence using purified plasmid DNA containing known amounts of cDNA as template in the PCR reactions. Plasmids containing specific sequences were purified from using QIAprep minipreps (Qiagen, Valencia, CA, USA). Copy numbers of plasmid DNA containing inserts were calculated based on the molecular mass of the plasmid and then converting to number of copies based on Avagodro's number (Li and Wang, 2000). A standard curve was constructed by plotting the number of copies as a function of the threshold cycle where product began to accumulate exponentially. The melting temperature, which is a measure of the GC content and length of the product, was used to identify the specificity of the PCR product. In addition, reaction products were separated on 1% agarose gels to verify product size. The cycle threshold from reactions containing unknown amounts of cDNA was converted to number of copies with the standard curves. The number of transcript copies was normalized to the amount of tissue (mg) used for RNA isolation.

Statistical analyses

A one-way analysis of variance (ANOVA) was used to compare the number of copies of myofibrillar mRNA (fast MHC, S_1 MHC and actin) calculated from real-time PCR. Values were log-transformed because of a high level of variability among samples and to correct for the correlation between mean and variance in these data (Neter et al., 1990). Pair-wise post-ANOVA comparisons were made using a Bonferroni test, with an experiment-wise error rate of 0.05. Simple linear regression was used to examine the correlation between the number of copies of mRNA for different myofibrillar genes, as well as for the correlation between TnT_1 and S_2 MHC from protein gels. Statview 5.0.1 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

SDS-PAGE and western blot analysis of myofibrillar proteins

Fibers displayed a characteristic assemblage of myofibrillar proteins, as previously summarized by Mykles (1985a,b, 1988, 1997). For fast fibers, these included the P_1 and P_2 isoforms of paramyosin, P75, TnT_2, TnI_1, TnI_3 and TnI_5 (Fig. 3B,C). Both S_1 and S_2 fibers possess only the P_2 isoform of paramyosin, lack P75 and possess TnT_3 and TnI_2 (Fig. 3B,C). A major difference between S_1 and S_2 fibers is that the S_2 fibers express the TnT_1 isoform, which has been used as marker for these fibers (Mykles, 1985b; Neil et al., 1993; Mykles et al., 2002; Fig. 3C). In addition, S_1 fibers generally possess the TnI_4 isoform in combination with TnI_2, while S_2 fibers express the

