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Fiber polymorphism in skeletal muscles of the American lobster, *Homarus americanus*: continuum between slow-twitch (S_1) and slow-tonic (S_2) fibers

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Summary

In recent years, an increasing number of studies has reported the existence of single fibers expressing more than one myosin heavy chain (MHC) isoform at the level of fiber proteins and/or mRNA. These mixed phenotype fibers, often termed hybrid fibers, are currently being recognized as the predominant fiber type in many muscles, and the implications of these findings are currently a topic of great interest. In a recent study, we reported single fibers from the cutter claw closer muscle of lobsters that demonstrated a gradation between the slow-twitch (S_1) and slow-tonic (S_2) muscle phenotype. In the present study, we focused on S_1 and S_2 fibers from the superficial abdominal muscles of the lobster as a model to study the continuum among muscle fiber types. Complementary DNAs (cDNA) encoding an S_2 isoform of myosin heavy chain (MHC) and an S_2 isoform of tropomyosin (Tm) were isolated from the superficial abdominal flexor muscles of adult lobsters. These identified sequences were used to design PCR primers used in conjunction with RT-PCR and real-time PCR to measure expression levels of these genes in small muscle samples and single fibers. The relative expression of the corresponding S_1 MHC and S_1 Tm isoforms was measured in the same samples with PCR primers designed according to previously identified sequences. In addition, we measured the relative proportions of MHC, troponin (Tn) T and I protein isoforms present in the same samples to examine the correlation of these proteins with one another and with the MHC and Tm mRNAs. These analyses revealed significant correlations among the different myofibrillar proteins, with the S_1 and S_2 fibers being characterized by a whole assemblage of myofibrillar isoforms. However, they also showed that small muscle samples, and more importantly single fibers, existed as a continuum from one phenotype to another. Most fibers possessed mixtures of mRNA for MHC isoforms that were unexpected based on protein analysis. These findings illustrate that muscle fibers in general may possess a phenotype that is intermediate between the extremes of 'pure' fiber types, not only at the MHC level but also in terms of whole myofibrillar assemblages. This study supports and extends our recent observations of mixed phenotype fibers in lobster claw and leg muscles. The existence of single fiber polymorphism in an invertebrate species underscores the generality of the phenomenon in skeletal muscles and emphasizes the need for an understanding of the proximal causes and physiological consequences of these intermediate fiber types.

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

Introduction

Skeletal muscles exhibit considerable variability in their physiological properties, with muscle fiber type being coupled to the mechanical requirements of a particular muscle. As such, muscle fiber type is an important functional determinant of the physiology of movement and locomotion, where different fiber types fulfill various mechanical needs of an organism (Rome et al., 1988; Rome and Lindstedt, 1997). Early studies of skeletal muscle properties attempted to sort out these different fiber types into discrete, functional categories to provide a meaningful foundation for understanding basic muscle biology. A number of criteria were used to classify different fiber types including contractile properties, histochemical assays and biochemical assays (Brooke and Kaiser, 1970; Barnard et al., 1971; Peter et al., 1972; Ariano et al., 1973). Although at least three distinct fiber types were identified based on these criteria, it remained unclear whether these were the only fiber types and whether single fibers were distinct or whether they existed as points on a continuum of fiber types (Brooke and Kaiser, 1970; Peter et al., 1972). More recently, specific myosin heavy chain (MHC) isoforms present in different muscles have been used to more precisely categorize muscle fiber types (reviewed by Pette et al., 1999; Caiozzo, 2002). Although single fibers expressing multiple MHC isoforms have sometimes been interpreted as being in the

process of switching from one type to another (Pette et al., 1999; Pette and Staron, 2000; Pette, 2001), Caiozzo et al. (2003) recently determined that a majority of fibers from various rat muscles were polymorphic, containing a mixture of two or more specific MHC isoforms. In fact, an increasing number of studies from several different vertebrate species has documented MHC polymorphism in single muscle fibers (reviewed by Stephenson, 2001). Whether muscle fibers represent distinct functional entities or whether each fiber phenotype is a complex blend of different myofibrillar isoforms is still a highly relevant question (Stephenson, 2001; Caiozzo, 2002; Caiozzo et al., 2003).

Like mammalian muscles, crustacean fibers are categorized into several different fiber types based on a number of criteria. Broadly, crustacean fibers are distinguished on the basis of structural characteristics (Jahromi and Atwood, 1969, 1971; Atwood, 1976; West, 1997), ATPase histochemistry (Ogonowski and Lang, 1979; Silverman et al., 1987), specific myofibrillar isoform assemblages (Mykles, 1985a,b, 1988, 1997) and the contractile properties of the fibers (Holmes et al., 1999). Currently, at least three different crustacean fiber types are recognized: fast, slow-twitch (S_1) and slow-tonic (S_2). In lobsters (*H. americanus*), fast fibers comprise the majority of the muscle mass in the cutter claw closer and in the deep abdominal flexor muscles. S_1 fibers are the exclusive fiber type in the crusher claw closer and are present in the claw and leg opener muscles, in the ventral musculature of the cutter claw closer and in the superficial flexors and extensors of the tail. S_2 fibers are present in the distal fibers of the cutter claw closer, claw and leg openers, as well as the superficial abdominal flexors and extensors. Previous studies have demonstrated that the superficial abdominal muscles of decapod crustaceans are comprised of both S_1 and S_2 fibers (Fowler and Neil, 1992; Neil et al., 1993).

In the current study, we focused on S_1 and S_2 fibers from the superficial abdominal muscles in the lobster as a model to study the continuum among muscle fiber types. We recently identified single fibers from the lobster claw closer that were polymorphic, expressing variable levels of both S_1 and S_2 MHC, as well as other myofibrillar isoforms (Medler and Mykles, 2003). In addition, we demonstrated that most lobster muscles co-expressed multiple MHC genes in muscles that had previously been viewed as distinct in their fiber types. These trends suggest that lobster muscle fibers, like the muscles of mammals and other vertebrates, often represent a point in a continuum of muscle phenotypes. While MHC is the most important determinant of the contractile properties of a muscle (Schiaffino and Reggiani, 1996), different fiber types exhibit whole assemblages of distinct myofibrillar isoforms (Schiaffino and Reggiani, 1996; Mykles, 1997). Because of the importance of these non-MHC proteins to muscle function, we examined the relative proportion of other myofibrillar isoforms (Tm, TnT, TnI) and how they were correlated in different fibers. For the present study, we obtained partial cDNAs for previously unidentified S_2 MHC and tropomyosin (Tm) isoforms and used sequence-specific PCR primers to measure the relative expression of S_1 and S_2 isoforms using real-time PCR. In addition, we used SDS-PAGE and western blotting to identify different isoforms of MHC, troponin T (TnT) and troponin I (TnI) in small muscle bundles and single muscle fibers. Together, these data provide information about the level of polymorphism present in crustacean muscle fibers and the correlation among different myofibrillar isoforms, at both the protein and mRNA levels.

Materials and methods

Animals and tissue preparation

Adult lobsters (*Homarus americanus* Milne-Edwards) were purchased locally, and muscles were harvested the same day. Superficial abdominal extensors and flexors were harvested from lobsters on ice. Muscle bundles were quickly frozen in liquid N_2 and then stored at –80°C until further preparation. Fiber bundles were divided into two pieces, one for RNA isolation and one for protein analysis. To obtain single muscle fibers, samples were then freeze-dried by centrifuging under a vacuum (Speed-Vac; 12 000 ***g***) for ~1 h. Upon drying, individual fibers were separated from the bundle with fine forceps under a dissecting microscope. Individual fibers were divided in two for RNA isolation and protein analysis. A total of 25 muscle bundles and 42 individual fibers taken from three lobsters was used in the analyses. Other muscles analyzed were harvested from animals used in another recent study (Medler and Mykles, 2003). These muscles include the slow (S_1) muscles of the crusher claw, the central fast muscles of the cutter claw, and the fast muscles of the deep abdominal flexors. A more detailed description of the different fibers present in the cutter claw is given in Medler and Mykles (2003). Only the central fast muscles from the cutter claw were used in the present study.

