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EARLY SPECIALIZATION OF THE SUPERFAST MYOSIN IN EXTRAOCULAR AND LARYNGEAL MUSCLES

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Summary

Extraocular muscle (EOM) exhibits high-velocity, low-tension contractions compared with other vertebrate striated muscles. These distinctive properties have been associated with a novel myosin heavy chain (MyHC) isoform, MyHC-EO. An atypical MyHC, MyHC IIL, has also been identified in laryngeal muscles that have similarly fast contractile properties. It co-migrates with MyHC-EO on high-resolution SDS gels, but appeared to be encoded by a different mRNA. We combined CNBr peptide maps and full-length cDNA sequences to show that rabbit muscle EO and IIL MyHCs are identical. Analysis of the 5′ untranslated region (5′UTR) of the mRNAs identified three variants that result from a combination of alternative splicing and multiple transcription initiation sites. This complex pattern of 5′UTRs has not been reported previously for MyHC genes. We identified the human homologue of the MyHC-EO gene in GenBank, and analyzed the 5′ upstream region, which revealed a paucity of muscle-specific transcription factor binding sites compared with the other MyHC genes. These features are likely to be critical to the unique regulation and tissue-specific expression of the MyHC-EO/IIL gene.

Phylogenetic analysis indicates that MyHC-EO/IIL diverged from an ancestral MyHC gene to generate the first specialized fast myosin. The catalytic S1 head domain is more closely related to the fast MyHCs, while the rod is more closely related to the slow/cardiac MyHCs. The exon boundaries of the MyHC-EO are identical to those of the embryonic MyHC gene and virtually identical to those of the α and β cardiac genes. This implies that most of the current exon boundaries were present in the ancestral gene, predating the duplications that generated the family of skeletal and cardiac myosin genes.

Key words: extraocular, laryngeal, muscle, 5′ untranslated region, exon, myosin heavy chain.

Introduction

The fibers in skeletal muscles are generally fast- or slow-contracting, and their distinctive physiological properties are conferred by specialized sets of contractile proteins. A primary determinant of the shortening velocity of a muscle fiber is the myosin heavy chain (MyHC), and multiple developmental, slow/cardiac and fast isoforms with distinctive properties have evolved. The extraocular muscles (EOMs) are distinguished from other skeletal muscles by faster contraction times (<10 ms) and lower twitch tensions (Close and Luff, 1974; Cooper and Eccles, 1930). These contractile properties are consistent with the hyperfast saccadic movements executed by extraocular muscle. The repertoire of EOM movements also includes the slow, low-tension contractions needed to keep distant objects in the visual field and permit convergence of the eyes for focusing (Barmack, 1977; Fuchs et al., 1985). These diverse functional requirements are fulfilled by six EOM fiber types that do not correspond to other muscle fiber types in either structure or protein composition (Chiarandini and Davidowitz, 1979; Spencer and Porter, 1988).

In addition to the six skeletal muscle MyHCs found in adult and developing muscles, extraocular muscles express a unique MyHC-EO, first identified by Wieczorek et al. (1985), as well as the α cardiac gene (Jacoby et al., 1990; Rushbrook et al., 1994). The human MyHC-EO gene has been mapped to the cluster of fast and developmental isoforms on human chromosome 17 (Winters et al., 1998), and its cDNA has been sequenced (Weiss et al., 1999). The novel MyHC-EO protein is distinguished from the other MyHC isoforms by its mobility on SDS–PAGE and unique antigenic sites, which have been used to detect the protein in EOM (Asmussen et al., 1993; Lucas et al., 1995; Sartore et al., 1987). *In situ* hybridization localized the mRNA to EOM and demonstrated that its expression is developmentally regulated and that it requires normal visual inputs during the critical period for visual maturation (Brueckner et al., 1996; Brueckner and Porter, 1998).

A protein with identical mobility on SDS–PAGE is also present in some laryngeal muscles, and immunological evidence indicates that MyHCs sharing an epitope are present in EO as well as laryngeal muscles (DelGaudio et al., 1995; Lucas et al.,

1995; Shiotani and Flint, 1998). The presence of a similar, potentially identical, MyHC isoform in laryngeal muscles is particularly interesting because these muscles also exhibit extremely fast contraction times and lower twitch tensions (Hall-Craggs, 1968; Martensson and Skoglund, 1964). A partial cDNA for this myosin, designated MHC2L, however, did not correspond exactly to the short sequence available for MyHC-EO (Merati et al., 1996; Wieczorek et al., 1985). As part of our effort to understand the contribution of MyHC-EO to the distinctive contractile properties of superfast muscle, we have determined the complete cDNA sequence of EO myosin from rabbit. In addition, to resolve the relationship between the EO and laryngeal MyHCs, we also cloned the novel myosin from rabbit laryngeal muscle and showed that the sequences are identical.

The very limited expression of MyHC-EO indicates that its transcription is regulated by different mechanisms from those of the other MyHCs. For the other isoforms, several important 5′ upstream enhancer and promoter regions have been identified. Within these regions, a number of transcription factor binding sites have been identified that specify MyHC expression in skeletal muscles and repress their expression in other tissues (Bouvagnet et al., 1987; Gupta et al., 1998; Huang and Liew, 1998; Shimizu et al., 1992; Takeda et al., 1992). They include the myogenic factors that bind E boxes, MyoD, myogenin, MRF4 and myf-5, members of the serum response factor family that bind CarG boxes, and MEF2, which recognizes AT-rich motifs (Buckingham, 1992; Olson, 1990). However, no molecular data are available on the potential upstream regulatory regions for the MyHC-EO gene. To this end, we determined the sequence of the 5′ untranslated region (5′UTR) and compared it with the human genomic sequence to identify regions that may contain the upstream regulatory elements responsible for the unique expression patterns of MyHC-EO.

Materials and methods

Tissue preparation and biochemical analysis

Muscles were dissected from dead 1.8–2.3 kg male New Zealand White rabbits and 200–300 g Sprague-Dawley rats and flash-frozen in liquid nitrogen. Myofibrils were prepared from frozen pulverized muscles (Briggs et al., 1987; Schachat et al., 1988). Myosin heavy chain isoforms were resolved on sodium dodecylsulfate (SDS) polyacrylamide gels as described by Talmadge and Roy (1993), with 7.4 % polyacrylamide and 35 % glycerol. The proteins were stained with Coomassie Brilliant Blue G. For CNBr peptide mapping, gels were stained briefly to locate the MyHC, and those portions of the gel were excised. The proteins were digested with CNBr in the gel slices, and the resulting peptides were resolved by SDS–PAGE (12 % gel) and stained with silver (Briggs et al., 1987; Pepinski, 1983).

Reverse transcription polymerase chain reaction and cDNA cloning

RNA was prepared from frozen pulverized EOM and thyroarytenoid (Chomczynski and Sacchi, 1987). These RNA preparations were used to generate libraries of MyHC cDNAs by reverse transcription and polymerase chain reaction (RT-PCR) amplification with the Titan One-Tube kit according to the manufacturer's instructions (Roche Molecular Biochemicals). Reverse transcription was performed at 50 °C for 30 min with a modified oligo-dT (RO4-oligodT) primer (Ennion et al., 1995), and MyHC-specific cDNAs were obtained by PCR with a primer from a highly conserved region of MyHC (MYHCcons-1F) and RO4 (see primer list below). The PCR reaction conditions were as follows: 2 min denaturation at 94 °C, then 23–28 cycles of 1 min at 94 °C, 1 min at 55 °C, and 1 min at 68 °C, followed by a final extension of 2 min at 68 °C. The PCR products were cloned into the PCRII vector (Invitrogen). The cloned DNA was purified with the Wizard miniprep kit (Promega) and then sequenced with an Applied Biosystems automated sequencer. The sequences were analyzed and compared with the Lasergene programs (DNASTAR, Inc.).