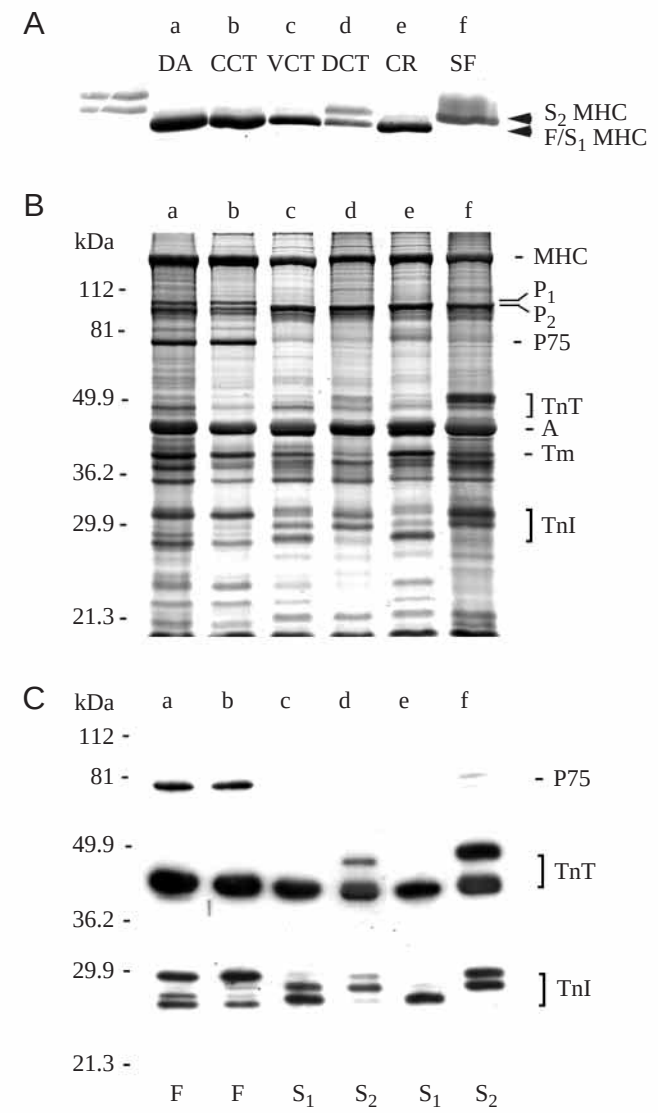

Fig. 3. Correspondence between myosin heavy chain (MHC) protein composition and other myofibrillar isoforms identified through SDS-PAGE and western blotting. (A) 8% silver-stained gel demonstrating different MHC isoforms present in various muscles. MHC isoforms could not be distinguished between the fast [deep abdominal flexor (DA) and central cutter (CCT)] and S_1 [ventral cutter (VCT) and crusher (CR)] muscles. However, the S_2 fibers [distal cutter (DCT) and superficial tail flexors (SF)] contained an additional isoform that migrated more slowly than the fast or S_1 isoforms (arrowheads mark positions of the isoforms). Some slow muscles contained a mixture of the S_1 and S_2 MHC isoforms (DCT), while others contained only the S_2 isoform (SF). The double band at the far left is rabbit MHC used as a control (Sigma). (B) 10% silver-stained gel showing other myofibrillar isoforms from the samples identified in A. Some of the dominant bands correspond to MHC, paramyosin isoforms P_1 and P_2, P75, troponin T (TnT), actin (A), tropomyosin (Tm) and troponin I (TnI). Fast fibers characteristically possess paramyosin isoforms P_1 and P_2, P75, TnT_2, TnI_1, TnI_3 and TnI_5. S_1 fibers possess P_2, TnT_3, TnI_2 and TnI_4. S_2 fibers are characterized by P_2, TnT_1, TnT_3, TnI_1 and TnI_2. (C) Composite of three western blots of the above samples probed with antibodies to P75, TnT and TnI. Most of the above isoform differences are observed when the blot is labeled with these antibodies.

TnI_1 isoform with TnI_2 (Fig. 3C). The fast MHC isoform (deep abdominal and cutter claw) and the slow MHC isoform from S_1 fibers (crusher claw and ventral cutter claw) could not be distinguished by SDS-PAGE (Fig. 3A; compare lanes a,b with c,e). Those fibers classified as S_2 based on the presence of TnT_1 (distal cutter claw and superficial abdominal flexor) possessed an isoform of MHC (S_2 MHC) with lower mobility than the other MHCs (Fig. 3A; lanes d,f). Using the S_2 MHC and TnT_1 as indicators, the fibers from the distal region of the cutter claw were found to be comprised of a mixture of S_1 and S_2 fibers (Fig. 4). Scanning densitometry was used to quantify the relative amounts of S_1 and S_2 MHCs, as well as the relative amounts of TnT_1 and TnT_3. A significant correlation was observed between the percentage of S_2 MHC and the percentage of TnT_1 in the fibers from the distal region of the cutter claw [% TnT_1=1.56+0.29(% S_2 MHC); P<0.0009, r^2=0.587]. The highest proportions of TnT_1 were observed in the fibers containing 100% of the S_2 MHC isoform and represented about 50% of the total TnT in those fibers (Fig. 4C).

