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Scallop striated and smooth muscle myosin heavy-chain isoforms are produced by alternative RNA splicing from a single gene

(catch muscle/polymerase chain reaction)

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ABSTRACT **We report here that the catch and striated adductor muscle myosin heavy-chain (MHC) isoforms of scallop (*Argopecten irradians*, previously *Aequipecten irradians*) are generated by alternative RNA splicing from a single gene. Scallop catch muscle cDNA and genomic DNA were amplified by PCR using primers based on the previously sequenced scallop striated muscle MHC cDNA. Mapping of the exon/intron borders and sequencing of a full-length catch muscle MHC in overlapping fragments revealed that the 24-kb gene encodes the MHC polypeptide in 27 exons and that four sets of tandem exon pairs are alternatively spliced into a striated and a catch MHC isoform. An additional alternative exon was identified in catch cDNA and is apparently spliced into a minor MHC isoform. The striated muscle-specific isoform is not expressed in other tissues, whereas the catch-type isoforms were also detected in various smooth muscles, but not in the striated one. Of the alternative exons, exons 5 and 6 encode part of the ATP-binding region and the 25-kDa/50-kDa proteolytic junction; exon 13 encodes part of one of the actin-binding regions and extends to the active site; exon 20 encodes the middle of the rod hinge region; exon 26 in the striated-specific sequence starts with the stop codon, whereas the catch-specific exon codes for an additional 10 residues. Differences between the alternative exons presumably determine the lower ATPase activity of smooth muscle myosin, contribute to the different structure of the striated and smooth muscle thick filaments, and may also be important for the molecular mechanism of the catch phenomenon.**

Myosins belong to a superfamily of proteins characterized by the presence of a conserved globular head domain attached to distinct tail domains (1, 2). The conventional myosin (or myosin II) is ubiquitously present in eukaryotic cells, although it is most extensively studied in striated muscle. Myosin II is composed of a pair of heavy chains and two pairs of light chains. The N-terminal halves of the heavy chains fold into two motor domains, while the rest of the chains dimerize to form an α-helical coiled-coil tail which is responsible for filament formation.

Distinct myosin heavy-chain (MHC) isoforms exist in different muscle and nonmuscle cells to perform numerous physiological functions. Divergent sequences in specific functional domains of MHC isoforms determine, at least in part, differences in contractile properties of distinct muscle types. Isoform diversity can be generated by either a multigene family or by regulated alternative RNA splicing from a single gene. In vertebrate skeletal and cardiac muscles, the MHC isoforms are encoded by a large multigene family (3, 4). The nematode *Caenorhabditis elegans* has four sarcomeric MHC genes (5). In contrast, fruit fly *Drosophila melanogaster* has only one muscle-specific MHC gene, and several specialized striated muscle isoforms arise by alternative splicing (6). Mammalian and avian smooth muscle MHC isoforms are also the products of differential RNA processing from a single gene (7–9). MHC isoform diversity in vertebrate and invertebrate nonmuscle cells is produced by both multiple genes and alternative splicing (10, 11).

Sequence analysis of MHCs has revealed that the vertebrate smooth muscle isoforms are more similar to nonmuscle MHCs than to the vertebrate or invertebrate striated isoforms (1, 2). This division may reflect important functional differences between striated muscle and smooth muscle/nonmuscle myosins. It is not known whether the above division can be expanded to invertebrate smooth muscle MHCs, which are not as well characterized as their vertebrate counterparts. The various smooth muscle fiber types in invertebrates are defined morphologically as "unstriated," and some of them appear to be more similar in structure and physiology to cross- or oblique-striated fibers than to vertebrate smooth muscles (12, 13).

Molluscs possess both phasic and tonic muscles. In scallop adductor muscles, the large phasic muscle is striated, and the tonic smooth muscle is divided into an intermediate and a catch portion. The catch muscle has a high paramyosin content and is capable of maintaining tension with a very low metabolic turnover (13). We have previously cloned and sequenced the striated muscle MHC isoform (14). Sequence analysis has indicated an overall similarity to vertebrate striated MHCs; nevertheless, regions within the head show higher homology to nonsarcomeric (e.g., vertebrate smooth muscle) MHCs, which are regulated molecules like the scallop myosin (14). In this paper we report that the MHC isoform in scallop catch fibers¶ is produced by alternative splicing from the same gene as the striated MHC. Four sets of tandem exon pairs are spliced into either a striated or a catch MHC isoform, in a mutually exclusive manner. Alternative exons presumably determine specific properties of the myosin isoforms in the two different muscle types. Our results also demonstrate that the scallop smooth and striated muscle MHCs are closely related, unlike their vertebrate counterparts. Preliminary reports of this work have appeared (15, 16).