From those sequences, additional isoform-specific primers were designed and used in a series of additional RT-PCR reactions to amplify overlapping clones that covered all but 600 base pairs (bp) at the 5′ end of the coding region. Annealing temperatures are listed with each primer pair, and extension times at 68 °C were determined by the expected product size (1 min kb^{-1}). Reactions were carried out with both EOM and thyroarytenoid RNA. All RT-PCR reactions were performed at least twice, and sequences were determined from 2–6 clones.

The rest of the coding region and the 5′ untranslated region of the mRNA were obtained by 5′RACE with the SMART cDNA synthesis kit (Clontech). This technique uses a specialized mechanism of adding the adapter primer that strongly enhances the yield of cDNAs that are fully extended to the mRNA cap site. Extraocular muscle RNA was reverse-transcribed with MYHC491R for 1 h at 42 °C. The resulting cDNAs were amplified by PCR with the SMART CDS primer and a nested primer MYHC279R to generate full-length products. The PCR condition were as follows: 1 min denaturation at 94 °C, then 33 cycles of 30 s at 94 °C, 30 s at 58 °C, and 2 min at 68 °C, followed by a final extension of 2 min at 68 °C. A broad band centered at the expected size (approximately 1000 bp) was gel-purified (Geneclean II, Bio101) and cloned as above.

The primers were as follows: RO4-oligodT (GGTCGACG-CATGCGGATCCATT$_{17}$), RO4 GGTCGACGCATGCGGA-TCCATT$_{17}$), MYHCcons-1F (AGAAGGCCAARAARGCC-AT); MYHC1450F (GGACAARAARCARMGNAAYTTYG) with MYHC2L-3R (GGTCCTCCTCAGCCTGGTACGTCAT) 60 °C; MYHC490F (GTTCAACCACCAYATGTTYGT) with MYHC1477R (GCCCTGGACTCTTTCTGAGCA) 56 °C; MYHC180F (GGAGARTCYGGNGCNGGNAA) with MYHC629R (GCTGCTCCAGCGTAGTTGGAA) 58 °C; MYHC491R (GCACGAACATGTGGTGGTTGAA), MYHC279–2R (GGGATGATAGCTCCTCTCACTAGA) 58 °C.

Computer analysis of nucleotide sequences

Nucleotide sequences were assembled and aligned with SEQMAN and MEGALIGN portions of Lasergene (DNAstar,

Inc.). For the phylogenetic analysis, MyHC sequences were truncated to omit 4–8 amino acid residues at the highly variable C terminus. The human genomic clone containing the EO gene (GenBank Accession no. AC00005291) was identified by submitting the rabbit EO cDNA sequence to a BLAST search through NCBI, NIH, and the exon organization of the human gene was predicted with the GENEQUEST module of the Lasergene program.

Results

Biochemical characterization of the novel myosin heavy chain in rabbit extraocular and laryngeal muscles

The myosin heavy chains of rabbit skeletal muscles were resolved by high-resolution SDS–gel electrophoresis as in Talmadge and Roy (1993). Fig. 1 identifies the fast and slow MyHC isoforms expressed in adult trunk and limb muscles: IIA and I in the slow soleus (Sol); IIB in adductor magnus (AM); and IIX/D in erector spinae (ES). Consistent with previous reports (Asmussen et al., 1993; DelGaudio et al., 1995), an additional band that migrates between MyHCs IIB and I is present in extraocular muscle (EOM). This EO myosin is not expressed in other skeletal muscles tested, including psoas, masseter, sternohyoid, sartorius, flexor carpii radialis and vastus intermedius. However, a protein of identical mobility is expressed in some laryngeal muscles, such as thyroarytenoid (Thyro), in which this isoform was designated MHC-IIL (DelGaudio et al., 1995).

Peptide mapping was performed to provide a more definitive comparison of the EO and IIL MyHC isoforms. The proteins were resolved on gels as described above, and gel slices containing a single MyHC isoforms were excised and treated with CNBr. The resulting peptides from IIB, EO, IIL and I MyHCs were electrophoresed on a 12.5 % gel, which separates peptides in the 100–10 kDa range (Fig. 2). The patterns of peptides from IIB and I are different from those in EO, confirming that EO MyHC is not closely related to them. However, the size and relative intensity of the peptides in EO and IIL are identical, indicating that these proteins are very similar if not identical.

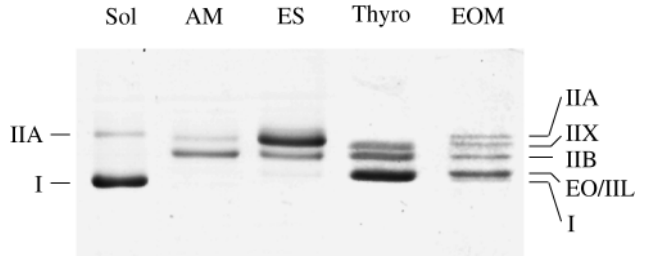

Fig. 1. Resolution of the novel myosin heavy chain (MyHC) in rabbit extraocular and laryngeal muscle. The positions of the adult skeletal muscle MyHCs IIX, IIB, IIA and I are shown in soleus (Sol), adductor magnus (AM) and erector spinae (ES). Additional MyHC bands are present in both the laryngeal muscle thyroarytenoid (Thyro) and extraocular muscle (EOM). They migrate at identical positions between IIB and I and are labeled EO/IIL. SDS–PAGE was performed as described in Materials and methods.

Full-length nucleotide sequence of EO MyHC

The limited 3′ coding and 3′UTR nucleotide sequence data available for MyHC-EO and MyHC-IIL indicated that they were not identical. To resolve this question, we determined the full nucleotide sequences encoding the EO and IIL MyHCs. cDNA clones were initially identified from myosin-specific cDNA libraries generated by RT-PCR. RNAs prepared from EOM and Thyro were reverse-transcribed with a composite oligo-dT adapter primer. To amplify all the MyHCs present in the RNA population, PCR amplification was performed with the adapter primer and an upstream primer derived from a highly conserved region of the MyHC. These cDNAs covered the 3′UTR and approximately 500 bp of the coding region. At least 15 clones from each library were analyzed and identified by comparing their DNA sequences with published rat and mouse MyHC sequences, because each MyHC isoform is highly conserved among different species, particularly in this 3′ region. The EOM library contained the assortment of adult and developmental MyHC sequences expected from protein characterization of these muscles, together with one additional sequence, which was identified as EO MyHC by comparison with shorter cDNAs from mouse and human muscle (Winters et al., 1998). Two different cDNA sequences were obtained

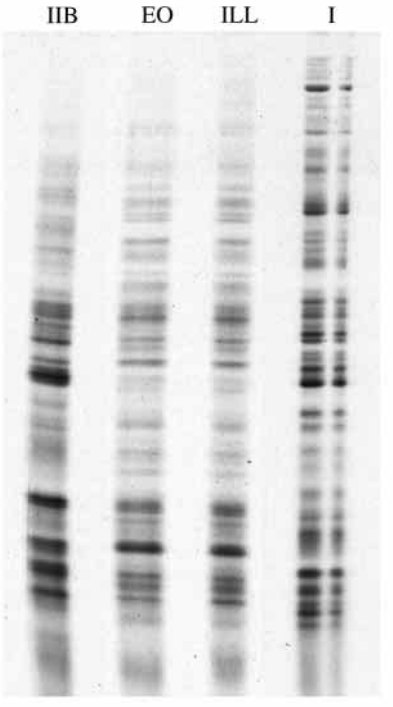

Fig. 2. CNBr peptide mapping of MyHC-EO/IIL. Gel slices containing individual myosin heavy chain (MyHCs) IIB, EO, IIL and I were treated with CNBr, and the resulting peptides were resolved by electrophoresis on a 12 % polyacrylamide–SDS gel and stained with silver. The patterns of protein fragments from EO and IIL are identical.

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Rabbit EOM/IIL ACGGATGCGGCCATGATGGCGGAGGAGCTGAAGAAGGAGCAGGACACCAGCGCCCACCTGGAGCGCATGAAGAAGAACCTGGAGCAGACGGTCAAGGACC  100
Rat MyHC-EO    ..T..C..C.....................................................T........G.....................A..G.......
Rat MyHC IIL   ..T..C..C.....................................................T........G.....................G.......