Isolation and sequencing of cDNAs encoding slow-tonic (S_2) MHC and Tm

A cDNA encoding the 3′ end of the coding region and 3′ UTR of an MHC-encoding gene was obtained using the 3′-RACE system (Invitrogen Inc., Carlsbad, CA, USA) and a gene-specific forward primer designed to a conserved region of the MHC sequence, as based upon the sequences of lobster fast and slow (S_1) sequences (5′-GAAGGCTAAGAAGGCC-ATGGTTGA-3′) (Medler and Mykles, 2003). Briefly, RNA was isolated from samples of superficial flexor muscles with TRIZOL (see Analysis of myofibrillar protein mRNAs). First-strand cDNA synthesis reactions contained 100 ng total RNA and 1 μl of 10 μmol l^{-1} Invitrogen Inc. adaptor primer (Cat. No. 10542-017): 5′-GGCCACGCGTCGACTAGTAC(T)17-3′. The other conditions of the first-strand synthesis reaction were the same as described below (see Analysis of myofibrillar protein mRNAs). Muscles used for RNA isolation were pre-screened with high-resolution SDS-PAGE to ensure that the muscle samples were exclusively expressing the S_2 MHC (see Analysis of myofibrillar proteins). PCR was carried out on approximately 10 ng cDNA using a universal amplification

primer (Invitrogen Inc.; Cat. No. 18382-010) as the reverse primer and the MHC-specific forward primer. PCR products were identified by separating samples on 1% agarose gels and staining the DNA with ethidium bromide. The PCR yielded two distinct products, one of approximately 1500 bp and the other of approximately 1000 bp. Both PCR products were purified by gel purification (Qiagen, Valencia, CA, USA) and cloned into the pCR 2.1 TOPO plasmid using TOPO TA cloning kit (Invitrogen Inc.). Transformed bacteria were grown overnight on pre-warmed MacKonkey ampicillin plates and were selected based on the inability to metabolize lactose. The presence of PCR inserts in the transformed colonies was confirmed by PCR using vector primers (M13 reverse and T7). Several positive clones containing inserts ranging from approximately 1 kb to 2 kb were selected for sequencing by Davis Sequencing (Davis, CA, USA). Of these clones, two were found to be a previously unidentified MHC that was presumed to code for an MHC protein in S_2 muscle fibers. Both strands of the MHC cDNA were ultimately obtained using a combination of vector primers and internal sequence-specific primers (Davis Sequencing).

A smaller cDNA insert yielded a DNA sequence for a previously unidentified tropomyosin cDNA bearing high identity to the fast and slow (S_1) tropomyosin sequence previously identified in the lobster (Mykles et al., 1998). It was later discovered that the last 12 bp on the 3′ end of the forward primer designed to amplify the MHC sequence shared 100% sequence identity to a region of the Tm sequence. This sequence similarity may stem from the fact that both the tail region of the MHC and the Tm proteins form coiled-coil structures. A full sequence was obtained by PCR using the universal amplification primer and a forward primer designed to the 5′ end of the published S_1 Tm sequence. This PCR product was cloned into the pCR 2.1 TOPO plasmid (Invitrogen Inc.) as described above. Both strands of this cDNA were obtained by sequencing with T7 and M13 vector primers and sequence-specific internal primers (Davis Sequencing).

Analysis of myofibrillar proteins

Muscle bundles 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 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. Individual freeze-dried fibers were placed directly in 50 μl SDS sample buffer and homogenized with a hand-held pestle that fitted directly into the 0.5 ml microcentrifuge tube.

For analysis of myofibrillar isoform assemblages, 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 the prepared samples using a Mini-Protean 3 gel system (Bio-Rad, Hercules, CA, USA). Samples were then electrophoretically 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) for at least 1 h and then incubated for at least 1 h with polyclonal antibodies raised in rabbits against purified TnT or TnI at 1:20 000 dilutions of antiserum in 0.05% Tween in TBS (TTBS: Sohn et al., 2000; Mykles et al., 2002). After several washes in TTBS, blots were incubated with biotinylated anti-rabbit IgG at 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 3) 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).

Analysis of myofibrillar protein mRNAs

RNA was isolated using TRIZOL reagent (Invitrogen Inc.). Fibers were homogenized in 1 ml TRIZOL using a hand-held glass homogenizer until the fiber was completely homogenized. 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 in 75% ethanol. After air-drying, samples were dissolved in water and quantified by measuring absorbance at a wavelength of 260 nm. The samples were then stored at –80°C. The samples were then treated with DNase (Invitrogen Inc.) for 15 min to remove any genomic DNA still in the samples. First-strand DNA was synthesized from total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen Inc.) using oligo (dT) primers. The 20 μl reaction contained 2.5 μg 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 RNA and 200 units of M-MLV reverse transcriptase.

PCR of MHC and Tm isoforms was performed using sequence-specific primers that amplified the specific isoform of interest. Forward primers were designed to anneal to regions of the open reading frame, while reverse primers annealed to unique non-coding regions of the 3′ UTR (Figs 1, 2; Table 1). Since the 3′ UTRs of the fast and S_1 isoforms are thought to be the same (Mykles et al., 1998), it is possible that the S_1 primers also amplified the fast Tm isoform. However, this possibility is unlikely since all of the fibers used in the current study were from slow muscles. Nonquantitative PCR was used to demonstrate the specificity of the primers, and purified plasmids containing the cDNA of interest were used as positive and negative controls. 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 stained with ethidium bromide. For figures, products from the individual reactions (S_1 and S_2 MHC, or S_1 and S_2 Tm) were combined before separation on agarose gels.

Real-time PCR was used to quantify the levels of S_1 MHC, S_2 MHC, S_1 Tm and S_2 Tm. The above protocol was used for the generation of cDNA from the isolated RNA. These samples were then amplified with primers specific to each isoform using The Light Cycler DNA Master SYBR Green I reaction mix (Roche Molecular Biochemicals, Indianapolis, IN, USA) and Cepheid Smart Cycler Instrument (Medler and Mykles, 2003;

Fig. 1. Alignment of cDNA sequences encoding part of the open reading frame (ORF) and 3′ untranslated region (UTR) for the slow-twitch (S_1), fast and slow-tonic (S_2) myosin heavy chains (MHC) to show sequence similarities and the regions of the S_1 and S_2 sequences amplified by PCR. Bases that match at least one of the other sequences are shaded, and the stop codons (TAA) are enclosed in boxes. Each sequence shares ~79% identity within the ORF. The 3′ UTR follows the stop codon and has low sequence identity. S_1 forward (S_1F), S_1 reverse (S_1R), S_2 forward (S_2F) and S_2 reverse (S_2R) MHC primer positions are indicated by arrows adjacent to the S_1 and S_2 sequences, respectively. Reverse PCR primers were designed to isoform-specific sequences in the UTR, while forward primers were designed to anneal to regions of relatively low sequence identity in the ORF. The fast and S_1 MHC sequences have been published previously (Cotton and Mykles, 1993; Medler and Mykles, 2003). The GenBank accession numbers for the MHC sequences are U03091 (fast), AY232598 (S_1) and AY521626 (S_2).