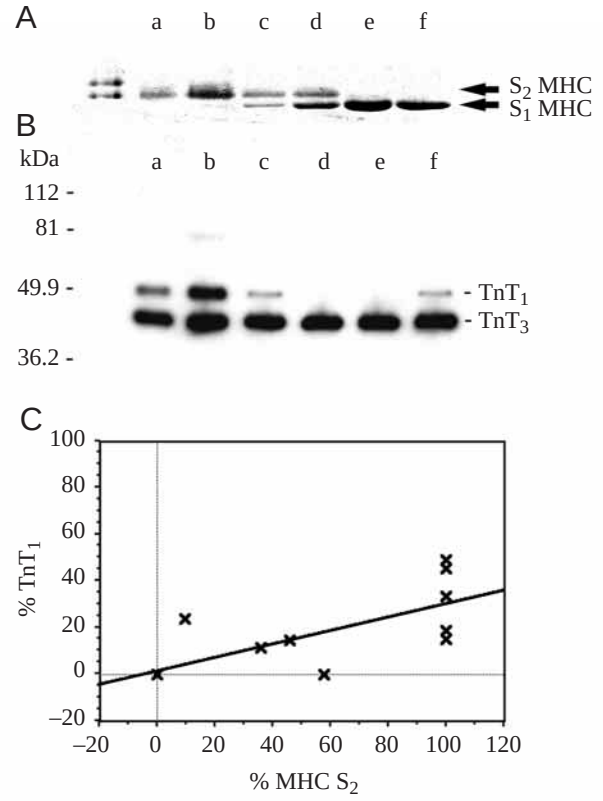

Fig. 4. Correlation between S_1 and S_2 myosin heavy chain (MHC) isoforms and troponin T_1 (TnT_1) found in slow fibers from the distal region of the cutter claw. (A) Representative fibers demonstrate that some fibers contain only the S_2 MHC isoform (a,b), some fibers contain both the S_1 and S_2 isoforms (c,d) and some fibers contain only the S_1 isoform (e,f). Rabbit MHC (Sigma) is the double band to the left of lane a. (B) Western blot probed with anti-TnT from the fibers shown in A. In general, the fibers containing larger amounts of the S_2 MHC isoform also contain a large proportion of TnT_1. Exceptions to this trend are observed in the fiber in lane f, which possesses only the S_1 MHC isoform but also contains TnT_1, and the fiber in lane d, which contains the S_2 MHC isoform but only has the TnT_3 isoform. (C) Correlation between the percentage of TnT existing as the T_1 isoform and the percentage of the S_2 MHC isoform found in different slow fibers. A significant correlation exists between TnT_1 and the S_2 isoform [% TnT_1=1.56+0.29(% S_2), r^2=0.59, P<0.0009]. While a correlation exists between these two markers for S_2 fibers, the relationship is not absolute and accounts for only ~60% of the variability in the proportion of TnT_1 in the fibers. These data support the notion that S_1 and S_2 fibers exist in a continuum between pure S_1 and S_2 fibers. It is also apparent that the TnT_1 isoform is never present by itself but, at most, accounts for about 50% of the TnT protein in a fiber.

Characterization of slow MHC and P75 cDNA

A cDNA encoding the C-terminal sequence of the S_1 MHC was isolated from an *H. americanus* slow muscle library made from mRNA isolated from crusher claw muscle (Fig. 2; GenBank accession no. AY232598). The cDNA was 1795 bp in length with an open reading frame of 1523 bp. The nucleotide sequence had highest sequence identity with the lobster fast MHC isoform encoding the C-terminal, or rod, region of the polypeptide (79% in the open reading frame; Cotton and Mykles, 1993; GenBank accession no. U03091; Fig. 2). The deduced amino acid sequence had 81% sequence identity (91% similarity) to the deduced amino acid sequence from the lobster fast MHC isoform (Fig. 5). In addition, the deduced amino acid sequence also matched a number of MHC sequences from a diverse group of animals, including *Drosophila melanogaster* (473 aa, 78% identity, 89% similarity; GenBank accession no. NM_078863), mollusks (*Loligo peali*: 505 aa, 62% identity, 80% similarity; GenBank accession no. AF042349; *Mytilus galloprovincialis*: 505 aa, 59% identity, 79% similarity; GenBank accession no. AJ249993), mammals (*Felix catus*: 505 aa, 54% identity, 76% similarity; GenBank accession no. U51472) and nematodes (*Caenorhabditis elegans*: 334 aa, 60% identity, 78% similarity; GenBank accession no. Z83107; *Toxocara canis*: 334 aa, 59% identity, 75% similarity; GenBank accession no. AJ306290).

Only a partial sequence of the P75 clone was obtained, due to technical difficulties with sequencing. However, one end of the clone was sequenced successfully and yielded enough information to design sequence-specific primers for PCR. This sequence was submitted to GenBank (accession no. AY302591) and the full sequence will be published when available.