Rabbit EOM/IIL TGCAGCACCGCCTGGACGAGGCCGAGCAGCTGGCGCTCAAGGGC---------GGCAAGAAGCAGATCCAGAAGCTGGAGAACCGGGTACGGGAGCTGGA  200
Rat MyHC-EO    ..........T...........T.............G.....T---------...................A......GC.A....G......T....
Rat MyHC IIL   ..........T..N........T.............G......CTGAAGGGC...................A......GC.A.A..G......T....

Rabbit EOM/IIL AACCGAGCTGGACGCGGAGCAGAAGAGGGGAGCCGAGGCTCTGAAGGGGGCCCACAAGTATGAACGCAAAGTCAAGGAAATGACGTACCAGGCTGAGGAG  300
Rat MyHC-EO    ..G.........T..A...........A.....T..A...................A.....G...........A..G.....T........C......
Rat MyHC IIL   ..G.........T..A...........A.....T..A.............G......A.....G...........A..G.....T........C......

Rabbit EOM/IIL GACCGCAAGAATTTTCTCAGGCTCCAGGACCTGGTGGACAAGCTGCAGGCCAAGGTCAAGTCCTACAAGAGGCAGGCTGAGGAGGCGGAGGAGCAGGCCA  400
Rat MyHC-EO    ..........CA.C...C.A.............A.................A..G...................A..A.....T........A....
Rat MyHC IIL   ..........CA.C...C.A.............A.................A..G...................A..A.....T........A....

Rabbit EOM/IIL ACACGCAGCTGTCCAGGTGCCGCAGAGTGCAGCACGAGCTGGAGGAGGCGGAGGAGAGAGGGCCGACATTGCCGAGTCCCAGGTCAACAAGCTCAGGGCCAA  500
Rat MyHC-EO    ....A.....A...........G..G..C.....T..A..A........T...........A..T..C..T.....T..A...........A........
Rat MyHC IIL   ....A.....A...........G..G..C.....T..A..A........T...........A..T..C..T.....T..A...........A........

Rabbit EOM/IIL GAGCCGAGACGTGGGGGGCCAGAAGATGGAAGAATGAGGCCCTCCTGAG-CTCATTGCCATGGGACACCTGCAGGAGAGAGGAGGGAACTGTGTGAGAAA  600
Rat MyHC-EO    ......T..T.....A.......................T....C...G...G.........C.....C........CA......GAC-..G..A...
Rat MyHC IIL   ......T..T.....A.......................T....C...G...G...A......C.....C........CA......GAC-..G..A...

Rabbit EOM/IIL TAAATTCTCCTAAATGCT--GCG
Rat MyHC-EO    ....CC.....GC..A..CAT
Rat MyHC IIL   ....CC.....GC..A..CAAA.
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Fig. 3. Alignment of the 3′ region of extraocular muscle myosin heavy chain (MyHC-EO) and MyHC-IIL cDNAs. Identical cDNA sequences were obtained from rabbit extraocular muscle (EOM) and IIL. The sequence of rat MyHC-EO determined here is essentially identical to the published rat MHC-IIL sequence (Merati et al., 1996). The additional nucleotides at position 150–158 of the rat MyHC-IIL match the preceding nine nucleotides and may reflect an inadvertent repetition of that sequence.

from the Thyro library and identified as MyHC-IIB and MyHC-IIL, which are identical to the MyHC-EO sequence reported here (Fig. 3). The rabbit MyHC-EO/IIL sequence reported here differs slightly from that of the published rat MyHC-IIL (Merati et al., 1996), so the rat MyHC-EO sequence was also determined. This sequence is essentially identical to the published MyHC-IIL, which confirms the identity of the rat MyHC-EO and -IIL.

This sequence information was used to generate overlapping clones and assemble the full-length sequence. Additional RT-PCR reactions were performed with RNA from both muscles, using combinations of isoform-specific and conserved primers to extend the sequences to the beginning of the coding region. Identical sequences of 5901 bp were obtained from EO and Thyro (GenBank Accession no. AF212147). Translation of the nucleotide sequence gives a protein of 1938 amino acid residues (Fig. 4), which is shown aligned with the translated human EO cDNA sequence (Weiss et al., 1999). The rabbit and human sequences are highly conserved, with 95.8 % identity of amino acids. This value is similar to the 96.1 % identity when the rabbit and human MyHC-2X/D genes are compared (not shown, GenBank Accession nos OCU3187 and AF111785). A higher degree of conservation between the human and rabbit sequences is present in regions known to be important for function of the MyHCs, including actin and ATP binding sites, which are nearly 100 % identical (Rayment et al., 1993). They both differ from the other MyHC isoforms in other regions that have been shown to impart distinctive functional characteristics including the unstructured loop 1, which influences the rate of ADP release, and loop 2, which influences ATPase activity (Kelley et al., 1993; Murphy and Spudich, 1998; Sweeney et al., 1998; Uyeda et al., 1994).

Exon organization of the human MyHC-EO gene

A BLAST search of GenBank sequences with the rabbit cDNA sequence identified a 200 kb human DNA clone among the high-throughput genome (HTG) sequences that contained the entire sequence of the human MyHC-EO gene. By comparison with the rabbit cDNA sequence, we have identified the predicted coding regions of the human gene and assembled an amino acid sequence that is nearly identical (99.7 % match) to the published sequence (Fig. 4) (Weiss et al., 1999). Each predicted exon is bounded by consensus donor and acceptor splice sites that define the exon–intron structure of the EO gene (Fig. 5). Composed of 39 coding and two noncoding exons, the human MyHC-EO gene spans more than 63 kb, which is approximately twice the length reported for other MyHC genes. The exon boundaries so defined are identical to those of the embryonic gene and very highly conserved with those of the α and β cardiac genes, the other MyHC genes for which this information is known. This high degree of conservation indicates that most of the exon boundaries were present in the ancestral gene before the gene duplications and chromosomal translocation that placed the fast and slow/cardiac genes on different chromosomes.

Structure of the 5′ untranslated region

This comparative analysis was also applied to the 5′UTR region of MyHC-EO, which was obtained by 5′RACE with rabbit EOM RNA. Surprisingly, three sequences of different length, designated A, B and C, were obtained (Fig. 6). Both A and B (166 and 136 nucleotides) are longer than the single 5′UTRs reported for other MyHCs. While B and C are identical to A over much of their length, they have an insertion of 50 nucleotides, suggesting that they result from alternative splicing. Sequences B and C are not likely to be incomplete

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Rabbit MSSDAEMAIFGEAAPYLRKPEKERIEAQNRPFDSKKACFAVDDKEMYVKGMIQSRENDKVTVKTLDDRTLTLNSDQVFPMNPPKFDKIEDMAMMTH 100
Human  .....................................VA.N.........T......I.......M....N..................... 100

Rabbit AVLYNLKERYAAWMIYTYSGLFCVTVNPYKWLPVYNPEVVTAYRGKKRQEAPPHIFSISDNAYQFMLTDRDNQSILITGESGAGKTVNTKRVIQYF 200
Human  .................................K....A..................................................... 200

Rabbit VTGDKKKEQQPGKMQGTLEDQIIQANPLLEAFGNAKTVRNDNSSRFGKFIRIHFGATGKLASADIETYLLEKSRVTFQLSSERSYHIFYQIMSNKK 300
Human  ........T.......................................................................................... 300

Rabbit DLLLISTNPFDFPFVSQGEVTVASIDDSEELLATDNAIDILGFSSEEKVGIYKLTGAVMHYGNMKFKQKQREEQAEPDGTEVADKAGYLMGLNSAE 400
Human  .......................................................................................... 400

Rabbit LCCPRVKVGNEYVTKGQNVQQVTNSVGALAKAVYEKMFLWMVTRINQQLDTKQPRQYFIGVLDIAGFEIFDFNSLEQLCINFTNEKLQQFFNHHMF 500
Human  .......................................................................................... 500