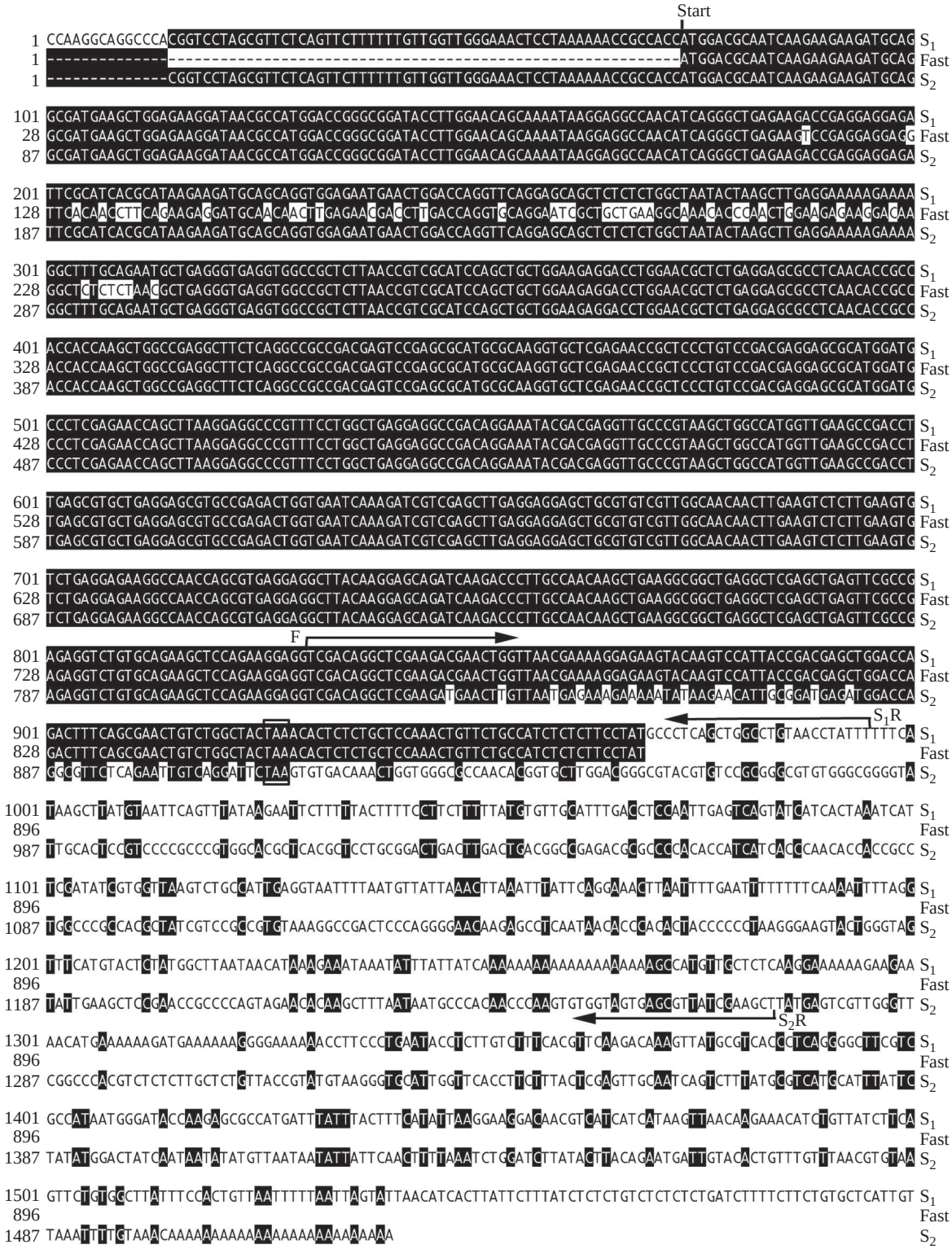

Fig. 2. Alignment of cDNA sequences encoding lobster muscle slow-twitch (S_1), fast and slow-tonic (S_2) tropomyosin (Tm). Bases that match at least one of the other sequences are shaded to indicate the sequence identities among the three isoforms, and the stop codons (TAA) are enclosed in a single box. The beginning of the start codon is indicated. The S_2 sequence shares 92% identity with the fast sequence within the ORF, while the S_1 and S_2 sequences share 97% identity. The S_1 and S_2 sequences are identical up to bp 833 of the S_2 sequence and are then divergent through the rest of the ORF and 3′ UTR. A common forward PCR primer was used to amplify both the S_1 and S_2 sequence, while S_1 reverse (S_1R) and S_2 reverse (S_2R) primers were designed to isoform-specific sequences within the 3′ UTR. The fast and S_1 Tm sequences have been published previously (Mykles et al., 1998). The GenBank accession numbers for the Tm sequences are AF034954 (fast), AF034953 (S_1) and AY521627 (S_2).

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

Sequence	Forward	Reverse
S_1 MHC*	CGAAACCAATGCTCTGAAGAACACC	GTAAGCATCAAGTACCGTCCAAGG
S_2 MHC	CGCCTCGAAGTTGAGGGTAAT	ACAGGTGTTACTTGTCGGTGTCGG
S_1 Tm	S_2 Tm forward primer	AAATAGGTTACAGGCCAGCTGAGG
S_2 Tm	TCGACAGGCTCGAAGATGAACTTG	AGCTTCGATAACGCTCACTACCAC

MHC, myosin heavy chain; Tm, tropomyosin; S_1, slow-twitch muscle; S_2, slow-tonic muscle.
*Primers for S_1 MHC are the same as used by Medler and Mykles (2003).

Yu and Mykles, 2003). A master reaction mix consisting of 1× Light Cycler DNA Master SYBR Green I, 2.5 mmol l^{-1} $MgCl_2$, 0.5 mmol l^{-1} of each primer and 0.8 mmol l^{-1} dNTP was prepared before adding 5 μl of the first-strand cDNA to yield a total reaction volume of 25 μl. PCR amplification consisted of denaturation of template and activation of HS Taq at 95°C for 5 min followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 6 s, and extension at 72°C for 30 s. A standard curve was constructed using purified plasmid with known amounts of cDNA template. Copy numbers of the plasmid were calculated based on the molecular mass of the plasmid and then converting to number of copies based on Avogadro's number (Li and Wang, 2000). The standard curves were created through serial dilutions, which were then plotted as a function of threshold cycle at which products began to accumulate exponentially. The product specificity was confirmed in two ways. First, the melting temperature was determined and used as a measure of G-C content. Second, samples were electrophoretically separated on 2% agarose gels to determine product size. The cycle threshold from reactions containing unknown amounts of cDNA was converted to number of copies using the established standard curves. The isoforms could then be correlated based on total amount of expression or based upon percent of expression relative to total expression of all isoforms coding for the same gene.

For the comparison of S_2 MHC expression among different muscle types, the number of copies of S_2 MHC mRNA was standardized to the total RNA in the samples, as previously described (Medler and Mykles, 2003). For the smaller samples from the superficial extensors and flexors, the amount of total RNA was too low to quantify accurately. For each of these samples, the amount of S_1 MHC mRNA relative to the amount of S_2 MHC mRNA from the same sample was used to assess the relative expression of these two genes. The same procedure was used for the S_1 and S_2 Tm mRNA measurements. Through use of these ratios, the S_1 and S_2 expression levels for both the MHC and Tm genes served as internal standards for each other.