MATERIALS AND METHODS

Amplification and Sequencing of Genomic DNA. Genomic DNA from bay scallop (*Argopecten irradians*) was prepared

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Abbreviations: MHC, myosin heavy chain; RLC, regulatory light chain; RT–PCR, reverse transcription–polymerase chain reaction.

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¶The amino acid sequence reported in this paper is deduced from nucleotide sequence deposited in the GenBank data base (accession no. U09782).

according to Ausubel *et al.* (17). One hundred nanograms of genomic DNA was amplified by PCR under standard conditions suggested by Perkin–Elmer, using primer pairs based on the cDNA sequence of striated muscle MHC (18): H85/H149 (nt 19–38 and 523–503); H95/H65 (nt 498–516 and 1143–1123); H86/H60 (nt 1135–1156 and 2893–2910); H84/H80 (nt 2222–2240 and 3820–3801); H83/H72 (nt 3817–3825 and 5946–5929); and H77/H69 (nt 5689–5706 and 6612–6596). Several other shorter fragments were also generated to facilitate subsequent DNA mapping and sequencing, either from genomic DNA or by nested PCR using the longer fragments as templates. When needed, sensitivity of PCR was improved with AmpliWax (Perkin–Elmer) and/or Perfect Match DNA polymerase enhancer (Stratagene) as suggested by the manufacturers. The amplified products were sequenced either directly, by the *fmol* DNA sequencing system (Promega), or after cloning of restriction fragments into pTZ19R vector (Pharmacia LKB) and using Sequenase 2.0 (United States Biochemical).

Reverse Transcription–Polymerase Chain Reaction (RT–PCR). Total RNA was isolated from scallop smooth adductor muscle and from other scallop tissues (foot, mantle, gonads, heart) by Tri Reagent (Molecular Research Center, Cincinnati) and reverse transcribed by the cDNA Cycle kit (Invitrogen) using random primers. MHC sequences were amplified by *Taq* polymerase (Promega) or *Pfu* polymerase (Stratagene) with the primers used for genomic DNA amplification, as well as with several additional MHC exon-specific primers (Fig. 1*A*). Isolated plasmid DNA from a cDNA library was also used as template for PCR cloning of MHC fragments. PCR products were sequenced directly with the *fmol* kit and in some cases with Sequenase after cloning them into pCRII vector (TA cloning system, Invitrogen).

Scallop Catch Muscle cDNA Library. An expression plasmid library was constructed from catch adductor muscle mRNA and screened with an anti-scallop myosin antibody (14).

RESULTS AND DISCUSSION

To study MHC isoform diversity, scallop smooth muscle cDNA and genomic DNA were amplified in parallel by PCR using oligonucleotide primers based on the previously sequences striated muscle MHC cDNA (14).

cDNA Sequence of a Scallop Catch Muscle MHC Isoform. The sequence of a partial MHC clone (C/2–7; Fig. 1*A*), isolated previously from a catch adductor muscle cDNA library, differed from the striated adductor MHC only in a stretch of 79 nt within the rod hinge-coding region (nt 3783–3861; from here on nucleotide numbering refers to the striated MHC cDNA sequence of ref. 18), indicating that the scallop tissue-specific MHC isoforms are generated by alternative splicing (15). To obtain the entire coding region of the catch MHC sequence, a PCR strategy was chosen. Based on the striated MHC cDNA sequence, six longer (Fig. 1*A*) and numerous shorter fragments were amplified by using total RNA for RT–PCR. Four divergent regions were identified in the full-length coding sequence after directly sequencing dozens of PCR products. Each region represents alternatively spliced exons of a single MHC gene (see details below).