Rabbit EEYKKEGIEWEFIDFGMDLAACIELIEKPMGIFSILEEECMFPKATDTSFKNKLYDQHLGKSNNFQKPKPAKGKAEAHFSLVHYAGTVDYNIAGWL 600
Human  .......................................................................................... 600

Rabbit DPLNETVVGLYQKSALKLLSFLFSNYAGAEAGDSGGSKKGGKKKGSSFQTVSAVFRENLNKLMTNLRSTHPHFVRCLIPNETKTPGVMDHYLVMHQ 700
Human  ............S.............T.................................................................. 700

Rabbit GVLEGIRICRKGFPSRILYADFKQRYRILNASAIPEGQFIDSKNASEKLLNSIDVDREQYRFGHTKVFFKAGLLGLLEEMRDEKLVTLMTRTQALC 800
Human  ...........................................................F...N.....................S...V. 800

Rabbit MRVEFKKMMERRDSIFCIQYNIRAFMNVKHWPWMNLFFKIKPLLKSAEAEKEMATMKEDFERAKEELARSEARRKELEEKMVTLLQEKNDLQLQVQ 900
Human  ......................S.....................................T..................S............. 900

Rabbit NLMDAEERCEGLIKSKIQLEAKVKELNERLEEEEEMNSDLVAKKRTLEDKCCSLKRDIDDLELTLTKVEKEKHATENKVKNLSEEMTALEENISKL 1000
Human  ................L........T...........E......N.....S..................................... 1000

Rabbit KSLQEAHQQTLDDLQVEEDKVNGLIKINAKLEQQTDDLEGSLEQEKKLRADLERVKRKLEGDLKMAQESIMDLENDKQQVEEKLKKKEFEISQLQT 1100
Human  ...................................................A.........S.........E...I..........L....A 1100

Rabbit EQVQSLQLQKKIKELQARTEELEEEIEAEHTLRAKIEKQRSDLARELEEISERLEEASGATSAQIEMNKKREAEFQKLRRDLEEATLQHEATAATL 1200
Human  ...H...F..........I.....................................................M.................. 1200

Rabbit ADSVAELGEQIDNLQRVKQKLEKEKSELKMEIDDLASNIETVSKSKSNVERMCRSVEDQFNEIKAKDDQQTQLIHDLNMQKARLQTQNGELNHQVE 1300
Human  ..............................M.....AL......I..T..T.....S......E.....................S.R.. 1300

Rabbit LISQLTKGKQALSQQLEEVKRQLEEETKAKNALAHALQSSRHDCDLLREQYEEEQEAKAELQRALSKANSEVAQWRTKYETDAIQRTEELEEAKKK 1400
Human  .......S....T.....L...M.........M.....................................K.................. 1400

Rabbit LQEAEENTETANSKCASLEKTKQRLQGEVDDLMLDLERANTACGTLDKKQRNFDKVLAEWKQKLDESQAELEAAQKESRALSTEIFKMRNAYEEVV 1500
Human  ......K.....................E...R....SH...A.................................S....L.......... 1500

Rabbit TLRRENKNLQEEIADLTEQMAETGKNLQEVEKTKKQVEQEKSDLQAALEEAEGSLEHEESKILRVQLELNQVKSELDRKLTEKDEEMEQLKRNSQR 1600
Human  .............S.....I.........A.....L.........V....V.................S.........VI.....I......... 1600

Rabbit LQSVLDAEIRSRNDALRLKKKMEGDLNEMEIQLGHASRQVAETQKHLRTVQGQLKDSQLHLDDALRSNEDLKEQLAMVERRNGLLQEELEEMKVAL 1700
Human  ................................SN..M....R.............................I........L.......... 1700

Rabbit RTRRLSEQELLDASDRVQLLHSQNTSLINTKRKLEADLAQCQAEVENSLQESRNAEEKAKKAITDAAMMAEELKKEQDTSAHLERMKKNLEQTVKD 1800
Human  ............................K.....I..........I.......................................... 1800

Rabbit LDEAEQLALKGGKKQIQKLENRVRELETELDAEQKRGAEALKGAHKYERKVKEMTYQAEEDRKNFLRLQDLVDKLQAKVKSYKRQAEEAEEQANTQ 1900
Human  ........................N...V.............................I............................. 1900

Rabbit RRVQHELEEAEERADIAESQVNKLRAKSRDVGGQKMEE                                                  1938
Human  ..........A.....................S.....                                                  1938
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Fig. 4. Derived amino acid sequence of extraocular muscle myosin heavy chain (MyHC-EO). Translation of the full-length MyHC-EO/IIL cDNA yields a protein of 1938 amino acids, which is virtually identical to the translated human MyHC-EO sequence (GenBank Accession no. AF111782).

extensions because there are TATA boxes approximately 30 bases upstream, which is typical of most transcription initiation sites. Comparison with the upstream sequence of the human gene shows that this region is remarkably conserved: 86.1 % of the nucleotides are identical, compared with 90.0 % and 74.2 % for the coding and 3′UTR regions, respectively. The TATA boxes upstream of B and C are also conserved in the human gene. The comparison also shows that the different rabbit 5′UTRs result from alternative splicing of exon 2. Sequences B and C are both made up of three exons, the same structure as the other MyHC mRNAs that have been characterized, α and β cardiac and the embryonic MyHC (Brand et al., 1991; Epp et al., 1993; Jaenicke et al., 1990; Liew et al., 1990; Strehler et al., 1986). Sequence A matches the 85 bp sequence

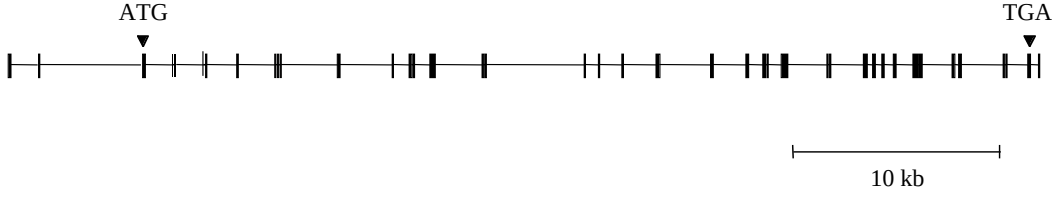

Fig. 5. Exon organization of the human extraocular muscle myosin heavy chain (MyHC-EO). The 41 exons are shown as vertical lines. The 5′ untranslated region (UTR) spans exons 1, 2 and part of exon 3. Exons 3–41 comprise the coding region and the 3′UTR. The start (ATG) and stop (TGA) signals for protein translation are marked with arrowheads.