Statistical analyses

A one-way analysis of variance (ANOVA) was used to compare the relative number of copies of myofibrillar mRNA and protein calculated from real-time PCR and western blots, respectively. Expression levels for the S_2 MHC isoform in different muscles were log-transformed because of high levels of variability among samples and to correct for the correlation between mean and variance in these data (Neter et al., 1990). The arcsin transformation is often used when comparisons are made among variables that are proportions (Neter et al., 1990), but we found that this transformation did not affect our comparisons. Consequently, the relative proportions of mRNAs and proteins were not transformed. 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 relative number of copies of mRNA for different myofibrillar genes, as well as for the correlations between TnT and TnI isoforms. Statview 5.0.1 (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

Results

Characterization of S_2 MHC and S_2 Tm cDNAs

A cDNA encoding the C-terminal sequence of the S_2 MHC was isolated from *H. americanus* superficial flexor muscle using a PCR-based cloning approach (Fig. 1; GenBank accession no. AY521626). The cDNA was 813 bp in length with an open reading frame (ORF) of 553 bp and a 3′ UTR of 260 bp. The nucleotide sequence had highest sequence identity with the lobster fast and S_1 MHC isoforms encoding the C-terminal rod region of the polypeptide (78% sequence identity to both fast and S_1 MHC in the ORF; Cotton and Mykles, 1993; Medler and Mykles, 2003; Fig. 1). The ORF of the S_2 sequence had approximately 75 additional base pairs following the corresponding sequences of the fast and S_1 isoforms.

A cDNA encoding the entire sequence of the S_2 Tm was isolated from *H. americanus* superficial flexor muscle using the same PCR-based cloning approach as used to obtain the S_2 MHC sequence (Fig. 2; GenBank accession no. AY521627). The cDNA was 1526 bp in length with an ORF of 855 bp and a 3′ UTR of 612 bp. The nucleotide sequence had highest sequence identity with the lobster fast and slow (S_1) Tm isoforms previously reported (Mykles et al., 1998). The sequence shared 97% identity with the S_1 Tm sequence in the ORF, differing only at the 3′ end of the ORF (bp 833–stop codon) and in the 3′ UTR. The sequence was 92% identical to the fast Tm, differing at both the 5′ (bp 174–299) and 3′ (bp 833–stop codon) ends of the ORF.

The deduced amino acid sequences for the three tropomyosin isoforms (S_1, fast and S_2) are shown in Fig. 3. As reported previously (Mykles et al., 1998), the fast and S_1

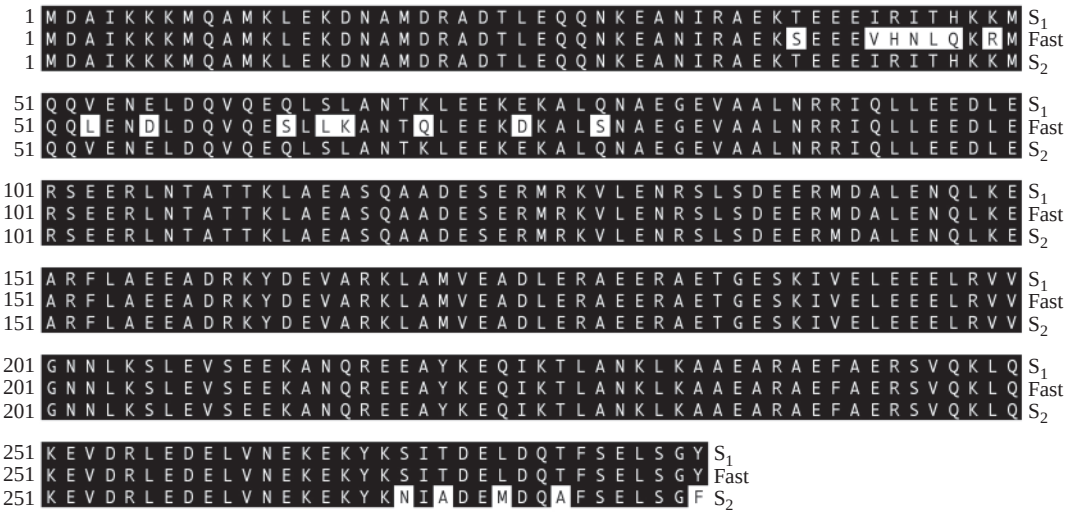

Fig. 3. Alignment of deduced amino acid sequences for the three tropomyosin isoforms (S_1, fast and S_2). As reported previously (Mykles et al., 1998), the fast and S_1 sequences are identical except in the region comprising residues 39–80, resulting in 95% identity. The S_2 and S_1 isoforms were identical except in five residues at the carboxy-terminal end of the molecule (residues 269–284), resulting in 98% identity between these two proteins.

sequences are identical except in the region comprising residues 39–80, resulting in 95% identity. The S_2 and S_1 isoforms were identical except in five residues at the C-terminal end of the molecule (residues 269–284), resulting in 98% identity between these two proteins.

Expression of S_2 MHC in different muscles

The S_2 MHC gene was found to be expressed in each of the muscles examined, with the highest expression being in the superficial muscles of the abdomen. Intermediate expression was observed in the slow (S_1) muscles of the claw, with the lowest expression being observed in the fast muscles of the claw and abdomen (Fig. 4). The average copy number per μg total RNA in the superficial muscles was five times higher than in the crusher claw muscles (9.58×10^7 *vs* 1.91×10^7), 147 times higher than in the cutter claw muscles (9.58×10^7 *vs* 6.51×10^5) and more than 500 times higher than in the deep abdominal flexors (9.58×10^7 *vs* 1.85×10^5).

Coexpression of the S_1 and S_2 MHC and Tm in superficial abdominal muscles

Bundles of fibers and single fibers alike were found to coexpress the S_1 and S_2 isoforms in single samples. This coexpression was evident for both the MHC and Tm isoforms. PCR with sequence-specific primers for these genes clearly demonstrated this coexpression, and simultaneous amplification with purified plasmid as template demonstrated that the primers were highly specific for each isoform (Fig. 5). In essentially every sample analyzed, co-expression of the S_1 and S_2 isoforms for both MHC and Tm was observed. Overall, the relative expression of the S_1 and S_2 MHC isoforms was significantly correlated with the S_1 and S_2 isoforms of Tm, although this relationship was non-linear (Fig. 6). The relative expression of the MHC isoforms ranged from nearly 100% of the S_1 isoform to nearly 100% of the S_2 isoform (Fig. 6). By contrast, the S_2 Tm isoform represented about 90% of Tm expression, even in fibers expressing the S_1 MHC isoform almost exclusively (Fig. 6).

Protein isoforms in different fibers

Relative amounts of protein isoforms were measured by SDS-PAGE and western blotting, followed by densitometry of the gels and X-ray films exposed during chemiluminescent detection of western blots. Protein isoforms for MHC, TnT and

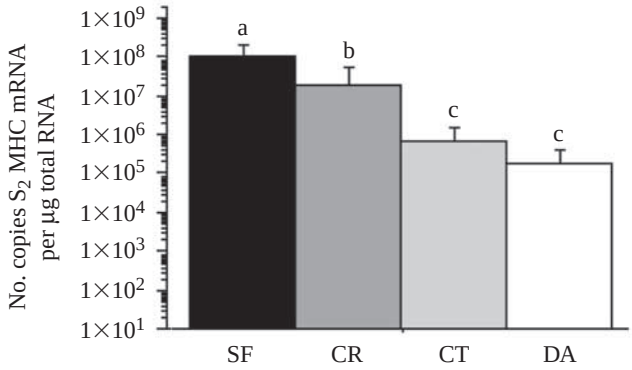

Fig. 4. Quantification of S_2 myosin heavy chain (MHC) mRNA by real-time PCR in different lobster muscle samples (fiber bundles). Expression was highest in the superficial flexors and extensors (SF), followed by the S_1 fibers of the crusher claw closer (CR) and then by the fast fibers of the cutter claw closer (CT) and deep abdominal flexors (DA). Differences were analyzed by ANOVA of log-transformed values of copy number per μg of total RNA. A Bonferroni *post-hoc* test was used to determine which means were significantly different from one another (represented by different lowercase letters). Values are means ± S.D. (*N*=9–11 samples per group).