Location of the alternative exons within the MHC sequence is shown in Fig. 1*A*. Exons 5 and 6 are consecutive exons in the head-coding region (nt 669–777 and 778–873). Exon 5c is 9 nt longer than exon 5s (exons designations c and s refer to catch and striated MHC isoforms, respectively). Exon 20c is the divergent sequence previously identified in C/2–7. The penultimate exon, exon 26c, is shorter by 8 nt than its alternative (nt 5959–6004).

We identified an additional MHC alternative exon by sequencing individual clones of a shorter fragment of the RT–PCR product H150/H60 (see Fig. 1*A*) from catch muscle. Apparently, exon 13c (nt 2095–2182) is spliced into a minor MHC isoform, since it was not detectable in sequencing ladders of directly sequenced PCR products (which may consist of a mixture of different molecules). PCR amplification with alternative exon-specific primers indicated that at least exons 6c and 20c were also present in this minor isoform

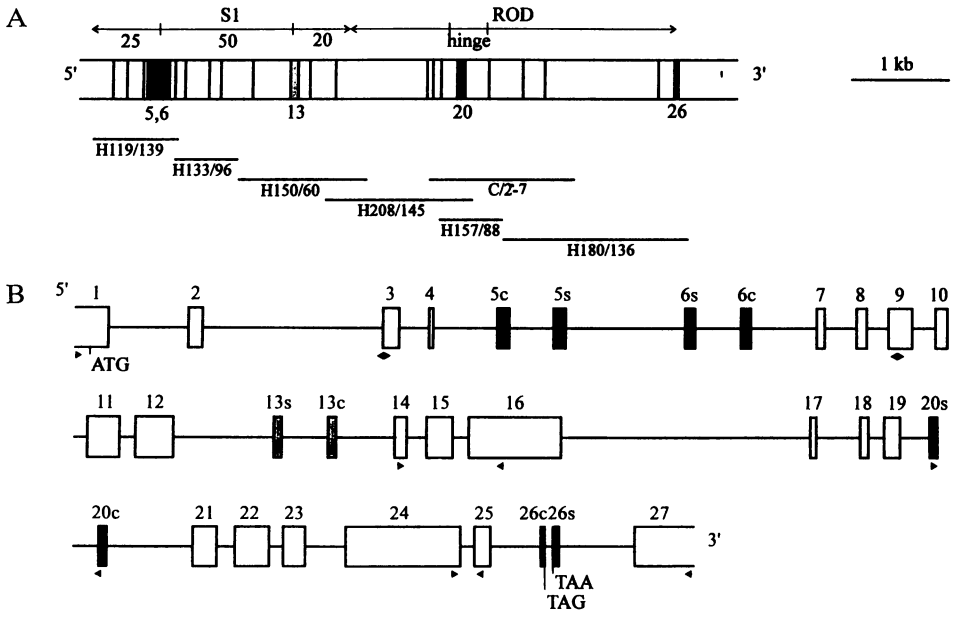

FIG. 1. Exon structure of the MHC sequence (*A*) and exon/intron map of the MHC gene (*B*). (*A*) Positions of the four "major" (black boxes) and one "minor" (gray box) alternative exon in scallop smooth adductor muscle isoforms are indicated. Only these exons are numbered. The main proteolytic fragments of MHC are shown above the map; 25, 50, and 20 indicate size (kDa) of subfragments of S1. Below the MHC exon structure, RT–PCR-amplified cDNA fragments are illustrated which cover the entire coding region, as well as a clone (C/2-7) isolated from a plasmid cDNA library. (*B*) Single line represents intervening sequences. Oligonucleotide primers used to amplify genomic DNA (see also *Materials and Methods*) are shown as arrowheads below the gene map. Exons designated s and c are spliced into striated and catch MHC isoforms, respectively. TAA and TAG termination codons in exons 26 are indicated. Maps are drawn to scale.

(data not shown). It should be noted, however, that exon-13c is spliced into a major catch MHC isoform in a closely related scallop species, *Placopecten magellanicus* (19).