Analysis of myofibrillar protein expression by RT-PCR

Expression of fast MHC, S_1 MHC, P75 and actin in different muscles was analyzed by RT-PCR. Single products of 315 bp, 453 bp, 401 bp and 176 bp, respectively, were obtained using oligonucleotide primers specific for the myofibrillar sequences (Table 1; Figs 6, 7). Fast fibers from the closer muscles in the cutter claw expressed not only fast MHC but also low levels of the S_1 MHC (Figs 6, 8). The fast fibers from the deep abdominal muscle did not exhibit any S_1 MHC expression

(Figs 6, 8). S_1 MHC expression in fast cutter claw fibers was highly variable but, on average, was about six orders of magnitude less than fast expression (Fig. 8). Actin expression was significantly lower than fast MHC expression in the deep abdominal muscles but not in the fast claw muscles (Fig. 8A,B). In general, actin expression was lower in the fast fibers, being, on average, about 1000-fold lower in the fast fiber types than in the S_1 fibers (Fig. 8). In some deep abdominal samples, actin expression was below the threshold of measurement (Fig. 6B, lane a). The various slow fibers predominately expressed the S_1 MHC and actin, with actin expressed at higher levels than S_1 MHC (Figs 7, 8C). The expression of fast MHC in the slow fibers was barely detectable by ethidium bromide staining (Fig. 7), but low levels were measured in the crusher claw closer muscles using real-time PCR (Fig. 8). Although fast MHC expression in S_1 fibers was variable, on average fast expression was 1000-fold less than S_1 MHC expression (Fig. 8). A significant correlation was observed between actin and S_1 MHC expression when values from both the cutter and crusher muscles were combined [log S_1 MHC=0.0437+0.716(log actin); P<0.0001, r^2=0.537] (Fig. 9). This correlation was not significant when made between expression levels from the S_1 muscles alone (P<0.52, r^2=0.027) but was significant for the fast cutter muscles alone (P<0.009, r^2=0.643).

Fig. 5. Alignment of deduced amino acid sequences for major overlapping portions of the ORFs of the fast and S_1 myosin heavy chain (MHC) sequences (amino acids that match exactly are shaded). The corresponding base pairs are indicated on the left. The deduced sequences from the fast and S_1 ORFs share 81% identity and 91% similarity in this region of the molecule. The S_1 and fast forward MHC PCR primers begin at positions 1193 bp and 938 bp, respectively.

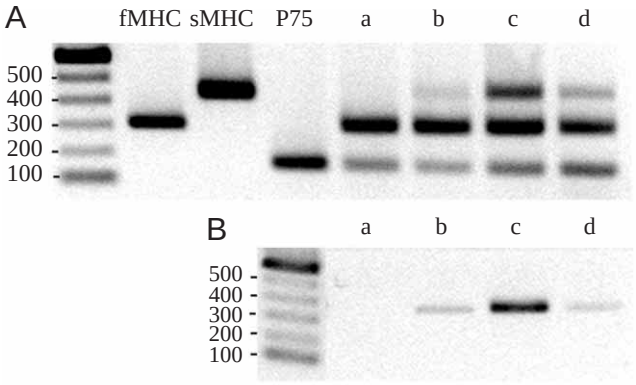

Fig. 6. Analysis of myosin heavy chain (MHC), actin and P75 expression in fast fibers by RT-PCR. (A) Individual reactions were run for each primer set (fast MHC, S_1 MHC or P75) and then products were combined and separated on the same gel. Plasmids containing target sequences (fast MHC, S_1 MHC or P75) were used as controls. (B) Separate reactions were run for actin and separated on a second gel. Deep abdominal flexor muscle (lane a) expressed fast MHC and P75, but actin expression was not detected. Other samples from these muscles expressed actin but at low levels compared with other fibers. Three individual fast fibers from the central region of the cutter claw (lanes b, c and d) expressed both fast and S_1 MHC, P75 and actin. (Inverse images of ethidium bromide-stained gels.)