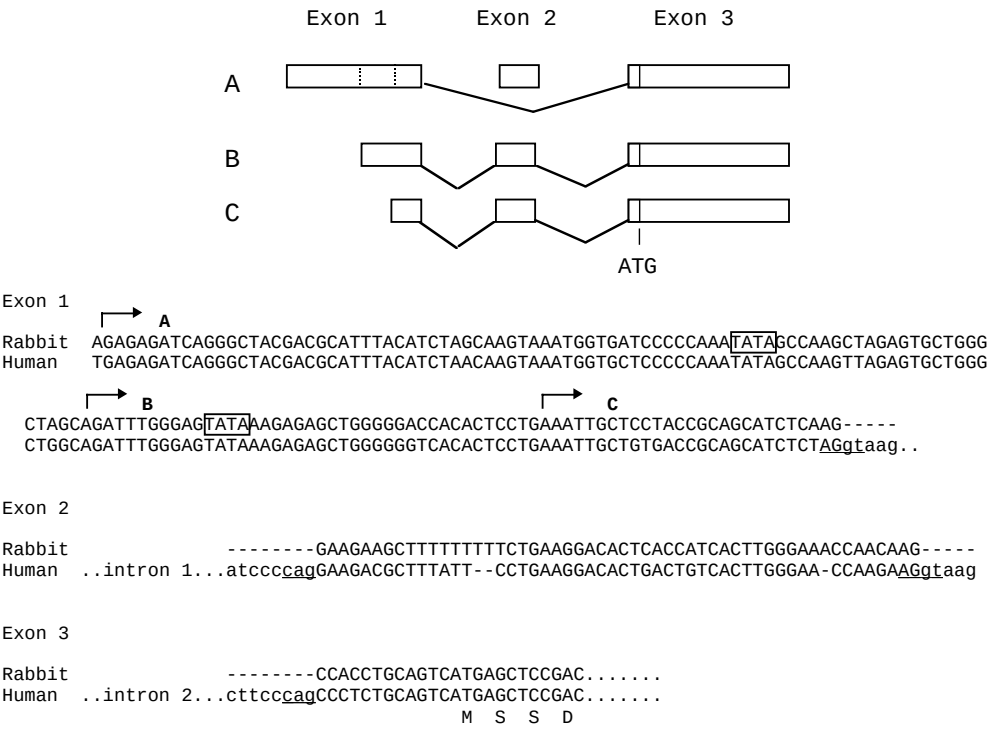

Fig. 6. Sequence and predicted genomic structure of the 5′ untranslated region of the extraocular myosin heavy chain gene. The alternative splicing pattern of transcript A skips exon 2. Transcription of B and C begin downstream of A in exon 1, and they both include exon 2. The nucleotide sequence of the rabbit cDNA is aligned with the human gene sequence to show the predicted exon boundaries. Short stretches of the introns are shown in lower case, and the consensus splice sites are marked by underlining. The locations of the transcription start sites A, B and C are indicated by horizontal arrows, and the TATA boxes upstream of B and C are boxed. Only part of exon 3 is shown.

in the human gene immediately upstream of B and continues to the end of that exon, but it then skips exon 2 and is directly linked to exon 3. These are the first reported examples of alternative splicing in the 5′UTR and of multiple transcription initiation sites in a myosin heavy chain gene. Similar features have been observed in a number of other genes, including muscle enolase, cardiac troponin T, acetylcholinesterase and heat shock protein 47, and in those cases, they are important for the developmental or regional regulation of gene expression (Giallongo et al., 1993; Jin et al., 1995; Li et al., 1993; Takechi et al., 1994). These features may therefore also be important for the unique regulation and limited expression of the MyHC-EO gene.

Phylogenetic analysis

Phylogenetic analysis was performed on the human myosin heavy chain sequences (Fig. 7) because only two of the rabbit MyHCs have been fully sequenced. The EO and embryonic myosins diverged from the fast lineage shortly after the fast and slow/cardiac myosins diverged but long before specialization of the fast isoforms. The structure of the myosin heavy chain is divided into several distinct functional regions, with the N-terminal 840 amino acid residues forming a compact structure termed S1, which has the actin-binding and ATPase domains, while most of the rest forms an extended coiled-coil rod. When these regions are compared separately, a somewhat different picture emerges. The same order of divergence is observed when the S1 region (residues 1–841) is analyzed separately, but for the rod, MyHC-EO/IIL appears to be more closely related to the slow/cardiac genes. The difference between the rod and S1 suggests that the two regions are under different selective pressures and that the MyHC-EO sequence, particularly in the S1 region, has evolved over time to meet its specific functional requirements. Selection in the rod may be less stringent, as long as the characteristic heptad repeat needed for the α-helical coiled-coil interaction is maintained (McLachlan and Karn, 1983; Stedman et al., 1990). The major difference is in the first 200 amino acid residues of the rod, roughly corresponding to the S2 region of MyHC, which has been implicated in modulating force transduction. These results indicate that MyHC-EO is the product of an ancient duplication that evolved a highly specialized function and that it was the first specialized fast MyHC gene.

Discussion

Although skeletal and cardiac muscles have a varied array of functions and contractile properties, they rely on the expression of relatively few myosin heavy chains, the fast IIA, IIB and IIX/D, and the α and β/slow cardiac isoforms. These few isoforms, expressed in combination with different thin filament proteins, provide sufficient range and fine-tuning of the contractile response to fulfill their diverse functions. In contrast, the contractile properties of the eye and laryngeal muscles are so distinctive that the full complement of myosin heavy chains, including the embryonic and perinatal forms, is not sufficient. These muscles, which exhibit faster contraction times and develop lower tension than other muscles (Close and Luff, 1974; Cooper and Eccles, 1930), require an additional specialized myosin isoform, MyHC-EO/IIL. In previous studies of the novel myosin isoforms in extraocular and laryngeal muscles, their gel electrophoretic and immunological properties indicated that they were closely related (DelGaudio et al., 1995; Lucas et al., 1995), but the reported nucleotide

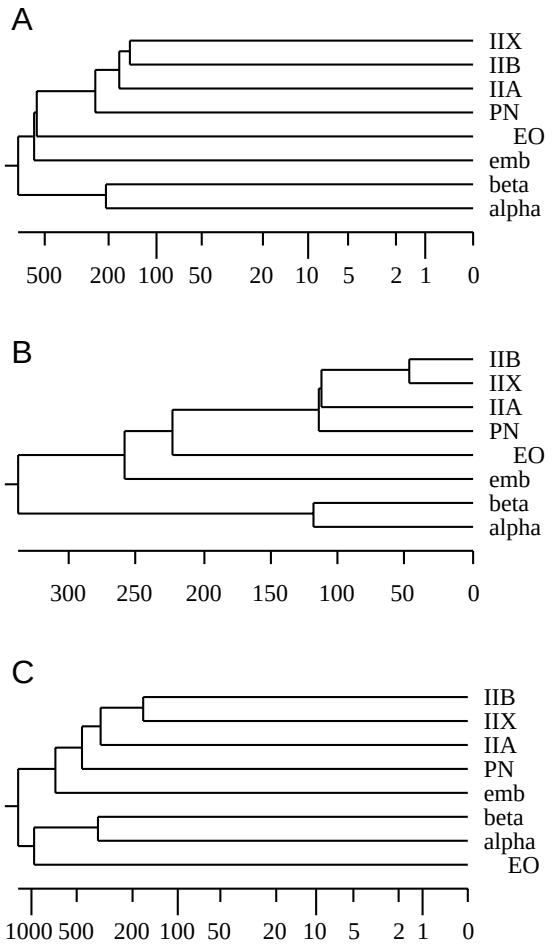

Fig. 7. Phylogenetic analysis. (A) Complete amino acid sequences of the six fast/developmental and two slow/cardiac human myosin heavy chains (MyHCs) were analyzed (GenBank Accession numbers: EO (extraocular), AF111782; IIB, AF111783; IIA, AF111784; IIX, AF111785; PN (perinatal), M36769; emb (embryonic), X13988; beta (β cardiac), M58018; alpha (α cardiac), D00943. The scale indicates the estimated number of substitutions. (B) The same order of divergence is observed when the S1 portion of the MyHC (amino acid residues 1–841) is analyzed separately. (C) Extraocular muscle myosin heavy chain appears to be more closely related to the β and α cardiac proteins when only the coiled-coil rod portion (approximately amino acid residues 842–1938) is analyzed.

sequences were short and not identical (Merati et al., 1996; Wieczorek et al., 1985), and it was not clear whether they were encoded by the same gene. Here, we show that the full-length cDNA sequences of MyHC-EO and MyHC-IIL are indeed identical (Fig. 3).