Structure of the MHC Gene. We were able to clone by PCR the entire coding region of the MHC gene in six large piece (see *Materials and Methods* and Fig. 1*B*). Each amplified fragment was extensively mapped by restriction enzyme digestion and partially sequenced to determine the location of the exon/intron borders. The map of the gene is shown in Fig. 1*B*. The ≈24-kb gene encodes the MHC mRNA in 27 exons. Five sets of tandem exon pairs are alternatively spliced into striated and catch muscle MHC isoforms, respectively. All introns have consensus trinucleotide borders: 5′-GT(A) . . . (C)AG-3′ (20). The large "intervening" sequences (e.g., after exons 2, 5s, and 16) may contain hitherto undetected alternative exons which are expressed in tissues other than the adductor muscle. Cytoplasmic MHC isoforms are probably also present in scallop, but genes in this class have not been found yet.

Table 1 shows the intron positions within the amino acid coding region. The intron position between the differentially expressed consecutive exon pairs 5s–6s and 5c–6c is notable: in the striated isoform it is at nt 777 (codon 212-0), while in the other spliced form it is shifted one codon toward the 3′ end. Comparison of intron positions of the scallop MHC gene with other MHC genes reveals several conserved borders, especially in the head-coding region (Table 1). In some cases (e.g., before exons 3, 5, 7, 8, and 12) these coincide with the border of a secondary-structure element observed in the crystal structure of the S1 fragment of chicken skeletal muscle myosin (22). More striking is the precise conservation of the boundaries of exon 20s and exon 20c with two alternative exons in the *Drosophila* MHC gene (6, 23). Interestingly, an insertional exon was identified, at the position corresponding to the 3′ end of exon 5c in the scallop catch sequence, in a chicken and mammalian smooth muscle MHC isoform (8, 9, 24) and in a nonmuscle MHC-B isoform (11). Alternative splicing near the 3′ end of the coding region has been found in *Drosophila* muscle and vertebrate smooth muscle MHC messages (6, 7, 23, 25).

Expression of MHC Isoforms in Various Scallop Tissues. The alternative exons are apparently processed in a coordinated manner. None of the differential exons of the striated MHC sequence were detected by means of direct sequencing of amplified catch cDNA, and *vice versa*. To check tissue-specific expression of particular exons, alternative-exon-specific primers were applied to RT–PCR of total RNA from striated muscle and various smooth muscles (catch, foot, mantle, gonads, heart). Fig. 2 shows representive results of the numerous PCRs we have performed. It is apparent that the striated muscle isoform is not expressed in significant amounts in catch muscle or other tissues. In contrast, the catch muscle isoform is expressed in other smooth muscles but not in striated muscle. With higher amounts of template cDNA for PCR, both alternative exons could be amplified in every tissue (data not shown); this indicates either cross contamination of the samples or a low basic level of expression that is detectable only due to the very high sensitivity PCR.

Comparison of the Peptide Sequences Encoded by the Alternative Exons. Exon 5 encodes part of the ATP-binding region—namely, the fully conserved phosphate-binding loop followed by a helix forming the base of the binding pocket (22)—and the highly divergent 25-kDa/50-kDa proteolytic junction (Fig. 3). The catch MHC exon has a divergent and 3-aa-longer junction sequence plus two residue differences in the helix. Exon 6 also encodes part of the nucleotide-binding pocket.

The minor alternative exon 13c encodes a putative actin-binding region, one strand from the seven-stranded β-sheet core of the head, and part of a surface loop forming one end of the nucleotide pocket (22).

Table 1. Intron positions within the MHC codyng region

Exon	Intron position	Intron size, bp	Conservation
1	66-0	≈800	Rat, Dro
2	114-0	≈1800	Rat, Dro, Nem
3	166-1	304	Rat
4	175-2	≈650	Rat
*5c**	*216-0*	≈450	(Smo, Non)
5s	212-0	≈1200	
6s	244-0	≈450	Rat, Dro
6c	*247-0*	≈650	
7	265-1	325	Rat, Dro, Nem
8	298-1	220	Rat
9	379-1	226	Rat
10	419-0	138	Rat
11	526-0	168	Rat, Dro, Nem
12	651-0	≈1000	Rat
13s	680-1	≈450	Rat
13c	*683-1*	≈600	
14	719-2	190	Rat, Dro
15	805-2	143	Rat
16	1110-0	≈2500	
17	1129-0	≈450	
18	1158-0	157	
19	1213-2	≈300	Dro
20s	1240-0	259	Rat, Dro
20c	*1243-0*	≈850	Dro
21	1322-0	180	Rat
22	1432-0	139	
23	1504-1	≈400	Rat
24	1883-0	139	Rat, Nem
25	1938-0	509	
*26c**	*3′-UTN*	87	
26s	3′-UTN	≈750	