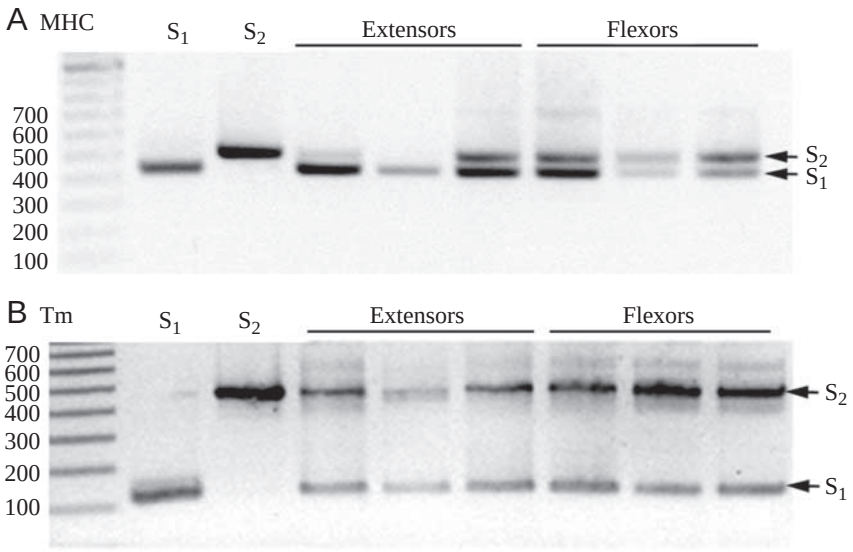

Fig. 5. Analysis of S_1 and S_2 myosin heavy chain (MHC) and tropomyosin (Tm) expression in single fibers from the superficial flexor and extensor muscles by RT-PCR. (A) Individual reactions were run for S_1 and S_2 MHC primers and then products were combined and separated on the same gel. Plasmids containing either the S_1 or S_2 MHC cDNA were used as controls. (B) Same analysis as in A, but using the S_1 and S_2 Tm primers. All of the fibers, with the exception of the second extensor in A, expressed both the S_1 and S_2 isoforms of MHC and Tm. (Inverse images of ethidium bromide-stained agarose gels.)

TnI in single fibers and bundles were present in a number of different combinations. These combinations followed predictable patterns that represented a continuum from what could be described as 'pure' S_1 to 'pure' S_2 fibers (Fig. 7). While multiple MHC isoforms were observed in muscle bundles, all of the single fibers analyzed were found to contain detectable levels of only single isoforms (Fig. 7). TnT_1 was found to be highest in S_2 fibers, while TnT_3 was the predominant TnT isoform in S_1 fibers (Figs 7–9). TnT_2 is only expressed in fast fibers (Mykles, 1985a, 1997). TnI_1 was a minor isoform observed in only a few samples and was not correlated with either TnT isoform (Fig. 9A). TnI_2 was the main TnI isoform in S_2 fibers (Figs 7, 8C), and TnI_2 content was inversely correlated with the proportion of TnT_3 in the fibers (positively correlated with TnT_1; Fig. 9B). TnI_3 content was found in reciprocal levels to TnI_2, being the main isoform in S_1 fibers (Figs 7, 8D), and was positively correlated with the proportion of TnT_3 (negatively correlated with TnT_1; Fig. 9C). TnI_4 was a minor isoform in the fibers and was found primarily in S_1 fibers, being positively correlated with TnT_3 concentration (Figs 7, 9D).

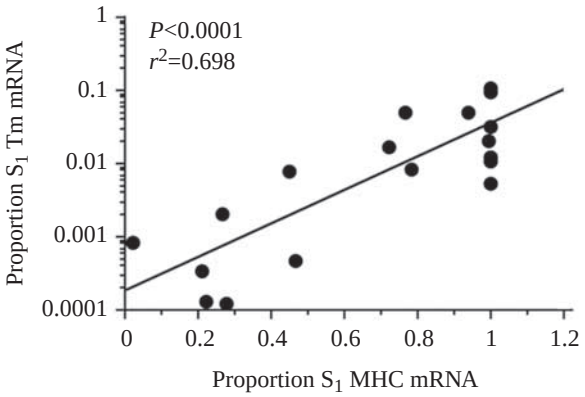

Fig. 6. Correlation between the relative proportion of S_1 myosin heavy chain (MHC) and S_1 tropomyosin (Tm) mRNA in single fibers (semi-log scale). The proportion of Tm expressed as the S_1 isoform was significantly ($P<0.0001$; $r^2=0.698$) correlated with the proportion of S_1 MHC expressed. The proportion of S_1 Tm was always less than that of S_2 Tm and no more than ~10% of the total Tm expressed, even in fibers classified as S_1 fibers. The correlation on a semi-log scale indicates that the relationship between Tm and MHC expression is non-linear ($\log y=2.35\log x-3.84$).

Major characteristics of S_1 and S_2 fibers

Fibers were classified as S_1, S_2 or intermediate based on MHC protein isoforms as identified by SDS-PAGE (Fig. 7). Based on this classification, several characteristic patterns were observed (Fig. 8). First, S_1 fibers expressed a higher proportion of the S_1 MHC mRNA, intermediate fibers contained slightly lower but non-significantly different levels, and S_2 fibers expressed a significantly lower proportion of the S_1 MHC mRNA (more S_2 MHC mRNA; Fig. 8A). Second, slow fibers contained TnT_1, TnT_3 or a combination of both isoforms. The S_1 fibers possessed a higher proportion of TnT_3, S_2 fibers contained significantly less TnT_3 (more TnT_1), while samples containing both isoforms were intermediate in the proportion of TnT_1 and TnT_3. Finally, lobster slow muscles contained varying amounts of TnI_1, TnI_2, TnI_3 and TnI_4 (Fig. 9). S_1 fibers primarily contained TnI_3, while the S_2 fibers mainly contained TnI_2. Intermediate samples contained intermediate values for both TnI_2 and TnI_3 (Figs 8C,D, 9).

Discussion

S_2 MHC and Tm cDNAs

This is the first report of DNA sequences coding for S_2 myofibrillar protein isoforms. The S_2 MHC cDNA contains the 3′ end of the ORF encoding the C-terminal end of the rod region of the MHC and the adjacent 3′ UTR of a gene encoding a previously unidentified MHC isoform. This cDNA shares highest sequence identity to the fast and S_1 MHCs previously cloned from lobster muscles (Cotton and Mykles, 1993;