The amino acid sequences of the human and rabbit MyHC-EO/IIL are quite highly conserved (Fig. 4), but they differ substantially from those of the other human fast and developmental isoforms (Weiss et al., 1999). In particular, MyHC-EO/IIL differs from the other isoforms in two flexible regions, loops 1 and 2, that form junctions between highly structured areas (Rayment et al., 1993). These regions are both important in determining the functional differences among isoforms. Loop 1, roughly residues 185–210, is adjacent to the nucleotide-binding pocket, and substitution in this region alters the kinetics by changing the rate of ADP release (Kelley et al., 1993; Murphy and Spudich, 1998; Sweeney et al., 1998). Loop 2, residues 660–675, is associated with differences in V_{max} (Uyeda et al., 1994). The high degree of conservation of MyHC-EO/IIL in human and rabbit is maintained even in these hypervariable regions, providing further evidence that the sequence variations in these regions are important for the functional differences among the isoforms. In other regions that are involved in nucleotide or actin binding, MyHC-EO/IIL shows more similarity with the faster isoforms, such as IIB, which would be consistent with the expectation that the contractile properties of EO MyHC will be fast or superfast.

Few sequence data are available on the myosins in other superfast muscles, such as those of the toadfish swim bladder or the male *Xenopus laevis* laryngeal muscles, which are both involved in the production of high-frequency sound, or myosin IIM, the jaw-closing myosin in masseter muscle of carnivores and some primates (Hoh et al., 1988; Rome et al., 1996; Rowlerson et al., 1981; Taylor et al., 1973; Wetzel and Kelley, 1983). Comparison of MyHC-EO with the partial sequence of MyHC-IIM (GenBank Accession no. U51472) shows that they are not closely related, which may not be surprising given the observation that those muscles develop high tension rather than the low-tension contractions of EO and laryngeal muscles (Taylor et al., 1973). Among the other superfast muscles that develop low tensions, the only data currently available are for a short 3′ MyHC sequence expressed in laryngeal muscle of male *Xenopus laevis* (Catz et al., 1992), which is indeed more closely related to MyHC-EO/IIL than to the other mammalian MyHC genes. Determining the contractile properties and resolving the relative contribution these amino acid differences make to the function of this myosin will require actual measurement of the catalytic properties of the myosin ATPase activity and the *in vitro* motility of EO myosin.

A link between the superfast, low-tension contractions and the kinetic properties of the myosin has been proposed by Rome et al. (1999). In a detailed analysis of the myosin crossbridge cycle in the superfast muscle of toadfish swim bladder, they found that the rate of crossbridge attachment was the same in toadfish white skeletal muscle and the swim bladder muscle, but that the rate of crossbridge detachment was 10 times greater in the swim bladder muscle. An increased detachment rate allows for faster relaxation and, by having fewer cycling crossbridges attached at any instant, it provides a mechanism linking faster contractions with lower force, a physiological property shared with extraocular and laryngeal muscles.

The kinetic properties of the individual myosin isoforms clearly play an important role in the contractile properties of such muscles, but there is commonly a synergy among the different systems that contribute to fiber mechanics, including the thin filament activating system and the Ca^{2+} release and reuptake systems (Schachat et al., 1987). In addition to expressing the EO-MyHC, extraocular and laryngeal muscle

also share other characteristics that promote superfast contractions. The small fiber size and extensive volume of sarcoplasmic reticulum allow for more rapid diffusion and reuptake of Ca^{2+} (Spencer and Porter, 1988), and biochemical analysis shows that parvalbumin, a cytoplasmic Ca^{2+}-binding protein that facilitates transport of Ca^{2+} from the myoplasm to the sarcoplasmic reticulum, and the sarcoplasmic Ca^{2+}-ATPase are amplified in both muscles (Blank and Schachat, 1999). This should result in a shorter-duration and lower-amplitude Ca^{2+} transient following neuronal stimulation, which almost certainly contributes to the short contraction time and reduced twitch tension of these muscles (Close and Luff, 1974; Cooper and Eccles, 1930). Interestingly, similar adaptations of the cytoplasmic Ca^{2+} reuptake system contribute to the longitudinal gradient of relaxation times in cod axial muscle (Thys et al., 1998), and increased activity of the sarcoplasmic Ca^{2+} reuptake system contributes to the axial gradient in scup muscle (Swank et al., 1997) and the rapid contractions of the toadfish bladder (Rome et al., 1996).

Definition of the predicted exon structure and the sequence of the 5′ untranslated region reveal several important features of the MyHC-EO gene that distinguish it from the other MyHC genes and may be critical for its regulation. Transcription of the rabbit MyHC-EO gene starts at several sites, which distinguishes it from the other muscle myosin heavy chain genes. In addition, transcript A may represent TATA-independent transcription since there is no consensus TATA box upstream of the corresponding position in the human gene (Fig. 6). Although it is possible that a TATA box is present in the rabbit gene, or that a corresponding transcript A is not made from the human gene, the remarkable level of conservation in this region suggests that TATA-independent transcription of the EO MyHC gene occurs. Relatively few genes exhibit TATA-independent transcription, and most of them are considered to be ‘housekeeping’ genes because they are expressed in many cell types. However, some TATA-less genes are regulated in cell- or tissue-specific ways, including the duck lactate dehydrogenase B/ε-crystallin gene, whose regulation differs in lens and cardiac muscles (Brunekreef et al., 1996; Kraft et al., 1993).

Another distinctive feature of the EO mRNA that may figure prominently in its regulation is the alternative splicing within the 5′UTR. Two of the mRNAs resemble those of other MyHC genes, which also result from TATA-dependent transcription and include the corresponding exon 2 (Brand et al., 1991; Epp et al., 1993; Jaenicke et al., 1990; Liew et al., 1990; Strehler et al., 1986). However, the absence of exon 2 in transcript A suggests that it may provide an independent means of regulating the MyHC-EO/IIL gene. Although alternative splicing of the 5′UTR has not been observed for other striated myosin heavy chain genes, such mRNAs are generated from a number of other genes, and the use of different splicing patterns is tightly regulated either developmentally or in a tissue-specific manner (Giallongo et al., 1993; Jin et al., 1995; Li et al., 1993; Takechi et al., 1994).

‘Data mining’ of GenBank with the rabbit EO MyHC cDNA sequence identified the human gene sequence determined as part of the Human Genome Project. Comparison of the two sequences identified the 5′-most exon of the human MyHC-EO/IIL gene and, from that, the potential upstream regulatory regions that can be tested for promoter or enhancer activity. For the embryonic, cardiac and IIB MyHC genes, a number of transcription factors that regulate muscle-specific expression have been identified, including the myogenic factors that bind E boxes, MyoD, myogenin and myf-5, as well as TEF, SP1, Oct1 and MEF2 (Bouvagnet et al., 1987; Gupta et al., 1998; Shimizu et al., 1992; Swoap, 1998; Takeda et al., 1992). Their binding sites are generally clustered in a proximal promoter region several hundred base pairs upstream of the transcription start site or in a distal enhancer region 1–2 kb upstream. Examination of the sequence upstream of the human MyHC-EO/IIL gene, however, revealed only one of these binding sites in the proximal region, an ‘E-box’, indicating that this gene is regulated either by regions located farther upstream or downstream of the typical MyHC gene promoter or by a novel set of transcription factors. An interesting possibility for the latter includes Dach2, Six1, Eya2 and Pax3, which are the vertebrate homologues of *Drosophila* factors that control eye development (Heanue et al., 1999). These factors also form a regulatory network expressed in developing vertebrate somites and capable of regulating expression of myogenic factors such as myogenin and MyoD.

The observation that the S1 catalytic domain of MyHC-EO more closely resembles the adult fast MyHC isoforms is consistent with the expectation that the contractile properties of MyHC-EO are fast or superfast. Their similarity might also suggest that MyHC-EO arose as a specialization of the fast MyHC-IIB gene, which encodes the myosin with the fastest known contractile properties. However, the phylogenetic analysis suggests a far more ancient origin, in which EO and embryonic MyHCs diverged from the fast MyHC precursor soon after the slow/cardiac myosins, but significantly before the specialization of the fast and perinatal genes. Additional evidence is provided by the identity of the 3′ exon boundaries of MyHC-EO/IIL, embryonic and α and β cardiac genes (Epp et al., 1993; Liew et al., 1990; Strehler et al., 1986), which contrasts with the structure shared by the fast MyHC genes (Schachat and Briggs, 1999). In the fast MyHC genes, the terminal exons all contain 150 bp of coding sequence, followed by the 3′UTR. In contrast, in MyHC-EO/IIL, this region exists as two exons, and these exon/intron boundaries are completely conserved in the embryonic as well as the α and β cardiac genes, indicating that they are derived from a common ancestral gene. The conservation of exon structures combined with the molecular phylogeny argue for an ancient origin of the MyHC-EO/IIL gene and a highly specialized function of the MyHC protein.