Intron position refers to the 3′ end of the previous exon. Position is shown by the codon number within the scallop striated MHC sequence (14) and by the intron phase (-0, -1, or -2). Asterisks indicate that the corresponding smooth muscle-specific exon has different length. Since exon 5c is 9 nt longer than exon 5s, the intron position in the catch MHC coding region are shifted 3 numbers up after this exon; moreover, the intron border after exon 5c is shifted one extra codon toward the 3′ end compared with its alternative pair; these numbers are in italics. 3′-UTR, 3′-untranslated region. Conservation means that the identical intron position exists in other sequenced MHC genes: Rat, rat embryonic skeletal muscle MHC (21); Dro, *Drosophila* sarcomeric MHC (6); Nem, *C. elegans unc-54* (5); Smo, rabbit smooth muscle MHC (8); Non, vertebrate nonmuscle MHC (11). The last two genes are in parentheses, indicating that only their partial sequence is known.

Amino acid replacements in alternative exons 5 and 6, and possibly in exons 13, presumably determine the significantly lower ATPase activities of scallop catch muscle myosin (27), since these are the only differences in the head domain of the MHC isoforms. One can consider the role of possible light-chain isoforms in modulating the ATPase cycle. Indeed, *Patinopecten yessoensis* has three regulatory light-chain (RLC) isoforms (27); reversible phosphorylation of the smooth adductor muscle isoform has been suggested to be important in regulation of each contraction (28). By urea electrophoresis, we have detected no RLC isoforms in *A. irradians*, two forms in *P. magellanicus*, and no essential light-chain isoform in either species (C. J. Perreault, A.J., L.N., and A.G.S.-G., unpublished results). By comparing the ATPase properties of RLC hybrid myosins from striated and catch muscles of two scallop species, we have found that the turnover rate is determined solely by the heavy chain (19). A longer 25-kDa/50-kDa junction sequence has been

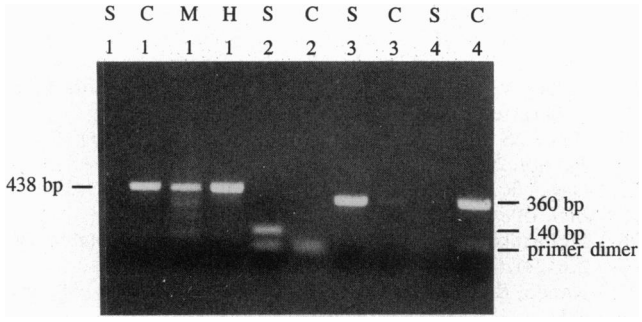

FIG. 2. RT–PCR of isoform-specific MHC exons. Total RNA from scallop striated muscle (S), catch muscle (C), mantle (M), and heart (H) was amplified by RT–PCR with catch muscle isoform-specific (1, 4) and striated muscle isoform-specific (2, 3) primers. The PCR products were electrophoresed in a 1% agarose gel and stained with ethidium bromide. Primer pairs used: 1, exon 2-specific (common, i.e. present in both isoforms) and exon 5c-specific; 2, exon 5s-specific and exon 6s-specific; 3, exon 17-specific (common) and exon 20s-specific; 4, exon 17-specific (common) and exon 20c-specific. There is no amplified product in the first lane (S1). "Primer dimer" is a PCR artifact.

identified in certain vertebrate smooth muscle (8, 9, 24) and nonmuscle MHC isoforms (11) which are generated by inclusion of an extra exon. The 7-aa insert confers functional differences between smooth muscle isoforms (9). How the flexible surface-loop sequence affects the properties of the ATPase center is not known.