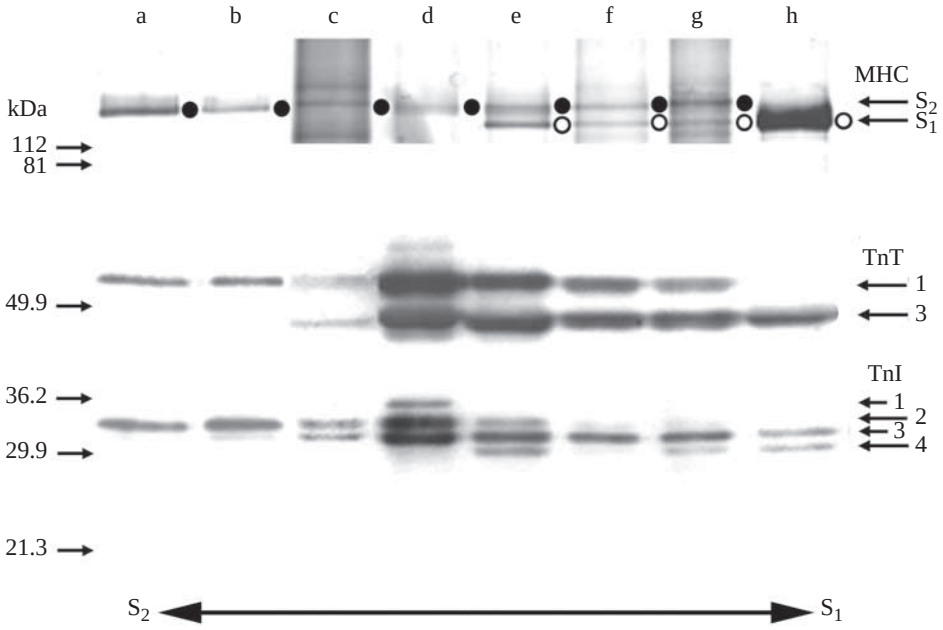

Fig. 7. Gradation between S_1 and S_2 muscle phenotypes identified through SDS-PAGE and western blotting, demonstrating the correlation among MHC, TnT and TnI isoforms. Predominantly S_2 muscle samples are on the left (lanes a,b), a predominantly S_1 sample is on the right (h), and samples following a gradation from S_2 to S_1 are in the middle (c–g) (a,b,h are single fibers, while c–g are fiber bundles). Myosin heavy chains (MHC) were identified by SDS-PAGE and silver staining. The S_2 MHC isoform has a lower electrophoretic mobility and is identified by a filled circle. The S_1 MHC has a higher mobility and is identified by an open circle. The four samples on the left (a–d) only possessed the S_2 MHC, the middle samples (e–g) had both S_1 and S_2 isoforms, and the last sample (h) possessed only the S_1 isoform. Troponin (Tn) T and I isoforms were identified by SDS-PAGE followed by western blotting. The TnT isoforms showed an orderly progression from TnT_1 (a,b) to both $TnT_{1,3}$ (c–g) to only TnT_3. The TnI isoforms showed a progression from TnI_2 (a,b) to $TnI_{2,3}$ (c–e), to TnI_3 (f–h). Various proportions of TnI_1 and TnI_4 were also observed in several of the samples (d,e,g,h).

Medler and Mykles, 2003), with nucleotide sequence identity among all three cDNAs being approximately 79% within the ORF of each cDNA. The fact that each of these genes possesses a unique 3′ UTR with low sequence identity provides a means to measure the expression of each of the three isoforms in different muscles. In a previous study (Medler and Mykles, 2003), we used this sequence divergence to measure different expression levels of the fast and S_1 MHCs in different muscles. Sequence-specific PCR primers have now been used to effectively measure the relative expression of the three different MHC isoforms. The S_2 MHC isoform was primarily expressed in the superficial flexors and extensors but was

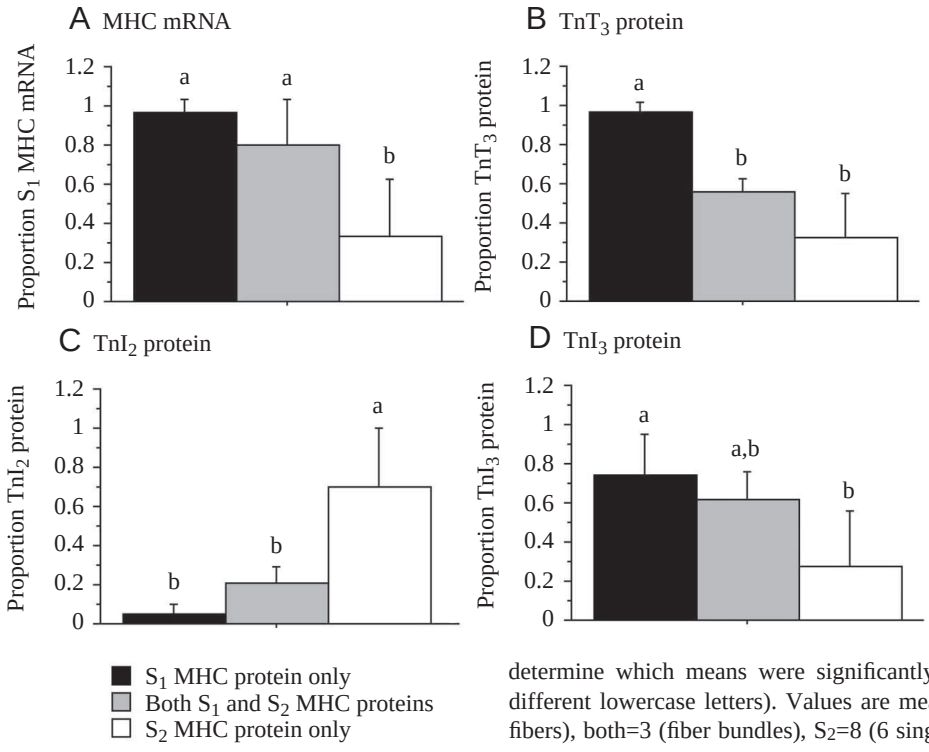

Fig. 8. Predominant isoforms in muscle samples (single fibers and bundles of fibers) classified by the myosin heavy chain (MHC) proteins present in the samples (S_1, S_2 or both MHC proteins). (A) The relative proportion of S_1 MHC mRNA was significantly higher in muscles with the S_1 MHC protein or both S_1 and S_2 proteins than in samples possessing only the S_2 MHC protein. Unexpectedly, samples characterized by the S_2 MHC at the protein level still expressed ~30% of their MHC mRNA as the S_1 isoform (~70% S_2 MHC). (B) S_1 fibers expressed significantly more troponin (Tn) T as the T_3 protein, while mixed samples and those with only the S_2 MHC expressed significantly less T_3 (more TnT_1). (C) TnI_2 was the predominant TnI isoform in the S_2 fibers, while (D) TnI_3 was the major isoform in S_1 fibers. Differences were analyzed by ANOVA, and a Bonferroni *post-hoc* test was used to determine which means were significantly different from one another (represented by different lowercase letters). Values are means ± S.D. [*N*=21 total samples: S_1=10 (single fibers), both=3 (fiber bundles), S_2=8 (6 single fibers, 2 fiber bundles)].