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References

Asmussen, G., Traub, I. and Pette, D. (1993). Electrophoretic analysis of myosin heavy chain isoform patterns in extraocular muscles of the rat. *FEBS Lett.* **335**, 243–245.

Barmack, N. H. (1977). Recruitment and suprathreshold frequency modulation of single extraocular muscle fibers in the rabbit. *J. Neurophysiol.* **40**, 779–790.

Blank, J. M. and Schachat, F. (1999). Extraocular and laryngeal muscles exhibit differential amplification of proteins involved in calcium homeostasis. *Mol. Biol. Cell* **10**, 246a.

Bouvagnet, P. F., Strehler, E. E., White, G. E., Strehler-Page, M. A., Nadal-Ginard, B. and Mahdavi, V. (1987). Multiple positive and negative 5′ regulatory elements control the cell-type-specific expression of the embryonic skeletal myosin heavy-chain gene. *Mol. Cell. Biol.* **7**, 4377–4389.

Brand, N. J., Dabhade, N., Yacoub, M. and Barton, P. J. (1991). Determination of the 5′ exon structure of the human cardiac alpha-myosin heavy chain gene. *Biochem. Biophys. Res. Commun.* **179**, 1255–1258.

Briggs, M. M., Lin, J. J. C. and Schachat, F. H. (1987). The extent of amino-terminal heterogeneity in rabbit fast skeletal muscle troponin T. *J. Muscle Res. Cell Motil.* **8**, 1–12.

Brueckner, J. K., Itkis, O. and Porter, J. D. (1996). Spatial and temporal patterns of myosin heavy chain expression in developing rat extraocular muscle. *J. Muscle Res. Cell Motil.* **17**, 297–312.

Brueckner, J. K. and Porter, J. D. (1998). Visual system maldevelopment disrupts extraocular muscle-specific myosin expression. *J. Appl. Physiol.* **85**, 584–592.

Brunekreef, G. A., Kraft, H. J., Schoenmakers, J. G. and Lubsen, N. H. (1996). The mechanism of recruitment of the lactate dehydrogenase-B/epsilon-crystallin gene by the duck lens. *J. Mol. Biol.* **262**, 629–639.

Buckingham, M. (1992). Making muscle in mammals. *Trends Genet.* **8**, 144–148.

Catz, D. S., Fischer, L. M., Moschella, M. C., Tobias, M. L. and Kelley, D. B. (1992). Sexually dimorphic expression of a laryngeal-specific androgen-regulated myosin heavy chain gene during *Xenopus laevis* development. *Dev. Biol.* **154**, 366–376.

Chiarandini, D. J. and Davidowitz, J. (1979). Structure and function of extraocular muscle fibers. In *Current Topics in Eye Research*, vol. 1 (ed. J. A. Zadunatsky and H. Davson), pp. 91–142. New York: Academic Press.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol chloroform extraction. *Analyt. Biochem.* **162**, 156–159.

Close, R. L. and Luff, A. R. (1974). Dynamic properties of inferior rectus muscle of the rat. *J. Physiol., Lond.* **236**, 259–270.

Cooper, S. and Eccles, J. (1930). The isometric responses of mammalian muscles. *J. Physiol., Lond.* **69**, 377–385.

DelGaudio, J. M., Sciote, J. J., Carroll, W. R. and Escalmado, R. M. (1995). Atypical myosin heavy chain in rat laryngeal muscle. *Ann. Otol. Rhinol. Laryngol.* **104**, 237–245.

Ennion, S., Sant'ana Pereira, J., Sargeant, A. J., Young, A. and Goldspink, G. (1995). Characterization of human skeletal muscle fibers according to the myosin heavy chains they express. *J. Muscle Res. Cell Motil.* **16**, 35–43.

Epp, T. A., Dixon, I. M., Wang, H. Y., Sole, M. J. and Liew, C. C. (1993). Structural organization of the human cardiac alpha-myosin heavy chain gene (MYH6). *Genomics* **18**, 505–509.

Fuchs, A. F., Kaneko, C. R. S. and Scudder, C. A. (1985). Brainstem control of saccadic eye movements. *Annu. Rev. Neurosci.* **8**, 307–337.

Giallongo, A., Venturella, S., Oliva, D., Barbieri, G., Rubino, P. and Feo, S. (1993). Structural features of the human gene for muscle-specific enolase. Differential splicing in the 5′-untranslated sequence generates two forms of mRNA. *Eur. J. Biochem.* **214**, 367–374.

Gupta, M., Zak, R., Libermann, T. A. and Gupta, M. P. (1998). Tissue-restricted expression of the cardiac alpha-myosin heavy chain gene is controlled by a downstream repressor element containing a palindrome of two ets-binding sites. *Mol. Cell. Biol.* **18**, 7243–7258.

Hall-Craggs, E. C. (1968). The contraction times and enzyme activity of two rabbit laryngeal muscles. *J. Anat.* **102**, 241–255.

Heanue, T. A., Reshef, R., Davis, R. J., Mardon, G., Oliver, G., Tomarev, S., Lassar, A. B. and Tabin, C. J. (1999). Synergistic regulation of vertebrate muscle development by Dach2, Eya2 and Six1, homologs of genes required for *Drosophila* eye formation. *Genes Dev.* **13**, 3231–3243.

Hoh, J. F., Hughes, S. and Hoy, J. F. (1988). Myogenic and neurogenic regulation of myosin gene expression in cat jaw-closing muscles regenerating in fast and slow limb muscle beds. *J. Muscle Res. Cell Motil.* **9**, 59–72.

Huang, W. Y. and Liew, C. C. (1998). Chromatin remodelling of the cardiac beta-myosin heavy chain gene. *Biochem. J.* **330**, 871–876.

Jacoby, J., Ko, K., Weiss, C. and Rushbrook, J. I. (1990). Systematic variation in myosin expression along extraocular muscle fibers of the adult rat. *J. Muscle Res. Cell Motil.* **11**, 25–40.

Jaenicke, T., Diederich, K. W., Haas, W., Schleich, J., Lichter, P., Pfordt, M., Bach, A. and Vosberg, H. P. (1990). The complete sequence of the human beta-myosin heavy chain gene and a comparative analysis of its product. *Genomics* **8**, 194–206.

Jin, J. P., Zhang, J. and Wang, J. (1995). An embryonic alternative transcription initiation site and the 5′-upstream structure of mouse cardiac troponin T gene. *Biochem. Biophys. Res. Commun.* **214**, 1168–1174.

Kelley, C. A., Takahashi, M., Yu, J. H. and Adelstein, R. S. (1993). An insert of seven amino acids confers functional differences between smooth muscle myosins from the intestines and vasculature. *J. Biol. Chem.* **268**, 12848–12854.

Kraft, H. J., Hendriks, W., de Jong, W. W., Lubsen, N. H. and Schoenmakers, J. G. (1993). Duck lactate dehydrogenase B/epsilon-crystallin gene. Lens recruitment of a GC-promoter. *J. Mol. Biol.* **229**, 849–859.

Li, Y., Camp, S., Rachinsky, T. L., Bongiorno, C. and Taylor, P. (1993). Promoter elements and transcriptional control of the mouse acetylcholinesterase gene. *J. Biol. Chem.* **268**, 3563–3572.

Liew, C. C., Sole, M. J., Yamauchi-Takihara, K., Kellam, B., Anderson, D. H., Lin, L. P. and Liew, J. C. (1990). Complete sequence and organization of the human cardiac beta-myosin heavy chain gene. *Nucleic Acids Res.* **18**, 3647–3651.