Discussion

MHC and myofibrillar isoform assemblages

Skeletal muscles are characterized using a number of criteria such as contractile properties, ultrastructure, oxidative capacity and distinct myofibrillar isoforms. Crustacean muscle fibers are consistent with other skeletal muscles in this respect and can be grouped using a number of characteristics to define distinct fiber types (Atwood, 1976; Silverman et al., 1987). In recent years, mammalian classification schemes have favored the type of MHC isoform expressed as a means of identifying various fiber types, since the rate of ATP hydrolysis is largely determined by the MHC isoform present (Schiaffino and Reggiani, 1996). Lobster muscles can also be segregated based on unique myofibrillar isoforms (Mykles, 1985a,b, 1997; Neil et al., 1993), including distinct MHC isoforms (Li and Mykles, 1990; Cotton and Mykles, 1993; Neil et al., 1993; Mykles, 1997). Previous studies of lobster muscles have documented a number of differences in myofibrillar proteins by SDS-PAGE, but this is the first study to identify unique MHC isoforms with one-dimensional SDS-PAGE. While the MHC found in S_2 fibers can be distinguished from other MHCs by these methods, the other MHC isoforms (fast and S_1) migrate with a similar mobility and cannot be distinguished from one another with these techniques. Nevertheless, we know that these MHCs are distinct based on partial digestion with proteases (Li and Mykles, 1990; Neil et al., 1993) as well as the nucleotide sequences reported here. By contrast, up to four MHCs can be

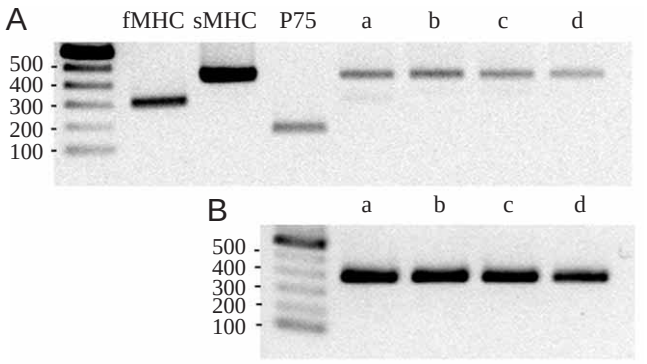

Fig. 7. Analysis of myosin heavy chain (MHC), actin and P75 expression in slow fibers by RT-PCR. Experimental arrangement is as in Fig. 6. Different slow fibers [distal cutter (lane a), ventral cutter (lane b), crusher (lane c), lateral superficial extensor (lane d)] predominantly expressed S_1 MHC and actin. The distal cutter (lane a) shows low amounts of fast MHC product. Actin expression (B) with this method appears to be higher than S_1 MHC expression (A). (Inverse images of ethidium bromide-stained gels.)

resolved from various crayfish muscles using similar methods (LaFramboise et al., 2000). Although little information is available about the number of MHC isoforms in crustaceans, the current identification of an S_2 MHC means that at least three isoforms are present in the lobster: fast, S_1 and S_2. Further work is needed to establish the relationship among these isoforms and those present in other crustacean muscles.

The contractile characteristics of lobster claw muscle fibers, determined both by fiber type and innervation pattern, can be used to categorize fibers as fast, intermediate or slow (Costello and Govind, 1983). Biochemical methods can also be employed to classify these fibers as fast, S_1 or S_2, respectively (Mykles, 1985a,b, 1988; Silverman et al., 1987). In the American lobster, S_1 fibers are known to possess higher myofibrillar ATPase activities than S_2 fibers (Mykles, 1988). Likewise, for the Norway lobster, S_1 and S_2 fibers have different contractile properties, with S_1 fibers contracting more quickly (Holmes et al., 1999). These differences apparently stem from the presence of the two distinct MHC isoforms as identified previously by partial digestion with α-chymotrypsin (Neil et al., 1993) and by SDS-PAGE in the present study. The amounts of S_1 and S_2 MHC isoforms in fibers from the distal region of the cutter claw are present in reciprocal levels, with some fibers possessing a near 1:1 ratio of the S_1 and S_2 isoforms (Fig. 4). Single fibers from other species under control conditions also contain multiple MHC isoforms, indicating that these mixtures are not only present in transitional fibers but represent the normal state of the muscles (Peuker and Pette, 1997; Lutz et al., 1998; Stevens et al., 1999; Lutz et al., 2001). In frog muscle, MHC isoform composition changes along the length of the fiber and is correlated with characteristic proportions of myosin light chain isoforms (Lutz et al., 2001). Further studies are currently underway to gain a better understanding of the significant level of polymorphism observed in the lobster muscles.