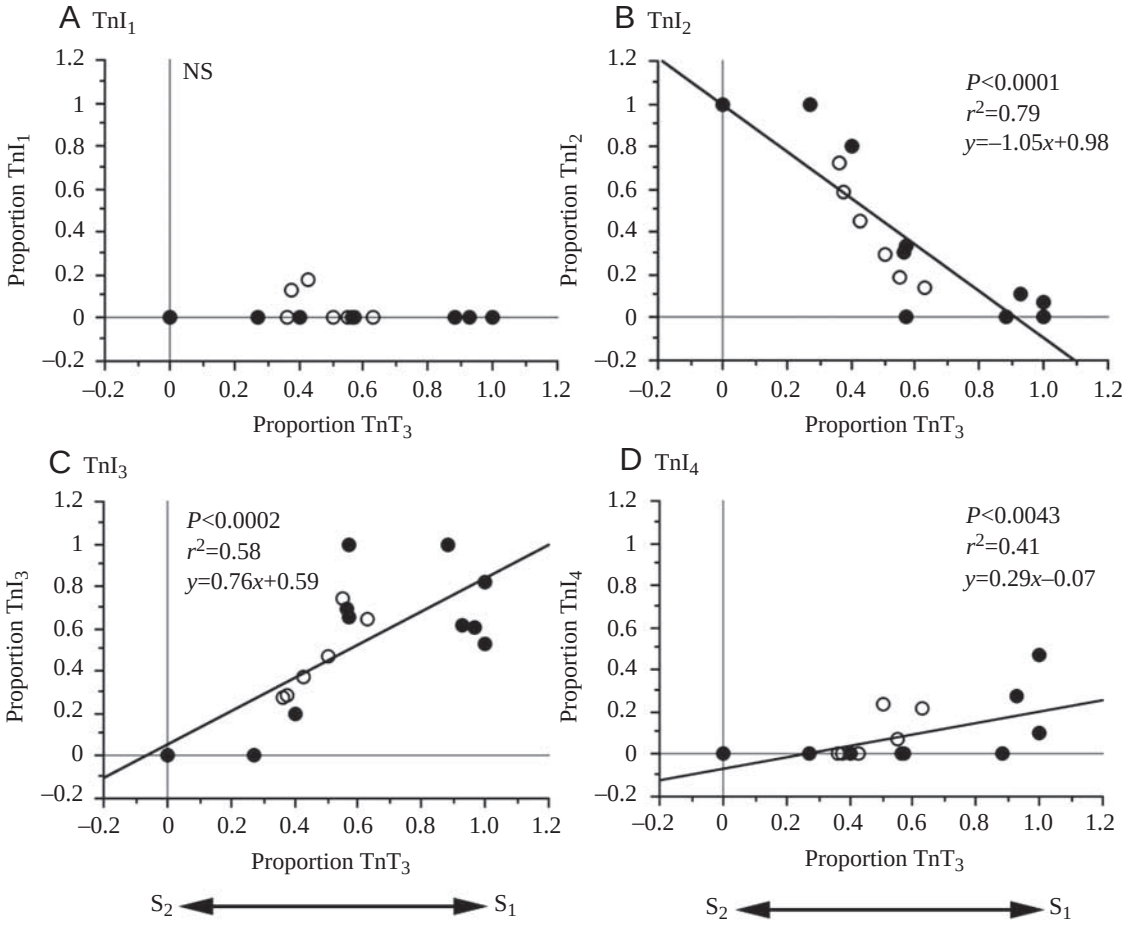

Fig. 9. Correlation between TnT and TnI protein isoforms. The proportion of each TnI isoform is plotted as a function of the proportion of TnT_3 (1 – proportion TnT_1). (A) TnI_1, which is expressed in fast muscles (Mykles, 1985a, 1997), was a minor isoform only observed in two samples and was not significantly correlated with TnT composition. (B) The relative amount of TnI_2 was negatively correlated with the proportion of TnT_3 (P<0.0001; r^2=0.79). (C) The relative amount of TnI_3 was positively correlated with the proportion of TnT_3 (P<0.002; r^2=0.58). (D) The proportion of TnI_4 was positively correlated with the proportion of TnT_3 (P<0.0043; r^2=0.41) but was present in lower amounts than TnI_3. Arrows at the bottom of the figure indicate that lower amounts of TnT_3 (left side of graphs) are characteristic of S_2 fibers, while greater amounts of TnT_3 (right side of graphs) are characteristic of S_1 fibers. Single fiber samples are indicated by filled circles, while samples from small bundles of fibers are represented by open circles.

also expressed at lower levels in other muscles (Fig. 4). Unexpectedly, the S_2 isoform was expressed at relatively high levels in fast muscles, although these levels were 150–500 times lower than in the superficial slow muscles (Fig. 4). We previously found that the S_1 MHC isoform was not expressed in the fast deep abdominal flexor muscles (Medler and Mykles, 2003). In later studies, we observed low levels of the S_1 MHC mRNA in some deep abdominal muscle samples (data not shown).

In addition, we report the entire sequence for a previously unidentified Tm gene that shares high sequence identity (more than 90% in the ORF) to the fast and S_1 Tm from lobster muscles (Mykles et al., 1998). Mass spectroscopy indicated that a third Tm isoform existed in some proportion in slow fibers (Mykles et al., 1998) and this is presumably that unidentified isoform (the predicted mass by mass spectroscopy is 32 884, and the estimated mass based on the deduced amino acid sequence is 32 877). The fast and S_1 isoforms are presumably produced from alternative splicing of exons in the 5′ end of the mRNA, while the rest of the sequence including the 3′ UTR is formed from the same exons (Mykles et al., 1998; Fig. 2). The S_2 Tm sequence is identical to the S_1 sequence, except that a different exon apparently comprises the 3′ end of the ORF and 3′ UTR (Fig. 2). The deduced amino acid sequence shows that the S_1 and S_2 isoforms differ by only five amino acids from residues 269–284, a region of the molecule important for head to tail overlap between sequential Tm molecules in the thin filament, as well as for TnT binding (Cho and Hitchcock-DeGregori, 1991; Perry, 2001). Alternative splicing of exons is used to generate different Tm isoforms in both vertebrate and invertebrate species (Basi et al., 1984; Cho and Hitchcock-DeGregori, 1991; Perry, 2001), and alternate exons in the C-terminal end of the molecule in particular have direct effects on contractile function (Cho and Hitchcock-

DeGregori, 1991; Perry, 2001). Physiological studies are needed to characterize the functional consequences of the alternate Tm isoforms in lobster muscle.

We used the sequence divergence in the 3′ UTR of the two isoforms to design reverse PCR primers to distinguish expression levels of the S_1 and S_2 isoforms. The relative expression of the S_1 and S_2 Tm isoforms was positively correlated with the expression of the S_1 and S_2 MHC isoforms, confirming that the newly identified sequence is an S_2 isoform of Tm. Interestingly, the S_2 isoform was expressed to a greater extent than the S_1 isoform, amounting to 90% or more in fibers that predominantly expressed the S_1 MHC isoform (Fig. 6).

Crustacean fiber types

Crustacean muscle fibers exist as a number of different fiber types, as identified by histochemistry, protein composition, ultrastructure and contractile properties (Jahromi and Atwood, 1969, 1971; Ogonowski and Lang, 1979; Mykles, 1985a,b, 1988, 1997; Silverman et al., 1987; West, 1997). In previous studies, S_1 fibers have been distinguished from S_2 fibers based on differences in myofibrillar isoform composition, histochemistry (Mykles, 1988; Fowler and Neil, 1992; Neil et al., 1993), sarcomere length (Neil et al., 1993), myofibrillar isoforms (Mykles, 1988; Neil et al., 1993), post-synaptic facilitation (Mykles et al., 2002) and *in vitro* shortening velocity (Holmes et al., 1999). The results of the current study are consistent with previous studies in several respects. MHC isoforms were identified based on different migration distances between the S_1 and S_2 isoforms, with the S_1 MHC having greater electrophoretic mobility (Fig. 7; Medler and Mykles, 2003). Interestingly, we did not identify any single fibers with both MHC proteins, even though all of the samples examined had a mixture of S_1 and S_2 MHC mRNA. Samples containing both MHC proteins were small bundles containing several fibers (Fig. 7, lanes e–g). In addition, the S_2 Tm isoform was found to be the predominant isoform, even in fibers that would be classified as S_1 based on other myofibrillar isoforms. In a previous study, mass spectroscopy data indicated that S_1 fibers from the lobster claw muscles contained approximately an equal mixture of the S_1 and S_2 Tm at the protein level (Mykles et al., 1998). It is possible that the S_1 fibers of the claw and those of the superficial abdominal extensors are not strictly equivalent but that the fibers from the abdomen are more S_2 in nature. Consistent with previous studies (Mykles, 1985a,b, 1988; Neil et al., 1993; Medler and Mykles, 2003), S_1 fibers were found to predominantly possess the TnT_3 protein isoform, while S_2 fibers had a higher level of the TnT_1 isoform (Figs 7–9). In previous studies, S_2 fibers were never found that possessed more than ~50% of the TnT_1 isoform, with the rest being composed of TnT_3 (Mykles, 1988; Medler and Mykles, 2003). In the current study, two fibers were found to contain 100% of the TnT_1 isoform (Fig. 7A,B). Moreover, we found that the relative amounts of TnT_1 and TnT_3 existed in a continuum, from fibers with 100% of TnT_1, to fibers with both isoforms, to fibers with 100% of TnT_3 (Figs 7–9). Previous studies have reported differences in TnI isoforms between S_1 and S_2 fibers (Mykles, 1985a,b, 1988; Neil et al., 1993), but in the current study we define these differences more precisely. TnI_2 was found to be the predominant protein isoform in S_2 fibers, while TnI_3 was the predominant isoform in S_1 fibers. In addition, TnI_4 became more abundant in S_1 fibers that had little TnT_1 (Figs 7, 9D). Isoforms associated with fast fibers (TnI_1 and TnI_5) were absent or extremely rare in both S_1 and S_2 fibers. The low levels of TnI_1 observed in a few muscle bundles (Fig. 9A) were unexpected, although a previous study reported some levels of TnI_1 in slow muscles of the Norway lobster (Neil et al., 1993). While S_1 and S_2 fibers could be distinguished based on the characteristic presence of the above isoforms, many intermediate fibers and bundles possessing a blend of isoforms formed a continuum from pure S_1 to pure S_2 fibers (Figs 5–9).