Lucas, C. A., Rughani, A. and Hoh, J. F. (1995). Expression of extraocular myosin heavy chain in rabbit laryngeal muscle. *J. Muscle Res. Cell Motil.* **16**, 368–378.

Martensson, A. and Skoglund, C. R. (1964). Contraction properties of intrinsic laryngeal muscles. *Acta Physiol. Scand.* **60**, 18–36.

McLachlan, A. D. and Karn, J. (1983). Periodic features in the amino acid sequence of nematode myosin rod. *J. Mol. Biol.* **164**, 605–626.

Merati, A. L., Bodine, S. C., Bennett, T., Jung, H. H., Furuta, H. and Ryan, A. F. (1996). Identification of a novel myosin heavy chain gene expressed in the rat larynx. *Biochim. Biophys. Acta* **1306**, 153–159.

Murphy, C. T. and Spudich, J. A. (1998). Dictyostelium myosin 25–50K loop substitutions specifically affect ADP release rates. *Biochemistry* **37**, 6738–6744.

Olson, E. N. (1990). MyoD family: a paradigm for development? *Genes Dev.* **4**, 1454–1461.

Pepinski, R. B. (1983). Localization of lipid–protein and protein–protein interactions within the murine retrovirus gag precursor by a novel peptide-mapping technique. *J. Biol. Chem.* **258**, 11229–11235.

Rayment, I., Rypniewski, W. R., Schmidt-Bäse, K., Smith, R., Tomchick, D. R., Benning, M. M., Winkelmann, D. A., Wesenberg, G. and Holden, H. M. (1993). Three-dimensional structure of myosin subfragment-1: a molecular motor. *Science* **261**, 50–58.

Rome, L. C., Cook, C., Syme, D. A., Connaughton, M. A., Ashley-Ross, M., Klimov, A., Tikunov, B. and Goldman, Y. E. (1999). Trading force for speed: why superfast crossbridge kinetics leads to superlow forces. *Proc. Natl. Acad. Sci. USA* **96**, 5826–5831.

Rome, L. C., Syme, D. A., Hollingworth, S., Lindstedt, S. L. and Baylor, S. M. (1996). The whistle and the rattle: the design of sound producing muscles. *Proc. Natl. Acad. Sci. USA* **93**, 8095–8100.

Rowlerson, A., Pope, B., Murray, J., Whalen, R. B. and Weeds, A. G. (1981). A novel myosin present in cat jaw-closing muscles. *J. Muscle Res. Cell Motil.* **2**, 415–438.

Rushbrook, J. I., Weiss, C., Ko, K., Feurman, M. H., Carleton, S., Ing, A. and Jacoby, J. (1994). Identification of alpha-cardiac myosin heavy chain mRNA and protein in extraocular muscle of the adult rabbit. *J. Muscle Res. Cell Motil.* **15**, 505–515.

Sartore, S., Mascarello, F., Rowlerson, A., Gorza, L., Ausoni, S., Vianello, M. and Schiaffino, S. (1987). Fibre types in extraocular muscles: a new myosin isoform in the fast fibres. *J. Muscle Res. Cell Motil.* **8**, 161–172.

Schachat, F. and Briggs, M. (1999). Identification of two patterns of exon organization in the human striated muscle myosin heavy chain genes. *Mol. Biol. Cell* **10**, 34a.

Schachat, F. H., Diamond, M. S. and Brandt, P. W. (1987). The effect of different troponin T-tropomyosin combinations on thin filament activation. *J. Mol. Biol.* **198**, 551–554.

Schachat, F., Williams, R. S. and Schnurr, C. A. (1988). Coordinate changes in fast thin filament and Z-line protein expression in the early response to chronic stimulation. *J. Biol. Chem.* **263**, 13975–13978.

Shimizu, N., Prior, G., Umeda, P. K. and Zak, R. (1992). cis-acting elements responsible for muscle-specific expression of the myosin heavy chain beta gene. *Nucleic Acids Res.* **20**, 1793–1799.

Shiotani, A. and Flint, P. W. (1998). Myosin heavy chain composition in rat laryngeal muscles after denervation. *Laryngoscope* **108**, 1225–1229.

Spencer, R. F. and Porter, J. D. (1988). Structural organization of the extraocular muscles. In *Neuroanatomy of the Oculomotor System* (ed. J. A. Buttner-Ennever), pp. 33–79. New York: Elsevier.

Stedman, H. H., Eller, M., Jullian, E. H., Fertels, S. H., Sarkar, S., Sylvester, J. E., Kelly, A. M. and Rubinstein, N. A. (1990). The human embryonic myosin heavy chain. Complete primary structure reveals evolutionary relationships with other developmental isoforms. *J. Biol. Chem.* **265**, 3568–3576.

Strehler, E. E., Strehler-Page, M. A., Perriard, J. C., Periasamy, M. and Nadal-Ginard, B. (1986). Complete nucleotide and encoded amino acid sequence of a mammalian myosin heavy chain gene. Evidence against intron-dependent evolution of the rod. *J. Mol. Biol.* **190**, 291–317.

Swank, D. M., Zhang, G. and Rome, L. C. (1997). Contraction kinetics of red muscle in scup: mechanism for variation in relaxation rate along the length of the fish. *J. Exp. Biol.* **200**, 1297–1307.

Sweeney, H. L., Rosenfeld, S. S., Brown, F., Faust, L., Smith, J., Xing, J., Stein, L. A. and Sellers, J. R. (1998). Kinetic tuning of myosin *via* a flexible loop adjacent to the nucleotide binding pocket. *J. Biol. Chem.* **273**, 6262–6270.

Swoap, S. J. (1998). *In vivo* analysis of the myosin heavy chain IIB promoter region. *Am. J. Physiol.* **274**, C681–C687.

Takechi, H., Hosokawa, N., Hirayoshi, K. and Nagata, K. (1994). Alternative 5′ splice site selection induced by heat shock. *Mol. Cell. Biol.* **14**, 567–575.

Takeda, S., North, D. L., Lakich, M. M., Russell, S. D. and Whalen, R. G. (1992). A possible regulatory role for conserved promoter motifs in an adult-specific muscle myosin gene from mouse. *J. Biol. Chem.* **267**, 16957–16967.

Talmadge, R. J. and Roy, R. R. (1993). Electrophoretic separation of rat skeletal muscle myosin heavy-chain isoforms. *J. Appl. Physiol.* **75**, 2337–2340.

Taylor, A., Cody, F. W. and Bosley, M. A. (1973). Histochemical and mechanical properties of the jaw muscles of the cat. *Exp. Neurol.* **38**, 99–109.

Thys, T. M., Blank, J. M. and Schachat, F. H. (1998). Rostral-caudal variation in troponin T and parvalbumin correlates with differences in relaxation rates of cod axial muscle. *J. Exp. Biol.* **201**, 2993–3001.

Uyeda, T. Q. P., Ruppel, K. M. and Spudich, J. A. (1994). Enzymatic activities correlate with chimaeric substitutions at the actin-binding face of myosin. *Nature* **368**, 567–569.

Weiss, A., Schiaffino, S. and Leinwand, L. A. (1999). Comparative sequence analysis of the complete human sarcomeric myosin heavy chain family: implications for functional diversity. *J. Mol. Biol.* **290**, 61–75.

Wetzel, D. M. and Kelley, D. B. (1983). Androgen and gonadotropin effects on male mate calls in South African clawed frogs, *Xenopus laevis*. *Horm. Behav.* **17**, 388–404.

Wieczorek, D. F., Periasamy, M., Butler-Browne, G., Whalen, R. G. and Nadal-Ginard, B. (1985). Co-expression of multiple myosin heavy chain genes, in addition to a tissue-specific one, in extraocular musculature. *J. Cell Biol.* **101**, 618–629.

Winters, L. M., Briggs, M. M. and Schachat, F. (1998). The human extraocular muscle myosin heavy chain gene (MYH13) maps to the cluster of fast and developmental myosin genes on chromosome 17. *Genomics* **54**, 188–189.