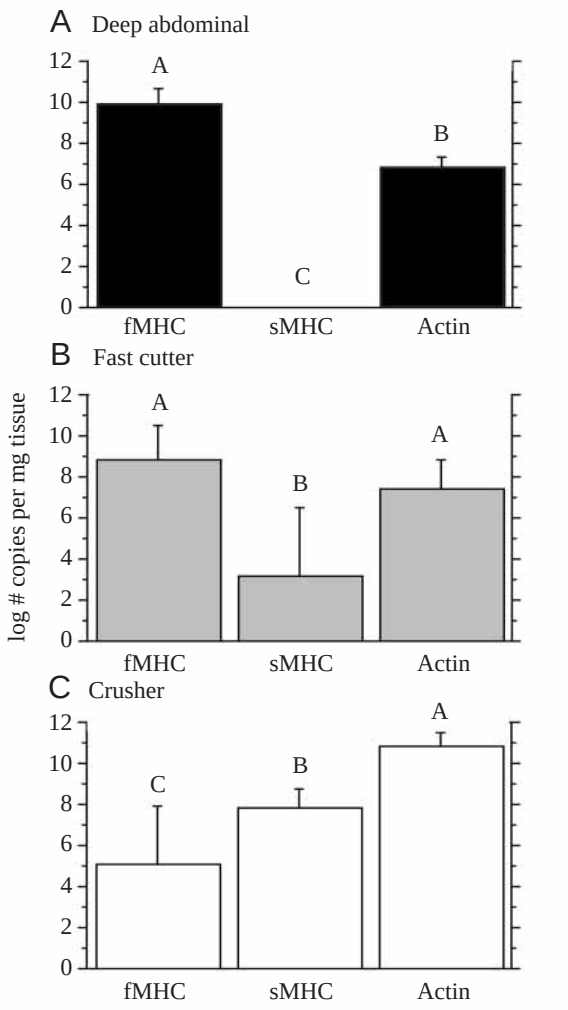

Fig. 8. Quantification of myosin heavy chain (MHC) and actin mRNAs by real-time PCR. (A) Deep abdominal muscles exclusively expressed fast MHC and significantly lower levels of actin. (B) Fast cutter claw muscles predominantly expressed fast MHC, but variable amounts of S_1 MHC were also detected. Actin expression was lower but not significantly different from the levels of fast MHC expression. (C) Crusher muscles expressed significantly more S_1 MHC than fast MHC. Actin expression was significantly higher than MHC expression in the slow muscles. Differences were analyzed by ANOVA of log-transformed values of copy number per mg of tissue. A Bonferroni *post-hoc* test was used to determine which means were different from one another (represented by different uppercase letters). Values are means ± S.D. (*N*=10 samples).

The relative concentration of the S_2 MHC isoform is significantly correlated with the proportion of the TnT_1 isoform (Fig. 3C), which has been used to define S_2 fibers (Mykles, 1985a,b, 1988; Neil et al., 1993). While some fibers contained the S_2 MHC isoform exclusively, the maximum amount of TnT_1 isoform was only a 50% mix with the T_3 isoform (Fig. 4C). Mykles (1988) reported that the TnT_1 and TnT_3 isoforms vary reciprocally in the S_1 and S_2 fibers of crabs and lobsters, but it appears that the maximum amount of the T_1 isoform is only about 50% of the total TnT concentration. The