Hybrid muscle fibers

Recent advances in the methodology used to identify different skeletal muscle fiber types have led to greater resolution of muscle phenotype in terms of the MHC isoforms present and copies of different mRNAs in individual fibers (Pette et al., 1999; Stevenson, 2001; Caiozzo, 2002; Caiozzo et al., 2003). Through these methods, a number of studies of several different mammalian and amphibian species have found that fibers expressing more than one MHC isoform are more common than once thought (Peuker and Pette, 1997; Lutz et al., 1998; Nguyen and Stephenson, 2002; Stevens et al., 1999; Lutz et al., 2001; Caiozzo et al., 2003). In fact, recent studies have demonstrated that these hybrid fibers can represent a higher proportion of fibers than the ‘pure’ fibers that were once thought to represent the general state of muscle fibers (Caiozzo et al., 2003; reviewed by Stephenson, 2001). We recently found that single fibers of the claw closer muscle in the lobster possess both S_1 and S_2 MHC isoforms (Medler and Mykles, 2003). However, in the current study we found that while single MHC isoforms were almost always identified at the protein level, essentially all of the fibers and bundles expressed a mixture of both S_1 and S_2 MHC mRNA, suggesting that the MHC protein isoforms may be regulated through translational (Jefferson and Kimball, 2001; Bolster et al., 2003) or post-translational control mechanisms. It is also possible that low levels of MHC proteins went undetected in the silver staining procedure, although samples with 30% or more of the S_1 MHC mRNA had no detectable amounts of the corresponding proteins (Fig. 8A). While MHC isoforms have received the greatest attention, additional factors including other myofibrillar proteins also contribute to muscle function and should not be overlooked (Bottinelli, 2001; Clark et al., 2002). For example, mutations of TnT associated with hypertrophic cardiomyopathy lead to significant increases in unloaded shortening velocity *in vitro* (Sweeney et al., 1998), and alternative splice variants of TnT in dragonfly flight muscles are associated with significant differences in muscle power output and flight performance (Fitzhugh and Marden, 1997; Marden et al., 1999, 2001). In the present study, mixtures

of the S_1 and S_2 Tm mRNA were observed in all of the fibers and bundles examined, and isoforms of TnT and TnI proteins were found to exist in intermediate ratios between the extremes of the pure S_1 fibers to pure S_2 fibers. Collectively, these findings are consistent with other recent observations suggesting that muscle fiber types in the lobster are not discrete entities but represent a continuum of fiber types (Mykles et al., 2002; Medler and Mykles, 2003). Intermediate phenotypes were regularly observed in single fibers, ruling out the possibility that fiber polymorphism was an artifact of multiple different fibers within a single sample (Figs 5, 6, 9). More broadly, the presence of intermediate fiber types in an invertebrate species indicates that mixed phenotype fibers are not a phenomenon limited to mammals and other vertebrates but are probably characteristic of skeletal muscles in general.

While we did not examine the anatomical correlates of fiber heterogeneity, a number of studies have reported differences in muscle composition along the length of fibers. Edman et al. (1985) observed differences in shortening velocity along the length of frog muscle fibers, and a recent study of toad muscle demonstrated that strain patterns vary along the length of the toad muscle fascicles (Ahn et al., 2003). These differences may be related to the variability in MHC and myosin light chain content that has been reported along the length of muscle fibers in *Rana pipiens* (Lutz et al., 2001), as well as to mechanical factors such as connective tissue properties and muscle architecture (Ahn et al., 2003). In birds, MHC isoforms are asymmetrically distributed within single muscle fibers, with neonatal isoforms being concentrated at the ends of the fibers (Bandman and Rosser, 2000). In future studies, we will examine differences that may exist at the protein and mRNA level along the length of the lobster fibers.

Continuum among fiber types

Two compelling patterns are apparent from the results of this study. As discussed above, fibers were found to contain a mixture of mRNA and protein isoforms, verifying the existence of hybrid fibers that are intermediate between what might be called pure fiber types. More importantly, these hybrid fibers existed along a continuum from pure S_1 to pure S_2 fibers. This continuum was observed not only in the expression of MHC (Figs 6–8) but also for Tm (Fig. 6) and TnT and TnI (Figs 7–9). At each end of the continuum, a characteristic myofibrillar assemblage of isoforms exists that typifies the pure S_1 and S_2 fibers. However, expression of these isoforms exists as a gradient from high expression to low expression and, in the span between the two extremes, fibers exist that possess isoforms characteristic of both the S_1 and the S_2 fibers. Recent studies have drawn attention to the commonplace of hybrid muscle fibers, and the recognition that fibers are not discrete entities but represent a whole range of subtly different phenotypes challenges our current understanding of fiber types in skeletal muscles (Stephenson, 2001; Caiozzo et al., 2003). What are the factors that influence muscle phenotype and allow for a continuum of physiological properties? In crustaceans, muscles are often controlled by multiple motor neurons (Atwood, 1976; Mykles et al., 2002), and this complexity probably contributes to fiber heterogeneity. Claw and leg opener muscles of crayfish and lobsters exhibit a continuum of phenotypes from S_1 to S_2 fibers that is correlated with the synaptic efficacy. Proximal and distal fibers are innervated by endplates that produce larger excitatory postsynaptic potentials (EPSPs) than the central fibers (Mykles et al., 2002). In clear correlation with synaptic properties, the proximal and distal fibers possess the TnT_1 isoform associated with S_2 fibers, while the central fibers are the S_1 phenotype (Mykles et al., 2002). This pattern supports the hypothesis that the physiology of the excitatory motor neuron has a direct effect on muscle phenotype and that gradations in neuronal activity may produce a gradation in muscle phenotype. Additionally, skeletal muscles are multinucleate cells, and recent studies have suggested that myonuclear domains may differ with respect to the genes that are expressed (Newlands et al., 1998; Allen et al., 1999). A study of transcriptional activity in cultured skeletal muscle fibers showed that gene expression occurred in pulses and that nuclei within a muscle cell were not active simultaneously (Newlands et al., 1998). Over the next several years, studies will reveal the contribution of these domains to the existence of hybrid muscle fibers and, more generally, the physiological relevance of single fiber polymorphism.

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