Cys-198 (replaced by Ala in smooth MHC), at the beginning of the 25-kDa/50-kDa junction, can be crosslinked to an ATP analogue in the presence of activating Ca^{2+} ions (29). Ser-178 is also thought to be involved in the Ca^{2+} regulation of scallop myosin (30). Although earlier studies indicate that the two adductor myosin isoforms bind Ca^{2+} in a similar fashion (31), a detailed study of the catch adduction myosin

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EXON-5
                          ATP-site      25/50 kDa junction
striated (176-211)   GESGAGKTENTKKVIMYLAKVACAVKKKDEEASDKK
catch                ---------S-------F----ANLY-QKQ-EPTTTHARA

EXON-6
                            ATP-site
striated (212-243)   EGSLEDQIIQANPVLEAYGNAKTTRNNNSSRF
catch                 SN------E-------F-----V--------

EXON-13
                       actin-site              ATP-site
striated (651-679)   ESLNKLMKNLYSTHPHFVRCIIPNELKQP
"minor" catch        -------C--RR-N----------LE-E-

EXON-20
                              S2-hinge
striated (1214-1239) LEKDKKDLKREMDDLESQMTHNMKNK
catch                S--E-QQ-RS-VE--QA-IQ-IS---
                     defgabc

EXON-25, -26
                          non-helical tailpiece
striated (1920-1938) ...RAKSSVSVQRSSVSVSASN*
catch                ...-------------------AAHVAHHHVE*
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FIG. 3. Comparison of the alternative exon sequences of scallop striated and smooth MHCs. The striated MHC amino acid sequences are from ref. 14; residue numbers are in parentheses. Exon borders were determined by sequencing amplified genomic DNA fragments. Functional sites are shown above the sequence. Dash indicates an identical amino acid. Bold letters represent fully conserved residues of known MHC sequences (14). Underlined are "regulation-sensitive" residues (14). In exon 20, *defgabc* illustrates one heptad repeat of the coiled-coil rod (26). Double underlined are residues with charge differences between the two sequences on the surface position of the coiled coil (*b*, *c*, and *f*). Asterisks indicate termination codons.

may reveal whether the alternative exons influence regulatory processes.

Exon 20 encodes the middle of the flexible hinge region within the rod. It covers almost exactly one 28-aa motif of the rod. These repeats have mostly charged amino acids on surface positions of the coiled-coil heptad repeats. The charge distribution along the repeats is thought to be essential in the ionic interactions between myosin molecules in the thick filament (26). It is noteworthy that the catch MHC sequence has four fewer charged residues, three of these occupying surface positions (*b*, *c*, and *f*; Fig. 3).

In *Drosophila* two alternatively processed hinge-coding exons are expressed in a highly tissue-specific fashion (6, 23). The conservation of these alternative exons in insects and molluscs suggests an important function for the hinge in the isoform-specific properties of MHCs. Alternative hinges may modulate contractile force through the proposed helix-coil melting of the hinge (32, 33) and may interact differentially with other myofibrillar proteins (e.g., paramyosin or a caldesmon-like protein; refs. 34 and 35); hence, this may contribute to the different structure of the thick filaments in scallop catch and striated adductor muscles.

Exon 26s starts with a stop codon, whereas exon 26c codes for an additional 10 aa; consequently, the coiled-coil tail of catch myosin terminates in a longer, 29-aa nonhelical tailpiece. Reversible phosphorylation of the tailpiece has been proposed to be involved in the molecular mechanism of catch contraction since it has been shown that in contrast to the scallop striated MHC, only the catch MHC is phosphorylated by an endogenous kinase, probably at Ser-1926 (36). The longer nonhelical tailpiece alone does not explain this difference, since the phosphorylatable MHC tailpiece from the anterior byssus retractor muscle of *Mytilus edulis* has the same length as the scallop striated tailpiece (14). Exon 26c is also expressed in scallop non-catch smooth muscles (data not shown), though it is not known whether phosphorylation occurs in these tissues. Besides its possible importance in the structural basis of catch contraction, the alternative tailpiece may also affect the assembly properties of myosin filaments in striated and smooth muscles.

Conclusion. We have partially characterized the muscle-specific MHC gene from the scallop *A. irradians*. The 24-kb gene encodes the MHC message in 27 exons; 4 of them are present as tandem exons pair and are alternatively spliced into two major isoforms in striated and catch muscle. A minor isoform was also identified containing an additional alternative exon. Alternative exons code for functionally important stretches of MHC sequence. Differences in these regions between MHC isoforms may influence or determine specific properties of myosins in physiologically different scallop muscles. Finally, our results demonstrate that the molluscan smooth and striated muscle MHCs are closely related sequences, unlike their vertebrate counterparts.

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