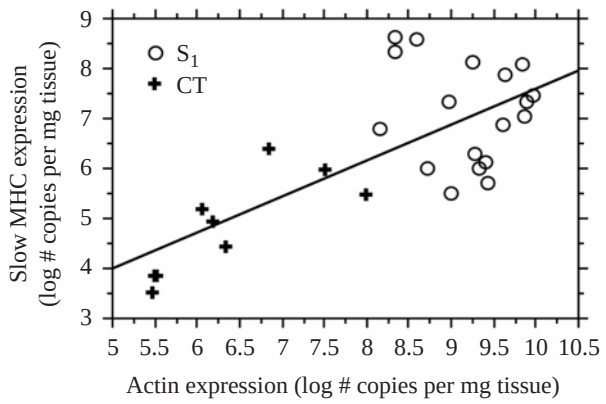

Fig. 9. Correlation between S_1 myosin heavy chain (MHC) expression and actin expression. A significant correlation was observed between S_1 MHC expression and actin expression: S_1 MHC expression=0.437+0.716(actin expression), r^2=0.537, P<0.0001. This correlation was dominated by the differences between the fast cutter fibers (CT) and the S_1 fibers. When these groups were analyzed separately, a significant correlation was still observed for the cutter fibers (r^2=0.643, P<0.009) but not for the S_1 fibers from the crusher claw muscles (r^2=0.027, P<0.52).

relatively loose correlation between TnT isoform assemblage and MHC composition (Fig. 4C; r^2=0.59) suggests that the S_1 and S_2 fibers are not completely distinct but probably exist as a continuum between pure S_1 and pure S_2 fibers. Consistent with this interpretation, the proximal fibers of the claw and leg opener muscles in crayfish and lobsters are S_2, while the central part of the muscles are S_1 (Mykles et al., 2002). The fibers between the proximal and central fibers appear to change gradually in their proportion of TnT isoforms, suggesting that the muscles exist in a continuum (Mykles et al., 2002). A physiological correlate of this continuum is that the more proximal fibers (S_2) show a greater degree of post-synaptic facilitation than the distal (S_1) fibers (Mykles et al., 2002). In the superficial abdominal flexors of *N. norvigicus*, the medial bundle of fibers is composed of S_2 fibers, while the lateral bundle is composed of S_1 fibers (Neil et al., 1993). While these two bundles exhibit considerable overlap anatomically, it is unclear whether individual fibers contain both the S_1 and S_2 MHC isoforms.

The fibers of the cutter claw closer muscle can be divided into anatomically distinct regions characterized by different types of fibers. The central region of the muscle is composed of fast fibers, while the ventral edge of the muscle is comprised of S_1 fibers (Mykles, 1985b), a pattern that is confirmed in the present study. In addition, Mykles (1985b) reported that a small proportion (10–15%) of the slow fibers in the cutter claw closer were S_2 fibers. In the present study, the S_2 fibers could be identified grossly as thin fibers forming a distinct bundle in the ventral aspect of the distal closer muscle (Fig. 1). This is similar to Mykles' description of a distal group of fibers in the claw of the land crab, as a wedge-shaped area occupied by small-diameter fibers (Mykles, 1988). Lang et al. (1980) demonstrated that fibers in this region of both the cutter and crusher claw muscles possess high oxidative capacity, consistent with the presence of S_2 fibers. As in a previous study (Mykles, 1985b), crusher fibers taken from the same region did not appear to form a distinct bundle and were classified as S_1 based on their myofibrillar isoform profile (not shown).

Analysis of myofibrillar gene expression with RT-PCR

An unexpected finding was the co-expression of fast and S_1 MHC genes in the same muscle tissue and even in single fibers. While the initial finding from RT-PCR indicated that this co-expression was primarily in the fast fibers of the cutter claw (Figs 6, 7), quantitative real-time PCR showed that the S_1 fibers also expressed the fast MHC (Fig. 8). It is unclear whether the expressed MHC isoforms are present at the protein level, since the fast and S_1 isoforms co-migrate on protein gels. While the closer muscles of the crusher claw are often considered to be 100% slow by biochemical methods (Mykles, 1985b, 1997), analysis of fiber type by sarcomere length and contractile properties indicates that a small portion of these fibers are fast (Jahromi and Atwood, 1971; Costello and Govind, 1983). Some of the expression of the minor MHC in both fast and slow claw muscles may be due to the presence of a minor population of such fibers. However, analysis of individual muscle fibers from the fast region of the cutter claw and from the crusher claw muscles reveals co-expression within single fibers. A significant number of fibers from rabbit, rat and frog muscles possess multiple MHC mRNAs, most of which are 'next neighbor' isoforms (Peuker and Pette, 1997; Lutz et al., 1998; Stevens et al., 1999). However, in some cases, MHC mRNA sequences were found in fibers that did not contain detectable levels of the corresponding proteins (Peuker and Pette, 1997; Stevens et al., 1999). Unloading of rat soleus muscle increases the proportion of these mismatched fibers (Stevens et al., 1999). It has been suggested that these discrepancies are evidence for both transcriptional and translational control of muscle phenotype (Peuker and Pette, 1997; Stevens et al., 1999). In this context, it seems likely that the low levels of expression of the unexpected MHC genes in the lobster may never actually be translated into functional proteins.

It is also possible that unidentified MHC isoforms hybridized with the primers designed for the deduced fast and S_1 MHC sequences. To date, only the S_1 and fast MHC isoforms have been cloned from the lobster and it is likely that other isoforms exist. As a case in point, the sequence encoding the S_2 MHC isoform identified in the present study has yet to be cloned. This protein is clearly co-expressed with the S_1 isoform in a population of slow fibers, and similar expression of unidentified sequences in other fibers may go undetected or result in non-specific amplification by one of the primer sets. Recently, the sequences of several crustacean MHC genes have been identified (Holmes et al., 2002) but these were all from the variable region of the myosin head, and the fast and S_1 MHC sequences of the lobster are from the rod region of the molecule, making direct comparisons impossible.

Each of the fiber types possessed a unique expression pattern with respect to the MHC isoforms and actin (Fig. 8). Interestingly, the phasic deep abdominal muscles expressed only the fast MHC isoform, with no S_1 MHC detected. These differences may be related to differences in the type of innervation, since the deep abdominal muscles are more uniformly phasic in their properties (Atwood, 1976; Cooper et al., 1998), while the claw closer muscles receive either phasic, tonic or both types of innervation (Atwood, 1976; Lang et al., 1980; Costello and Govind, 1983). Other crustacean muscles demonstrate definite correlations between muscle phenotype and innervation (Günzel et al., 1993; Mykles et al., 2002). Another major difference between fiber types is that the S_1 fibers contained significantly higher levels of actin mRNA than the fast fibers. Consistent with this, slow fibers have a thin to thick filament ratio of about six, while fast fiber have a ratio of about three (Jahromi and Atwood, 1969, 1971). A significant correlation was observed between S_1 MHC and actin in all fibers expressing the S_1 MHC isoform. However, this pattern was observed over a very broad range of isoform expression in fast and slow fibers and was not significant when examined over the narrower range of actin expressed in the slow fibers. This correlation and the relationship between the S_2 MHC and TnT_1 may reflect a functional linkage between individual isoforms within a whole myofibrillar protein assemblage. These isoforms are not typically interchanged in isolation but exist as part of an entire population of protein isoforms that characterize a particular muscle type (Schiaffino and Reggiani, 1996; Mykles, 1997).

Conclusions

This study extends our understanding of the differences among skeletal muscle fiber types in crustaceans at a molecular level of organization. Although fast and S_1 MHC isoform expression patterns can be quantified and correlated with the expression of other myofibrillar proteins, these isoforms co-migrate in SDS-PAGE gels and cannot be distinguished by these methods. However, another MHC associated with S_2 fibers can clearly be distinguished from the S_1 MHC isoform and coexists with this isoform in many fibers. The relative concentration of the S_2 MHC isoform was positively correlated with the TnT_1 isoform, which has been considered diagnostic for S_2 fibers. The gradation between muscle fiber types like that seen with the S_1 and S_2 fibers provides an opportunity to examine the determinants of muscle phenotype, including differences in fiber innervation pattern. Similar MHC polymorphism has been detected in mammalian and frog muscles in recent years, but the functional implications of this gradation in muscle phenotype is still unclear. Further work is needed to identify the gene sequences encoding other MHC isoforms, such as the S_2 MHC. Current studies are focusing on the correlation between S_1 MHC mRNA and S_1 and S_2 MHC proteins in superficial abdominal muscles. The methods used in the present study can also be applied to other questions of muscle diversity and plasticity in crustaceans. For example, the developing lobster claw muscles undergo a process of fiber switching during development that represents a natural model of skeletal muscle plasticity (Govind et al., 1987; Govind, 1992; Mykles, 1997). We are using real-time PCR to examine the changes in myofibrillar gene expression that may contribute to the fiber switching process in juvenile claw muscles.

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